Zebrafish Development and Disease Model Laboratory

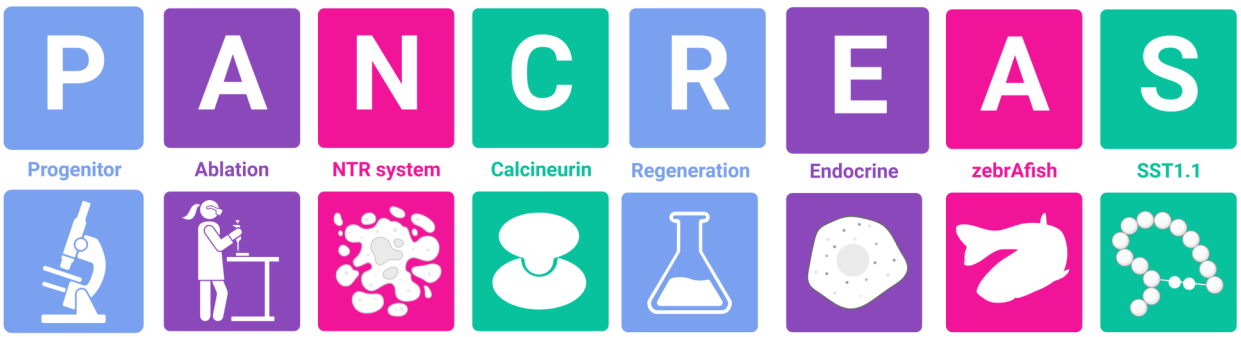
Faculty of Sciences

University of Liège

Unravelling the Contribution of Ductal Progenitors during Beta cell Regeneration in Zebrafish: a focus on Calcineurin

LAURA MASSOZ

Thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Sciences



LIÈGE université
GIGA institute

fnrs
LA LIBERTÉ DE CHERCHER

Zebrafish Development & Disease Models

Supervised by: **Dr. Isabelle Manfroid**

Academic year 2022-2023

Zebrafish Development and Disease Model Laboratory

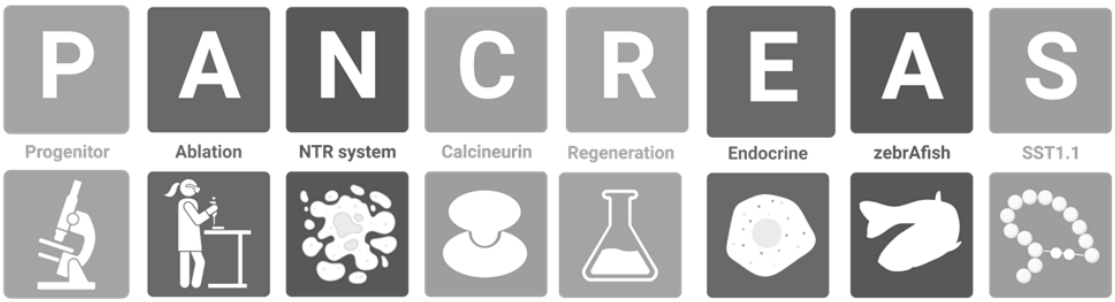
Faculty of Sciences

University of Liège

UNRAVELLING THE CONTRIBUTION OF DUCTAL PROGENITORS DURING BETA CELL REGENERATION IN ZEBRAFISH: A FOCUS ON CALCINEURIN

LAURA MASSOZ

Thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Sciences



LIÈGE université
GIGA institute

fnrs
LA LIBERTÉ DE CHERCHER

Zebrafish Development & Disease Models

Supervised by: **Dr. Isabelle Manfroid**

Co-supervised by: **Dr. Marianne Voz**

Academic year 2022-2023

Source of funding

Acknowledgments

General acknowledgments

I would like to thank all members of the jury, Dr. Staels, Dr. Pal Singh, Dr. Muller, Dr. Lacroix and Dr. Thiry for their guidance and evaluation of my work. Your kind advices help improve the quality of the present manuscript. I am truly grateful for your expertise and valuable time in assessing my thesis.

I also express my gratitude to the members of my thesis committee whom followed my work through the years. Dr. Hanson, Dr. Lacroix, Dr. Voz and Dr. Thiry, I thank you not only for your advices and guidance from the beginning of the thesis but also for your implication in my project and for your support.

I am grateful to Dr. Peers, Dr. Voz and Dr. Manfroid for giving me the opportunity to first pursue my master thesis and then my thesis within their laboratory. You gave allowed me to work and evolve in a lab with a great atmosphere. Moreover, you taught me how to work with the amazing model that is the zebrafish. I also thank you for your guidance during the weekly lab meetings, which help to improve my communications skills.

Remerciements personnels

Cette thèse m'a offert l'opportunité de répondre partiellement à une question qui m'a captivé : comment ce petit zebrafish parvient-il à régénérer ses cellules bêta ? Dans la poursuite de réponses, le périple m'a permis de dépasser mes propres limites. Cette thèse a été une expérience enrichissante, tant sur le plan scientifique que personnel. Au fil de mon parcours, j'ai eu le privilège de croiser la route de nombreuses personnes exceptionnelles, certaines m'accompagnant brièvement et d'autres me soutenant tout au long du chemin. C'est grâce au soutien et à l'engagement de ces individus que ce travail a pu voir le jour, et je tiens à les remercier.

Je tiens tout d'abord à exprimer ma plus sincère gratitude envers ma promotrice, le Dr. Isabelle Manfroid. Sans son aide et sa détermination, ce projet n'aurait jamais pu voir le jour. Je vous remercie infiniment pour votre implication et ce dès le début, notamment dans la préparation du FRIA. Je vous suis reconnaissante de m'avoir permis de devenir une meilleure scientifique, en me laissant le temps de me poser les bonnes questions et en me guidant vers les réponses. Je vous remercie aussi de la confiance que vous m'avez accordée, de m'avoir laissé travailler de manière indépendante tout en étant toujours disponible pour la moindre question ou analyse. Le projet calcineurine a été semé d'embûches mais grâce à de longues heures de réflexion ensemble, nous avons obtenu un article dont je suis fière et j'espère qu'il vous rendra également fière. C'est grâce à votre enseignement que j'ai pu gérer la fin de thèse et la mener à terme. Au-delà du côté purement scientifique, je vous remercie pour votre bienveillance et votre gentillesse à mon égard. Je pense sincèrement que je n'aurais pu réaliser un tel travail avec une autre personne.

Je tiens également à remercier toute l'équipe d'Isabelle, la « regeneration team ». Dès mon arrivée au labo, j'ai été très chaleureusement accueillie par Jordane (Jojo) et David. Je vous remercie tous les deux de m'avoir guidé pendant mes débuts au labo et de m'avoir passé votre savoir. Jojo, mon expérience n'aurait pas été pareille sans ton sourire et tes encouragements. Je te remercie de m'avoir appris toutes ces techniques de manière rigoureuse mais toujours dans la bonne humeur. David, je te remercie aussi pour tes enseignements, de m'avoir aidé à prendre confiance en moi et de m'avoir poussé à calculer les dilutions de tête. Et sans oublier, 4800 RPZ… L'équipe s'est ensuite agrandie avec l'arrivée de Claudio et Marie. Claudio, without knowing, you help me improve my English skills. It was good having an international student in the team. Thank you for your help in all these Pokemon raids. Marie, ma co-neuneu, la deuxième Claudette, ton arrivée au laboratoire a été plus que bénéfique à bien des égards. Parce ce qu'on partageait souvent les mêmes galères, ça faisait du bien d'avoir quelqu'un à qui parler tant des résultats que de tous les autres aspects de la thèse. Merci de m'avoir appris tes petites astuces (du timer !) et tous les moments agréables passés ensemble.

Notre petite équipe régénération s'intègre dans le labo ZDDM dirigé en plus d'Isabelle, par Marianne et Bernard qui forment ensemble une bonne équipe. Tous les trois apportent une énergie et une facette différente, qui permet au labo d'être tel qu'on le connait aujourd'hui. Je tiens particulièrement à remercier Marianne, qui s'est retrouvée à devenir ma co-promotrice

cette dernière année. Merci pour ta relecture minutieuse de l'article calcineurine, d'avoir apporté ce regard un peu externe, qui m'a aidé à éclaircir le texte et surtout à faire plus de liens. Je te remercie aussi de m'avoir donné ce petit coup de pied dont j'avais besoin quand je n'osais pas appuyer sur « submit » pour l'article. Bien que notre collaboration n'aura pas été sans désaccords, je te suis reconnaissante pour ta franchise et pour toujours avoir écouté mes arguments. Au-delà du point de vue purement scientifique, j'apprécie ton enthousiasme ainsi que ton côté direct et ton humour. Sans oublier, ces petits « échanges » de potins quand nous étions au microscope. Je remercie également Bernard qui a toujours des conseils avisés lors des lab meetings. Merci pour ta relecture de la revue, tes conseils ont contribué à l'améliorer. Je te suis également reconnaissante de ton aide pour le mini-projet nfat pendant les révisions.

Je remercie également les membres de l'équipe, qui plus que des collègues, sont devenus des amis. Vous avez tous contribué à cette ambiance enrichissante, qui a fait que c'était un réel plaisir de venir travailler à vos côtés au laboratoire. Comme dirait Marianne, nous n'avons pas besoin de team buildings mais plutôt « de-building » car nous sommes déjà trop proches ! Merci pour ces conversations, ces moments de rire, nos sorties en dehors du labo et surtout votre soutien. Parce qu'on forme une équipe soudée, il y aura toujours quelqu'un pour prendre bien soin des poissons. Chiara, Chirara, Kiaraoké, avec qui j'ai partagé bons nombres d'aventures (C !). Je n'oublierai pas tes danses endiablées, notamment au concert de Dua Lipa. Colin, mon frère, mon bro', partenaire de café, merci pour ces discussions, et ce « co-voiturage » dans lequel c'est presque toujours ton tour de conduire. Bien sûr, je ne pourrais oublier Caroline, qui a décidé qu'elle serait mon petit poulain. Tu as en même temps ces côtés adorables et petit dragon et c'est pour cela qu'on t'adore. Merci pour ces petites conversations matinales. Vous êtes tous les trois des personnes exceptionnelles et je vous souhaite beaucoup de courage et de réussite pour achever vos thèses. Mes remerciements vont également à Lydie, entre autres pour la relecture de l'article calcineurine ainsi que de m'avoir aidé pour la collaboration. Ses questions pointues aux lab meetings m'ont aidées à peaufiner mon travail. Je souhaite la bienvenue aux 3 nouveaux membres du labo, Romane, Elisa et Manon et leur souhaite du courage pour la suite.

Quelques stagiaires ont rejoint momentanément l'équipe du ZDDM. Parmi eux Célia, Cécé, la première étudiante que j'ai encadrée, a contribué à ce projet. Parce que je te faisais bien souvent revenir les week-ends, pour toutes ces heures au confocal (pendant lesquelles tu étais bien contente que je te laisse tranquille !), et ton implication dans le projet, merci Cécé. Je tiens également à remercier Rémi, membre aussi de la regeneration team. Merci pour la bonne humeur et l'air frais que tu as apporté au laboratoire. Sans oublier Arthur, qui a réussi à me faire comprendre quelques notions de bioinfo, et toujours partant pour une blague. Amel, cela a été un réel plaisir de te rencontrer et c'est toujours un plaisir de passer du temps avec toi et ta famille. Ton amitié qui ne s'est pas érodée avec le temps, m'est précieuse.

Je remercie également tous les membres des laboratoires voisins, des équipes de Marc, Ingrid et Patricia, grâce à qui la bonne humeur règne au labo. Vous avez tous contribué à cette ambiance agréable au laboratoire. Ratish and Gustavo, I am grateful that we began our thesis together and share our experiences along the way. Thanh always " still alive", thank you for your kindness and always sharing interesting papers. Hanh and Que, the best guides in Viet

Nam, many thanks for making me discover the vietnamese culture, food and landscapes, and for all these great moments together no matter the country we were. Hanh, we spend so much amazing time together, thank you for being my friend. Je ne pourrais pas oublier ma besta Aurélie, si douée en peinture de zebra. Je remercie aussi Sébastien pour ces nombreux conseils, sa gentillesse et sa jovialité. Amandine, toujours partante pour une petite conversation au détour d'un couloir. Marco, for fitting so well in sometimes the craziest environment in the lab. Ma petite Déborah (ou Jessica pour les intimes), ma Lauracolyte, qui est presque un petit zebra, merci pour tous ces moments passés ensemble. Pour toutes ces soirées de Noël organisées ensemble, ces petites danses de la joie, ces concerts, et encore au moins 1000 autres moments partagés, merci. Jordane, Blondie non seulement pour tous ces rires, ces moments de joies qui mettent du baume au cœur, mais aussi pour être une oreille si attentive, merci. Tu rends le monde autour de toi plus beau, à grand coups de paillettes. On a tellement partagé pendant ces quelques années, tu es une personne exceptionnelle et je te souhaite tout le meilleur. Parce que ABH hanhan, je te fais à toi et bébé Mila plein de bisous baleine et paillettes.

Je remercie aussi grandement les membres des plateformes d'imagerie et anciennement zebrafish pour leur aide inestimable. J'aimerais remercier plus particulièrement Sandra pour sa disponibilité et Alexandre pour être toujours prêt à apporter son aide. Merci Alex pour être toujours aussi impliqué lorsque l'on vient te voir avec un nouveau problème, tes conseils pour rendre les analyses d'image plus efficaces (même si avec les canaux c'est compliqué !) et bien sûr, ta sympathie. Je remercie aussi grandement Hélène, Virginie, Ophélie et Denis pour être toujours à l'écoute quand j'avais besoin d'aide pour les poissons. Merci Denis pour t'être si bien occupé de mes poissons, de me rabrouer quand j'oublie une étiquette et ce même si je dois me cacher quand je viens nourrir un peu plus mes bébés. Je remercie également la plateforme bioinformatique, et plus particulièrement Arnaud pour son aide et pour avoir si bien conservé les données.

Mes remerciements les plus sincères vont également à Emmanuel Dejardin, non seulement pour son aide avec la bourse EOS mais aussi pour s'être soucié de mon avancement. Je vous remercie pour votre gentillesse ainsi que tous les conseils avisés. Je vous suis également reconnaissante de m'avoir donné cet important élan de motivation quand j'en ai eu le plus besoin.

Au-delà du GIGA, je tiens également à remercier les Drs. Anne Grappin-Botton et Yung Hae Kim pour le temps qu'elles ont consacré sur le projet calcineurine.

Pendant mon parcours universitaire, j'ai eu la chance de rencontrer des personnes exceptionnelles qui sont devenu des amis proches. Parce que vous m'amener tous ce côté doux et pétillant de la vie, j'aimerais vous adresser ces quelques mots. Florie, certaines rencontres nous marquent à vie et ta rencontre est l'une d'entre d'elle. Parce que tu m'as appris à vivre la vie plus spontanément, parce que on est pas des mijoles, et parce qu'on est pas les mêmes, merci. Je remercie également Garcia non seulement pour son amitié mais aussi pour son soutien de fer en toutes circonstances et ce depuis toutes ces années. Je remercie également Déborah, qui s'est aussi lancée dans l'aventure de la thèse. Nos moments de rires peuvent adoucir même les plus rudes journées. Je remercie également Nani pour toujours écouter sans le moindre jugement et pour sa gentillesse infinie. Axel, mon partenaire de bibli pendant 5 ans, ce lamentin, le seul capable de nous déchiffrer avec Florie quand le débit de parole est accéléré par nos rires. Sans oublier Charly, pour ton petit grain de folie et ton amitié, Maurane pour ta

gentillesse et ta bienveillance, Cédric mon premier ami à l'univ, merci. En dehors de l'université, je ne pourrais bien sûr oublier notre quatuor avec les Girls. Valentine, ma plus vieille amitié, peut-être parfois loin des yeux mais toujours près du cœur. Camcam et Méla toujours présentes et partantes pour nos soirées entre filles, merci.

J'aimerais remercier plus particulièrement mes deux amis, Jérémie et Renaud rencontrés au labo, pour leur présence quotidienne et leur soutien sans faille. Parce qu'on se comprend ; parce qu'on partage tout, nos joies comme nos peines, que ce soit un problème scientifique ou personnel ; parce que vous apportez un tourbillon enivrant de folie dans ma vie, merci. Renaud mon ami, je ne compte plus les bêtises, les moments de rire, mais j'aimerais te remercier de ton intérêt, ton enthousiasme et de ton implication, à chaque fois que je te parle d'un problème. Bien souvent nos journées se terminent par une conf-call, pendant laquelle on retrace les nouvelles du jour, et permet de nous défouler de nos journées. And at last, Jérémie, mon bestie, nous avons traversé ensemble toutes les épreuves de nos thèses. C'est peu dire que ta présence a réussi à alléger tous les moments difficiles. Ton courage, ta persévérance et ton soucis du détail représentent un modèle pour moi. Merci pour ton soutien, tes danses dans le labo et tous ces rires. Notre amitié s'est forgée bien au-delà du labo, d'abord quand on regardait GoT, puis dans toutes les aventures et voyages ensemble. Tu m'as fait découvrir de nouvelles saveurs de la vie et je t'en remercie. Même si tu es bien plus têtu que moi (c'est écrit donc c'est vrai), tu es une personne exceptionnelle, gentille, sensible et joviale qui amène une vague de bonne humeur partout où tu vas. Il n'y aurait pas assez de place dans une thèse pour parler de toutes nos aventures, donc simplement, merci pour tout.

Cette dernière année a été marquée par ton arrivée, Laurent. Tu m'as amené une bouée de sauvetage et une bouffée d'air frais dans les moments les plus difficiles. Tu formes une bulle de bonheur autour de moi. Merci d'essayer de comprendre ce que je raconte quand je parle des résultats et merci de ton soutien.

Finalement, mes derniers remerciements sont pour ma famille. Merci pour toutes les petites choses de la vie que vous avez faites pour moi, quand je ne pouvais pas m'éloigner du labo. Sydney, mon mini-moi et Jason, parce qu'une relation frère/sœurs est indescriptible. Jamais vous n'aurez le dernier morceau de chocolat, mais si vous avez besoin d'un rein… Karel et Thierry qui ont rejoint notre cercle, pour plus de bons moments. Damien et Laety qui m'ont donné le plus beau cadeau. Parce que la vie apporte des joies inattendues, comme les rires de ma filleule Emy et de mon neveu Eliot. Enfin mes derniers mots sont pour toi, maman. Parce que j'aurais toujours besoin de ton soutien.

Je dédie cette thèse à la mémoire de mon père, Francis Massoz.

« Tu n'es plus là où j'étais mais tu es partout où je suis. »

Victor Hugo

Diabetes is a global epidemic affecting nearly 10% of the population worldwide. The disease is coupled with a significant loss of the β cell mass, leading to dysregulation of the glycemia. Stimulating β cell regeneration holds great promise as a potential treatment for curing diabetic patients. Observations in diabetic patients and studies in mouse models of pancreatic injury showed that mammalian pancreatic cells exhibit plasticity towards insulin production. However, the regeneration process is slow and inefficient, posing challenges in understanding the underlying mechanisms. In contrast, the zebrafish, a model organism known for its remarkable tissue regeneration capabilities, can spontaneously and efficiently regenerate β cells after their destruction. This raises the intriguing question of how the zebrafish is able to regenerate β cells. Therefore, the goal of our laboratory is to unravel the cellular and molecular mechanisms of β cell regeneration in zebrafish. In this thesis, we focus on investigating the contribution and the mechanisms underlying regeneration from pancreatic progenitors residing within the ductal tree.

To gain insights into the mechanisms of β cell regeneration from ductal progenitors, we conducted transcriptomic profiling of ductal cells following β cell ablation. Our data revealed intriguing candidates, including the protein phosphatase calcineurin and the tumour suppressor p53. We demonstrated that repression of calcineurin accelerates β cell regeneration, whereas overactivation of calcineurin suppresses regeneration, leading to dysregulation of blood glucose levels. Specifically, calcineurin repression enhances ductal cell proliferation and the subsequent formation of endocrine progenitors, ultimately depleting the progenitor pool. Overall, our findings indicate that calcineurin finely regulates the balance between progenitor proliferation and endocrine differentiation, ensuring proper β cell regeneration. Calcineurin emerges as a crucial guardian of the progenitor pool. Conversely, our results also highlight the importance of p53 in promoting ductal cell proliferation and subsequent β cell regeneration, as demonstrated by the inhibitory effect of the p53 inhibitor, PFTα.

Furthermore, we observed that regenerated β cells exhibit a bi-hormonal phenotype, co-expressing the somatostatin1.1 and insulin hormones. We discovered that the δ1.1 sub-population rapidly converts into insulin-producing cells. Notably, these cells also originate from the ducts, suggesting that the two cellular sources of regenerated β cells are not mutually exclusive but rather coordinately involved.

Our study uncovers novel cellular and molecular mechanisms underlying β cell regeneration in zebrafish. These findings shed light on the pivotal role of progenitor proliferation and the contribution of δ1.1 cells in this process. Collectively, these insights provide valuable clues that may contribute to improving β cell regeneration in mammals.

Le diabète est une épidémie mondiale touchant près de 10% de la population. La maladie est associée à une perte significative de la masse des cellules bêta, entrainant une dysrégulation de la glycémie. La stimulation de la régénération des cellules bêta représente une promesse en tant que traitement potentiel pour guérir les patients diabétiques. Les observations chez les patients diabétiques ainsi que les études sur des modèles murins de lésions pancréatiques ont montré que les cellules pancréatiques des mammifères présentent une certaine plasticité envers la production de l'insuline. Cependant, la régénération est lente et inefficace, ce qui pose des défis pour comprendre les mécanismes sous-jacents. En revanche, le poisson-zèbre, un organisme modèle réputé pour ses remarquables capacités de régénération, peut régénérer spontanément et efficacement les cellules bêta après leur destruction. Cela soulève la question de savoir comment le poisson-zèbre parvient à régénérer les cellules bêta. Ainsi, l'objectif principal de notre laboratoire est de déterminer les mécanismes cellulaires et moléculaires de la régénération des cellules bêta chez le poisson-zèbre. Dans cette thèse, nous nous concentrons sur l'étude des mécanismes de régénération à partir des progéniteurs pancréatiques présents dans l'arbre canalaire. Pour comprendre ces mécanismes, nous avons réalisé un profilage transcriptomique des cellules des canaux après l'ablation des cellules bêta. Ces données ont révélé des candidats intéressants, notamment la phosphatase calcineurine et le suppresseur de tumeur p53. Nous avons démontré que la répression de calcineurine accélère la régénération des cellules bêta, tandis que la surexpression de calcineurine la diminue, entraînant une dysrégulation de la glycémie. Plus spécifiquement, la répression de calcineurine favorise la prolifération des cellules canalaires et la formation ultérieure de progéniteurs endocriniens, épuisant ainsi le pool de progéniteurs. Dans l'ensemble, nos résultats indiquent que calcineurine régule finement l'équilibre entre la prolifération des progéniteurs et la différenciation endocrine, assurant ainsi une régénération adéquate des cellules bêta. Calcineurine apparaît comme un gardien du pool de progéniteurs. D’autre part, nos résultats mettent également en évidence l'importance de p53 dans la promotion de la prolifération des cellules des canaux et la régénération des cellules bêta, comme en témoigne l'effet répresseur de l'inhibiteur de p53, le PFTα.

De plus, nous avons observé que les cellules bêta régénérées présentent un phénotype bi-hormonal, exprimant à la fois les hormones somatostatine1.1 et insuline. Nous avons montré que la sous-population δ1.1 se convertit rapidement en cellules productrices d'insuline. Ces cellules bi-hormonales proviennent également des canaux, ce qui suggère que les deux sources cellulaires des cellules bêta régénérées ne sont pas mutuellement exclusives, mais plutôt coordonnées.

Notre étude révèle de nouveaux mécanismes cellulaires et moléculaires sous-jacents à la régénération des cellules bêta chez le poisson-zèbre. Ces résultats mettent en lumière le rôle central de la prolifération des progéniteurs et la contribution des cellules δ1.1 dans ce processus. Dans l'ensemble, ces découvertes fournissent des éléments importants qui pourraient contribuer à stimuler la régénération des cellules bêta chez les mammifères.

List of Contents

List of Abbreviations

bHLH: basic helix loop helix
CAC: Centroacinar cell

CaN: Calcineurin
CTL: Control
DE: Differentially expressed
DMSO: dimethyl sulfoxide
dpf : days post fertilization
dpt : days post treatment
GFP: Green fluorescent protein
GSEA : Gene Set Enrichment Analysis
hpf: hours post fertilization
hESC: human embryonic stem cell
hiPSC: human incuced pluripotent stem cell
IHC : Immunihistochemistry
Ins: Insulin
MTZ: Metronidazole
NFP : Nifurpirinol
NTR: nitroreductase
PDL: Pancreatic duct ligation
PFA : Paraformaldehyde

PI: Principal Islet
PNC : pancreatic Notch cell
Px: Partial pancreatectomy
RNA-seq : RNA sequencing
ROS: Reactive oxygen species
STZ : Streptozotocin

SI : Secondary islet
T1D : Type 1 diabetes
T2D: Type 2 diabetes
Tg : transgenic
WT: Wild type

I. Introduction

1. The pancreas : structure and function

The pancreas, which is located behind the stomach and is connected to the duodenum through the Wirsung and Santorini ducts (**Figure 1**), is an amphicrine gland that plays a dual role in the digestive system. On one hand, its exocrine function aids in nutrient digestion, while on the other hand, its endocrine function is crucial for hormonal and metabolic regulation.

1.1.The exocrine pancreas

The exocrine compartment is composed approximatively 85-90% of the pancreas. It comprises two major cells types : the acinar and the ductal cells. The acinar cells are clustered in functional unit called acinus (from Latin, meaning “grape”) (**Figure 1**). These acini are responsible for the production, the storage and the secretion of digestive enzymes. They are classified into three major categories, α-amylases, lipases and proteases, which are respectively responsible for the hydrolysis of carbohydrates, fatty acids and proteins. The acinar cells secrete inactive precursor of enzymes, called zymogens or pro-enzyme. Once in the duodenum, the pro-enzymes are activated through proteolytic cleavages in order to carry out their digestive function. The trypsinogen is firstly cleaved by an endopeptidase into its activated form, the trypsin, which can subsequently activate the others pro-enzymes. The secretion of inactive enzymes is a protection mechanism that prevents a putative over-secretion of enzymes to digest the pancreas itself. However, in some pathologic cases as an obstruction of the ductal network, a premature activation of the trypsinogen lead to acute pancreatitis. These patients have an increased risk to develop diseases such as exocrine pancreas insufficiency, pancreatic cancer or diabetes ((Lee and Papachristou 2019), for review).

The ductal network is comprised several sub-types of ducts, which are classified according to their localisation from the acinus and according to their histology. Intercalated ducts receive the secretion from the acinus and are connected to intralobular ducts. These latter ducts are themselves connected to interlobular ducts, which eventually discharge the exocrine secretion into the principal pancreatic duct, before entering the duodenum. In addition to allow the flow of the pancreatic enzymes to the duodenum, the ductal cells, as exocrine cells, produce and secrete mucins protecting the ducts against the digestive enzymes, and several ions (HCO_3^- , Na^+, K^+, Cl^-) that neutralize the acidic pH of the chyme coming from the stomach.

The junction between the acinus and the ductal network is established by a specialized ductal cell, the so-called centroacinar cell (CAC). These cells are present at the centre of every acinus, at the terminal duct. Interestingly, the centroacinar cells show characteristic features of progenitor (Seymour et al. 2007) and seem to retain some progenitor potential in the adult pancreas (Rovira et al. 2010).

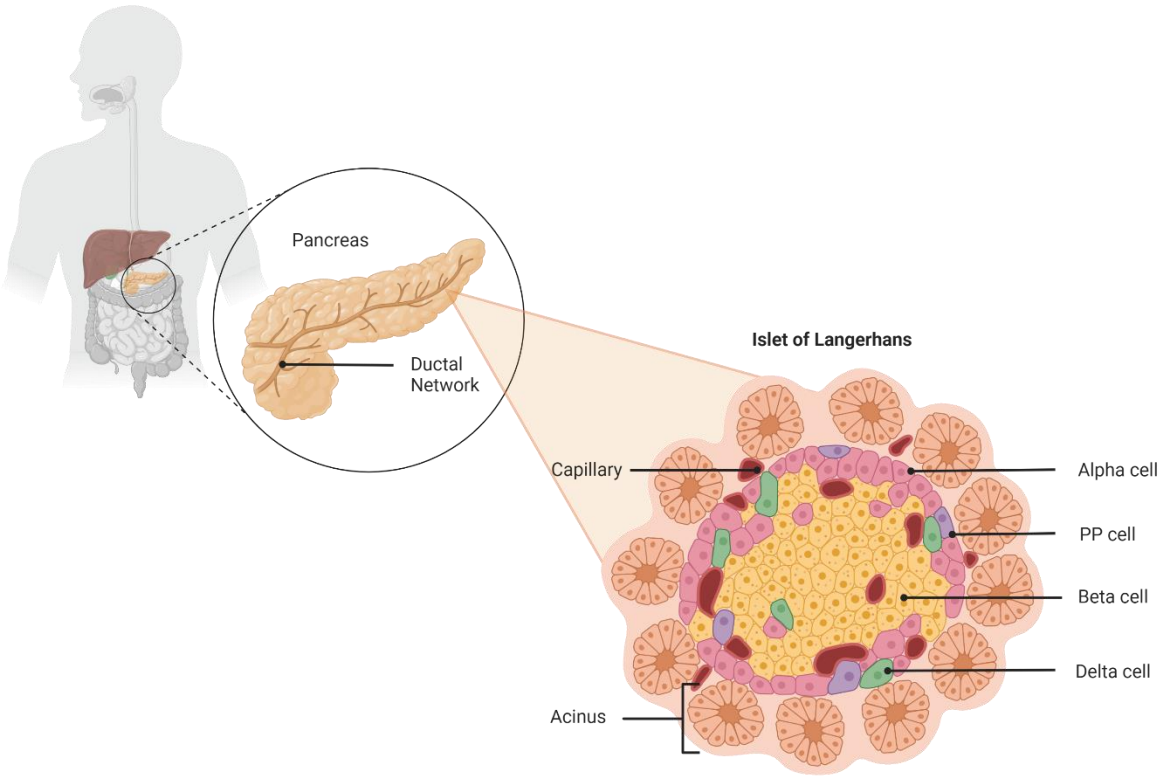


Figure 1 *: Anatomy and histology of the pancreas*

The pancreas is an organ located behind the stomach, which connects to the duodenum through the Wirsung duct. It is composed of two distinct functional parts: the exocrine and the endocrine compartments. The exocrine compartment of the pancreas is responsible for secreting digestive enzymes. It comprises acinar cells, which produce and secrete enzymes, and ductal cells, which transport the enzymes to the duodenum. The endocrine part of the pancreas consists of specialized cells grouped in islets of Langerhans, which are dispersed throughout the pancreas. The islets are composed of several types of cells, including α cells (which secrete glucagon), PP cells (which secrete polypeptide pancreatic), β cells (which secrete insulin), ε cells (which secrete ghrelin), and δ cells (which secrete somatostatin). These cells work together to regulate the glycemia and ensure proper glucose utilization.

1.2.The endocrine pancreas

While the endocrine part represents only 5% of the pancreas, its function is critical for the general homeostasis. The endocrine pancreas consists of hormone-producing cells grouped in islets, called Langerhans islets in human, which are dispersed within the exocrine pancreas. The islets are composed of highly specialized cells that are classified according to the hormone they predominantly produce. These islets are highly vascularized and highly innervated mini-organs. A great amount of capillary surrounds and infiltrates each islet, enabling glucose sensing and hormones secretion into the bloodstream.

At least five hormone-producing cell types can be found within the islets (α, β , δ, ε, and γ) (**Figure 1**). The α cells and β cells respectively produce glucagon and insulin, which are crucial in glucose homeostasis. The δ cells produce the somatostatin hormone, which one can repress both glucagon and insulin secretion. The ε cells produce ghrelin, implicated in the control of appetite and subsequent food intake. The γ cells, also called PP-cells, produce the pancreatic polypeptide (PP). Its function is still not completely understood, but it regulates both endocrine and exocrine pancreatic secretion, satiety and gastrointestinal motility.

The architecture of the islets is critical for its function due to cell-cell communication. Of note, the composition and organization of the endocrine cells within the islets differ between species (Steiner et al. 2010). In human, the architecture of the islet is heterogenous depending on the localization of the islet (Bonner-Weir, Sullivan, and Weir 2015). β cells represent half of the islet while α cells and δ cells respectively represent 40% and 10% of the total islet. E and γ are quite rare. In mice, the islet is structured with a core of β cells (representing 60-80% of the islet) surrounded by a mantel composed of the others endocrine cells (10-20% of α cells, 10% of δ and 1% of ε and γ) (Cabrera et al. 2006) (**Figure 2**).

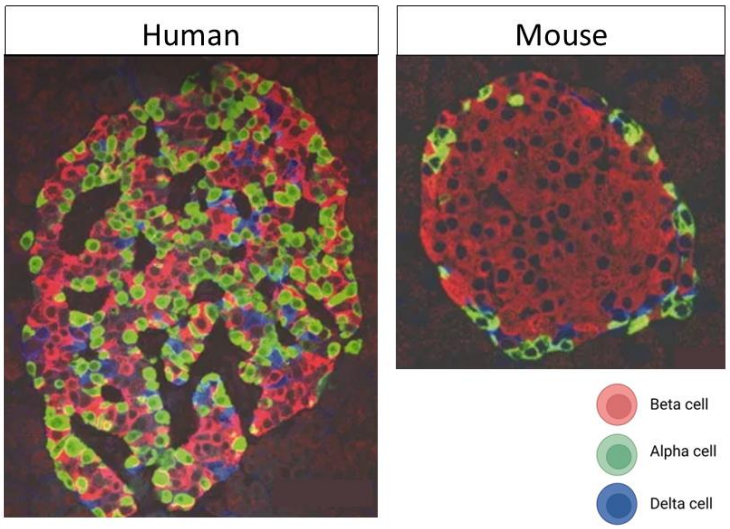


Figure 2 *: Architecture of endocrine islets in human and mouse*

Immunostaining of human and mouse pancreatic endocrine islet, showing different distribution and composition of endocrine cells. β cells are labelled in red, α cells in green and δ cells blue. Adapted from (Cabrera et al. 2006)

1.3.Glycemia regulation

The glucose is the principal source of energy of the cell. Therefore, it is critical to maintain the blood glucose concentration, or glycemia, at the homeostatic level *i.e.* around 90mg/dL in human. A sufficient amount of glucose should be available for the correct function of the cells but an excessive concentration of glucose leads to glucose toxicity. The glycemia is mainly regulated by the insulin-producing β cells and the glucagon-producing α cells (**Figure 3**).

When the concentration of blood glucose increases postprandial, up to 140mg/dL in healthy patients, the glucose enters into the β cells through the low affinity glucose transporter GLUT2 (**Figure 4**). Inside the β cell, glucose is metabolized into ATP. The increased ratio of ATP/ADP will trigger the closing of the K^{+}_{ATP} channels, leading to membrane depolarisation. This electric signal induces the opening of Ca^{2+} channels. The resulting high concentration of Ca^{2+} inside the β cell enables the exocytosis of the insulin vesicles and results in the secretion of insulin into the bloodstream. The insulin is an hypoglycaemic hormone, acting on the peripheral tissues (skeletal muscles, liver, adipose tissue, etc.) to induce the glucose entry inside the cell, to stimulate glycogen and fat formation and to inhibit neoglucogenesis, resulting in a decrease of the glycemia. On the opposite, in case of a low glycemia, the glucose will still be able to enter inside the α cells through high affinity glucose transporter resulting in membrane depolarization

and glucagon secretion. The glucagon hormone triggers the breakdown of the glycogen stored in the liver into glucose, leading to an elevation of the glycemia.

While insulin and glucagon are master regulators of the glycemia, they are not the only hormones or signals controlling this process. Other neural signals or hormones, as the somatostatin produced by the δ cells, can modulate the glycemia by acting on the α and β cells. Dysregulation of this dynamic process, leading to chronic hyperglycaemia, is one characteristic of a metabolic disease, diabetes mellitus.

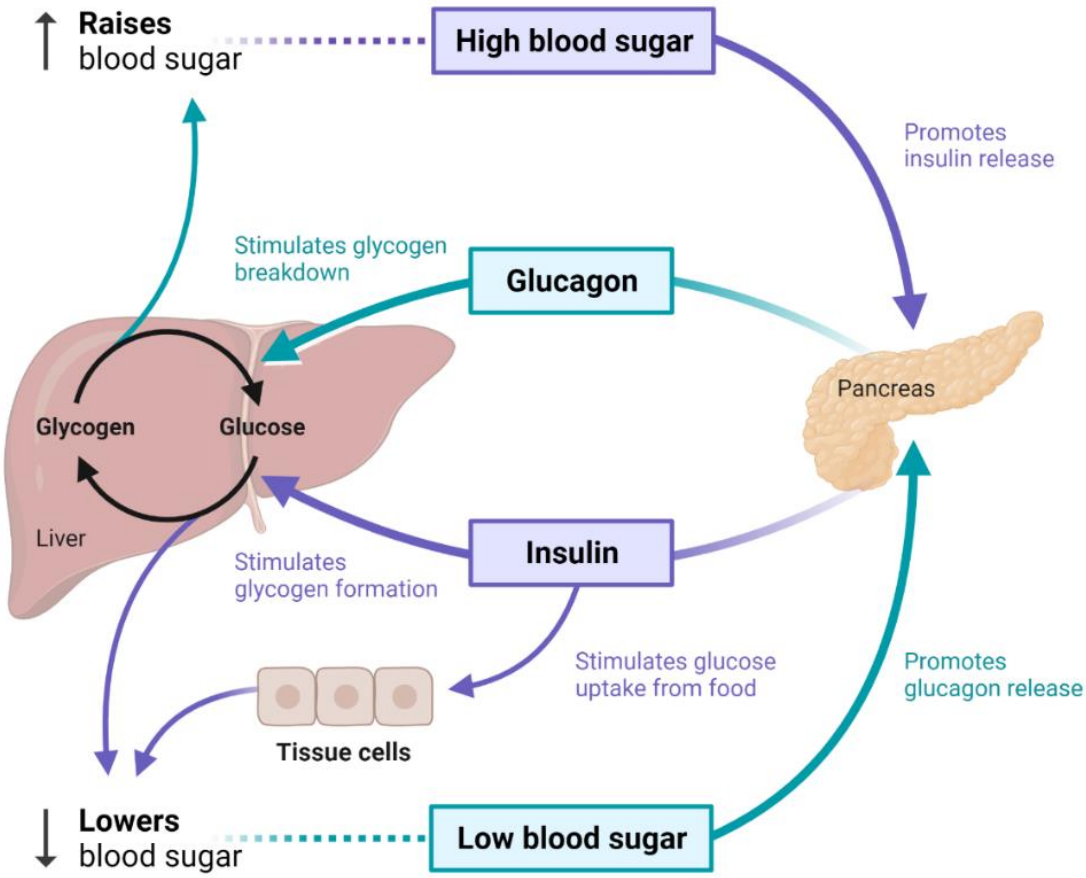


Figure 3 *: Regulation of the glycemia*

The level of glucose in the blood, also known as glycemia, is primarily regulated by two pancreatic hormones that have opposing effects: insulin and glucagon. When the glycemia is high, the pancreas secretes insulin. Insulin promotes glucose uptake and storage in the liver, muscle, and fat tissues, to lower the blood glucose level and maintains it within a normal range. On the other hand, when the glycemia is low, the pancreas secretes glucagon. Glucagon triggers the breakdown of glycogen stored in the liver to release glucose into the bloodstream and raises the blood glucose level.

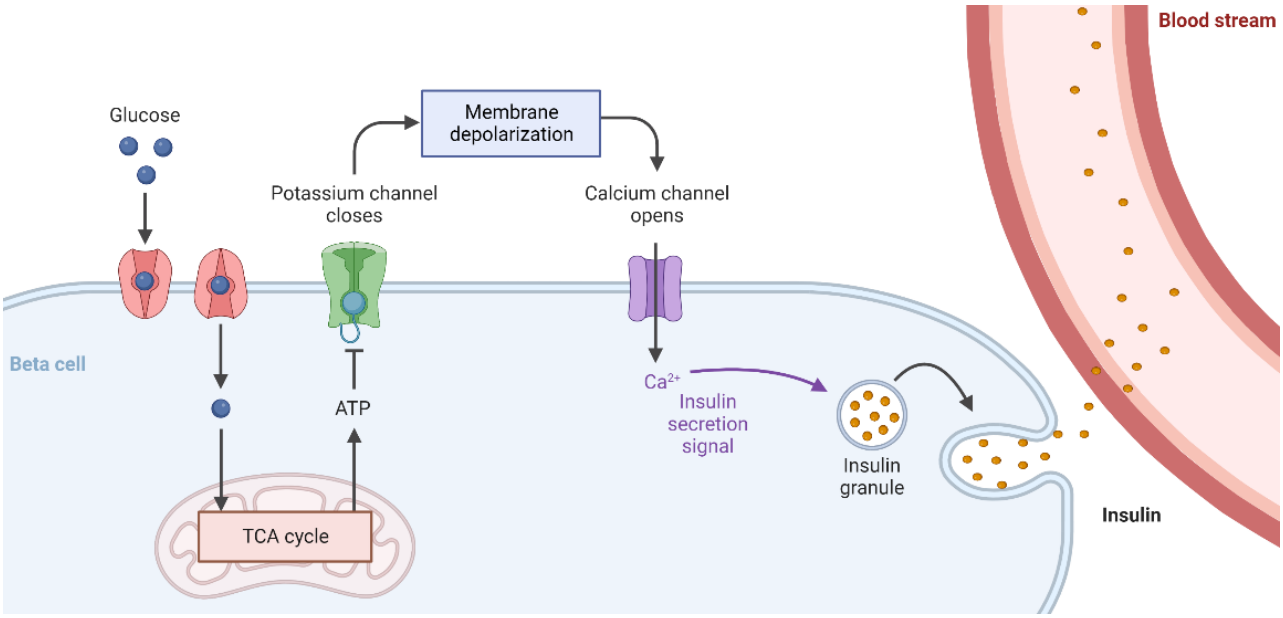


Figure 4 *: Insulin secretion*

Glucose enters the β cell through the glucose receptor GLUT2 and is then metabolized by the TCA cycle to produce ATP. When glucose concentration is high, ATP production increases, leading to a shift in the ADP/ATP balance. This shift results in the closure of the K_{ATP} channel, which increases the concentration of K^+ ions inside the β cell. As a result of the subsequent membrane depolarization, voltage-dependent calcium channels open, allowing Ca^{2+} ions to enter the cell. Finally, the entry of Ca^{2+} ions triggers the exocytosis of insulin vesicles, leading to the release of insulin into the bloodstream.

1.4.A zoom on β cells and insulin signalling

The pancreatic β cells are highly specialized cells, which can be described as cell factories for insulin production. The discovery of the insulin hormone by Frederick Banting and Charles Best in 1921 marked a pivotal moment in the history of medicine. This hormone is a peptide of 51 amino acids and 5.8 kDa in human (Weiss, Steiner, and Philipson 2014). The gene *INS* is transcribed as preproinsulin, which after processing through the endoplasmic reticulum and the Golgi apparatus, results in a mature form of 2 peptide chains attached by disulfide bonds, the insulin, and a peptide called the C-peptide. After maturation, the insulin is stocked into cytosolic vesicles upon secretion (Weiss, Steiner, and Philipson 2014). Of note, the C-peptide is often used as a biomarker of β cell function.

Once the insulin hormone is secreted, it can bind the transmembrane insulin receptor (IR) (**Figure 5**). This receptor is part of the family of tyrosine kinase receptors, whose activation initiates several signalling pathways and thus has several cellular effects. Thereby, insulin signalling not only triggers glucose uptake but activates as well metabolic and growth pathways.

Insulin binding to IR causes its auto-phosphorylation, subsequently leading to the Insulin Receptor Substrates 1 and 2 (IRS) phosphorylation inside the target cell. These phosphorylated forms of IRS initiate the two major branches of the insulin pathway : the PI3K (phosphoinositol 3-kinase)/Akt signalling and the MAPK (mitogen activated protein kinase) signalling.

Upon activation of PI3K by IRS1, PI3K phosphorylates PIP2 (phosphatidylinositol 4,5-bisphosphate) to generate PIP3 (phosphatidylinositol 3,4,5-triphosphate). PIP3 then recruits PDK1 (3-phosphoinositide dependent protein kinase-1) and its phosphorylated form activates the protein kinase Akt (also called PKB). Akt is a major mediator in cell signalling and is able to activate several pathways. In this case, Akt is responsible for the translocation of GLUT4 to the membrane, increasing glucose uptake. In parallel, Akt also activates GS (Glycogen Synthase), leading to glycogen synthesis, as well as mTORC1 and thus subsequent protein synthesis. Akt promotes also cell survival and cell cycle by inhibition of the transcription factors FOXO (Forkhead box O).

Activation of MAPK signalling through the kinases MEK and ERK promotes cell cycle and cellular differentiation.

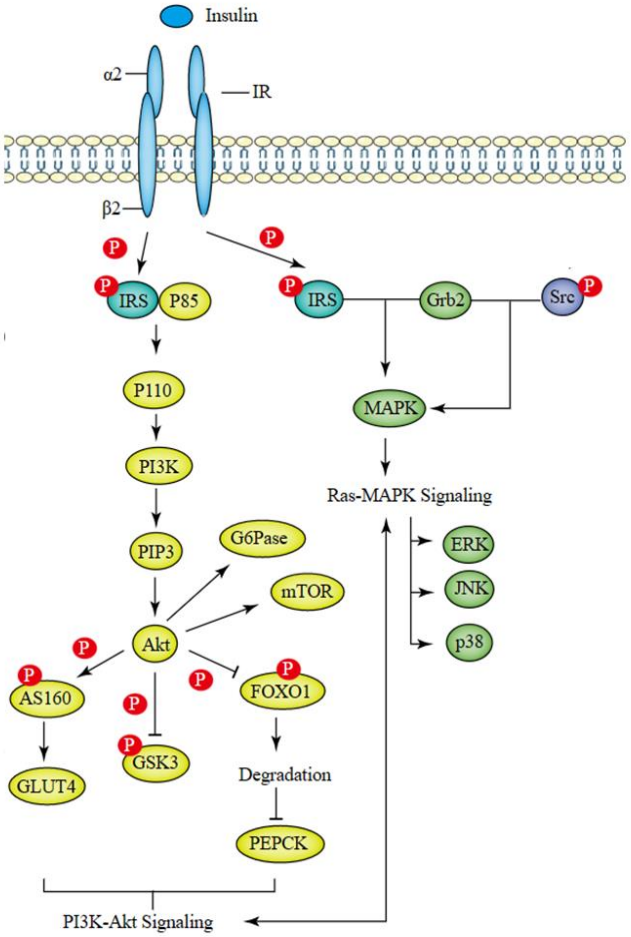


Figure 5 *: Insulin signalling pathway*

Binding of insulin to IR receptor activates PI3K signalling (in yellow), leading to the fusion of GLUT4 vesicles with the cell membrane to enable glucose entry as well as protein and glycogen synthesis. In parallel to PI3K, the insulin activates MAPK signalling (in green), promoting cellular growth and proliferation. Adapted from https://www.creative-diagnostics.com/insulin-signaling-pathway.htm

1.5.A zoom on the α and δ cells

In the same way than β cells are insulin factories, the α and δ cells are specialized in glucagon and somatostatin production, respectively. Glucagon, named for its role as a "glucose agonist", is a peptide hormone of 29 amino acids with a molecular weight of 3.4 kDa. The *GCG* gene is transcribed and translated as preproglucagon, which will mature through the reticulum endoplasmic and Golgi apparatus into the mature form of the glucagon peptide. Of note, the *gcg* gene is not only expressed in pancreatic α cells, but can be also transcribed in the intestine and in the brain where processing of preglucagon leads to the production of the incretin glucagon like peptide 1 (GLP-1) (Rix et al. 2015). The glucagon signalling is mediated by the G protein-coupled receptor glucagon receptor. Its activation by the binding of glucagon subsequently leads to cAMP production, PKA activation that will phosphorylates transcription factors to enable their function. Hence, the main roles of glucagon are stimulation of glycogenolysis (the degradation of glycogen into free glucose) and inhibition of glycogenesis (formation of glycogen) (Rix et al. 2015).

The somatostatin hormone was first isolated from the hypothalamus and it inhibits the secretion of growth hormone (GH). Therefore, the hormone was initially known as ‘GH release-inhibiting hormone’ and then renamed somatostatin to illustrate its general growth-inhibiting effect. Somatostatin is produced in the pancreatic δ cell and in the gastrointestinal tract. The peptide hormone is derived from the maturation of preprosomatostatin that undergoes processing into two somatostatin peptides: SST-28 (28 amino acids) and SST-14 (14 amino acids). The latter is the major form found in the δ cells (Rorsman and Huising 2018). The five different SST receptors are part of the G protein–coupled receptor family, leading to the activation of several signalling pathways. Somatostatin has mainly anti-secretory actions on several cell types as the α and β cells, the pancreatic exocrine cells (Theodoropoulou and Stalla 2013).

2. Diabetes mellitus

2.1.Generalities

The word diabetes roots from ancient Greek “διαβήτης” literally meaning “ passing through”, which describes the principal symptoms of the disease : the polydipsia and the polyuria. From the appearance of these symptoms, the average life expectancy was only of a few weeks.
There are two major groups of disease named after these symptoms ; diabetes insipidus and diabetes mellitus. Diabetes insipidus, named after the non-sweet taste of the urine, result of the dysregulation of the vasopressin hormone. This disease will not be discussed further in this present work.

Diabetes mellitus, named after the sweet taste of the urine, is a metabolic disease characterized by a dysregulation of the glucose metabolism. The term diabetes generally refers to diabetes mellitus in this work. A blood glucose test revealing a glycemia over 200 mg/dL suggest diabetes. In 2021, 537 million adults are affected with this disease worldwide, which account for over 10% of the global population (Sun et al. 2022). According to the WHO, diabetes mellitus is the first non-contagious pandemic around the world (https://www.who.int/news-room/fact-sheets/detail/diabetes).

The disease is divided into sub-groups according to their aetiology. We can cite among them : type 1 diabetes (T1D), type 2 diabetes (T2D), gestational diabetes, LADA and MODY. The common feature between these groups is a chronic hyperglycaemia. The excess of glucose increases the production of ROS by the mitochondria and thus oxidative stress, causing cellular damages. Therefore, the glucotoxicity leads to several micro- and macro- vascular complications associated with diabetes. Micro-vascular complications include retinopathy, nephropathy, neuropathy, which are specific to diabetes. Macro-vascular complications are not specific to diabetes, nevertheless diabetic patients have an increased risk to develop coronary heart disease, cerebrovascular disease or peripheral artery disease (Katsarou et al. 2017).

2.2.Type 1 Diabetes Mellitus (T1D)

Type 1 diabetes (T1D) accounts for approximatively 10% of the all diabetic cases. Previously thought as a childhood disease, 50% of the newly diagnosed patients are actually adults. T1D is an auto-immune disease, meaning that the immune system attacks the β cells, resulting in loss of functional β cells and thus insulin deficiency. Auto-antibodies targeting β cells, such as anti-insulin, anti-glutamate decarboxylase (GAD65), or anti-zinc transporter 8 (ZNT8), are detected in majority of patients (Ilonen et al. 2013; Ziegler et al. 2013). In the early stages of T1D, while auto-antibodies are present, there is no insulin deficiency yet. Therefore, the symptoms appear at the later stage, when the β-cell mass has already collapsed leading to a late diagnosis.

Although the exact aetiology is not yet fully understood, T1D is strongly associated with predisposing genetic components. The major proportion of the genetic risks are found within the variants of the HLA-II (Human Leucocyte Antigen Class II) haplotype, implicated in antigen presentation. Both HLA DR4-DQ8 and DR3-DQ2 haplotypes predispose in developing auto-antibodies (DiMeglio, Evans-Molina, and Oram 2018). In addition, Genome Wide Association Studies (GWAS) linked T1D with non-HLA variants, which are mostly associated with the immune system (Pociot and Lernmark 2016). Still, the penetrance of these genetic factors is quite low and they are not sufficient to trigger the disease. Environmental factors as non-healthy lifestyle, maternal factors, viral infections or gut microbiota are associated with islet autoimmunity or diabetes (Norris, Johnson, and Stene 2020 for review). T1D is actually a multi-factorial disease and it is the combination of events that triggers the appearance of auto-antibodies.

2.3.Type 2 Diabetes Mellitus (T2D)

Type 2 Diabetes (T2D) accounts for about 90% of the patients diagnosed with diabetes (Scully 2012). While the pathophysiology remains unclear, the main characteristics of T2D are peripheral insulin resistance (Roden and Shulman 2019) and β cell dysfunction (Cohrs et al. 2020). Due to insulin resistance, peripheral organs such as the muscles, the adipose tissue or the liver are not able to sense properly the insulin. Therefore, the glucose cannot enter inside the cells resulting into hyperglycaemia. At first, insulin secretion is stimulated to balance the chronic hyperglycaemia. B cell mass and insulin production transiently increase. In

combination with β cell dysfunction, the increasing demand of insulin causes exhaustion of the β cells, leading to their dedifferentiation or apoptosis (A. E. Butler et al. 2003).

T2D is a multifactorial disease involving environmental factors and genetic components. Environmental factors as obesity, sedentary lifestyle or even the composition of the gut microbiota strongly increase the risk to develop insulin resistance and T2D (Galicia-Garcia et al. 2020). Traditionally thought to be mainly an environmental disease, this last decade genetics studies revealed the important contribution of the heritability. First studies identified *INSR, IRS1* and *KCNJ11* locus as linked to the disease (Duggirala et al. 1999). More recently, GWAS studies point out hundreds of genetic variants, which pre-dispose to T2D. Interestingly, most of these variants are found in genes important for β cell physiology such as *CAMK1D, ATP5G1* or *IGF2BP2* (Dupuis et al. 2010; Voight et al. 2011; Xue et al. 2018), underlying that β cell dysfunction is critical in T2D pathophysiology.

2.4.Others forms of diabetes

The most common complication during pregnancy is gestational diabetes. During the gestation, the β cell mass extends to respond to an increased metabolic demand. However, when the adaptive changes of the endocrine pancreas cannot overcome the increasing metabolic demand, it results in insulin resistance and gestational diabetes. Although this condition resolves after birth, the risk to develop insulin resistance, obesity and T2D is multiplied in both mother and child (McIntyre et al. 2019).

Latent Autoimmune Diabetes in Adults (LADA) is a slow progressing autoimmune form of diabetes. Because this form shares characteristics from both T1D and T2D, it is also called type 1.5 diabetes. Notably, patients exhibit the presence of auto-antibodies, they present insulin resistance, and yet retain the ability to produce low amounts of insulin.

Maturity Onset Diabetes of the Young (MODY) is a rare form of diabetes that is strictly monogenic and rely on mutations in critical genes for β cell function and glucose homeostasis. Mutations leading to MODY has been reported in 14 different genes : *HNF4A, GCK, HNF1A, HNF4A, HNF1B, NEUROD1, KLF11, CEL, PAX4, INS, BLK, ABCC8, KCNJ11* and *APPL1*.

3. Treatments of Diabetes Mellitus

3.1. Current therapy

A century ago , the first patient was treated with exogenous insulin injection (Sims et al. 2021). This treatment has been a revolution, considerably prolonging the lifetime of diabetic patients. Since then, the development of recombinant insulin, long lasting vs short acting insulin analogues and insulin pumps considerably enhanced the quality of life of patients (Katsarou et al. 2017). Exogenous insulin injection is the common treatment for T1D and MODY, and can be used in severe cases of T2D. Combined with a healthy lifestyle, it enables the management of the glycemia and reduces the risk to develop the complications associated with diabetes. However, exogenous regulation of the glycaemic physiology is challenging and patients often undergo hyper- and hypo- glycemia episodes.

In T2D patients, an adaptation of their lifestyle is the first suggestion. Weight loss, exercise and healthy lifestyle can improve the management and even possibly reverse the disease. However, medication is often needed to manage the disease and to decrease the risk to develop related complications. Several groups of hypoglycaemic drugs act on insulin secretion or the sensitivity to insulin. Among them, the most commonly used medication is a biguanide, the metformin. It inhibits gluconeogenesis and improves the sensitivity of target cells to insulin. Other molecules have hypoglycaemic effects, as example, sulfonylureas and meglitinides improve the glycemia by stimulation of insulin secretion (Marín-Peñalver et al. 2016). These last years, incretins as Glucagon Like Peptide 1 (GLP-1) receptor agonist are more and more widely used in T2D patients. These drugs help to reduce the glycemia by increasing insulin secretion and by lengthening the emptying of the stomach, reducing calories intake (Nauck et al. 2021).

While all these medications improve the regulation of the glycemia and manage diabetes, these are palliatives treatments. With the exception of type 2 diabetes (T2D), which can potentially be reversed through significant weight loss and the adoption of a healthy lifestyle, diabetes remains an incurable disease nowadays. A strategy to cure the disease would be to replace the β-cells. For some patients, all treatments listed above, including exogenous insulin injections are not sufficient for a proper glycaemic control. In these extreme cases, pancreas or islet cell transplantation from cadaveric donor can be performed based on the Edmonton protocol (Shapiro et al. 2000, 2006). Whereas the transplanted patients do not rely on insulin injections for a few years, the procedure is very rare due the need of immunosuppressive drugs and the

lack of donors. A healthy human pancreas typically contains around 10 billion β cells (Bonner-Weir, Sullivan, and Weir 2015), necessitating the transplantation of a significant number of islets and as a consequence, often requiring the purification of β cells from multiple donors. Thereby, there is undeniably a need for an alternative and reliable source of β cells.

3.2. Future therapies

In parallel to the research to constantly improve current diabetic treatments, several strategies are explored to restore glucose homeostasis and thereby cure diabetes. For autoimmune diabetes, the ideal approach would be to tackle the immune system to avoid β cell destruction. This could be achieve by immunotherapy, for instance by modulation of T-cells or inhibition of auto-antigens. Whereas some of these therapies could delay β cell destruction, patients still need insulin injections and improvement of immunotherapies are still under investigation (Ni et al. 2019, for review).

Nonetheless, diabetes is often diagnosed at a late stage of the disease, when the β cell mass has already collapsed. Then, β cell replacement is required. Stem cells represent an attractive source, theoretically unlimited, of cells. The first insulin expressing cells were produced *in vitro* from human embryonic stem cells (hESCs) by d'Amour and colleagues (D'Amour et al. 2006). The five-steps protocol is based on *in vivo* developmental differentiating stages of endocrine cells (D'Amour et al. 2006). A slight modification in the protocol followed by transplantation in mice, allowed for the cells to secrete insulin in response to glucose (Kroon et al. 2008) (**Figure 6**). Importantly, these insulin expressing cells could decrease the hyperglycaemia in the streptozotocin diabetic model (see section 4.4) in mice (Kroon et al. 2008). Based on those two initials studies, many groups contribute on improving protocols to produce β cell-like *in vitro* (H. Liu et al. 2021; Pagliuca et al. 2014; Rezania et al. 2014; Velazco-Cruz et al. 2019). Another significant advancement has been achieved through the utilization of induced pluripotent stem cells (iPSCs). In addition to alleviate ethical concerns, the use of pluripotent cells offers the potential to produce β-cell-like cells derived directly from somatic cells of patients (Millman et al. 2016), avoiding rejection of the transplanted cells. Furthermore, encapsulation devices to protect the engrafted cells from the immune system and to enable vascularisation of the islets to sense glucose and secrete hormones are currently developed (Shapiro et al. 2021). Although preliminary results of clinical trial are encouraging, as C-peptide could be detected in diabetic patients (Shapiro et al. 2021), stem cell therapy still face up limitations and challenges. A considerable limitation is the heterogeneity (Veres et al. 2019) of

the derived cells. Some cells are indeed not fully mature, leading to less metabolically functional cells that have tendency to form teratomas. Han and colleagues recently reported a case of aggressive teratoma in patient transplanted with β cell-like cells derived from hiPs (Han et al. 2022). In conclusion, substantial progress has been made in the field of stem cell therapy for diabetes. While more research is needed to face these challenges, this approach is promising.

Another attractive strategy to replace the β cells in diabetic patients, by-passing *in vitro* manipulations, would be to stimulate β cell neogenesis *in situ*. Combined with the inhibition of their destruction, regeneration of β cells represents an effective option to cure diabetes. β cell regeneration is the main focus of this present work.

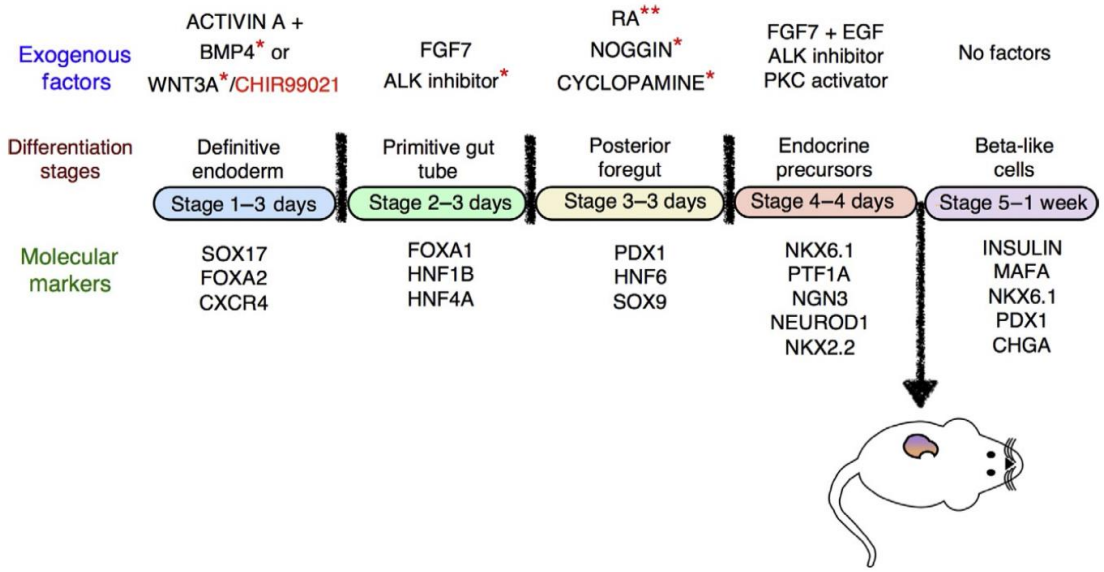


Figure 6 *: In vitro stem cell differentiation towards β cell like* From (Kinkel and Prince 2009).

Overview of stem cell differentiation protocols towards insulin producing cell in five stages, showing the exogenous factors added to media, to recapitulate the steps of β cell differentiation with the molecular markers used to assess the progression of differentiation.

4. β cell regeneration in mammals

4.1.Generalities

"Regeneration is defined as the ability to regrow an organ or a tissue destroyed by degeneration or injury. Humankind has been fascinated with regeneration abilities since the times of the Ancient Greece. In Greek mythology, one of the labours of Hercules was to kill the Hydra, which is able to regrow two heads when one is ablated. In another myth, Prometheus' liver is renewed every night. However, it was only in the late seventeenth century that scholars paid formal attention to regeneration. Abraham Trembley became a pioneer in this field with his work on fresh water polyps. He described that after cutting a polyp in pieces, each of them was able to regrow an entire organism. He named the polyp "hydra" for its regenerative capacities. After that, regenerative biology had a major influence in the history of biological sciences as it contributed to legitimize biology as an experimental discipline rather than a descriptive science. In recent decades, new technologies such as imaging, genetic engineering and stem cells have enabled the development of regenerative biology, which laid the foundation of a new branch of medicine, *i.e.*, regenerative medicine. Many human diseases and pathologies such as diabetes, Alzheimer's disease, blindness, heart failure or spine injuries, today incurable, could be cured if functional tissues or cells could be restored by regeneration." (Extract from (Massoz, Dupont, and Manfroid 2021), see the complete review in supplemental).

Despite humans having relatively limited regenerative capacities compared to other organisms, some tissues and organs demonstrate the ability to regenerate under specific circumstances. This includes both regeneration during normal homeostasis, which ensures continuous tissue maintenance and renewal, as well as regeneration upon injury to repair or restore damaged tissues. Examples of such regenerative tissues in human include the epidermis, intestine, and liver, even in adulthood. Regenerative mechanisms encompass several cellular mechanisms such as the proliferation of remaining cells, cellular reprogramming or trans-differentiation, and the neogenesis of new cells from stem cells or progenitors, underlying re-activation of developmental processes.

4.2.Pancreas development : a focus on β cell neogenesis

To decipher the regenerative mechanisms of β cells, it is relevant to understand the development of the pancreas and more specifically the events leading to β cell neogenesis. Here is a short review on pancreas embryogenesis, focusing on β cell formation.

By the end of the gastrulation, the three embryonic layers -the endoderm, the mesoderm and the ectoderm- are established. The pancreas organogenesis results from the evagination of a ventral and a dorsal bud from the foregut endoderm, occurring around embryonic day 9 (E9) in mice, which will fuse to form the future pancreas at E11.5 (**Figure 7**). The patterning and the specification of the pancreas is governed by many molecules signal from the surrounding tissues *i.e.* the meso- and ectoderm and the notochord such as FGF10 (Fibroblast Growth Factor), BMP7 (Bone Morphogenic Protein) or RA (Retinoic acid). Combined with regulation from signalling pathways, for example Notch signalling, which control the expression of several transcription factors, these signals orchestrate the development of the mature pancreas from the primitive endoderm (Pan and Wright 2011; Stanger and Hebrok 2013).

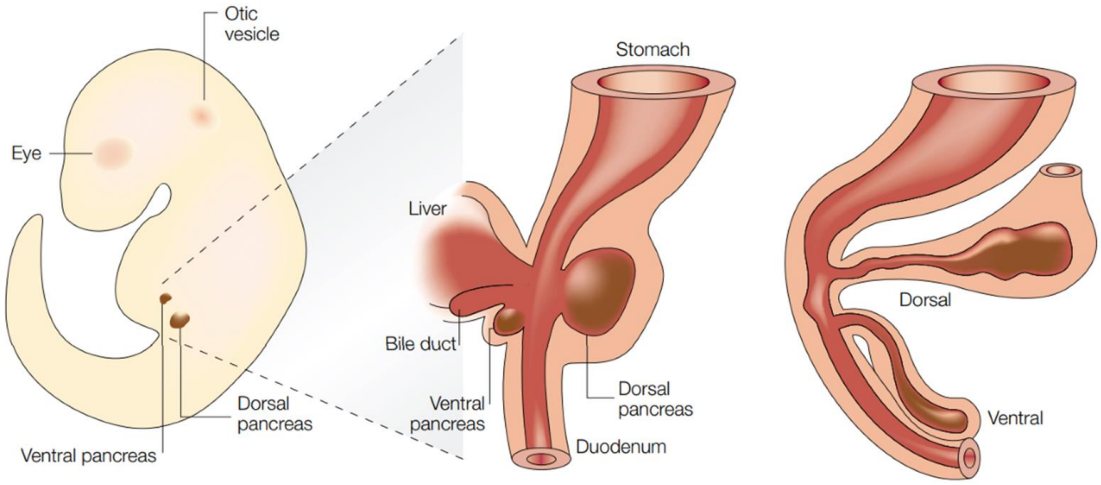


Figure 7 *: Pancreas organogenesis in mouse.* Adapted from (Edlund 2002).

Formation of the pancreatic ventral and dorsal buds, which will fuse to form the whole pancreas in the mouse embryo.

The pancreatic buds contains proliferating multipotent progenitors cells (MPCs), which will give rise to all the endocrine and exocrine cells. These MPCs express the transcription factor *PDX1* (Pancreatic Duodenal Homeobox 1), considered as the first pancreatic marker (Offield et al. 1996). The endocrine cells are generated in two waves in mice, during the first and the second transition. This first wave consists of an early differentiation of endocrine cells, mainly α cells, which can be detected even before reorganization of the dorsal bulk of MPCs, the luminogenesis. This process will lead to the formation of the pancreatic plexus in shape of tubular network structures (L. Flasse, Schewin, and Grapin-Botton 2021). Progenitors cells are organised in a branched epithelium, which is segregated into trunk and tip regions (**Figure 8**). Tip regions will be committed to acinar cells fate. These tips cells are characterised by the expression of *Pdx1*, *Ptf1a* (Pancreatic Transcription Factor 1a) and *Cpa1* (Carboxipeptidase 1a) (Stanger and Hebrok 2013). Multipotent progenitors also express others transcription factors as *Nkx6.1* (homeobox protein NK-homolog 6.1), *Sox9* (SRY-Box9) or *Hnf1b* (Hepatocyte Nuclear Factor 1-β). These transcription factors are determinant for the progenitors and their specification. For example, SOX9 maintains the pool of pancreatic progenitor cells (Seymour et al. 2007). Trunk regions were initially referred as primitive ducts and consist of bi-potent progenitors, giving rise either to endocrine or ductal cells. The second transition consists of massive formation of acinar, endocrine and ductal cells from the pancreatic epithelium (Pan and Wright 2011; Stanger and Hebrok 2013).

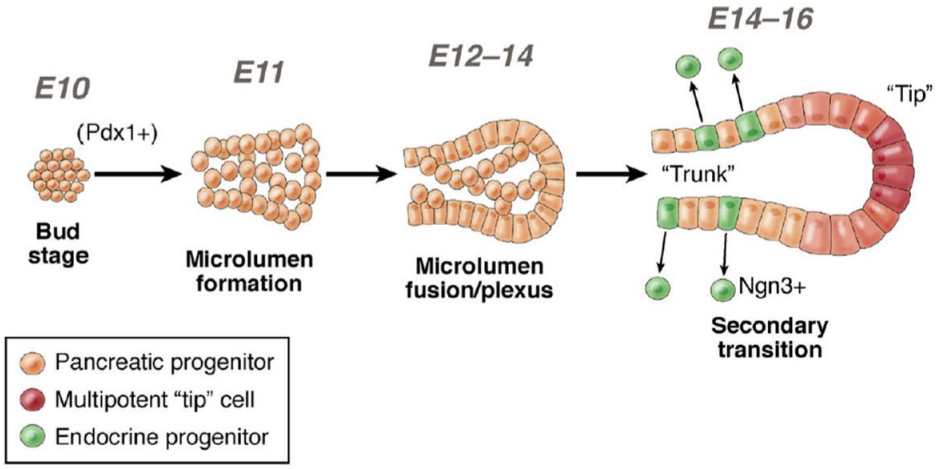


Figure 8 : *Pancreas morphogenesis : formation of plexus with trunks and tips regions* (Stanger and Hebrok 2013)

Pancreatic progenitors PDX1+ organize to form microlumens, which then evolve into a plexus. By E14-16, the tip of this plexus comprises multipotent progenitors, which subsequently undergo commitment to acinar cells. The trunks of the plexus consist of bi-potent progenitor cells. The fate of these cells is determined by the expression of *Ngn3*. Those expressing *Ngn3* are committed to the endocrine fate and undergo delamination to form islets. The remaining cells within the plexus form the ductal tree.

NEUROG3 (Neurogenin3) is the key transcription factor in endocrine differentiation in mammals (Schwitzgebel et al. 2000). It initiates the cascade of transcription factors such as *Neurod1*, *Pax4* or *Isl1*, leading to endocrine differentiation. NEUROG3 is part of the ARP/ASCL (Atonal Related Proteins/ Achaete Scute-Like) family. These transcription factors contain a basic Helix-Loop-Helix (bHLH) domain, responsible for dimerization of the protein and for DNA biding. Their expression is repressed by transcriptional targets of Notch pathway, the Hairy and enhancer of Split (HES) family. Fundamentally, Notch signalling repress the endocrine program notably in bi-potent progenitors of the trunk regions. Down-regulation of Notch signalling in bi-potent progenitors enable an increased expression of *Neurog3*, and thus endocrine differentiation.

The specification towards a particular endocrine cell type occurs at the level of the progenitor and is dependent of a permissive window during the embryogenesis. *Neurog3* expression indeed drives progenitor differentiation into the different endocrine lineage depending on its timing of activation. While the early activation of *Neurog3* (around E9) leads to α cell differentiation, its later expression induces the second wave of endocrine differentiation, including α, β and δ cells around E13.5 and E15.5. Several transcription factors are involved in cellular differentiation. *Pax4* expression is necessary for β and δ cell differentiation while *Arx* leads towards α cell fate. *Nkx6.1* is express in the MPCs, its expression is then restricted to β cells in adult and is essential for both β cell differentiation and function. A scheme of transcription factors involved in pancreatic cells differentiation is displayed at **Figure 9**. During late differentiation, the endocrine progenitors delaminate from the trunk regions. Differentiated endocrine cells then proliferate to form islets.

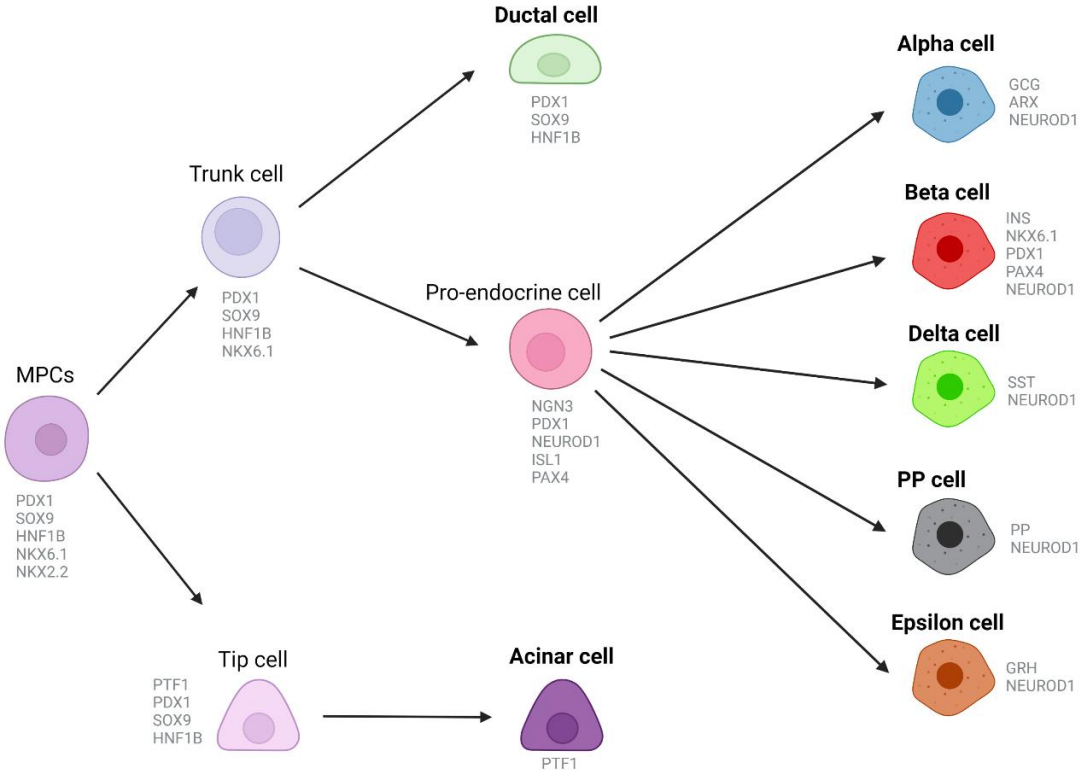


Figure 9 *: Summary of key transcription factors involved in pancreatic cell differentiation in mammal*

Multipotent pancreatic Cells (MPCs) PDX1+ will either be committed to tip cells PTF1+ giving rise to acinar cells. Trunks cells are characterised by SOX9, HNF1B and NKX6.1 expression. NGN3+ cells are committed to endocrine fate whereas they loose SOX9. Remaining SOX9+ cells give rise to ductal cells.

4.3.Evidences for intrinsic capacity of β cell regeneration in human

Although the human pancreatic β cells mass do not spontaneously regrow after a loss, the exploration of a regenerative potential brings some promising clues supporting this capacity.

In exceptional conditions, as during pregnancy or in obesity, the β cell mass can increase to respond to a higher metabolic demand (Bouwens and Rooman 2005). This observation was surprising since β cell proliferation occurs only at a slow rate during the childhood and in the healthy adult, the β cell mass is stable and no β cell proliferation nor neogenesis is observed. Nevertheless, the fact that the β cell mass can increase when the metabolic demand is higher could underline a presumptive capacity for β cell proliferation or neogenesis in the adult (Bouwens and Rooman 2005).

Another promising observation supporting the regenerative capacity, is the detection of few insulin+ cells or C-peptide in long-lasting T1D patients. Given the strong autoimmune attack destroying the β cells, it is unlikely that they elude the immune system (Meier et al. 2005). Therefore, the presence of few β cells in these patients could be explained by an attempt of regeneration. Supporting this hypothesis, insulin+ cells have been found associated with the pancreatic ducts in pancreas-transplanted T1D patients showing autoimmune reaction (Pagola et al. 2008). This fact raises the point that β cell neogenesis could occur from the ductal network as during the development of the pancreas. Moreover, another study pointed out that insulin+ cells near the ductal tree also express the pancreatic progenitor cell markers *PDX1* and *NEUROG3* in T2D patients (Yoneda et al. 2013). The authors claim that β cell neogenesis is the major process to compensate β cell loss rather than proliferation (Yoneda et al. 2013). Interestingly, some pancreas of T2D patients contain polyhormonal cells (Cinti et al. 2016; Yoneda et al. 2013). It has been suggested that they could either originate from β cells that would de-differentiate in diabetic conditions or they could result from trans-differentiation of other endocrine cells, as an attempt to produce insulin (Moin and Butler 2019).

Altogether, these observations raise the possibility of a putative capacity of regeneration that could be stimulated to regrow the β cell mass in human.

4.4.Murine models of pancreas injury

In order to study mechanisms or presumptive β cell regeneration, several murine models of diabetes or pancreas injury have been developed. One commonly used genetic model is the Non Obese Diabetic (NOD) mice, which spontaneously develop T1D. The mechanisms of β cell destruction are similar to human diabetic patients, since it involves a T-cell mediated response and auto-antibodies (as auto-INS, auto-GAD and auto-Zn8) (Serreze and Leiter 1994). Another important model is the Akita mice, which also spontaneously show T1D characteristics. β cell failure is due to a mutation within the gene coding for insulin, causing its misfolding and subsequent ER stress, ultimately leading to β cell death (Oyadomari, Araki, and Mori 2002). While these genetic models are widely used to study the pathophysiology of T1D, ER stress and islets transplantation, the recurrent destruction of β cells is not ideal to study events of regeneration. Thereby, it was useful to develop models of pancreas injury that would allow regenerative mechanisms. The different models can be classified in surgical, chemical and genetic/chemical pancreas injuries (**Figure 10**).

Surgical methods include Partial Pancreatectomy (Px) and Pancreatic Duct Ligation (PDL). Partial pancreatectomy consists of the resection of 50-80% of the pancreas, resulting in a dramatic loss of all pancreatic cell types, including β cells and causes glucose intolerance (Bonner-Weir et al. 1993). This model showed important regenerative capacities as new pancreatic lobes were formed and showed proliferation of acinar cells and β cells. In addition, an increased proliferation of ductal cells occurs in specific areas, called “proliferating ductules” (Bonner-Weir et al. 1993; Li et al. 2010). The PDL technique involves the ligation of the main pancreatic duct, leading to the accumulation of the pancreatic enzymes and subsequent destruction of the acinar compartment and a strong inflammation in the obstructed part of the pancreas (De Groef et al. 2015; Wang, Klöppel, and Bouwens 1995). Despite the absence of direct β cell destruction or alteration in glucose homeostasis, this model highlights compelling evidence of β cell regeneration (see section 4.5.3.).

Chemicals as alloxan or streptozotocin (STZ) are toxic glucose analogues, which can enter inside β cells via the glucose transporter GLUT2. Inside the β cells, these two toxins inhibit insulin secretion and act as genotoxic agents by producing extra amount of Reactive Oxygen Species (ROS). STZ through its alkylating activity causes DNA strand breaks. Both STZ and alloxan lead to β cell death. Using different concentrations of treatments, the degree of β cells destruction can be controlled. Moreover, several rounds of β cell ablation can be performed by

repeating the treatments. However, since the GLUT2 transporter is also found in hepatic and kidney cells, these substances can be toxic to both the liver and kidneys.

Genetics methods are based on the expression of a suicide transgene specifically in the β cells. For this purpose, the diphteria toxin A (DTA) is widely used. It leads to cell apoptosis by inhibiting protein synthesis. To induce specific β cell destruction, the transgenic RIP-DTR mice that express the diphteria toxin receptor (DTR) under the control of the insulin promoter (RIP), are treated with DTA. The model offers several advantages, including the specificity of β cell destruction and the ability to induce their destruction whenever needed (Herrera et al. 1994).

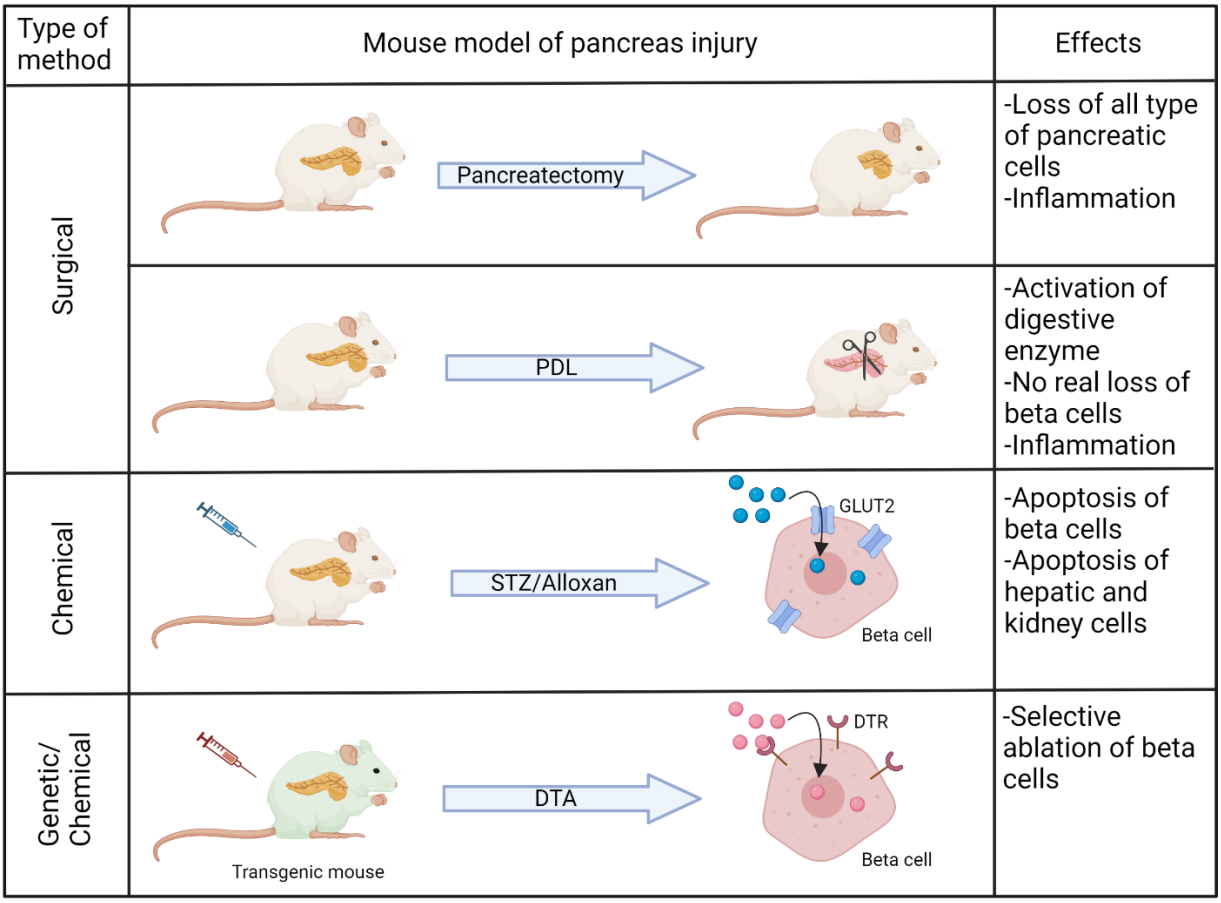


Figure 10 *: Common mouse models of pancreatic injury used to study β cell regeneration*

Various methods to induce β cell regeneration have been developed, each with distinct characteristics. Surgical approaches involve procedures such as pancreatectomy and pancreatic duct ligation (PDL). Chemical methods leverage the presence of GLUT2 on β cells and utilize toxic compounds like Streptozotocin (STZ) or Alloxan, which can enter the cells through these channels. Additionally, genetic/chemical methods utilize the expression of the DTR receptor, allowing for DTA entry upon treatment.

4.5.Pancreatic cellular sources of β cell regeneration

Using the models of pancreas injury outlined in **Figure 10**, several studies identified the contribution of different cellular sources for β cell regeneration within the pancreas (**Figure 11**). Importantly, the source of regeneration seems to depend on the type of injury (Aguayo-Mazzucato and Bonner-Weir 2018), the details are described below. The mechanisms include 1) proliferation of remaining β cells (Dor et al. 2004) ; 2) transdifferentiation of α cells (Thorel, Népote, et al. 2010), δ cells (Chera et al. 2014b), γ cells (Perez-Frances et al. 2021) or acinar cells (Pan et al. 2013) ; 3) neogenesis from putative ductal progenitors (Al-Hasani et al. 2013; Inada et al. 2008; X. Xu et al. 2008).

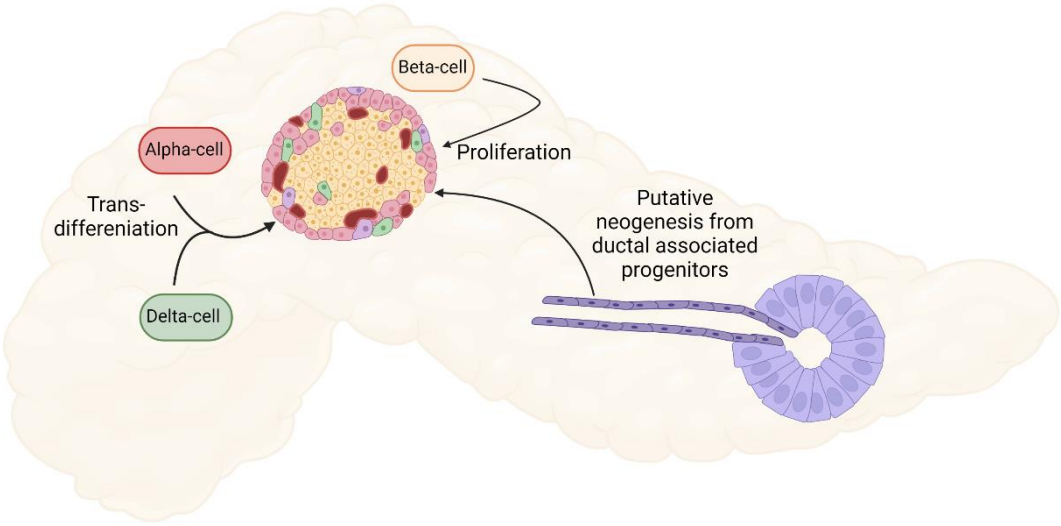


Figure 11 *: Origins of spontaneous β cell regeneration in mouse models of pancreas injury*

Several pancreatic cells show the capacity to spontaneously give rise to new insulin producing β cell in mouse model of pancreas injury. Regenerated β cell can originate from α- or δ- cells trans-differentiation, proliferation of remaining β cells or neogenesis from putative progenitors residing within the ducts.

4.5.1. β cell proliferation

During normal growth, β cell replication is a major process to increase the β cell mass. However the proliferative potential of β cell rapidly decreases after birth and the vast majority of the β cells are quiescent in adult rodent (Teta et al. 2005) as well in human (P. C. Butler et al. 2007). Nonetheless, several studies reported activation of β cell replication in injured pancreas of young rodents, suggesting regeneration could occur via β cell proliferation.

For instance, in response to partial pancreatectomy, young rats showed an increased proportion of incorporation of BrdU in β cells (Bonner-Weir et al. 1993). Consistent with this observation, low doses of STZ is a well-known model that increases β cell proliferation (Teta et al. 2005). In addition, β cell proliferation has been reported in the PDL model (Wang, Klöppel, and Bouwens 1995) and in the DTA system that ablated 70% of β cells (Nir, Melton, and Dor 2007). Importantly, genetic tracing confirmed that new β cells originated from remaining β cells (Dor et al. 2004). Of note, while Dor *et al.* concluded that the major part of the regenerated β cells come from proliferation, others reported neogenesis in addition to proliferating β cells (Van De Casteele et al. 2013; Wang, Klöppel, and Bouwens 1995). Altogether, these studies showed a capacity of β cell regeneration by proliferation in injury models that allow the persistence of a significant number of β cells.

However, these experiments were performed on young rodents. Older individuals (from one year old individuals) did not show proliferation, even after partial pancreatectomy or STZ (Rankin and Kushner 2009), underlying the lower regenerative potential with aging. The understanding of this lost ability of proliferation in older individuals could be a key to enhance regeneration in adults. As example, β cells show an age-related accumulation of cell cycle inhibitors as p16Ink4a, which is related to cellular senescence. Suppression of p16Ink4a could enhance the potential of proliferation in adult mice after STZ treatments (Krishnamurthy et al. 2006). On the other hand, human β cells seem even more refractory to proliferation than murine β cells, increasing the difficulty of translation towards human (Aguayo-Mazzucato and Bonner-Weir 2018). Even though it may be difficult to increase β cell proliferation, it could provide significant benefits for patients who retain β cells (in the first stages of diabetes) or it could be combined with induction of neogenesis, as in embryogenesis to expand the β cell mass.

4.5.2. Transdifferentiation of other pancreatic cell types

Polyhormonal cells expressing insulin and glucagon and/or somatostatin were found in diabetic patients (Cinti et al. 2016; Yoneda et al. 2013), raising the possibility that endocrine cells retain a plasticity enabling them to convert into β cell-like. Herrera's laboratory was the first to demonstrate that α and δ cells can spontaneously reform insulin producing cells by transdifferentiation in the DTA ablation model (Chera et al. 2014b; Thorel, Népote, et al. 2010). Adult mice could regenerate around 10% of their β cell mass by ten months upon near total ablation (Thorel, Népote, et al. 2010). Importantly, the mice needed insulin injections only during the 5 first months to prevent them dying from the acute hyperglycaemia. Lineage tracing experiments showed that the regenerated β cells result from α cell transdifferentiation (Thorel, Népote, et al. 2010). In juvenile mice, β cell regeneration is more efficient since 20% of the β cell mass was reformed upon 4 months after ablation (Chera et al. 2014b). Interestingly, these β cells did not originate from α cells but rather come from the δ cells. These latter de-differentiate and proliferate in response to β cell destruction and finally activate *Neurog3* expression (Chera et al. 2014b). As such, the process of β cell regeneration is age dependent in mice.

Collombat and colleagues showed that the ectopic expression of *Pax4* in α cells (Al-Hasani et al. 2013) or in δ cells (Druelle et al. 2017), triggers their transdifferentiation into β cells in adult mice (Al-Hasani et al. 2013). In the same manner, loss of *Arx* in the α cells results in a β cell like phenotype (Courtney et al. 2013). Importantly, the modified α and δ cells could improve β cell regeneration in response to STZ treatments. More recently, the same group showed that the neurotransmitter GABA (gamma-aminobutyric acid) promote α to β cell transdifferentiation, enhancing β cell regeneration in the STZ mice model (Ben-Othman et al. 2017).

4.5.3. Neogenesis from the ducts

Another possible source of β cells could be *de novo* formation from pancreatic progenitors, thought to reside in the ductal compartment of the pancreas in adult. The hypothesis of the persistence of pancreatic progenitors in the ducts partially stems from our comprehension of endocrine cell differentiation during embryonic development, when endocrine cells arise from bi-potent progenitor domains within the ductal tree. Furthermore, ductal cells maintain the expression of some progenitor markers such as *Sox9* (Seymour et al. 2007) or *Hnf1b* (Solar et

al. 2009a). Still in support of this hypothesis, some diabetic patients exhibit elevated ductal cell proliferation and the presence of adjacent ins+ cells (see above 4.3). The progenitor capacity of the ducts was investigated in *in vitro* experiments. Primary cultures of ductal cell demonstrated their capacity to give rise to endocrine cells (Bonner-Weir et al. 2000). The rest of this section is dedicated to *in vivo* experiments.

Lineage tracing experiments have not yet provided conclusive evidence for the generation of new β cells from the ductal cells in adult. In a study conducted by Inada and colleagues, transgenic mice expressing Cre driven by the human Carbonic Anhydrase II (CAII) promoter, which is known to be active in the ductal cells, were used to investigate the origin of new β cells in pancreatic ductal ligation (PDL) (Inada et al. 2008). The study showed that in this model, the CAII+ cells have the capacity to differentiate into β cells (Inada et al. 2008). In contrast, two other studies using the promoters of *Sox9* (Kopp et al. 2011) or *Hnf1b* (Solar et al. 2009a) as drivers after PDL or alloxan-induced ablation, failed to replicate these findings. In line with this, a recent study aimed at tracing β cells and non-β cells (including others endocrine cells and ducts) after different types of pancreas injuries (Zhao et al. 2021). They showed that new β cells come from remaining β cells rather than from neogenesis after PDL, partial pancreatectomy or STZ (Zhao et al. 2021). In contrast to Inada study, these three studies therefore concluded that ducts are not the main source of β cell in the adult. These differences could be explained by the choice of the driver, their mosaicist expression or the heterogeneity of ductal cells. Nonetheless, even in cases where neogenesis was not observed, reactivation of the pro-endocrine marker NEUROG3 has been detected in the ducts (Kopp et al. 2011; Solar et al. 2009a), indicating a response from the ductal compartment to pancreas injury. The detection of *Neurog3* expression is relevant since a small population of ductal cells that express *Neurog3* has been shown to participate in β cell neogenesis in cases of diabetes (Van De Casteele et al. 2013).

The importance of ductal cells in maintaining endocrine cell mass has been underscored in the series of papers by Collombat and colleagues, which focus on the transdifferentiation of α and δ cells (Al-Hasani et al. 2013; Ben-Othman et al. 2017; Courtney et al. 2013; Druelle et al. 2017). In their models that induce α/δ cell transdifferentiation (see section 4.5.2) there is a need to compensate the lack of the endocrine cells. As a consequence, ductal cells enter in proliferation, re-express *Neurog3* and give rise to new α/δ cells (Al-Hasani et al. 2013; Druelle et al. 2017), as shown for α cells in lineage tracing experiments using the *Hnf1b* driver (Al-Hasani et al. 2013). As such, ductal cells give rise to β cells through an intermediate α cell stage.

Importantly, these findings emphasize that, even in cases of transdifferentiation, progenitor cells are needed to maintain the overall endocrine cell population.

In recent years, a population of human ductal cells ALK3^{+}/PDX1^{+}/CAII^{-} has been proposed as pancreatic progenitors (Qadir et al. 2018). Of note, ALK3 is used as a surrogate marker for the receptor BMPR1A and this population excludes CAII+ cells, in contrast to (Inada et al. 2008) study. To isolate these cells, the authors used the P2RY1+ as surrogate marker for PDX1. Single-cell RNA sequencing data of this ALK3^{+}/PDX1^{+}/CAII^{-} ductal population further supported their progenitor identity (Qadir et al. 2020). Furthermore, when these human cells were implanted into the kidneys of immunodeficient mice, they spontaneously differentiated into all the exocrine and endocrine pancreatic cell types (Qadir et al. 2020), underlying their multipotency. Interestingly, some ALK3+/PDX1+ cells have also been found in the pancreas of diabetic patients (Qadir et al. 2020). These studies provide strong and convincing evidences for the existence of pancreatic progenitor cells within the ducts.

Collectively, these studies indicate that ductal cells possess the intrinsic ability to differentiate into all pancreatic cell types, even in adulthood. However, this capacity appears to be repressed as neogenesis does not spontaneously occur. Conversely, some studies reported plasticity of endocrine cells, as illustrated by their trans-differentiation to produce insulin. Even in these cases, the spontaneous regeneration was slow and limited. Hopefully, targeting the regenerative mechanisms holds the potential to stimulate these processes, leading to the efficient generation of new β cells and potentially reversing diabetes.

Nevertheless, deciphering regenerative mechanisms in models that exhibit limited regenerative capacities is challenging. Therefore, studying regenerative model organisms that possess robust regenerative abilities, such as the zebrafish, can be highly advantageous for unravelling the intricate mechanisms of regeneration. The zebrafish is widely recognized as a powerful model for studying tissue regeneration.

5. Zebrafish

5.1.Generalities

The zebrafish (*Danio rerio*) is a small freshwater fish native to South Asia and a member of the Cyprinidae family within the Cypriniformes order. The zebrafish gets its name from the blue stripes that adorn its body (**Figure 12**).

In the 1960s, biologist Georges Streisinger was a pioneer in introducing and breeding zebrafish in the laboratory (Marinaga 1990; Streisinger et al. 1981). The zebrafish quickly became a valuable model organism for studying vertebrate development due to its external embryonic development and the transparency of the embryos (Kimmel 1989). Its success is also due to its small size (3 to 4 cm), rendering it easier and less expensive to house and breed compared to mammals. Additionally, the zebrafish has a rapid development, with organogenesis complete by six days post-fertilization (6 dpf) after only three days of embryonic development (**Figure 13**). Zebrafish are capable of breeding after only three months and produce numerous offspring, further contributing to their usefulness as a model organism.

Despite that the zebrafish genome has been duplicated, resulting in several orthologs for some human genes, the complete sequencing of its genome revealed important similarities with the human genome (Howe et al. 2013). 71.4% of the human genes indeed possess at least one ortholog in the zebrafish genome. This proportion is even higher for the disease-linked genes, whose 82% can be related to at least one zebrafish orthologue (Howe et al. 2013). Over the past decade, advancements in technologies like CRISPR/Cas9 and TALENs for creating mutants and transgenic lines (Choe et al. 2021), high-resolution microscopy, and high-throughput drug screenings, faster and easier in zebrafish, contributed to revolutionize biomedical research.

One of the key advantages of the zebrafish model is its remarkable regenerative abilities, which enable it to rapidly and efficiently regrow various tissues including fins, retina, heart and pancreatic β cells, following injury (Gemberling et al. 2013). This feature has quickly established it as a powerful model for studying complex tissue regeneration (Marques, Lupi, and Mercader 2019).

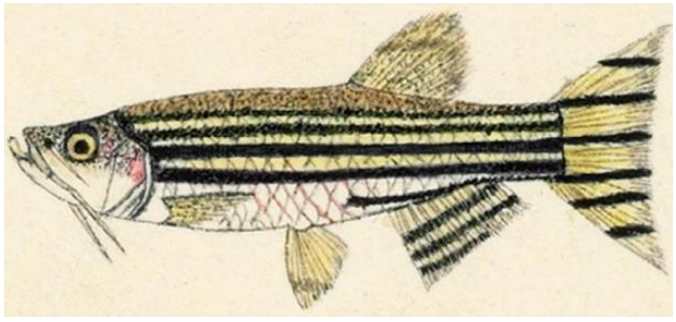


Figure 12 *: Drawing of a zebrafish by Francis Hamilton, the first scientist who described the zebrafish in 1822. The Trustees of the Natural History Museum, London, United Kingdom*

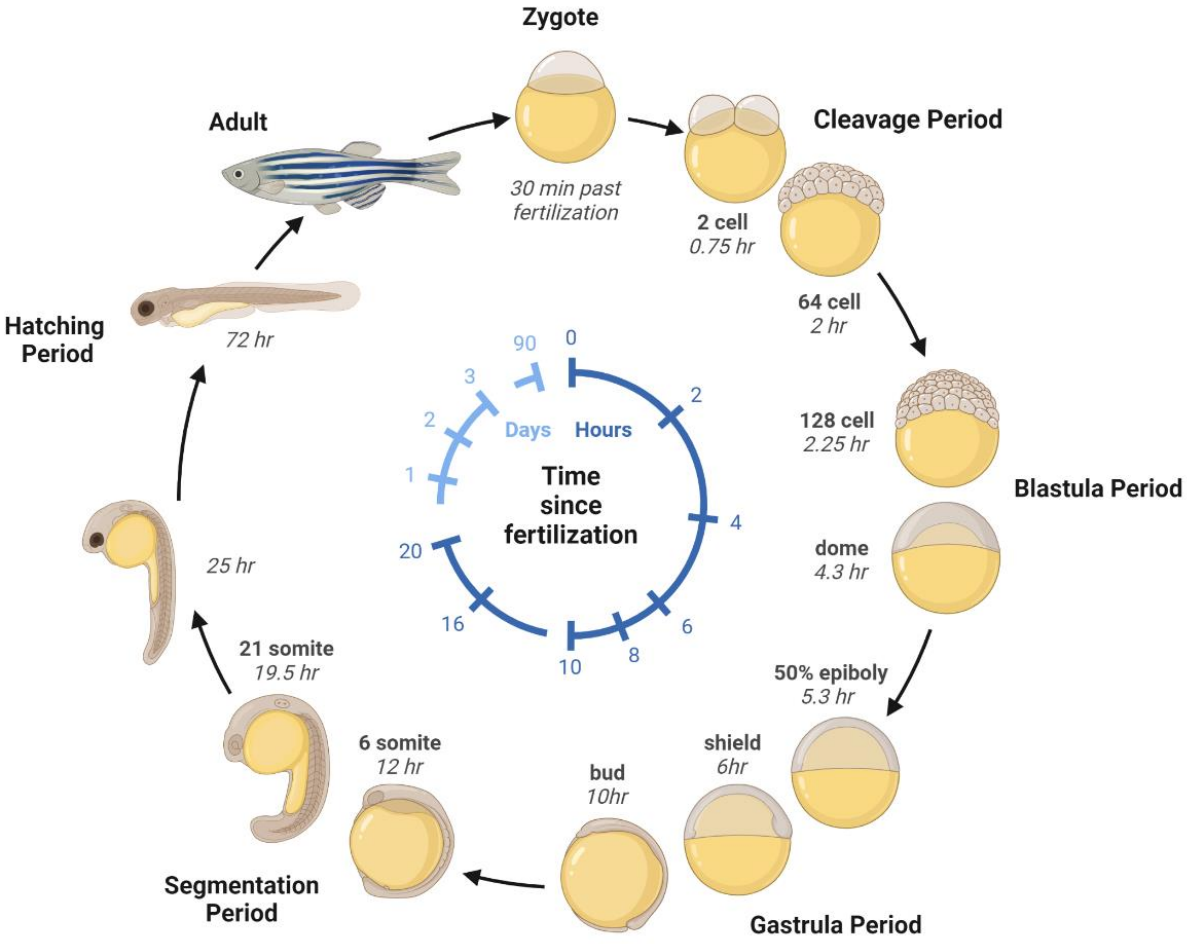


Figure 13 *: Life cycle of Danio rerio*

5.2.Zebrafish as a powerful model of regeneration

In the contrary to human and more generally mammals, some organisms have surprising regenerative capabilities. “Invertebrates and phylogenetically primitive vertebrates are able to regenerate full tissues after injury. Even though species with strong regenerative capacities are non-uniformly widespread across the phylogenetic tree, simpler organisms generally perform better in this respect (Elliott and Sánchez Alvarado 2018). For this reason, it has been assumed that regenerative potential has been lost during evolution. However, in the last years, several studies have revealed that regenerative mechanisms are still present in mammals but are latent or dormant, and thus it would be possible to stimulate them (Maden 2018). This is why elucidating the regenerative mechanisms in competent species is important to permanently cure patients. Classical models of regeneration are found in invertebrate and vertebrate phylum such as the hydra, planarian, drosophila, zebrafish, axolotl and newt.” (Extract from (Massoz, Dupont, and Manfroid 2021)). The zebrafish model can therefore be exploited to uncover mechanism of complex tissue regeneration to possibly re-active these mechanisms in mammals (**Figure 14**).

As a proof of concept, several studies could show improvement of regeneration in mammals, underlying the successful translation of findings from zebrafish to mammals (Massoz, Dupont, and Manfroid 2021). The examples of the involvement of *Ascl1a* and *Lin28* in retinal regeneration and miRNAs in cardiomyocyte regeneration illustrate the relevance of this approach (**Figure 14**). Briefly, genes coding for both transcription factor *Ascl1a* (Fausett, Gumerson, and Goldman 2008) and the RNA binding protein *Lin28* (Ramachandran R., Fausett B.V. 2010) are expressed in response to injury in zebrafish and their expression is necessary for retinal regeneration. Interestingly, these genes are not expressed in mice. Transgenic mediated expression of *ascl1a* could activate retinal regeneration in juvenile mice (Ueki et al. 2015) and co-expression of *ascl1a* and *lin28* could restore retinal cells after injury even in adult mice (Elsaeidi et al. 2018).

Another elegant example is illustrated by the identification of miRNAs necessary for cardiac regeneration (**Figure 14**), using different approaches. On one hand, the comparison of miRNAs expression between zebrafish cardiomyocytes after injury and non-injured zebrafish showed that miR-99/100, let-7a/c are repressed during regeneration (Aguirre et al. 2014). On the other hand, comparison between miRNA profiling between zebrafish cardiomyocytes and mice cardiomyocytes after injury revealed that miR-26a is repressed in zebrafish but not in mice (Crippa et al. 2016). Repression of these miRNA (miR-99/100, let-7a/c and miR-26a) in mice improve cardiomyocyte proliferation and subsequent regeneration (Aguirre et al. 2014; Crippa et al. 2016), highlighting potential clinical targets to stimulate cardiac regeneration (**Figure 14**).

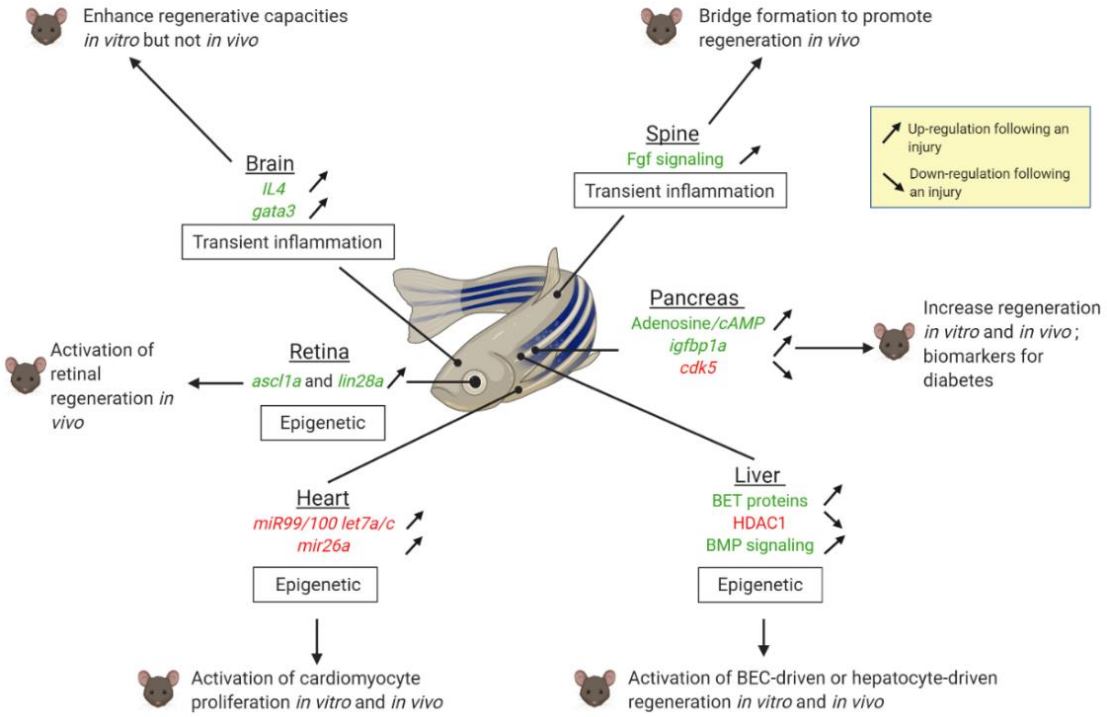


Figure 14 *: Overview of regenerative mechanisms identified in zebrafish that led to improvement of regeneration of several tissues in mammals.*

The up-headed (vs. back-headed) arrows mean that the expression is upregulated (vs. downregulated) in zebrafish after injury. Factors highlighted in green exert positive effect in regeneration, those in red impair regeneration. From (Massoz, Dupont, and Manfroid 2021)

5.3.The zebrafish pancreas

5.3.1. Anatomy and physiology

Similar to mammals, the pancreas of zebrafish contains endocrine islets and ducts dispersed within acinar tissue. These islets are composed of the same endocrine cells (β-, α-, δ-, and epsilon cells) as in mammals, although PP cells have not been described in zebrafish. However, a notable difference in the zebrafish pancreas is the presence of a principal islet (PI) located in the head of the pancreas, accompanied by numerous secondary islets (SI) that are typically smaller and scattered along the ducts in the pancreatic tail (Kinkel and Prince 2009) (**Figure 15)**. It is worth mentioning that the duct organization in this system is similar to that of mammals, with CACs located at the acinus junction, and intra- followed by extra-pancreatic ducts branching out from there (Parsons et al. 2009).

In the adult zebrafish, the fasting glycemia typically is around 50 mg/dL. It is worth noting that the dynamic regulation of glycemia in zebrafish is comparable to that of mammals, as demonstrated by glucose tolerance tests (Eames et al., 2010). Endocrine cells are responsible for regulating glycemia from an early larval stage, indicating the early functionality of the principal islet (Jurczyk et al., 2011). Furthermore, the β cells in zebrafish are essential for glucose metabolism, as their destruction leads to hyperglycaemia (Moss et al., 2009). To conclude, both the anatomy and the physiology of the zebrafish pancreas are quite similar to the mammalian pancreas.

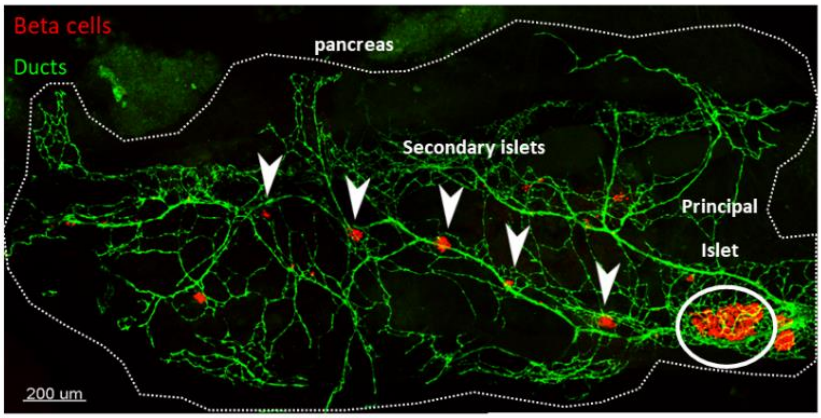


Figure 15 *: Immunohistochemistry of zebrafish pancreas*

Pancreas of 2 months-old zebrafish showing β cells in red and the ductal network in green. The picture shows the presence of a principal islet in the head of the pancreas and several secondary islets in the pancreatic tail that are closely associated with the ducts.

5.3.2. Development : a focus on the second wave of endocrine cells formation

The embryonic development of the zebrafish pancreas bears striking similarities to that of mammals, as it is regulated by the same master signalling pathways that govern the progression from organ specification to cellular differentiation (Kinkel and Prince 2009). Moreover, the cascade of transcription factors that leads to cell differentiation is largely conserved between zebrafish and mammals, with only minor variations.

Similar to mammals, the zebrafish pancreas originates from two endodermal buds. The dorsal bud emerges at 24 hpf (hours post fertilization) and eventually fuses with the ventral bud that emerges later, around 34 hpf. The cells derived from the dorsal bud exclusively give rise to the first wave of endocrine cells in the principal islet (**Figure 16**). Meanwhile, the ventral bud gives rise to all pancreatic cells, including exocrine acinar and ductal cells, as well as the second wave of endocrine cells. The latter contribute to additional cells in the principal islet and secondary islets (Kinkel and Prince 2009). As such, the ventral bud consists of multipotent pancreatic cells (MPCs) that express among others *pdx1* (Biemar et al. 2001), *ptf1a* (Zecchin et al. 2004), *sox9b* (Manfroid et al. 2012) and *nkx6.1* (Binot et al. 2010) likewise mammalian MPCs (**Figure 17**). Later in the development, the ductal epithelium cells express *sox9b*, *nkx6.1*, exhibit Notch activity (Parsons et al. 2009), and are able to give rise either to ductal or endocrine cells. This domain is similar to the bi-potential trunk in mouse, underlying the similarity of the second wave in zebrafish and the second transition occurring in mammals (Parsons et al. 2009).

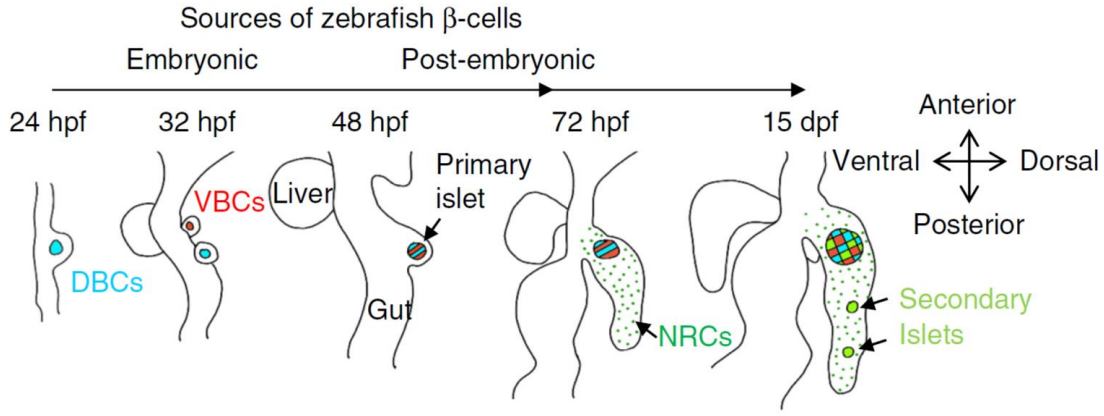


Figure 16 *: Morphogenesis of the zebrafish pancreas*

The progenitors from the ventral bud (VBCs) will exclusively give rise to the first wave of endocrine cells in the principal islet. Progenitors from the dorsal bud (DBCs) give rise to all pancreatic cell types, including endocrine cells from the primary islet and from the secondary islets. NRCs are Notch Responsive Cells. From (Singh et al. 2017).

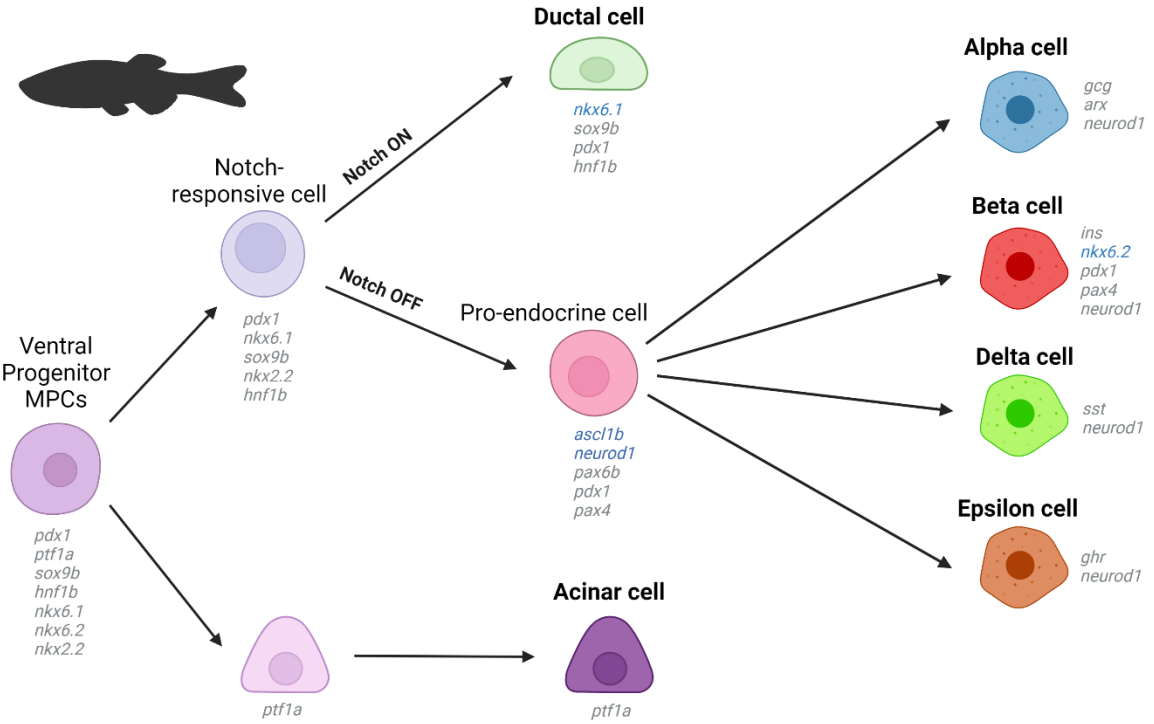


Figure 17 : Summary of key transcription factors involved in pancreatic cells specification in zebrafish

This scheme put the emphasis on the second wave of endocrine cell formation, from the ventral progenitors. Differences with mammals are underlined by the blue colour. The ventral progenitor cells are multipotent and give rise to all pancreatic cell types. Notch responsive cells give rise either to ductal cell when Notch signalling is sustained or endocrine cells when Notch is repressed. On the opposite to mammals, *nkx6.1* is expressed in ductal cells and not in β cells, in which nkx6.2 is expressed. Overall, the cascade of transcription factor is quite similar compared to mammals.

Notch signalling plays a crucial role in maintaining the expression of *sox9b* in pancreatic progenitor cells (Manfroid et al. 2012), which results in the repression of endocrine cell differentiation and the induction of the ductal fate. More precisely, Ninov and colleagues showed that the level of Notch activity determines the behaviour of the progenitor ductal cells (Ninov, Borius, and Stainier 2012). While a high level of Notch activity keeps the progenitor in a quiescent state, its downregulation enables the progenitor to enter the cell cycle and its complete repression leads to endocrine differentiation (Ninov, Borius, and Stainier 2012). Notch constrains the endocrine program by repressing the expression of the transcription factor Neurog3 in mammals (see section 4.2.). *Neurog3* is not expressed in the zebrafish pancreas and the endocrine differentiation is induced by two others transcription factor of the same family, *Ascl1b* and *Neurod1* (L. C. Flasse, Pirson, et al. 2013). *Ascl1b* is the first endocrine marker

expressed in the pancreas and initiates the endocrine cascade. While *ascl1b* expression is transient, *Neurod1* maintains the endocrine program and is still expressed in mature endocrine cells (L. C. Flasse, Pirson, et al. 2013). Of note, Notch represses *ascl1b* and *neurod1* (L. C. Flasse, Pirson, et al. 2013; Aurélie P. Ghaye et al. 2015) expression and sustains *nkx6.1* expression in zebrafish (Aurélie P. Ghaye et al. 2015).

Whereas *nkx6.1* is expressed in MPCs (Binot et al. 2010) this transcription factor is not expressed in mature β cells in zebrafish, in contrast to mouse. Its paralog *Nkx6.2* may fulfil the role of NKX6.1 in the function and identity of the β cells. Also, unlike in mammals, the progenitor markers *nkx6.1* and *pdx1* remain expressed in the ductal tree until adulthood, and the Notch pathway is still essential for maintaining *nkx6.1* expression (Aurélie P. Ghaye et al. 2015).

6. β cell regeneration in zebrafish

Given the incredible regenerative abilities of the zebrafish, it is not surprising that it can spontaneously regenerate its β cells after destruction not only in larvae (Curado et al. 2007; Harsan Pisharath et al. 2007) but even in the adult (Moss et al. 2009). First regenerated β cells are detected within 10 days and the glycemia is normalized around two weeks after β cell ablation (Aurélie P. Ghaye et al. 2015; Moss et al. 2009). To study this process, several techniques of pancreas injury have been developed in zebrafish, including pancreatectomy, STZ (Moss et al. 2009), the DTA system (Ninov, Borius, and Stainier 2012) or nitroreductase (NTR) system. The latter is widely used in zebrafish to induce ablation of several cell types (Curado et al. 2007).

6.1.Nitroreductase mediated β cell ablation

The nitroreductase (NTR) is an enzyme that reduces nitro- functional group from nitroaromatic substrates leading to the production of cytotoxic products. This enzyme is not naturally expressed in zebrafish, which has allowed for the development of a conditional and selected cell ablation method. It involves the expression of the bacterial NTR under a tissue-specific promoter, *i.e.* a suicide transgene, and the administration of a pro-drug (**Figure 18**). The reduction of the pro-drug by the NTR results in the formation of reactive nitroso group causing DNA damage and ultimately leading to the apoptosis of the targeted cells (Curado et al. 2007).

The pro-drug metronidazole (MTZ) rapidly became the most common drug used for that system since no diffusible products are formed after its reduction, enabling specific ablation of target cells without by-stander effects (Curado et al. 2007; Harsan Pisharath et al. 2007). Depending the cell type to ablate, the concentration and the duration of MTZ treatment differs. Of note, the concentration commonly used (*i.e.* 10 mM) is close to general toxicity (Mathias et al. 2014).

To ablate the β cells, the transgenic line *Tg(ins:NTR*-mCherry)* can be used. This line enables the expression of a mutated form of the bacterial NTR to improve its efficiency (Mathias et al. 2014) under the control of the insulin promoter, therefore in β cells (Bergemann et al. 2018; Harsan Pisharath et al. 2007). After MTZ treatment, β cells generate ROS (Kulkarni et al. 2018) and the apoptosis is activated as seen by caspase3 detection.

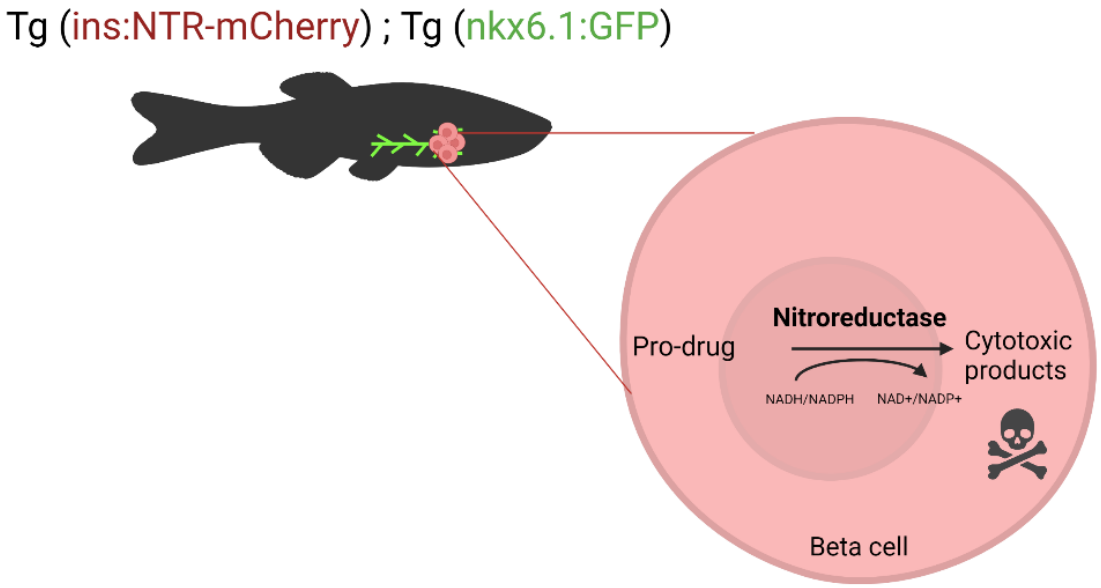


Figure 18 *: Nitroreductase mediated β cell ablation in transgenic zebrafish*

6.2.Cellular origins of regenerated β cells

Pioneer studies in zebrafish have demonstrated the spontaneous regeneration of β cells (Curado et al. 2007; Moss et al. 2009; Harsan Pisharath et al. 2007), sparking interest in the underlying cellular and molecular mechanisms. Subsequent research has focused on revealing the origin of regenerated β cells and performing chemical and genetic screenings to enhance even further the regenerative potential. Likewise in mammals, regenerated β cells can arise from the proliferation of remaining β cells (Andersson et al. 2012) or the transdifferentiation of α cells in zebrafish (Ye et al. 2015). However, unlike mammals, zebrafish possess ductal-associated progenitors capable of generating new β cells even in adulthood (Delaspre et al. 2015; Aurélie P. Ghaye et al. 2015) (**Figure 19**). It is worth noting that the NTR-mediated ablation system was used to identify all these cellular sources in zebrafish, a distinction from mammals, where the origin of β cells is influenced by the type of pancreas injury (see sections 4.4. and 4.5.).

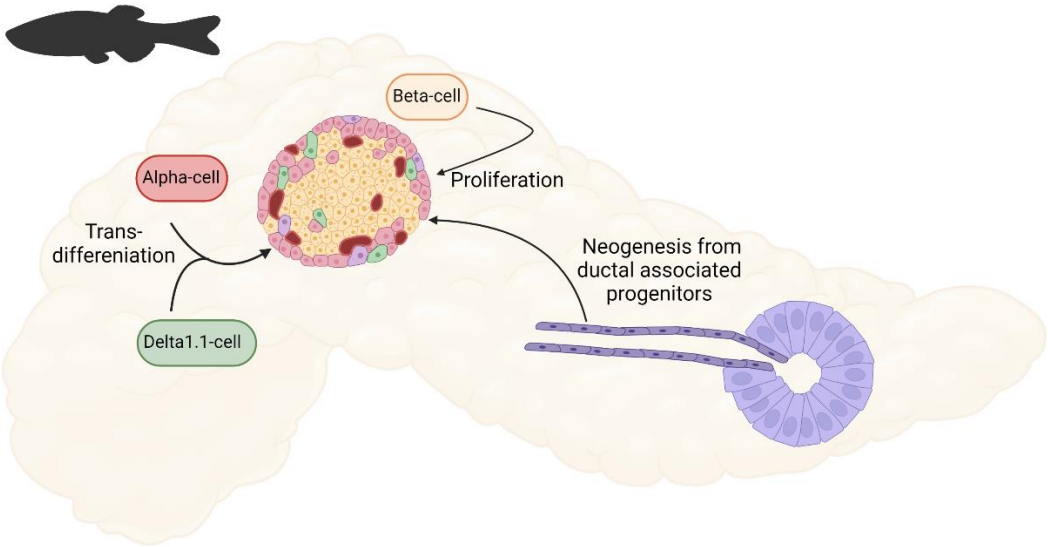


Figure 19 *: Origins of spontaneous β cell regeneration in zebrafish*

After β cells ablation, new regenerated β cells can arise from the proliferation of remaining β cells, conversion of α or δ1.1 endocrine cells and from neogenesis of ductal associated progenitors.

6.2.1. β cell proliferation

β cell proliferation is a crucial process during embryogenesis to expand their population. It is noteworthy that zebrafish β cells retain their proliferative capacity even after embryonic development (Singh et al. 2017), which is not the case for most mammals. Previous studies using zebrafish larvae have identified various signalling pathways, such as retinoic acid and glucocorticoid, that can enhance β cell proliferation (Tsuji et al. 2014).

On the other hand, interesting studies have focused on exploring the regenerative potential of β cells. For example, Andersson et al. performed a high-throughput chemical screening using zebrafish larvae and the NTR/MTZ ablation system to identify compounds able to increase β cell regeneration in the principal islet (PI) (Andersson et al. 2012). The most potent drug was NECA (50-N-ethylcarboxamidoadenosine), an adenosine agonist activating GPCR signalling. NECA promoted β cell regeneration by increasing their proliferation, as demonstrated by EdU incorporation (Andersson et al. 2012). Later, an independent study identified another compound, the PIAA ((E)-3-(3-phenylbenzo[c]isoxazol-5-yl) acrylic acid), that stimulates the production of cAMP resulting in an increase of β cell regeneration by proliferation (J. Xu et al. 2018). Notably, both NECA and PIAA were able to enhance β cell regeneration in STZ-treated mice (Andersson et al. 2012; J. Xu et al. 2018). Recently, another chemical screening performed by Andersson's team identified a SIK inhibitor promoting β proliferation after ablation in zebrafish larvae, mediated by the UPR (Unfolded Protein Response) (Charbord et al. 2021). It is worth noting that those studies focused on larval stage, when only the principal islet is present.

Taken together, those studies have demonstrated that β cell replication is a source of regenerated β cells in zebrafish larvae, and identified potential pharmacological compounds that could be used for further clinical studies.

6.2.2. α cell trans-differentiation

The α cells represent an important source for β regeneration after severe ablation in mammals (Thorel, Népote, et al. 2010). In the same manner, lineage tracing experiments revealed that α cells are able to transdifferentiate into β cells in the NTR-mediated ablation system in zebrafish (Ye et al. 2015). Specifically, the study found that in zebrafish larvae, α cell conversion and

neogenesis from progenitors were the primary sources of β cell regeneration, rather than β cell proliferation. Interestingly, very few sst2+/ins+ were detected after ablation. The authors conclude that δ cells do not seem to retain the same plasticity than α cells. The same study points out that α cell conversion is dependent to glucagon and GLP-1 since the knock down of *gcg* alters the efficiency of the process. However, high glycemia does not impact α cell conversion, suggesting that glucagon and GLP-1 do not regulate α cells conversion through the control of glycemia (Ye et al. 2015).

Transcriptomic profiling of zebrafish islets isolated after NTR-mediated ablation identified some secreted proteins that could potentially enhance β cell regeneration (J. Lu et al. 2016). One such protein, insulin-like growth factor (Igf) binding-protein 1 (igfbp1), was found to increase α cell transdifferentiation when overexpressed via transgenesis in zebrafish larvae. Notably, the effect was sustain even in one-month-old zebrafish, indicating that α cells retain their plasticity beyond embryogenesis (J. Lu et al. 2016). Additionally, IGFBP1 also promoted α cell transdifferentiation in mouse and human islets *ex vivo*, underlying the possibility of translation towards mammals. Interestingly, the analysis of a prospective study showed that patients with insulin resistance have lower levels of IGFBP1 while those with higher levels of IGFBP1 present a lower risk to develop T2D (J. Lu et al. 2016). As such, IGFBP1 could be a potential biomarker for insulin resistance in addition to be a good candidate to improve β cell regeneration in clinical studies.

6.2.3. Neogenesis from ductal- associated progenitors

Although the persistence of pancreatic progenitors within the ductal network in the adult is still debated in mammals (see section 4.5.3), those cells do exist in zebrafish. One of the first clue in favour for a the progenitor capacity of the ductal cells was underlined thanks to *sox9b-/-* mutant zebrafish (Manfroid et al. 2012). The deletion of *sox9* indeed provokes abnormal duct morphogenesis and impaired β cell regeneration in the larvae. Notably, activation of Notch signalling is necessary to sustain Sox9b expression in the ductal cells (Manfroid et al. 2012). In line with this, Ninov and colleagues could show by genetic lineage tracing that ductal cells give rise to new endocrine cells following constitutive β cell ablation in the zebrafish larvae (Ninov et al. 2013). Moreover, the study highlights that this process is dependent of the nutrient uptake, through the activation of mTor (mammalian Target of Rapamycin) pathway that will then

repress Notch signalling (**Figure 20**) to induce β cell formation and regeneration (Ninov et al. 2013).

Although these studies have demonstrated the existence of progenitors within the ducts, these experiments were conducted in larvae, in which cells may retain greater plasticity due to ongoing development. Interestingly, a subset of cells within the ductal tree, the centro-acinar cells (CACs), remain Notch-responsive even in adulthood (Delaspre et al. 2015). Genetic lineage tracing using the Tg (*tp1:CreEr^{T2}*) line, which labels Notch-responsive cells, revealed that CACs give rise to new β cells after NTR-mediated ablation or partial pancreatectomy in adult zebrafish (Delaspre et al. 2015). Simultaneously, our laboratory found the same results using non-genetic lineage tracing (Aurélie P. Ghaye et al. 2015). Additionally, we demonstrated using the Tg (*nkx6.1:GFP*) line labelling all ductal tree even in adulthood, that ductal nkx6.1+ cells proliferate in response to β cell ablation, suggesting that the progenitor capacity is not restricted to CACs (Aurélie P. Ghaye et al. 2015). Altogether, both studies showed that ductal cells retain progenitor capacity during adulthood and that these cells can generate new β cells in response to their ablation (Delaspre et al. 2015; Aurélie P. Ghaye et al. 2015).

Further studies from Andersson's group performed chemical or genetic screening on islets cells from zebrafish larvae to ultimately enhance β cell regeneration. As such, *CDK5* inhibition (with roscovitine or DRF053) (K. C. Liu et al. 2017) as well as folinic acid treatment (Karampelias et al. 2021) could increase β cell regeneration not only in larvae but in one-month old zebrafish as well. Moreover, inhibition of *MNK2* MAPK, consistent with the role of mTOR, was found to enhance β cell regeneration by increasing protein synthesis (Karampelias et al. 2022). Importantly, in all three studies, the increased regeneration was due to endocrine differentiation of ductal cells without any impact on ductal cell proliferation. Of note, the effect of these signalling could be translated in mammalian model of endocrine cell formation or β cell regeneration, underlying their relevance for further studies.

Collectively, these studies established that pancreatic progenitors persist within the ducts of adult zebrafish and are the source of β cell neogenesis after their ablation. Even though some interesting studies uncover molecular mechanisms of this process, all the screenings were performed on larvae. Moreover, genetic screenings only took into account the principal islet. As such, there is still a need to establish the molecular mechanisms of β cell neogenesis from ductal progenitors in a regenerative context.

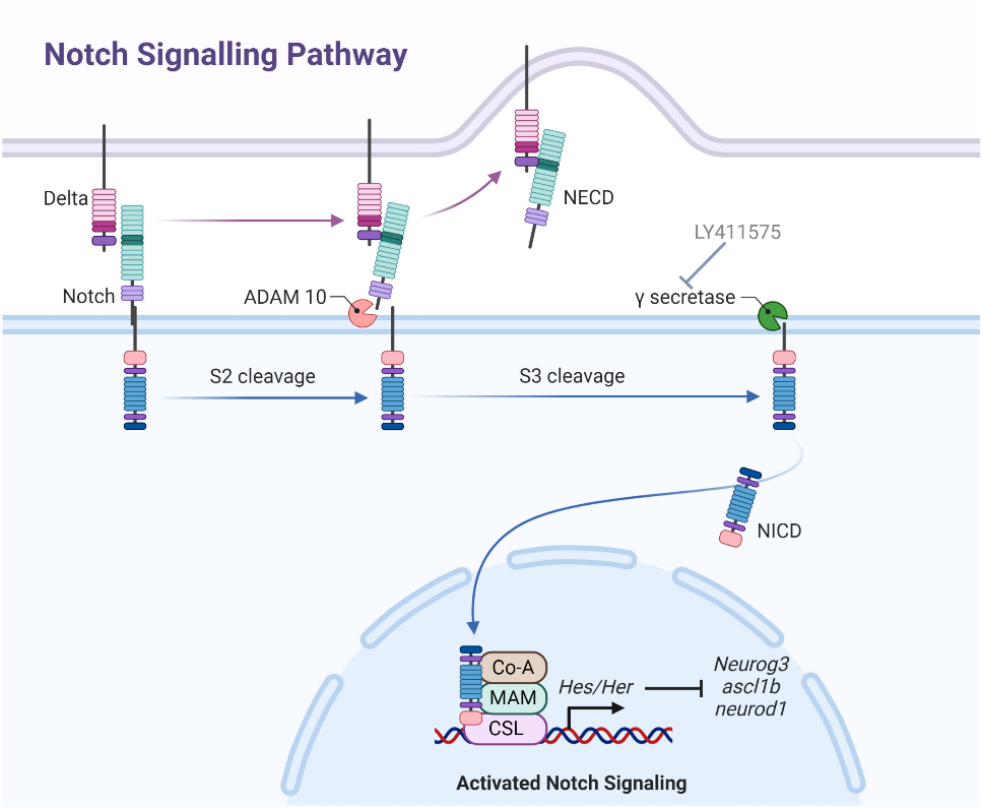


Figure 20 *: Notch signalling pathway*

The Notch receptor is a heterodimer composed of an extracellular domain and an intramembrane/intracellular domain. Binding of a ligand (Δ) to the Notch receptor triggers two proteolytic cleavages of the receptor. The first cleavage is carried out by the ADAM protease, and the second is carried out by the γ-secretase. Of note, several inhibitor of the γ-secretase are used to repress Notch pathway. The intracellular domain of the Notch receptor (NICD) is then released into the cytoplasm and enters the nucleus. NICD with its co-factors can activate transcription of target genes as hes/her genes, which are repressor of ARP/ASCL transcription factor (as *Neurog3* in mammals and *ascl1b, neurod1* in zebrafish) necessary for endocrine differentiation.

6.3. Calcineurin

In this study, we identified Calcineurin (CaN) as a candidate in the study of β cell regeneration (see section Results). CaN, previously known as protein phosphatase 2B (PP2B), is a calcium and calmodulin dependent serine/threonine protein phosphatase. The structure of CaN is highly conserved across eukaryotes, from yeast to humans.

The enzyme is composed of two subunits: the catalytic subunit, which contains the catalytic domain and an auto-inhibitory arm, and the regulatory subunit, which possesses binding sites for calcium and calmodulin. When intracellular calcium levels rise, Ca^{2+} ions attach directly to the regulatory subunit and calmodulin, causing a conformational change that activates the enzyme by shifting the auto-inhibitory arm away from the catalytic site (Roy and Cyert 2020). The catalytic unit of CaN has actually three isoforms, namely αCaN, βCaN, and γCaN that are structurally similar. While the αCaN and βCaN, coded respectively by PPP3CA and PPP3CB, are expressed ubiquitously, αCaN is dominant in neurons, and βCaN is more expressed in other cell types. Initially believed to be exclusively expressed in the testes, PPP3CC codes for the γCaN isoform and is actually expressed at low levels in other tissues (Creamer 2020).

CaN is able to dephosphorylate a wide range of substrates, including transcription factors, receptors and ion channels, cytoskeletal proteins, and other signalling proteins (Creamer 2020). The CaN canonical pathway involves dephosphorylation of the transcription factor NFAT (Nuclear factor of activated T-cells), enabling its entry inside the nucleus to activate transcription of its target genes (**Figure 21**). The CaN/NFAT pathway was first known for its role in T-cell activation (Oh-hora and rao 2009). However, CaN is important in a wide range of biological processes as the cell cycle (Masaki and Shimada 2022), cardiac hypertrophy or fin regeneration in zebrafish (Kujawski et al. 2014).

FK506, also known as tacrolimus, and Cyclosporin A (CsA) are both inhibitors of the CaN enzyme. Their mechanism of action involves binding to specific immunophilins, namely FKBP for FK506 and cyclophilins for CsA, forming complexes that competitively inhibit CaN. As a result, the nuclear factor of activated T cells (NFAT) is inhibited, which ultimately suppresses the immune response (J. Liu et al. 1991). These drugs are thus commonly used in transplanted patients to prevent organ rejection (Venegoni 2002), and they are used as efficient inhibitors of CaN, even in zebrafish (Kujawski et al. 2014).

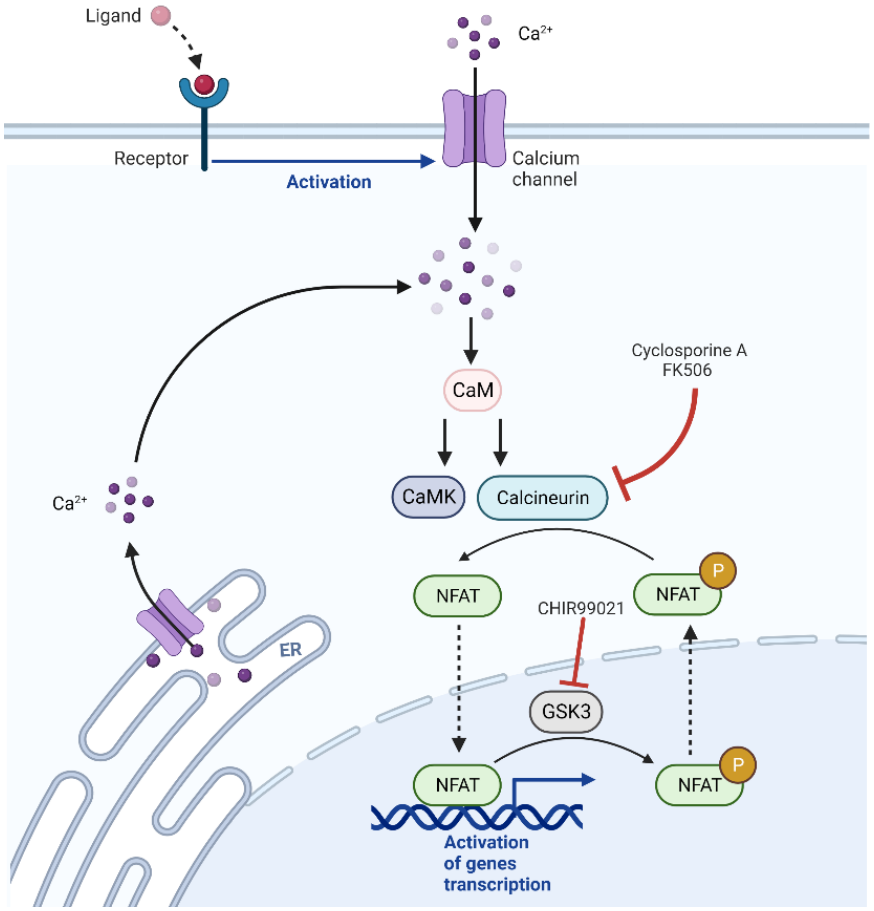


Figure 21 *: Calcineurin/NFAT canonical signalling*

CaN is activated by binding of Ca^{2+} and calmodulin. Once activated, CaN dephosphorylates NFAT, enabling its entry in the nucleus to activate transcription of targeted genes. Kinases within the nucleus as GSK3 phosphorylates NFAT to inactivate it. Calcineurin inhibitors as Cyclosporine A and FK506 are often used to repress the pathway.

In our study, we identified CaN in the context of a cellular senescence transcriptomic signature. In aging or in response to a stress, the cellular senescence mechanism restricts the proliferation of the damaged cells by inducing permanent cell cycle arrest. These senescent cells acquire a secretory function known as the Senescence Associated Secretory Phenotype (SASP) and they produce several cytokines, proteases and growth factors for cell-cell communication. The accumulation of senescent cells is usually correlated with aging related disease (Van Deursen 2014).

6.4.P53

The protein p53 is a well-known tumour suppressor, which can also be referred as the guardian of the genome. p53 is involved in cell cycle regulation, DNA repair and apoptosis. When errors of replication happen during the DNA duplication, p53 is then stabilized and activates DNA repair mechanisms or apoptosis in order to avoid mutations and keep DNA integrity. Notably, the *p53* gene is the most frequently altered gene in human cancer, underlying its importance. In addition to its role in apoptosis induction, p53 emerged as an important regulator of cell metabolism and cellular growth (Vousden and Ryan 2009). Metabolic stress as nucleotide or amino acid starvation, activates p53 to promote cell survival (Maddocks et al. 2013; Pelletier et al. 2020; Shiraki et al. 2014).

During the first steps of tissue regeneration, *p53* expression is generally repressed to enable cell proliferation as illustrated in the zebrafish heart (Shoffner et al. 2020a) or in the liver in mouse or zebrafish (He et al. 2022; Stepniak et al. 2006). However, other studies showed that DNA repair mechanisms (Sousounis et al. 2020) and p53 (Di Giovanni et al. 2006) are necessary for proper regeneration. Overall, these studies underline a dual role of p53 in complex tissue regeneration, depending on the tissue or the stage of regeneration (Charni et al. 2017).

II. Objectives

In contrast to mammals, the zebrafish possess the intrinsic ability to efficiently regenerate β cells after their loss. Moreover, it is well established in zebrafish that pancreatic progenitors remain within the ductal tree even in adulthood. As such, it is interesting to uncover mechanisms of β cell regeneration from ductal progenitors in zebrafish, which could ultimately lead to improvement of β cell regeneration in mammals.

The main objective of our laboratory is to unravel cellular and molecular mechanisms of β cell regeneration in zebrafish. To trigger regeneration, we first need to ablate the β cells. To this end, we use the NTR mediated ablation model. However, we observed that this system was not optimal since the efficiency of ablation was variable between individuals. As a consequence, we first needed to improve the NTR system for effective and reliable β cell ablation.

1) **The first goal of this study was to improve β cell regeneration in the NTR system.**

To uncover regenerative mechanisms from ductal progenitors, the transcriptomic profile of ductal cells during β cell in regeneration in the adult zebrafish was previously established in the laboratory. These data highlight putative regulators of β cell regeneration. Amongst the candidates, we decided to focus mainly on the study of calcineurin function, which is the main part of the present thesis. Secondly, we also studied the implication of another interesting candidate, the tumour suppressor p53.

2) **Determine the role of the phosphatase calcineurin and p53 in β cell regeneration from ductal progenitors.**

Intriguingly, it has been observed in the laboratory that regenerated β cells co-express the insulin and the somatostatin hormone. Subsequent study identified that these cells actually express *sst1.1*. We next wanted to determine the role of the δ sst1.1 population and the origin of bi-hormonal sst1.1+/ins+ cell during β cell regeneration.

3) **Determine the origin of bi-hormonal sst1.1+/ins+ cells that appeared in response to β cell loss.**

III. Results

1. Improvement of β cell ablation in the NTR system

To investigate the role of signalling pathways during β cell regeneration, we designed an experimental pipeline using zebrafish larvae to perform functional tests. However, we encountered issues with the low consistency and efficiency of β cell ablation using the NTR/MTZ system. In addition, the concentration of MTZ required for cell ablation was close to general toxicity, which increased mortality when combined with other treatments.

To address these challenges, we needed to improve the efficiency of ablation to enable quantitative experiments. By switching to another prodrug, the nifurpirinol (NFP), instead of MTZ, we observed improved consistency and efficiency of β cell ablation using the NTR system. Our findings were published in the Wound Repair and Regeneration journal in 2018, in an article titled "Nifurpirinol: A more potent and reliable substrate compared to metronidazole for nitroreductase-mediated cell ablations" (Bergemann et al. 2018). I contributed to this study by conducting experiments on the *Tg(ins:NTR-mCherry)* larvae, which confirmed the superior efficacy of NFP over MTZ. Importantly, the concentration of NFP used for the ablation did not cause toxic effects.

Wound Repair and Regeneration

BRIEF COMMUNICATION

Nifurpirinol: A more potent and reliable substrate compared to metronidazole for nitroreductase-mediated cell ablations

David Bergemann, MSc; Laura Massoz, MSc; Jordane Bourdouxhe, BBMS; Claudio A. Carril Pardo, MSc; Marianne L. Voz, PhD; Bernard Peers, PhD; Isabelle Manfroid, PhD

Zebrafish Development and Disease Models Laboratory/GIGA, University of Liège, Liege, Belgium

Reprint requests:
Isabelle Manfroid, Avenue de l'Hôpital 1, B34, 4000 Liège, Belgium.
Tel: 1 3243663374;
Fax: 1 3243664198;
Email: isabelle.manfroid@uliege.be

Manuscript received: November 27, 2017
Accepted in final form: April 10, 2018

DOI:10.1111/wrr.12633

ABSTRACT

The zebrafish is a popular animal model with well-known regenerative capabilities. To study regeneration in this fish, the nitroreductase/metronidazole-mediated system is widely used for targeted ablation of various cell types. Nevertheless, we highlight here some variability in ablation efficiencies with the metronidazole prodrug that led us to search for a more efficient and reliable compound. Herein, we present nifurpirinol, another nitroaromatic antibiotic, as a more potent prodrug compared to metronidazole to trigger cell-ablation in nitroreductase expressing transgenic models. We show that nifurpirinol induces robust and reliable ablations at concentrations 2,000 fold lower than metronidazole and three times below its own toxic concentration. We confirmed the efficiency of nifurpirinol in triggering massive ablation of three different cell types: the pancreatic beta cells, osteoblasts, and dopaminergic neurons. Our results identify nifurpirinol as a very potent prodrug for the nitroreductase-mediated ablation system and suggest that its use could be extended to many other cell types, especially if difficult to ablate, or when combined pharmacological treatments are desired.

One prerequisite to conduct regeneration studies is to develop robust and reproducible ablation models of the tissue/cell type of interest. Zebrafish has become a widely used model organism for the study of regeneration due to its potent regenerative capabilities of the heart,[1] spinal cord,[2] or pancreas,[3,4] to only cite those organs among others. A decade ago, a chemo-genetically inducible ablation model had been developed to address developmental and regeneration processes in zebrafish.[3,4] This system involves the targeted expression of a bacterial nitroreductase (NTR) under a cell-specific promoter and the treatment with a prodrug that becomes reduced by NTR into cytotoxic products, thus, leading to apoptosis of the targeted cell type. Since then, this method has been applied to a variety of cell types in zebrafish (reviewed in Ref. 5). The prodrug used with the NTR system is the nitroaromatic antibiotic metronidazole (MTZ), thanks notably to the lack of bystander effect allowing selective cell-specific ablation, and also presumably because of its widespread use in therapy making it a vastly documented antibiotic.

Nevertheless, this system harbors some limitations linked to MTZ. First, the commonly used 10 mM/24 hours treatment regimen appears not effective enough to ablate efficiently certain cell types, like neurons[6] or germline cells[7] needing 3 and 5 days treatment, respectively. Additionally, 10 mM concentration has proved to be toxic during such prolonged treatments and this concentration is only 1.5 times inferior to its general toxic levels even after the 24 hours standard treatment.[8] This toxicity may also hamper combined treatments to investigate possible pharmacological compounds for their influence on regeneration.

Here, we aimed at improving ablations by the NTR system and characterized another nitroaromatic compound as new NTR substrate. Its efficiency has been compared to that of metronidazole in NTR-mediated cell ablations in zebrafish. We tested its capacity to act as a NTR substrate in three different transgenic zebrafish lines expressing NTR, for example, in pancreatic beta cells, osteoblasts, and dopaminergic neurons. We also assessed the toxicity of this compound and verified the regenerative capacity of pancreatic beta cells after their ablation with this novel prodrug.

DMSO	Dimethyl sulfoxyde
dpf	Days postfertilization
dpt	Days posttreatment
IHC	ImmunoHistoChemistry
MTZ	Metronidazole
NFP	Nifurpirinol
NTR	Nitroreductase
WT	Wild-type

METHODS

Zebrafish maintenance and transgenic lines

Zebrafish (*Danio rerio*) were raised and cared for according to standard protocols. All animal work has been conducted according to national guidelines and approved by the ethical committee of the University of Liège (Protocol Numbers 14-1662 and 16-1872). *Tg(ins:NTR*-P2A-mCherry)*^{ulg034} was generated using the multisite Gateway three-fragment vector construction kit (Invitrogen, Carlsbad, CA). Briefly, the NTR* (T41Q/N71S/F124T) sequence was amplified from plasmid pBK 2xNRSE-2xzHB9-5xUAS-TagYFP-T2A-NTR(T41Q; N71S;F124T)[8] (gift from Jeff S Mumm), P2A-mCherry sequence was added by overlapping PCR before being cloned into pDONRP2R-P3. 897 pb of the *danio rerio insulin* promoter was cloned with forward primer 5′-GTATCTATAGTTGAACATGAAAGCAT-3′ and reverse primer 5′-GGTCACACTGACACAAACACACA-3′. The final construct in pDestTol2pA[9] has been injected with transposase into wild type (WT) AB embryos. *Tg(osx:mCherry-NTR)*^{pd46} was produced in another laboratory.[10] *Tg(ins:dsRed)*^{m1018}[11] was provided by Didier Stainier. *Tg(dat:CFP-NTR)*[12] (Zfin reference Tg*(slc6a3:CFP-NTR))* was given by Marc Ekker.

Drug preparation and treatments

Control treatments consisted of E3 medium containing 0.2% DMSO (or up to 0.8% DMSO in the toxicity assessment). MTZ (Sigma M1547; St. Louis, MO) working solutions were directly prepared in E3 medium with 0.2% DMSO. Nifurpirinol (Dr Ehrenstorfer GmbH, Augsburg, Germany) stock solution (2.5 mM) was prepared in DMSO before dilution in E3 medium at the given concentrations (see Results). Both solutions were prepared protected from light. Adult fish were bathed for 30 minutes in 25x Baktopur direct (Sera 02590) before being treated in 2.5 μM Nifurpirinol. Larvae and adults were treated for 18 hours in the dark.

Sample processing and imaging

Adult fish fasted for 18 hours were sacrificed at 3 days post-treatment (dpt), and their glycemia was measured as previously described.[13] *Tg(osx:mcherry-NTR)* and wild type (WT) larvae were live imaged under a Leica M165 FC binocular. *Tg(ins:NTR*-P2A-mCherry)* and *Tg(dat:CFP-NTR)* larvae were fixed in 4% PFA at 4 °C overnight before IHC. Adult digestive tracts were processed for paraffin embedding after fixation. IHC on whole larvae and on paraffin sections were performed as previously described.[13] Antibodies used: guinea-pig anti-insulin (Dako A0564; Glostrup, Denmark) 1:500, polyclonal rabbit anti-mCherry/dsRed (Living Colors DsRed Polyclonal Antibody, Clontech 632496; Mountain View, CA) 1:500, rabbit anti-somatostatin (Dako A0566) 1:200, mouse anti-glucagon (Sigma G2654) 1:300, Rabbit Anti-active Caspase-3 (BD Pharmingen 559565; San Jose, CA) 1:500, monoclonal anti-CFP (Living Colors Monoclonal Anitbody JL-8, Clontech 632381) 1:500, and corresponding Alexa Fluor secondary antibodies (Molecular Probes, Sunnyvale, CA). All samples were imaged on a Leica SP5 confocal microscope.

Analysis and data representation

Confocal images were analyzed using Imaris7.2.3 (Bitplane, Zurich, Switzerland). Islet cells (alpha, beta and delta) and dopaminergic neurons of the diencephalon were counted manually plane by plane. Graphs and statistics were done in GraphPad Prism 5 GaphPad, La Jolla, CA. Kruskal–Wallis with Dunn's multiple comparison post-test or Mann–Whitney tests were applied. *p*-Values are shown as *<0.05, **<0.01, ***<0.001.

RESULTS

More efficient and robust pancreatic beta cell ablations with nifurpirinol compared to metronidazole

As NFP is, like MTZ, a nitroaromatic antibiotic compound (Figure 1A), we postulated that it could, therefore, act as substrate for the bacterial NTR. It is also widely used in domestic aquaculture and is rapidly and well absorbed from the water.[14] To test the capacity of NFP to induce cell ablation, we produced a zebrafish line expressing the enhanced version of NTR[15] in pancreatic beta cells, the *Tg(ins:NTR*-P2A-mCherry)* line, in which NTR* stands for the mutated NTR. We treated adult *Tg(ins:NTR*-P2A-mCherry)* fish with NFP and measured their glycemia at 3 days post-treatment (dpt) as a readout. Strong hyperglycemia was detected in fish treated with NFP (523 ± 113 mg/dL, $n = 8$) as compared to control fish (65 ±13 mg/dL, $n = 9$; Figure 1B). Near absence of beta cells was confirmed by immunohistological analyzes (Figure 1B'). This indicates that NFP is able to induce pancreatic beta cell ablation and thus acts as a substrate for NTR-mediated cell ablation.

To compare the ablation efficiency of NFP vs. MTZ, we applied the gold-standard protocol for pancreatic beta cells ablation,[3,4] treating *Tg(ins:NTR*-P2A-mCherry)* larvae at 3 days postfertilization (dpf) with a range of concentrations for the two compounds and analyzing ablation at 4 dpf (1 dpt; Figure 1C). Quantification of beta cell number shows that both prodrugs induce ablation in a concentration-dependent manner. However, even the highest dose of MTZ (10 mM) still results in incomplete ablation (mean 3.6 ± 3.2 vs. 35.1 ± 4.9 cells in CTL) compared to control larvae, and strong inter-individual heterogeneity is observed. In contrast to MTZ, NFP treatment consistently induced a robust decrease in beta cell mass already at 2.5 μM (mean 2.8 ± 1.2 cells) and ablation was complete at 5 μM (mean 0.8 ± 1.4 cells; Figure 1D). NFP treatment showed less variation between individuals when compared to MTZ (SD = 1.4 vs. 3.2). Activated-caspase3 labeling (Figure 1D) confirmed that NFP treatment induces ablation through apoptosis as previously reported with MTZ.[3] Altogether, these data show that NFP (5 μM) triggers a stronger and more robust ablation of beta cells than MTZ and at much lower concentrations, that is, 2,000 times (5 μM for NFP compared to 10 mM for MTZ).

Nifurpirinol triggers ablation at subtoxic concentrations and without bystander effect

As the use of NFP has never been described in zebrafish so far, we assessed its general toxicity on nontransgenic larvae.

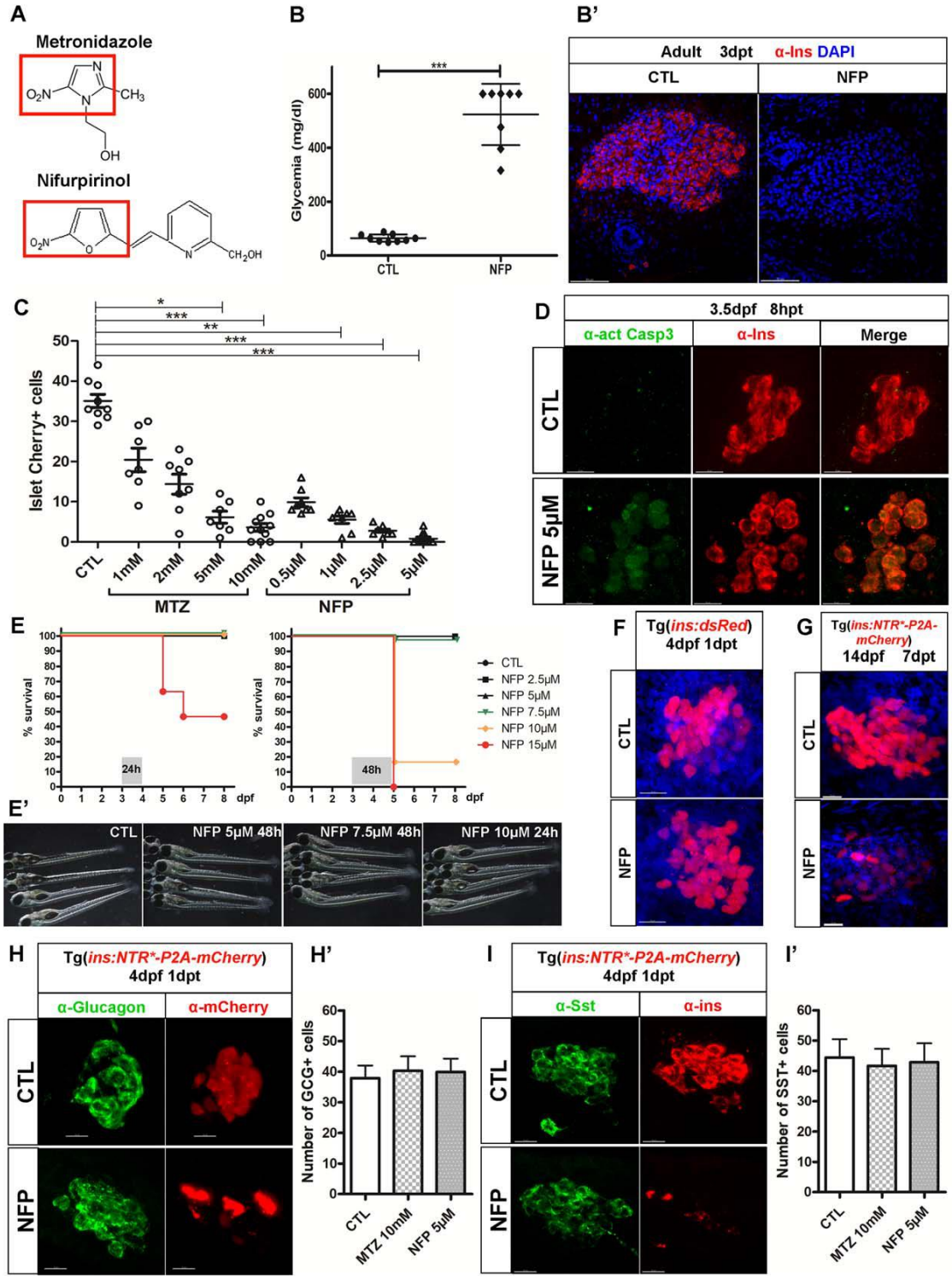

A
Metronidazole
Nifurpirinol
B
Glycemia (mg/dl)
CTL
NFP
B'
Adult 3dpt α-Ins DAPI
CTL
NFP
C
Islet Cherry+ cells
CTL
1mM
2mM
5mM
10mM
0.5μM
1μM
2.5μM
5μM
MTZ
NFP
D
3.5dpf 8hpt
α-act Casp3
α-Ins
Merge
CTL
NFP 5μM
E
% survival
24h
48h
dpf
CTL
NFP 2.5μM
NFP 5μM
NFP 7.5μM
NFP 10μM
NFP 15μM
E'
CTL
NFP 5μM 48h
NFP 7.5μM 48h
NFP 10μM 24h
F
Tg(ins:dsRed)
4dpf 1dpt
G
Tg(ins:NTR*-P2A-mCherry)
14dpf 7dpt
H
Tg(ins:NTR*-P2A-mCherry)
4dpf 1dpt
α-Glucagon
α-mCherry
H'
Number of GCG+ cells
CTL
MTZ 10mM
NFP 5μM
I
Tg(ins:NTR*-P2A-mCherry)
4dpf 1dpt
α-Sst
α-ins
I'
Number of SST+ cells
CTL
MTZ 10mM
NFP 5μM

Figure 1. Nifurpirinol acts as a more efficient substrate than MTZ for NTR-mediated pancreatic beta cell ablation. (A) Structure of metronidazole (MTZ) and nifurpirinol (NFP), showing the presence of a nitroaromatic group (red rectangle). (B) Glycemia (mg/dL) of CTL ($n = 9$) and NFP ($n = 8$)-treated adult zebrafish at 3 dpt. Glycemia was set to 600 when exceeding detection limit. $p < 0.0001$, Mann–Whitney test. Mean ±SD. (B') Cross-section through corresponding adult CTL and ablated islets. Beta cells stained for insulin (red), nuclei (Dapi), scale bar = 50 μm. (C) Dose-response of ablation according to MTZ and NFP concentrations assessed by quantification of islet Cherry+ cells in 4 dpf larvae treated from 3 to 4 dpf. Kruskal–Wallis $p < 0.0001$ and Dunn's post hoc test (*=$p < 0.05$, **=$p < 0.01$, ***=$p < 0.001$). $n = 8$ for 1–2 mM MTZ and 0.5, 5 μM NFP, $n = 9$ for CTL and 1–2.5-5 μM NFP, $n = 7$ for 5 mM MTZ, $n = 11$ 10 mM MTZ. (D) Apoptotic beta cells (red) determined by activated caspase3 labeling (green) of 3.5 dpf larvae, 8 hours posttreatment (hpt) with DMSO (CTL) or 5 μM NFP (NFP) (z-stack). Scale Bar = 10 μm. (E–E') General toxicity of NFP in WT larvae. (E) Survival curves of WT larvae treated at 3 dpf with increasing concentrations of NFP for 24 hours and 48 hours and monitored until 8 dpf ($n = 30$/group). (E') Corresponding morphological aspects of live imaged larvae at 8 dpf. (F) Absence of toxicity of NFP on beta cells (red) shown in representative principal islets of 4 dpf *Tg(ins:dsRed)* larvae lacking NTR, treated from 3 to 4 dpf with DMSO (CTL) and 5 μM NFP. (G) Regenerated beta cells (red, NFP) in a principal islet of a 14 dpf *Tg(ins:NTR*-P2A-mCherry)* larva (treatment from 7 to 8 dpf, compared to a control islet (CTL), confocal z-stack. (H–I') Bystander effect assessed in *Tg(ins:NTR*-P2A-mCherry)*. (H) Representative confocal images of CTL and NFP islets showing alpha cells (green) and beta cells (red). (H') Amount of alpha cells in principal islet determined by number of glucagon positive cells in 4 dpf larvae treated from 3 to 4 dpf with DMSO (CTL), MTZ, or NFP. (I) Representative confocal images of CTL and NFP condition showing delta cells (green) and beta cells (red). (I') Amount of delta cells in principal islet assessed by the number of somatostatin positive cells in 4 dpf larvae treated from 3 to 4 dpf with DMSO (CTL), MTZ or NFP.

By treating WT larvae from 3 to 4 or from 3 to 5 dpf with increasing concentrations of NFP and analyzing their survival until 8 dpf, we established that general toxicity appears at 15 μM with a 24-hours treatment regimen and at 10 μM for a 48-hours treatment regimen (Figure 1E). Accordingly, larvae treated for 48 hours with 5 or 7.5 μM did not show any morphological defects (Figure 1E'). We also treated Tg(*ins:dsRed*) larvae, that do not encode for NTR, with 5 μM NFP from 3 to 4 dpf and could not detect any effect on beta cells as compared to controls (Figure 1F), indicating that NFP has no direct adverse effect on beta cells. This indicates that NFP triggers beta cell destruction specifically through the NTR system, at effective concentrations at least 2 times lower than its toxic dose.

To ensure that NFP is permissive for regeneration, *Tg*(*ins:NTR*-P2A-mCherry*) larvae were analyzed 7 days after ablation with 5 μM NFP treatment. Treated larvae showed islet beta cells, most of them bearing weak mCherry, indicative of newly formed beta cells (Figure 1G). Thus, ablation with NFP does not interfere with intrinsic regeneration mechanisms.

To further validate the use of NFP in NTR-mediated cell ablations, we assessed its potential bystander effects (i.e., the possibility that the viability of neighboring cells could be affected by diffusible toxic metabolites) by analyzing a potential impact on surrounding alpha cells, which have previously been shown to be unaffected by 10 mM MTZ,[3] and on delta cells. Quantification of alpha cells (glucagon marker, Figure 1H–H') and delta cells (somatostatin marker, Figure 1I–I') of *Tg*(*ins:NTR*-P2A-mCherry*) larvae did not reveal any significant difference between CTL, MTZ (10 mM), and NFP (5 μM) treatments. Corresponding confocal images did not show any morphological impact on both cell types. Furthermore, the absence of bystander effect is supported by the observation that all apoptotic cells in the islet, as revealed by anti-activated caspase3, are beta cells (see Figure 1D). Together, these data indicate that the NFP-mediated ablation is specific to the targeted cells without any apparent bystander effect.

Nifurpirinol triggers efficient ablation in different cell types

Finally, we assessed if NTR-mediated cell ablation with NFP is extendable to other cell types. For that purpose, we examined if NFP could trigger ablation in two additional transgenic lines driving expression of the NTR enzyme in osteoblasts and neurons respectively. In *Tg*(*osx:mCherry-NTR*), the *osx* promoter drives expression of NTR and mCherry specifically in osteoblats.[10] NFP-treated larvae displayed only cell debris (and potential mCherry-loaded macrophages) in the structures labeled at this stage (Cleitrum [Cl], Dento-Maxilar [Dm], Opercular [Op]), as compared to CTL larvae (Figure 2A), indicating massive destruction of osteoblasts by NFP. This shows that NTR-mediated cell ablation using NFP is not limited to our beta cell model.

To determine if NFP could improve ablations of cell types that are difficult to ablate, we used the *Tg(dat:CFP-NTR)* which expresses NTR and CFP in dopaminergic neurons under the control of the *dat* (*slc3a6*) promoter. With these fish, even a 72-hours MTZ regimen only lead to moderate ablation (50–60%).[12] To compare MTZ and NFP efficiencies in inducing dopaminergic neuron ablation, we treated *Tg(dat:CFP-NTR)* larvae at 3 dpf for 48 hours with 7.5 mM MTZ or 5 μM NFP (Figure 2B). Normal non ablated dat:CFP-NTR+ cells are identified by a round-shaped nucleus surrounded by a compact cytoplasm (yellow asterisks, Figure 2B). Although there is a reduction in the number of CFP-positive dopaminergic neurons in both cases, MTZ-treated larvae still harbor abundant normal cells compared to NFP (yellow asterisks, Figure 2B). In contrast, NFP-treated larvae show more cell debris and large CFP+ cells with a large cytoplasm and irregular nucleus (yellow arrows, Figure 2B) which are presumably phagocytic cells having engulfed apoptotic CFP neurons (as described in Ref. 16). Furthermore, NFP-treated larvae display much fewer cells with a normal morphology as compared to MTZ-treated larvae, underpinning a more efficient ablation with NFP. Quantification of all CFP+

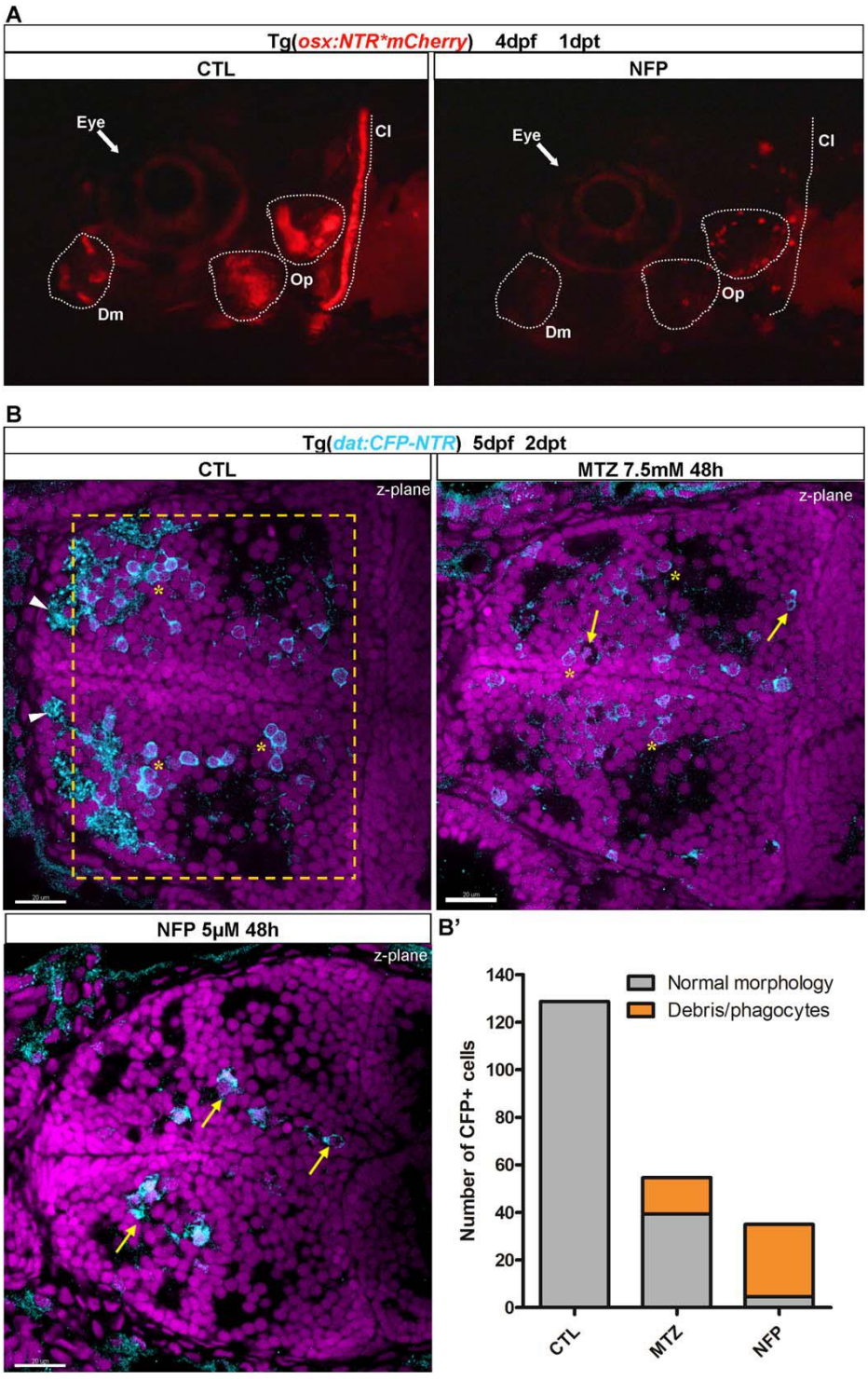

A
Tg(osx:NTR*mCherry) 4dpf 1dpt
CTL
NFP
Eye
Cl
Op
Dm
B
Tg(dat:CFP-NTR) 5dpf 2dpt
CTL
MTZ 7.5mM 48h
NFP 5µM 48h
z-plane
B'
Normal morphology
Debris/phagocytes
Number of CFP+ cells
140
120
100
80
60
40
20
0
CTL
MTZ
NFP

Figure 2. Nifurpirinol induces effective ablations in other NTR-mediated ablation models. (A) Ablation of osteoblasts with NFP in 4 dpf *Tg(osx:mcherry-NTR)* treated from 3 to 4 dpf, compared to control (CTL) (stereomicroscope live images). Bone pieces: Cleitrum (Cl), Dento-Maxilar (Dm), Opercular (Op). (B) Comparison of ablation efficiency of MTZ and NFP on dopaminergic neurons (Cyan) in the diencephalon of *Tg(dat:CFP-NTR)* treated with 7.5 mM MTZ or 5 μM NFP from 3 to 5 dpf. Representative individuals are shown with one representative z-plane. Dapi for nuclei staining is shown in magenta. Yellow arrows point at debris of dopaminergic neurons and at CFP+ phagocytes. Yellow asterisks point at viable CFP+ cells. Confocal images, scale bar = 20 μm. Anterior to the left. (B') Graph showing the number of CFP+ cells subdivided in cells presenting a normal morphology (gray bars) and debris/phagocytes (orange bars), quantified in the region delimitated by the yellow frame ($z = 40$ μm depth). Kruskal–Wallis $p < 0.0001$ and Dunn's post hoc test: CTL versus NFP normal cells $p < 0.001$; CTL vs. NFP debris/phagocytes $p < 0.001$; CTL versus MTZ: ns. The nuclei-free CFP+ anterior region in CTL samples was not taken into account in the quantifications (white arrowheads).

cells (Figure 2B') shows that MTZ treated larvae ($n = 8$) still harbor ~30% (in gray, mean 39 vs. 129 cells in CTL) of morphologically intact dopaminergic neurons as compared to control ($n = 8$), while NFP ($n = 7$) treated larvae are mostly devoid of morphologically normal cells. The nuclei-free CFP+ anterior region in CTL samples has not been taken into account in the quantifications (white arrow heads). Similar results were already observed after 24 hours treatment with NFP (not shown), suggesting that efficient ablation of dopaminergic neurons can be achieved with NFP after shorter treatments compared to MTZ. Together, these results indicate that NFP can efficiently trigger NTR-mediated cell ablations in different models and might be especially of interest in cell types which need nearly toxic MTZ conditions to induce ablation.

DISCUSSION

To improve ablations with the NTR system, we identified the nitroaromatic antibiotic nifurpirinol (NFP) as a novel and potent substrate for the NTR enzyme and showed its efficiency in three different cell types. NFP triggers cell-specific ablation at much lower concentration than MTZ (2000 x) and at doses at least twofold below its toxic concentrations, NFP-mediated ablation is performed through apoptosis and does not induce bystander effect. These results make NFP a more efficient and versatile prodrug compared to MTZ for NTR-mediated cell ablations and regeneration studies.

NFP shows not only more efficiency but also more reproducible ablations between individuals, as illustrated by the dose response on beta cell ablation. Indeed, in our hands, MTZ shows high variability in ablation efficiencies and needs thorough screening to ensure effective ablations. This is a considerable issue when performing high throughput studies or in adult fish where ablation efficiency is difficult to monitor precisely prior to analysis of regeneration. Furthermore, NFP is already effective at 2.5 μM and can be used at concentrations up to 10 μM for 24 hours without deleterious effect on viability. In contrast, the most efficient dose of MTZ, 10 mM, still yields inconsistent ablations but cannot be increased because of toxicity issues.[8] A potent and nontoxic NTR substrate is desirable when other manipulations such as combined treatments with other pharmacological drugs to investigate regenerative processes are performed. A range of 2.5 to 10 μM of effective concentrations also means that NFP offers the choice to determine the optimal dose for ablation which might be cell-dependent. Our study shows that the NFP/NTR combination is effective not only for pancreatic beta cells but is extendable to other ablation models like osteoblasts and, most importantly, for dopaminergic neurons for which ablation appears tricky and harsher MTZ regimens proved to be mandatory.[12]

Historically, the NTR-mediated cell ablation method was first developed in a cell culture system[17] before being adapted in mammals[18] and then seemed to be very sporadically used in the latter whereas it was taken over by the zebrafish community. This might be explained by the fact that initial studies used the prodrug CB1954 which has a strong bystander effect.[19] For about 10 years the model has been developed in zebrafish with a multitude of cell-specific transgenic lines and has seen significant improvement with the enzymatic NTR mutant.[8] Here, we propose NFP as a novel potent prodrug to overcome limitations such as poor/inconsistent ablation efficiencies and toxicity linked to MTZ. In light of its widespread use in zebrafish and of such improvements, it would also be commendable to reconsider the system for wider use in mammal models.

ACKNOWLEDGMENTS

We would like to thank the GIGA-Zebrafish (H. Pendeville) and GIGA-imaging facility (S. Ormenese and J-J. Goval). We are grateful to Jeff .S. Mumm for providing the plasmid construct containing the triple NTR mutant. We thank Didier Stainier for generous gift of transgenic line Tg(*ins:dsRed*), Marc Muller and Jörg Renn for providing and advice on transgenic line Tg(*osx:mCherryNTR*), and Marc Ekker for kindly providing Tg(*dat:CFP-NTR*).

Source of Funding: Université de Liège, Fonds Spéciaux de la Recherche- Crédit classique 0000005312; Belgian Fonds pour la Formation à la Recherche dans l'Industrie et dans l'Agriculture (FRIA), 0000009232; Belgian Fonds De La Recherche Scientifique FRS – FNRS, Crédit de recherche 0000006539, Mandat chercheur qualifié 0000009017. D. Bergemann was supported by FRIA (Fonds pour la Formation à la Recherche dans l'Industrie et dans l'Agriculture) and the Léon Fredericq Funds (University of Liège). I. Manfroid, M. Voz and B. Peers are "Chercheur Qualifié" at FRS-FNRS. C. Carril Pardo is supported by a doctoral Becas Chile fellowship from CONYCIT. This work was also supported by the ARC program of the University of Liège (Action de Recherches Concertées).

Conflicts of Interest: The authors have no conflict of interest to declare.

REFERENCES

1. Poss KD, Wilson LG, Keating MT. Heart regeneration in zebrafish. *Science* 2002; 298: 2188–90.

2. Becker T, Wullimann MF, Becker CG, Bernhardt RR, Schachner M. Axonal regrowth after spinal cord transection in adult zebrafish. *J Comp Neurol* 1997; 377: 577–95.
3. Curado S, Anderson RM, Jungblut B, Mumm J, Schroeter E, Stainier DY. Conditional targeted cell ablation in zebrafish: a new tool for regeneration studies. *Dev Dyn* 2007; 236: 1025–35.
4. Pisharath H, Rhee JM, Swanson MA, Leach SD, Parsons MJ. Targeted ablation of beta cells in the embryonic zebrafish pancreas using *E. coli* nitroreductase. *Mech Dev* 2007; 124: 218–29.
5. White DT, Mumm JS. The nitroreductase system of inducible targeted ablation facilitates cell-specific regenerative studies in zebrafish. *Methods* 2013; 62: 232–40.
6. Lambert AM, Bonkowsky JL, Masino MA. The conserved dopaminergic diencephalospinal tract mediates vertebrate locomotor development in zebrafish larvae. *J Neurosci* 2012; 32: 13488–500.
7. Hsu C-C, Hou M-F, Hong J-R, Wu J-L, Her GM. Inducible male infertility by targeted cell ablation in zebrafish testis. *Mar Biotechnol* 2010; 12: 466–78.
8. Mathias JR, Zhang Z, Saxena MT, Mumm JS. Enhanced cell-specific ablation in zebrafish using a triple mutant of *Escherichia coli* nitroreductase. *Zebrafish* 2014; 11: 85–97.
9. Kwan KM, Fujimoto E, Grabher C, Mangum BD, Hardy ME, Campbell DS, et al. The Tol2kit: a multisite gateway-based construction kit forTol2 transposon transgenesis constructs. *Dev Dyn* 2007; 236: 3088–99.
10. Singh SP, Holdway JE, Poss KD. Regeneration of amputated zebrafish fin rays from de novo osteoblasts. *Dev Cell* 2012; 22: 879–86.
11. Anderson RM, Bosch JA, Goll MG, Hesselson D, Dong PDS, Shin D, et al. Loss of Dnmt1 catalytic activity reveals multiple roles for DNA methylation during pancreas development and regeneration. *Dev Biol* 2009; 334: 213–23.
12. Godoy R, Noble S, Yoon K, Anisman H, Ekker M. Chemogenetic ablation of dopaminergic neurons leads to transient locomotor impairments in zebrafish larvae. *J Neurochem* 2015; 135: 249–60.
13. Ghaye AP, Bergemann D, Tarifeño-Saldivia E, Flasse LC, Von Berg V, Peers B, et al. Progenitor potential of nkx6.1-expressing cells throughout zebrafish life and during beta cell regeneration. *BMC Biol* 2015; 13: 70.
14. S M, Takase Y, Kubota SS. The absorption and distribution of a chemotherapeutic agent, P-7138 in fishes. *Bull Jap Soc Fish* 1968; 34: 1118.
15. Provost E, Rhee J, Leach SD. Viral 2A peptides allow expression of multiple proteins from a single ORF in transgenic zebrafish embryos. *Genesis* 2007; 45: 625–9.
16. van Ham TJ, Brady CA, Kalicharan RD, Oosterhof N, Kuipers J, Veenstra-Algra A, et al. Intravital correlated microscopy reveals differential macrophage and microglial dynamics during resolution of neuroinflammation. *Dis Model Mech* 2014; 7: 857–69.
17. Bridgewater JA, Springer CJ, Knox RJ, Minton NP, Michael NP, Collins MK. Expression of the bacterial nitroreductase enzyme in mammalian cells renders them selectively sensitive to killing by the prodrug CB1954. *Eur J Cancer* 1995; 31: 2362–70.
18. Clark AJ, Iwobi M, Cui W, Crompton M, Harold G, Hobbs S, et al. Selective cell ablation in transgenic mice expression *E. coli* nitroreductase. *Gene Ther* 1997; 4: 101–10.
19. Bridgewater JA, Knox RJ, Pitts JD, Collins MK, Springer CJ. The bystander effect of the nitroreductase/CB1954 enzyme/prodrug system is due to a cell-permeable metabolite. *Hum Gene Ther* 1997; 8: 709–17.

2. Unravelling the role of candidate signalling pathways in β cell regeneration from the ducts

In order to get an overview of molecular pathways regulating β cell regeneration, the transcriptomic profile of ductal cell during β cell regeneration was previously established in the laboratory by David Bergemann. These data showed a strong proliferative response and counteracting mechanisms as the cellular senescence and p53. Among genes regulated in these pathways, calcineurin (CaN) appeared as an compelling candidate. The main goal of this present work was to investigate the function of CaN in β cell regeneration from ductal progenitors. Furthermore, we next investigated the function of p53 in β cell regeneration.

2.1. Calcineurin

We showed that the phosphatase calcineurin fine tunes the balance between progenitor proliferation and endocrine differentiation to ensure a proper regenerative response. These data are presented here in an article entitled " Negative cell cycle regulation by Calcineurin is necessary for proper β cell regeneration in zebrafish" currently under review for eLife. I contributed to this work by designing the experiments, carrying out every experiments excepted the RNA-sequencing, analysing the data and writing the manuscript.

Negative cell cycle regulation by Calcineurin is necessary for proper β cell regeneration in zebrafish

Laura Massoz[1*], David Bergemann[1], Arnaud Lavergne[1,2], Célia Reynders[1], Caroline Désiront[1], Chiara Goossens[1], Lydie Flasse[1], Bernard Peers[1], Marianne L. Voz[1] and Isabelle Manfroid[1]

Author Affiliations

[1]Zebrafish Development and Disease Models laboratory, GIGA-Stem Cells, University of Liège, Liège, Belgium

[2]GIGA-Genomics core facility, GIGA, University of Liège, Liège, Belgium

*Corresponding author:

Email address: laura.massoz@doct.uliege.be

Abstract

Stimulation of pancreatic β cell regeneration could be a therapeutic lead to treat diabetes. Unlike humans, the zebrafish can efficiently regenerate β cells, notably from ductal pancreatic progenitors. To gain insight into the molecular pathways involved in this process, we established the transcriptomic profile of the ductal cells after β cell ablation in the adult zebrafish. These data highlighted the protein phosphatase calcineurin as a new potential modulator of β cell regeneration. We showed that calcineurin overexpression abolished the regenerative response, leading to glycemia dysregulation. On the opposite, calcineurin inhibition increased ductal cell proliferation and subsequent β cell regeneration. Interestingly, the enhanced proliferation of the progenitors was paradoxically coupled with their exhaustion. This suggests that the proliferating progenitors are next entering in differentiation. Calcineurin appears as a guardian, which prevents an excessive progenitor proliferation to preserve the pool of progenitors. Altogether, our findings reveal calcineurin as a key player in the balance between proliferation and differentiation to enable a proper β cell regeneration.

Introduction

Blood glucose homeostasis is tightly controlled by pancreatic endocrine cells. Insulin-producing β cells work in close association with α cells, which secrete glucagon, and δ cells secreting somatostatin, to maintain normal glycemia. The insulin plays a critical role in this process as it is the only hormone able to lower the glycemia. B cell loss is a hallmark of type 1 and of late stages of type 2 diabetes, leading to chronic hyperglycemia. B cell destruction is largely irreversible in human and in mammals, making the disease incurable nowadays. Nevertheless, studies in diabetic mice models uncovered promising evidences of slight recovery of β cells via regeneration. For example, new β cells can arise from the replication of remaining β cells (Dor et al. 2004). Different models of pancreas injuries revealed the plasticity of mammalian pancreatic cells. Actually, differentiated endocrine cells such as α cells (Thorel, Nepote, et al. 2010) and δ cells (Chera et al. 2014a) can reprogram and convert into insulin-producing cells, a process that is age dependent. Another possible source of β cells could be de *novo* formation from pancreatic progenitors residing in the ductal compartment of the adult pancreas (X. Xu et al. 2008). Lineage tracing experiments could not clearly highlight new β cells arising from the ductal cells in the adult (Solar et al. 2009b; X. Xu et al. 2008; Zhao et al. 2021). However, expression of the pro-endocrine marker Neurog3 was detected in the ducts in different mouse models of pancreas regeneration (Courtney et al. 2013; Kopp et al. 2011; X. Xu et al. 2008). In addition, a rare population of ductal cells expressing Neurog3 has been

reported to contribute to β cell neogenesis during diabetes (Gribben et al. 2021). A recent study of single cell RNA sequencing reveal a sub-population of human ductal cells that are able to give rise to all the pancreatic cell types, including β cells, following implantation in mice (Qadir et al. 2020). Altogether, these findings suggest that mammalian pancreatic ducts possess the intrinsic capacity to (re)generate β cells even though this process is poorly efficient and slow, especially in adults. In contrast to mammals, zebrafish possess remarkable capacity of regeneration, independently of its age (Choi et al. 2014; Gemberling et al. 2013; Yona Goldshmit et al. 2012; Kroehne et al. 2011; Moss et al. 2009; Poss, Wilson, and Keating 2002). This model can therefore be exploited to identify and characterize regenerative mechanisms and ultimately induce regeneration in mammals. Based on regenerative mechanisms identified in zebrafish, several studies succeed to improve regeneration in mammals, underlying the possibility of translation from zebrafish to mammals ((Fausett, Gumerson, and Goldman 2008; Y Goldshmit et al. 2015; Ko et al. 2016; Mashkaryan et al. 2020; Papadimitriou et al. 2018) and (Massoz, Dupont, and Manfroid 2021), for review).

In zebrafish, the nitroreductase (NTR)/nitroaromatic prodrug system is widely used. In this technique, the combination of cell type-specific NTR expression and nitroaromatic prodrug exposure allow for controlled and targeted cell ablation (Curado et al. 2007; Harshan Pisharath 2007). When NTR is expressed under the control of the *insulin* (*ins*) promoter (*Tg*(*ins:NTR-mCherry*) (Harsan Pisharath et al. 2007)), β cell destruction is complete within 3 days following the treatment in adult fish, which correlate with a peak of hyperglycemia (Delaspre et al. 2015; Aurélie P. Ghaye et al. 2015). The regeneration of β cells upon ablation is spontaneous and fast and the glycemia is normalized within two weeks [13], [29]. Similar to mice, new β cells can arise through proliferation of surviving β cells (Andersson et al. 2012) as well as through the contribution of α cells (Helker et al. 2019; Ye et al. 2015) and δ cells (Carril Pardo et al. 2022; Singh et al. 2022), underscoring the overall conservation of these processes from zebrafish to mammals. Nevertheless, unlike mammals, the presence of pancreatic progenitors in the ducts is well established in both larval (Ninov et al. 2013) and adult zebrafish (Delaspre et al. 2015; Aurélie P. Ghaye et al. 2015). Lineage tracing experiments pointed out that ductal cells and centroacinar cells (CACs) give rise to new β cells (Delaspre et al. 2015; Ninov et al. 2013). In adults, duct-derived β cells start to be detected between 7 and 10 days following β cell ablation (Delaspre et al. 2015; Aurélie P. Ghaye et al. 2015).

Notch signaling is a key player regulating the differentiation of duct-associated progenitors into endocrine cells. Larval duct cells as well as adult CACs display strong Notch activity (Delaspre

et al. 2015; Parsons et al. 2009). This signaling pathway has a central role in β cell genesis during both development (Ninov, Borius, and Stainier 2012) and regeneration (Ninov et al. 2013) by repressing endocrine differentiation. In zebrafish, different levels of Notch activity determine the behavior of the pancreatic progenitors. While a high level of Notch activity maintains cells in quiescence, a moderate level induces the entry in the cell cycle and proliferation whereas a low level drives endocrine differentiation of the progenitor cells (Ninov, Borius, and Stainier 2012). A steep decrease of Notch activity pushes the progenitors to differentiate prematurely, bypassing the amplification step and leading to their depletion (Ninov, Borius, and Stainier 2012). Repression of the Notch signaling by mTor, activated by glucose and nutrients, hence promotes β cell formation and regeneration from ductal progenitors (Ninov et al. 2013). While Notch and mTor signaling are crucial for this process, there is still a need to establish a global view of the molecular mechanisms regulating β cell regeneration.

To identify early events regulating ductal-derived β cell regeneration, we determined the transcriptomic signature of ductal cells from adult zebrafish following β cell destruction. Our data highlighted an upregulation of the Calcineurin (CaN) pathway. To elucidate CaN function in β cell regeneration, we both repressed and activated CaN pathway. We showed that CaN regulates β cell neogenesis in the ducts during regeneration, by modulating progenitor proliferation. Together, our findings underline that CaN fine tunes the balance between progenitor proliferation and β cell differentiation to guarantee proper regeneration.

Results

Transcriptomic profiling of ductal cells after β cell destruction highlights regulation of calcineurin pathway

To gain a better understanding of the molecular mechanisms underlying the regeneration of β cells from the ducts, we determined the transcriptional landscape of ductal cells by RNA-sequencing after β cell ablation in adults. To selectively ablate the β cells, we used the *Tg*(*ins:NTR-mCherry*) transgenic fish and the ductal cells were labelled through the *Tg*(*nkx6.1:GFP*) reporter line (Aurélie P. Ghaye et al. 2015) in which GFP marks the ductal tree and associated multipotent pancreatic progenitors (Aurélie P. Ghaye et al. 2015). More precisely, 3 to 4 fishes were treated with the pro-drug MTZ at 10mM overnight, to induce ablation of β cells or with DMSO for the non-ablated controls. Ablation was confirmed by blood glucose measurement before collection of the pancreas. To capture the early events triggered

by the destruction of β cells, we generated the transcriptome of the ducts 3 days post ablation treatment (dpt), *i.e.* before β cell neogenesis. Differential gene expression analysis revealed that 1866 genes are up-regulated and 1515 genes down-regulated in the ductal cell of fish treated with MTZ compared to control ($pAdj < 0.05$). According to Gene Ontology analysis, the most enriched pathways among the up-regulated genes were DNA replication and cell cycle (**Figure 1A**). This further corroborates our previous findings and those of others regarding the activation of duct-associated progenitors' proliferation in response to β cell ablation (Delaspre et al. 2015; Aurélie P. Ghaye et al. 2015). As expected, the Notch pathway was enriched in the down-regulated genes (Ninov et al. 2013; Ninov, Borius, and Stainier 2012) (**Figure 1B**). Intriguing in a context of strong proliferative response, we found an enrichment of the cellular senescence signature (**Figure 1A**). We looked further in the genes associated with this signature.

Figure 1

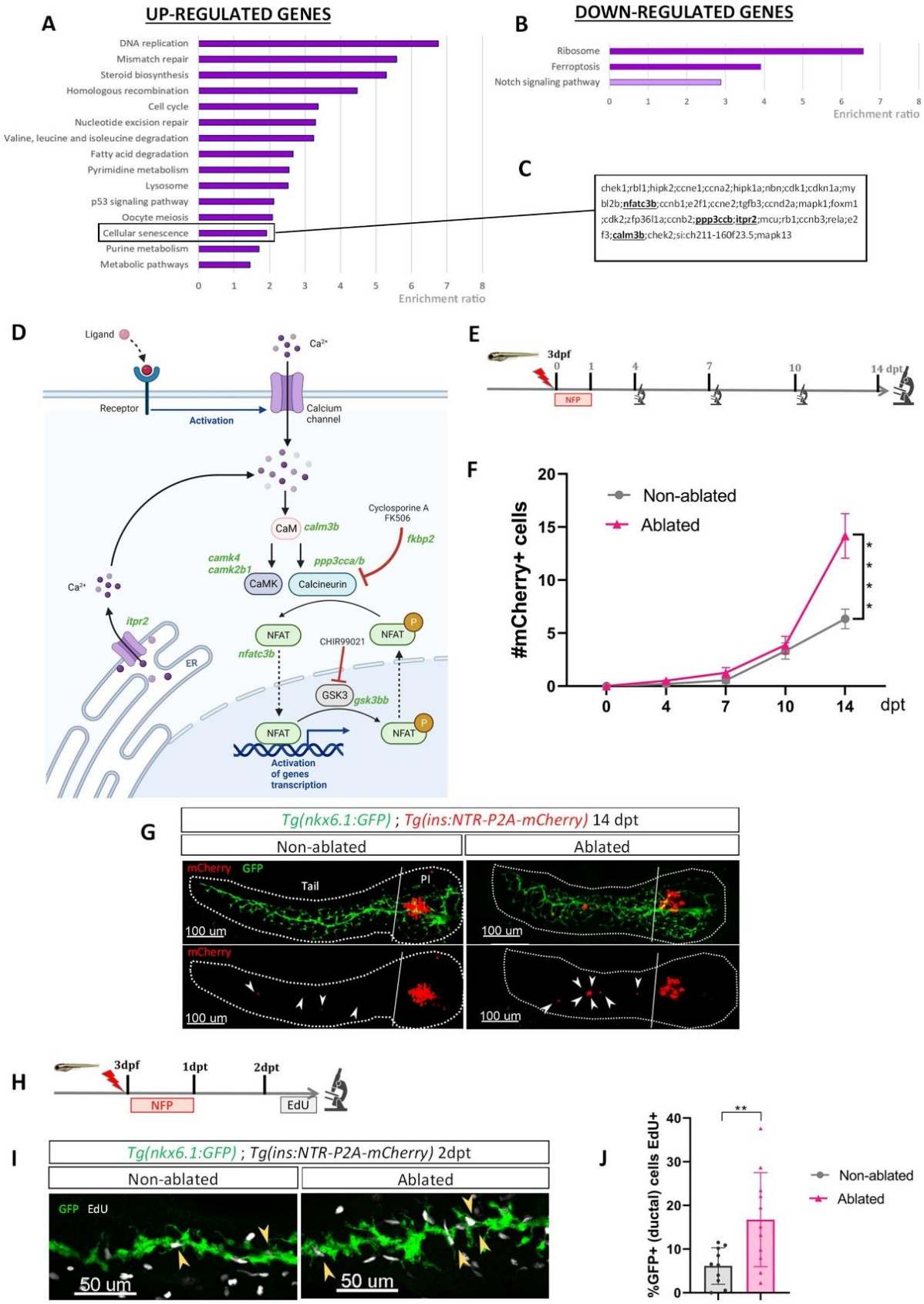


Figure 1 : Transcriptomic profiling of ductal cells during β cell regeneration and validation in larvae

A-B) Enrichment ratio of selected non-redundant signatures of KEGG pathways overrepresented in ductal cells after β cells ablation (UP – A and DOWN -B) compared to ductal cells without β cells ablation. Gene Ontology (GO) terms were identified using ORA analysis by WebGestalt (Liao et al., 2019) using the list of DE genes provided by DESeq. The light color for Notch pathway means p-value = 0.11.

C) List of genes associated with the signature of cellular senescence from A-B. Genes related to CaN pathway are in bold.

D) Calcineurin canonical pathway with genes in green that are modulated in transcriptomic data from A-B.

E) Experimental design for regeneration test in larvae. Briefly, after nifurpirinol treatment from 3 to 4dpf, larvae were fixed and analyzed at 4-7-10 and 14 days post treatment (dpt).

F) Graph representing the mean number of mCherry+ β cells in the pancreatic tail of Tg(ins:NTR-P2A-mCherry) ; Tg(nkx6.1:GFP) at 0-4-7-10 and 14 dpt. The gray spheres represent non-ablated conditions and the pink triangles the ablated condition. Data are presented as mean values ± SEM. One-way ANOVA test with Tukey's multiple comparison test, ****p-value<0.0005.

G) Whole mount fluorescent immunohistochemistry (GFP and mCherry) of the pancreas of Tg(ins:NTR-P2A-mCherry) ; Tg(nkx6.1:GFP) larvae at 14dpt. 3D projection (stack) of one non-ablated and one ablated representative samples. The principal islet (PI) and the pancreatic tail are shown. Arrows point out mCherry+ β cells in the pancreatic tail. Scale 100µM.

H) Experimental design for EdU assay in larvae. After NFP treatment for 3 to 4dpf, larvae were exposed to EdU at 2dpt before fixation for analysis.

I) Whole mount fluorescent immunohistochemistry (GFP and EdU) of the pancreatic tail of Tg(ins:NTR-P2A-mCherry) ; Tg(nkx6.1:GFP) larvae at 2dpt. 3D projection (stack) of one non-ablated and one ablated representative samples. Arrows point out GFP+ duct cells EdU+ in the pancreatic tail. Scale 50µM.

J) Barplot representing the percentage of GFP+ ductal cells which incorporated EdU+ in non-ablated and ablated conditions. Data are presented as mean values ± SD. T-test. **p-value<0.005.

Among them were found several components of the calcineurin signaling pathway such as *nfatc3b*, *ppp3ccb* (the catalytic subunit of Calcineurin*), itpr2* and *calm3b* **(Figure 1C)**. In addition to these genes related to cellular senescence, our RNAseq studies revealed the modulation of other genes from the CaN canonical pathway (**Figure 1D, genes in green**), underlying its potential role in β cell regeneration. Calcineurin (CaN) is a highly conserved calcium/calmodulin dependent Ser/Thr phosphatase, involved in numerous biological process including fin regeneration and β cell function, (Cao et al. 2021; Kujawski et al. 2014; McMillan et al. 2018; Tornini et al. 2016). This prompted us to investigate the role of CaN in β cell regeneration

Previous RNAseq data performed in our laboratory indicate that CaN (*ppp3cca/b*) and NFATc3 (*nfatc3a/b*) are mainly expressed in endocrine cells (Tarifeño-Saldivia et al. 2017) (**Figure 1A supplemental**), which is in accordance with the role of CaN/NFAT signaling in β cells (Heit et al. 2006). CaN genes (*ppp3cca/b*) as well as *nfatc3b,* are express at lower levels in the ducts at basal state but their expression is induced in response to β cell destruction (**Figure 1B supplemental**).

Calcineurin activity regulates the ductal regenerative response

To evaluate the role of CaN in β cell regeneration, more specifically derived from ductal progenitors, we have chosen to use young larvae, where regenerated β cells in the pancreatic tail arise exclusively from the ducts (Ninov et al. 2013). In response to β cells ablation, the intrapancreatic ducts undergo a ductal regenerative response whereby differentiation toward the endocrine fate is increased (Ninov et al. 2013). We first determined the rate of β cell neogenesis from the ducts in response to a single acute ablation of β cells as we performed in adults. We treated *Tg*(*ins:NTR-P2A-mCherry*); *Tg*(*nkx6.1:GFP*) larvae with nifurpirinol (NFP) from 3 to 4 dpf, mCherry+ β cells were quantified in the GFP+ ducts present in the tail at several time points: 4, 7, 10 and 14 dpt (**Figure 1E**). Duct-associated β cells started to be detected in non-ablated larvae between 7 and 10 dpt (**Figure 1F**) and the number of β cells slowly increased until 14 dpt (**Figure 1F, 1G**). In ablated larvae, the increase became more pronounced from 10 dpt onwards (**Figure 1F, 1G**), indicating faster endocrine differentiation. This experiment establishes that the ductal regenerative response is detectable between 10 and 14 days after the ablation of β cells, performed at 3 dpf. We next wanted to determine if ductal cell proliferation is activated in response to β cell destruction in larvae as in adult fish. We exposed *Tg(ins:NTR-P2A-mCherry); Tg(nkx6.1:GFP)* larvae to EdU the second day following ablation (**Figure 1H**). In ablated larvae, the proportion of GFP+ ductal cells EdU+ (in S-phase) was higher compared

to non-ablated larvae (**Figure 1I, 1J**). This result shows that acute β cell ablation in larvae rapidly activates ductal cell proliferation, as previously reported in adult zebrafish.

As our transcriptomic data from adult zebrafish revealed modulation of CaN pathway at 3 dpt, we treated *Tg*(*ins:NTR-P2A-mCherry*); *Tg*(*nkx6.1:GFP*) larvae from 1 to 3 dpt after β cell ablation, with a CaN inhibitor, the Cyclosporin A (CsA) (Kujawski et al. 2014) (**Figure 2A**). The number of newly formed β cells was monitored in the tail from 4 to 14 dpt. CsA enhanced β cell formation at 10 dpt (**Figure 2B-D)**. However, this effect appears to be transient since no discernible difference was observed between the control and CsA-treated larvae in regeneration at the latest time point, suggesting an acceleration of the regenerative response (**Figure 2B, Figure 2A supplemental**). Interestingly, CsA did not affect β cell differentiation in non-ablated larvae indicating that CsA only acts in a regenerative context (**Figure 2B-D)**. Of note, CsA increased as well the number of regenerated β cells in the principal islet (**Figure 2B supplemental**).

We next tested the effect of CsA on endocrine progenitors in a regenerative context. We induced regeneration in Tg(*neurod1:GFP*) larvae where the GFP is expressed in both endocrine progenitors and mature endocrine cells. We first assessed generation of GFP cells at different time points (**Figure 2D supplemental)** and showed that CsA induced an increase of neurod1+ cells from 4 dpt. The increase was still detectable at least until 10dpt (**Figure 2E, Figure 2E supplemental**). To determine if these additional cells result from their own proliferation, we performed a pulse of EdU just before analysis (**Figure 2D supplemental**). We observed that CsA did not affect the neurod1+ proliferation rate that is very low at these stages (**Figure 2F**). As a consequence, the effect of CsA cannot be explained by endocrine cell proliferation but rather by neogenesis from progenitors. As CsA affects pro-endocrine cells formation, we next wondered if the increased cell formation induced by CsA is specific to β cells. Treatment with CsA was performed as previously and δ and α cells were detected by immunofluorescence. Interestingly, CsA did not affect α nor δ1.1 cells neogenesis in response to β cell ablation (**Figure 2F-G supplemental**). Overall, these experiments showed that CsA affects specifically the β cells and their endocrine progenitors.

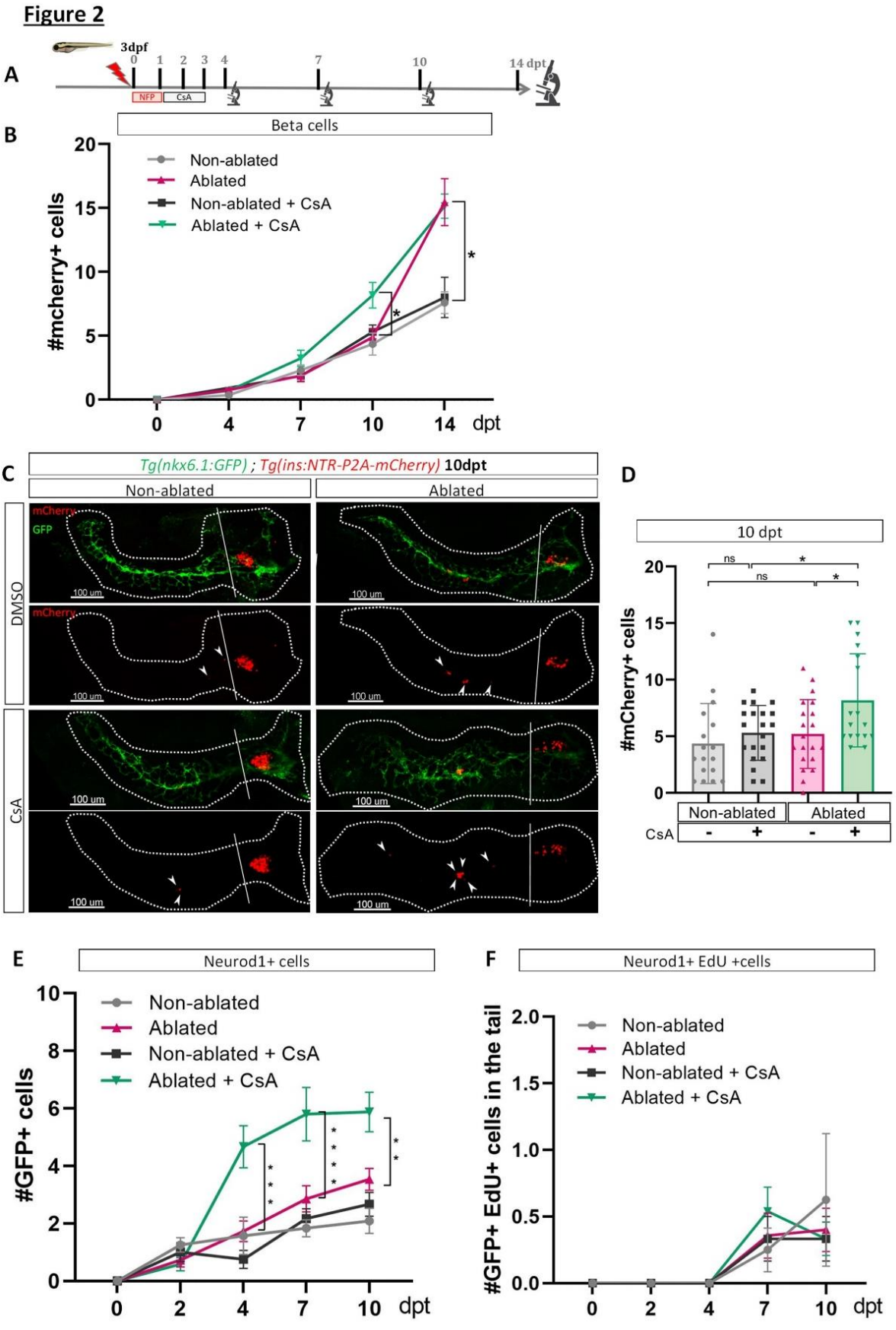


Figure 2 : *Calcineurin inhibition with CsA increases the ductal regenerative response*

A) Experimental design for regeneration test in larvae with CsA treatment. Briefly, after nifurpirinol treatment from 3 to 4dpf, larvae were treated with CsA from 1 to 3dpt and fixed and analyzed at 4-7-10 and 14 days post treatment (dpt).

B) Graph representing the mean number of mCherry+ β cells in the pancreatic tail of *Tg(ins:NTR-P2A-mCherry) ; Tg(nkx6.1:GFP)* at 0-4-7-10 and 14 dpt. The gray spheres represent non-ablated condition ; the pink triangles represent the ablated condition ; the black squares CsA condition and inverted green triangles ablated + CsA condition. Data are presented as mean values ± SEM. Two-way ANOVA test with Sidak's multiple comparisons test, *p-value<0.05.

C) Whole mount fluorescent immunohistochemistry (GFP and mCherry) of the pancreas of *Tg(ins:NTR-P2A-mCherry) ; Tg(nkx6.1:GFP)* larvae at 10dpt. 3D projection (stack) of non-ablated and ablated larvae treated with DMSO or CsA representative samples. The principal islet (PI) and the pancreatic tail are shown. Arrows point out mCherry+ β cells in the pancreatic tail. Scale 100µM.

D) Barplot representing the number of number of mCherry+ β cells in the pancreatic tail of *Tg(ins:NTR-P2A-mCherry) ; Tg(nkx6.1:GFP)* larvae at 10dpt. The gray spheres represent non-ablated condition ; the pink triangles represent the ablated condition ; the black squares CsA condition and inverted green triangles ablated + CsA condition. Data are presented as mean values ± SD. Two-way ANOVA with Tukey's multiple comparison test, *p-value<0.05.

E) Graph representing the mean number of GFP+ neurod1+ cells in the pancreatic tail of *Tg(ins:NTR-P2A-mCherry) ; Tg(neurod1:GFP)* at 0-4-7 and 10 dpt. The gray spheres represent non-ablated condition ; the pink triangles represent the ablated condition ; the black squares CsA condition and inverted green triangles ablated + CsA condition. Data are presented as mean values ± SEM. Two-way ANOVA test with Sidak's multiple comparisons test, **p-value<0.005; *** p-value<0.0005; **** p-value<0.00005.

F) Graph representing the mean number of GFP+ neurod1 EdU+ cells in the pancreatic tail of *Tg(ins:NTR-P2A-mCherry) ; Tg(neurod1:GFP)* at 0-4-7 and 10 dpt. The gray spheres represent non-ablated condition ; the pink triangles represent the ablated condition ; the black squares CsA condition and inverted green triangles ablated + CsA condition. Data are presented as mean values ± SEM.

CaN over-activation abolishes the regenerative response

We then wondered if an opposite regulation of CaN *i.e.* its activation impacts as well the regenerative response. To that end, we generated a transgenic line Tg(*hsp70:GFP-P2A-ppp3ccCA*) that allows ubiquitous expression of a constitutively active form of CaN^{CA} (*ppp3cc*^{CA}) upon heat shocks. β cell ablation was triggered in Tg(*hsp70:GFP-P2A-ppp3cc*^{CA}); Tg(*ins:NTR-P2A-mCherry*); Tg(*nkx6.1:GFP*) larvae from 3 to 4 dpf and CaN^{CA} expression was induced by four successive heat-shocks from 1 to 3 dpt (**Figure 3A**). The overexpression of CaN^{CA} after ablation impaired the regenerative response at 14 dpt (**Figure 3B-C**). Similar results were obtained with Tg(*UAS:GFP-P2A-ppp3cc*^{CA}); Tg(*cftr:gal4*)[45] ; Tg(*ins:NTR-P2A-mCherry*) larvae in which CaN^{CA} is constitutively and specifically overexpressed in the ducts within the pancreas (**Figure 3D-E**). Importantly, the structure of the ducts was similar in CaN^{CA} overexpressing larvae compared to Tg(*nkx6.1:GFP*) controls (**Figure 3D**), suggesting that the suppression of the regenerative response in CaN^{CA}-overexpressing larvae was not due to morphogenetic defects during ductal growth. This result strongly suggest that CaN acts directly in the ducts to regulate β cell regeneration while it is not necessary for normal β cell differentiation.

Figure 3

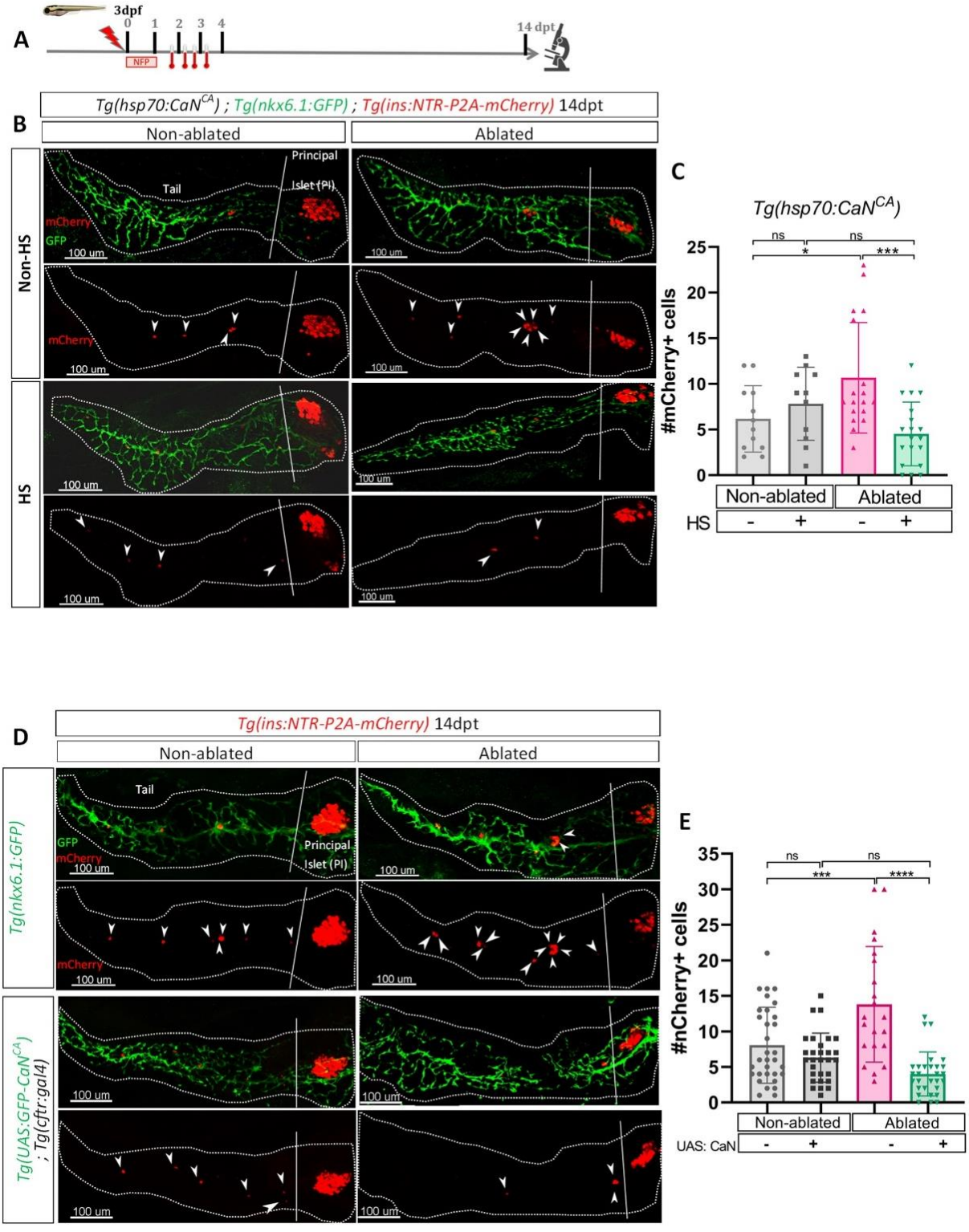


Figure 3: *Transgenic mediated overexpression of calcineurin abolish the ductal regenerative response*

A) Experimental design for regeneration test in larvae with heat-shocks. Briefly, after nifurpirinol treatment from 3 to 4dpf, four heat shock were performed from 1 to 3dpt and larvae were fixed and analyzed at 14dpt.

B) Whole mount fluorescent immunohistochemistry (GFP and mCherry) of the pancreas of *Tg(hsp70:CaN^{CA})*; *Tg(ins:NTR-P2A-mCherry); Tg(nkx6.1:GFP)* larvae at 14dpt. 3D projection (stack) of one non-ablated and one ablated with or without heat-shock representative samples. The principal islet (PI) and the pancreatic tail are showed. Arrows point out mCherry+ β cells in the pancreatic tail. Scale 100µM.

C) Barplot representing the mean number of mCherry+ β cells in the pancreatic tail of *Tg(hsp70:CaN^{CA})*; *Tg(ins:NTR-P2A-mCherry); Tg(nkx6.1:GFP)* larvae at 14dpt. The gray spheres represent non-ablated condition ; the pink triangles represent the ablated condition ; the black squares heat-shock condition and inverted green triangles ablated + heat-shock condition. Data are presented as mean values ± SD. Two-way ANOVA with Tukey's multiple comparisons test, *p-value<0.05, *** p-value<0.0005, ns = non-significant.

D) Whole mount fluorescent immunohistochemistry (GFP and mCherry) of the pancreas of larvae at 14dpt. 3D projection (stack) of *Tg(ins:NTR-P2A-mCherry) ; Tg(nkx6.1:GFP)* one non-ablated and one ablated representative control samples and *Tg(UAS:CaN^{CA})*; *Tg(ins:NTR-P2A-mCherry) ; Tg(cftr:gal4)* one non-ablated and one ablated representative samples. The principal islet (PI) and the pancreatic tail are showed. Arrows point out mCherry+ β cells in the pancreatic tail. Scale 100µM.

E) Barplot representing the mean number of mCherry+ β cells in the pancreatic tail of larvae at 14dpt. The gray spheres represent non-ablated *Tg(ins:NTR-P2A-mCherry) ; Tg(nkx6.1:GFP)* condition ; the pink triangles represent the ablated *Tg(ins:NTR-P2A-mCherry) ; Tg(nkx6.1:GFP)* condition ; the black squares non-ablated *Tg(UAS:CaN^{CA})*; *Tg(ins:NTR-P2A-mCherry) ; Tg(cftr:gal4)* condition and inverted green triangles ablated *Tg(UAS:CaN^{CA})*; *Tg(ins:NTR-P2A-mCherry) ; Tg(cftr:gal4)* condition. Data are presented as mean values ± SD. Two-way ANOVA with Tukey's multiple comparison test, *** means p-value<0.0005, **** p-value<0.00005, ns = non-significant.

CaN regulates β cell differentiation induced by Notch inhibition in absence of regeneration

Our transcriptomic data showed that the Notch pathway is downregulated in ductal cells during β cell regeneration (**Figure 1**). As the level of Notch activity determines the behavior of ductal cells (Ninov et al. 2013) from quiescence to proliferation and subsequently to β cell differentiation, we tested whether calcineurin acts together with the Notch pathway on a common pool of ductal progenitors. To inhibit the Notch pathway, we treated larvae with several concentrations of the gamma-secretase inhibitor LY411575 from 3 to 4dpf in absence of regeneration. The activity of CaN was inhibited by CsA during the same timeframe (**Figure 4A**). As previously, we used reporter lines for β and ductal cells *Tg*(*ins:NTR-P2A-mCherry*); *Tg*(*nkx6.1:GFP*) and the number of secondary β cells was analyzed at 6dpf (**Figure 4A**). As expected, the number of β cells progressively rose as the concentration of the Notch inhibitor increased (**Figure 4B**). Combined treatment with CsA fostered the differentiation of β cells between 1 and 10 µM LY411575 but did not result in further increase at 15 µM LY411575 (**Figure 4B**), suggesting that CaN is important within a permissive window of Notch activity. Since we observed the highest synergistic effect at 5µM of LY411575, we used this concentration for the following experiments (**Figure 4C-D**). It is worth noting that combined treatment of LY411575 (5uM) and another CaN inhibitor, FK506, resulted in the same synergistic increase of β cell differentiation (**Figure 4A-B supplemental**), confirming that the effect is due to CaN inhibition. The combined effect of Notch and CaN inhibition is transient as it is not observed at 7dpf anymore (**Figure 4E**). Therefore, as observed in regenerative conditions (**Figure 2B**), CaN inhibition accelerates β cell neogenesis induced by Notch repression.

To determine to which extent CaN pathway can modulate Notch induced β cell neogenesis, we activated CaN^{CA} overexpression in *Tg*(*hsp70:GFP-P2A-ppp3cc*^{CA}*); Tg*(*ins:NTR-P2A-mCherry*) larvae by an heat-shock at 3 dpf and treated them with LY411575 (**Figure 4C supplemental)**. CaN^{CA} overexpression resulted in a lowered β cell formation induced by Notch inhibition (**Figure 4D supplemental**), revealing that CaN activation counterbalanced the effects of Notch inhibition. Using our previous settings of Notch and CaN inhibition, we next wondered if the canonical pathway downstream of CaN was involved in the enhancement of β cell differentiation. To activate NFAT, a well-known target of CaN, we used CHIR99021 allowing a stabilization of the active form of NFAT (**Figure 1D**). We found that CHIR99021 rescued the effect of CsA (**Figure 4E supplemental**), suggesting that CaN inhibition increases

β cell neogenesis at least partially by the regulation of NFAT. Overall, these results reveal that CaN regulates β cell formation in pro-endocrinogenic context, such as induced by a low level of Notch activity. Moreover, it suggests that both CaN and Notch pathway act on a common pool of ductal progenitors to regulate β cell neogenesis.

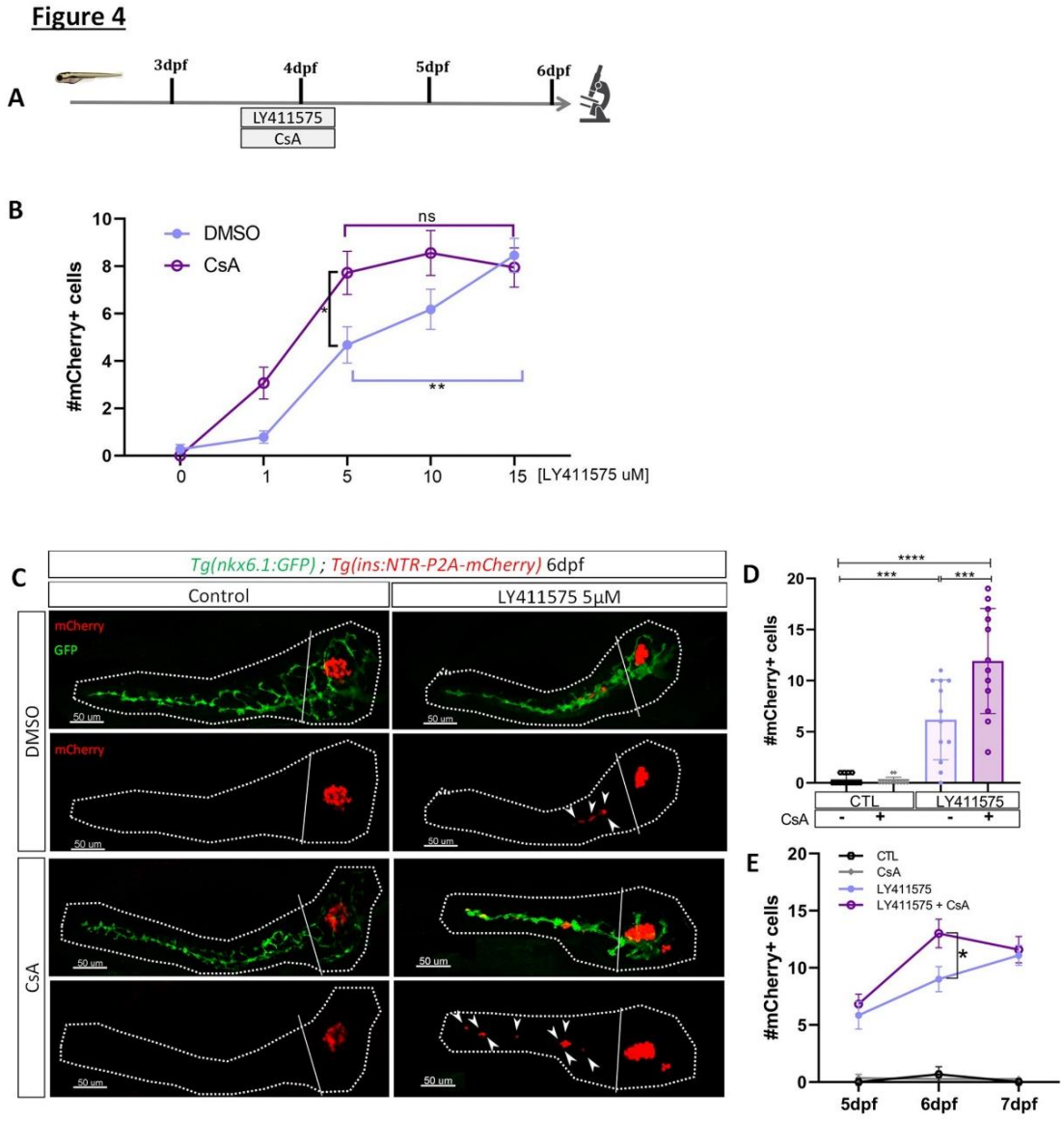


Figure 4: *CaN repression potentializes the effect of Notch inhibition on β cell formation*

- **A)** Experimental design for Notch inhibition test in non-ablated condition. Larvae were treated concomitantly with LY411575 (Notch inhibitor) and CsA from 3 to 4dpf and were fixed and analyzed at 6dpf.
- **B)** Graph representing the mean number of mCherry+ β cells in the pancreatic tail of *Tg(ins:NTR-P2A-mCherry) ; Tg(nkx6.1:GFP)* larvae at 6 dpf depending the concentration of LY411575. The blue dots represent LY411575; and purple combination of LY411575 and CsA. Data are presented as mean values ± SEM. Two-way ANOVA test with Sidak's multiple comparison test, *p-value<0.05, **p-value<0.05, ns = non-significant.
- **C)** Whole mount fluorescent immunohistochemistry (GFP and mCherry) of the pancreas of *Tg(ins:NTR-P2A-mCherry) ; Tg(nkx6.1:GFP)* larvae at 6dpf. 3D projection (stack) of one control (without any

treatment) ; one CsA-treated ; one LY411757-treated and one with both CsA and LY411575 treated larvae. The principal islet (PI) and the pancreatic tail are showed. Arrows point out mCherry+ β cells in the pancreatic tail. Scale 50µM.

D) Barplot representing the mean number of mCherry+ β cells in the pancreatic tail of *Tg(ins:NTR-P2A-mCherry) ; Tg(nkx6.1:GFP)* larvae at 6dpf. The black dots represent the control; gray CsA treatment ;blue LY411575; and purple combination of LY411575 and CsA. Data are presented as mean values ± SD. Two-way ANOVA with Tukey multiple comparison test, ***p-value<0.0005, ****p-value<0.00005.

E) Graph representing the mean number of mCherry+ β cells in the pancreatic tail of *Tg(ins:NTR-P2A-mCherry) ; Tg(nkx6.1:GFP)* larvae at 5-6-7 dpf. The black dots represent the control; gray CsA treatment ;blue LY411575; and purple combination of LY411575 and CsA. Data are presented as mean values ± SEM. Two-way ANOVA test with Sidak's multiple comparison test, *p-value<0.05.

CaN controls the proliferation of duct-associated progenitors induced by Notch inhibition

Given that we observed the most significant increase in β cell formation with CsA when Notch activity was mildly repressed (**Figure 4B-D**), and since mild Notch activity has been shown to promote progenitor amplification (Ninov, Borius, and Stainier 2012), it suggests that CaN acts at this level. To explore this possibility, we exposed briefly *Tg*(*nkx6.1:GFP*) larvae to EdU after mild Notch (LY411575 5µM) and CaN inhibition (**Figure 5A**). As expected, Notch inhibition increased the proportion of proliferating EdU+ GFP+ duct cells at 4dpf (**Figure 5B-C**) and decreased the amount of ductal progenitors 2 days later (**Figure 5D**), which is concomitant with the increase of β cell differentiation (**Figure 4C-D**). Interestingly, the combined inhibition with CsA further increased these proportions (**Figure 5B-D**). In contrast, CaN inhibition alone had no effect on basal proliferation or on the number of ductal progenitors (**Figure 5B-D**). Incidentally, at 4dpf, while the proliferation is increased (**Figure 5C**), the number of ductal cells remained the same in all conditions (**Figure 5A supplemental**), suggesting that ductal cells have not yet left the cell cycle to differentiate. In comparison, after stronger Notch inhibition (15µM), the ductal cells are already depleted at 4dpf (**Figure 5B supplemental**), as they directly differentiate without entering the cell cycle (Ninov, Borius, and Stainier 2012). In these conditions, CsA could therefore not enhance ductal progenitor proliferation and thus β cell formation (**Figure 4B**). These results show that CaN and Notch pathways act together on the proliferation of the ductal progenitors to prevent their exhaustion.

Figure 5

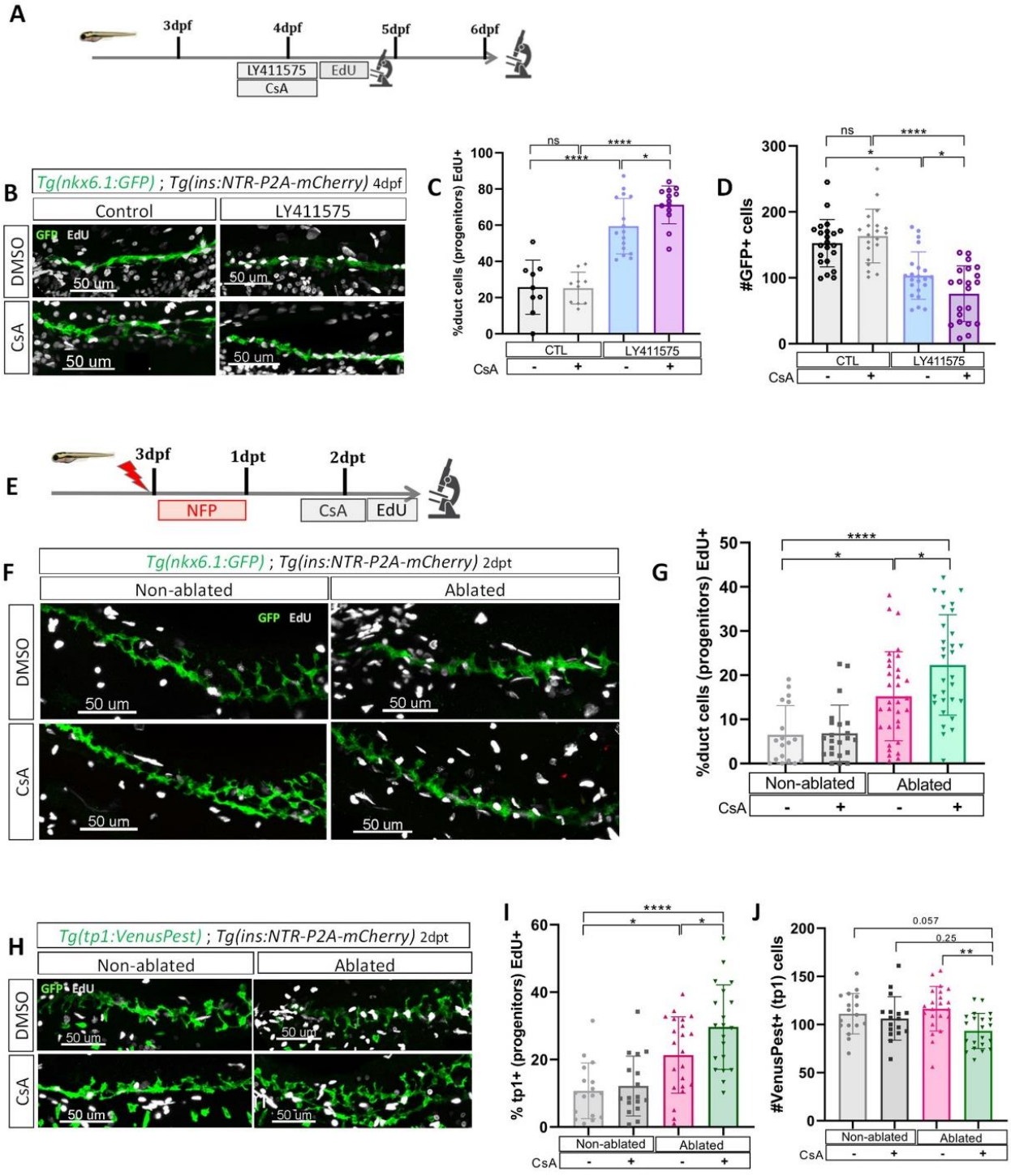


Figure 5: *CaN repression increases the proportion of duct proliferating cells*

- **A)** Experimental design for EdU assay in Notch test. Larvae were treated concomitantly with LY411575 (Notch inhibitor) and CsA from 3 to 4dpf and then briefly treated with EdU before fixation and analysis at 4 dpf or at 6dpf.
- **B)** Whole mount fluorescent immunohistochemistry (GFP and EdU) of the pancreatic tail of *Tg(ins:NTR-P2A-mCherry) ; Tg(nkx6.1:GFP)* larvae at 4dpf. 3D projection (stack) of one control (without any treatment), one with CsA only, one with LY411575 only and one with both CsA and LY411757 representative samples. Scale 50µM.

C) Barplot representing the percentage of GFP+ ductal cells which incorporated EdU+ in pancreatic tail of *Tg(ins:NTR-P2A-mCherry) ; Tg(nkx6.1:GFP)* larvae for the Notch test. The black dots represent the control; gray CsA treatment ;blue LY411575; and purple combination of LY411575 and CsA. Data are presented as mean values ± SD. T-test. Two-way ANOVA test with Tukey's multiple comparisons test, *p-value<0.05 ; ****p-value<0.00005 ; ns = non-significant.

D) Barplot representing the number of GFP+ ductal cells which in pancreatic tail of *Tg(ins:NTR-P2A-mCherry) ; Tg(nkx6.1:GFP)* larvae at 6dpf for the Notch test. The black dots represent the control; gray CsA treatment ;blue LY411575; and purple combination of LY411575 and CsA. Data are presented as mean values ± SD. T-test. Two-way ANOVA test with Tukey's multiple comparisons test, *p-value<0.05 ; ****p-value<0.00005 ; ns = non-significant.

E) Experimental design for EdU assay in regeneration. Larvae were treated with nifurpirinol for β cell ablation from 3 to 4dpf then with CsA from 4 to 5dpf and then briefly treated with EdU before fixation and analysis.

F) Whole mount fluorescent immunohistochemistry (GFP and EdU) of the pancreatic tail of *Tg(ins:NTR-P2A-mCherry) ; Tg(nkx6.1:GFP)* larvae at 5dpf. 3D projection (stack) of one representative sample of non-ablated or ablated with or without CsA are shown. Scale 50µM.

G) Barplot representing the percentage of GFP+ ductal cells that incorporated EdU+ in pancreatic tail of *Tg(ins:NTR-P2A-mCherry) ; Tg(nkx6.1:GFP)* larvae at 5dpf. The gray spheres represent non-ablated condition ; the pink triangles the ablated condition ; the black squares CsA condition and inverted green triangles ablated + CsA condition. Data are presented as mean values ± SD. Two-way ANOVA test with Tukey's multiple comparisons test, *p-value<0.05 ; ****p-value<0.00005 ; ns = non-significant.

H) Whole mount fluorescent immunohistochemistry (VenusPest and EdU) of the pancreatic tail of *Tg(ins:NTR-P2A-mCherry) ; Tg(tp1:VenusPest)* larvae at 5dpf. 3D projection (stack) of one representative sample of non-ablated or ablated with or without CsA are shown. Scale 50µM.

I) Barplot representing the percentage of GFP+ ductal cells that incorporated EdU+ in pancreatic tail of *Tg(ins:NTR-P2A-mCherry) ; Tg(tp1:VenusPest)* larvae at 5dpf. The gray spheres represent non-ablated condition ; the pink triangles the ablated condition ; the black squares CsA condition and inverted green triangles ablated + CsA condition. Data are presented as mean values ± sd. Two-way ANOVA test with Tukey multiple comparisons test, *p-value<0.05 ; ****p-value<0.00005 ; ns means non-significant.

J) Barplot representing the number of VenusPest+ ductal cells that incorporated EdU+ in pancreatic tail of *Tg(ins:NTR-P2A-mCherry) ; Tg(tp1:VenusPest)* larvae at 5dpf. The gray spheres represent non-ablated condition ; the pink triangles the ablated condition ; the black squares CsA condition and inverted green triangles ablated + CsA condition. Data are presented as mean values ± SD. Two-way ANOVA test with Tukey's multiple comparisons test, **p-value<0.005.

CaN prevents exhaustion of Notch responsive progenitors during β cell regeneration

Taken together, our results indicate that CaN plays a role in regulating the proliferation of ductal progenitors in contexts that are permissive for β cell differentiation. To demonstrate that CaN regulates ductal progenitor proliferation in a similar manner to Notch inhibition but during regeneration, we exposed *Tg(ins:NTR-P2A-mCherry)*; *Tg(nkx6.1:GFP)* larvae after ablation and CsA treatment (**Figure 5E**). CaN inhibition enhanced the proliferation of ductal cells in ablated larvae (**Figure 5F-G**). To next determine if CaN acts on the Notch-responsive progenitors in β cell regeneration, we then used *Tg(tp1:VenusPest)* Notch reporter line. At larval stages the vast majority of ductal cells are Notch responsive and the reporter line marks all the progenitors within the ductal tree (Parsons et al. 2009). We treated *Tg(ins:NTR-P2A-mCherry)*; *Tg(tp1:VenusPest)* as described above (**Figure 5E**). In ablated-larvae, CsA increased tp1+ ductal cell proliferation (**Figure 5H-I**). Moreover, CsA induced a reduction of tp1+ ductal cells in ablated larvae (**Figure 5 H-J**), suggesting an exhaustion of the Notch responsive progenitors, in accordance with premature β cell differentiation we observed (**Figure 2B-4D**). Lastly, CsA does not affect tp1+ cells in non-ablated larvae showing that CaN inhibition did not directly affect Notch signaling (**Figure 5J**). Those results suggest that CaN fine tunes the balance between proliferation of the progenitors and their differentiation to prevent their exhaustion during β cell regeneration.

CaN regulation is functionally relevant in adult zebrafish

To further expand upon our findings and investigate their relevance in a non-developmental context, we next ought to determine whether CaN function is maintained in older zebrafish. We used 2 months old *Tg*(*ins:NTR-P2A-mCherry*); *Tg*(*nkx6.1:GFP*) juveniles fish to perform β cell ablation followed by CsA treatments. We analyzed the number of small islets (up to 5 cells) at 7 and 10dpt. At 7dpt, CsA increased the number of small islets in ablated juveniles (**Figure 6A-B**) showing that CaN inhibition enhances β cell regeneration in juvenile zebrafish, as in larvae. It is noteworthy that, as in larvae, we highlighted an acceleration of β cell regeneration. The increase number of small islets is indeed transient as it not observe anymore at 10dpt (**Figure 6C**).

Next, we determined the functional impact of CaN overexpression or inhibition by assessing the glycemia at 7, 10 and 14 after β cell ablation. Overexpression of CaN^{CA} using either *Tg(hsp70:GFP-P2A-ppp3cc*^{CA}*)* or *Tg*(*cftr:gal4*); *Tg*(*UAS:GFP-P2A-ppp3cc*^{CA}*),* led to an

increased glycemia at both 7 and 10dpt (**Figure 6D-E)**, before recovery at 14 dpt (**Figure 6A supplemental**). This indicates that the overexpression of CaN impedes the recovery of glycemia induced by β cell regeneration. However, CaN inhibition did not seem to further improve the glycemia (**Figure 6D-E**) probably because the glycemia was already low at 7dpt (77 mg/dL on average) compared to non-ablated control (mg/dl) (**Figure 6D-E**). Altogether, these results show that in adult zebrafish also, CaN regulation is necessary to enable β cell regeneration and for proper recovery of the glycemia after β cell loss.

Figure 6

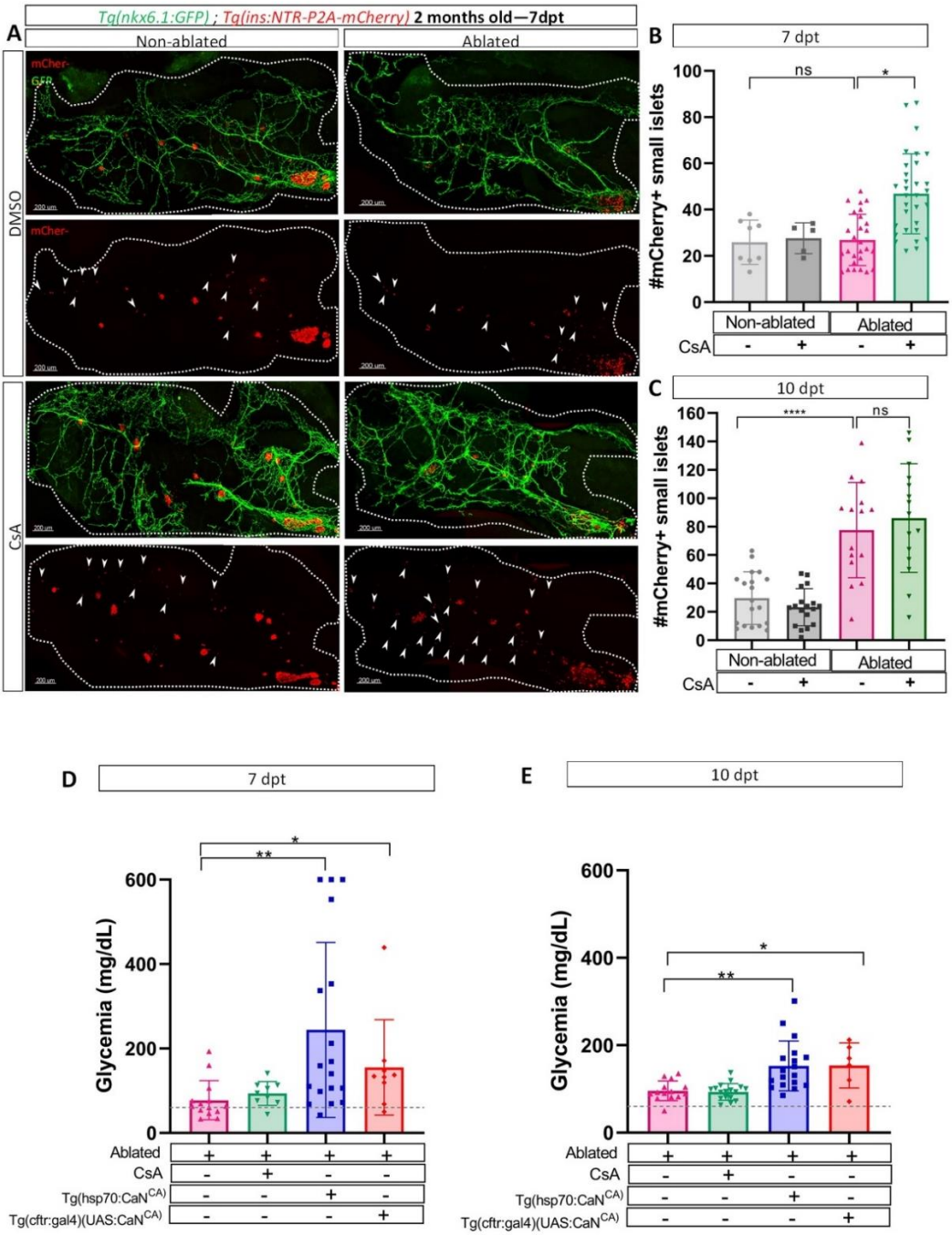


Figure 6: *CaN regulation is important in juveniles/adults and necessary for correct glycemia recovery*

A) Whole mount fluorescent immunohistochemistry (GFP and mCherry) of the pancreas of *Tg(ins:NTR-P2A-mCherry) ; Tg(nkx6.1:GFP)* 2 months old zebrafish at 7dpt. 3D projection (stack) of non-ablated and ablated larvae treated with DMSO or CsA representative samples. The principal islet (PI) and the pancreatic tail are shown. Arrows point out mCherry+ β cells in the pancreatic tail. Scale 200µM

B-C) Barplot representing the number of number of mCherry+ small secondary islets (=<5 cells) in the pancreatic tail of *Tg(ins:NTR-P2A-mCherry) ; Tg(nkx6.1:GFP)* 2 months old zebrafish at 7 **(B)** and 10dpt **(C).** The gray spheres represent non-ablated condition ; the pink triangles represent the ablated condition ; the black squares CsA condition and inverted green triangles ablated + CsA condition. Data are presented as mean values ± SD. Two-way ANOVA with Tukey's multiple comparison test, *p-value<0.05.

D-E) Barplot representing the glycemia (mg/dL) of *Tg(ins:NTR-P2A-mCherry) ;* months old adult zebrafish at 7 **(D)** and 10dpt **(E).** The pink triangles represent the ablated condition ; the inverted green triangles ablated + CsA condition; the blue squares *Tg(hsp70:CaN^{CA})* after heat shocks ; the orange lozenges *Tg(UAS:CaN^{CA})*; *Tg(cftr:gal4)*. The grey line represents the mean glycemia of controls (non-ablated) fish. Data are presented as mean values ± SD. One-way ANOVA with Tukey's multiple comparison test, *p-value<0.05; **p-value<0.005.

Discussion

The present study reveals the importance of the protein phosphatase CaN as a new player in β cell regeneration in zebrafish. We demonstrate that CaN act on ductal-associated progenitor cells by balancing proliferation and endocrine differentiation. In addition, we show the interplay between CaN and Notch signalling, a master regulator of β cell regeneration.

Previous drug and genetic screening using zebrafish larvae enabled the identification of several regulators of β cell regeneration from different pancreatic cellular sources. For example, adenosine has been shown to stimulate β cell replication (Andersson et al. 2012) and , igfbp1a (J. Lu et al. 2016) and TGFb suppression (Helker et al. 2019) promote α-to-β cell transdifferentiation. As for cdk5 inhibition and folinic acid/Folr1, they promote β cell regeneration from the pancreatic ducts (Karampelias et al. 2021; K. C. Liu et al. 2017). Here, to identify novel regulators of β cell regeneration specifically from pancreatic ducts, we carried out a transcriptomic profiling of duct cells following β cell ablation in the adult zebrafish. Transcriptomic analyses show that the regulated genes encompass most of the genes and pathways identified in previous studies (igfbp1, mTor, Notch, etc.), underlying the importance of those actors in β cell regeneration. Our data reveal also that DNA replication is the most enriched signature attesting that duct cells undergo a potent proliferative response after the destruction of β cells.

Besides these expected signatures, our transcriptomic data uncover the unanticipated upregulation of numerous genes implicated in DNA repair and cell cycle arrest. These signatures might indicate that highly proliferating ductal cells activate counteracting mechanisms. Among the genes regulated in those signatures, we focused on CaN and determined its role in β cell regeneration. Pharmacological inhibition of CaN increases the proliferation of duct cells induced by β cell ablation, resulting in an acceleration of β cell regeneration in the ducts. Consistently, transgene-mediated CaN overactivation abolishes the regenerative response. Importantly, the inhibition of regeneration is observed when CaN is overexpressed either ubiquitously or selectively in *cftr*-expressing ductal cells indicating that the role of CaN in β cell regeneration is intrinsic to the ducts. Based on functional assays in larvae, we not only confirm the activation of the proliferation of ductal cells soon after β cell ablation, but also that the rate of progenitor proliferation is carefully controlled by CaN in order to achieve proper and timely regeneration of β cells. Our data are consistent with earlier studies reporting a role of CaN in proliferation dynamics during fin regeneration. In the regenerating fin, low CaN activity is found in the proximal region of the blastema characterized by a high rate of proliferation and regeneration and its activity increases distally where lower proliferation is observed (Cao et al. 2021; Kujawski et al. 2014; Tornini et al. 2016). It was suggested that CaN controls blastemal cell progeny divisions (Tornini et al. 2016). In human, the importance of CaN in proliferation is also highlighted in organ transplanted patients. When patients are treated with Cyclosporin A (*i.e.* the CaN inhibitor we used in this study) as immunosuppressive drug they indeed present an increased risk of skin cancer, notably due to keratinocyte senescence inhibition (Wu et al. 2010).

It has been shown that Notch inhibitory treatments switch progenitors from proliferative self-renewing to premature differentiation, leading to progenitor depletion (Ninov, Borius, and Stainier 2012; Parsons et al. 2009). Our study reveals that this phenomenon is further exacerbated by CaN inhibition. Importantly, during normal larval development in absence of Notch inhibitory treatment, CaN does not affect basal ductal proliferation nor β cell differentiation. Hence, Notch signalling has to be repressed to detect the effect of CaN on the progenitors, suggesting that CaN acts downstream of Notch pathway. In differentiating keratinocytes, CaN cooperates with Notch signalling to regulate p21/*cdkn1a* (which is upregulated in the ducts at 3 dpt), cell cycle withdrawal and differentiation (Mammucari et al. 2005). These studies show that CaN acts in association with Notch signalling on progenitors proliferation and on their differentiation.

Based on our data, we build the model depicted in **Figure 7A**. Our study suggests that CaN acts in competent progenitor cells and that this competence is determined by Notch signalling. When Notch is repressed to a mild level, the progenitor enter into the cell cycle and acquire a pro-endocrinogenic competence (Ninov, Borius, and Stainier 2012). CaN acts on these progenitors to tone down an excessive proliferation and avoid the exhaustion of these progenitors. CaN is therefore a guardian of the progenitor population. CaN inhibition both increases progenitor proliferation and induces their depletion, suggesting a switch to a symmetric division resulting in two daughter cells entering in endocrine differentiation.

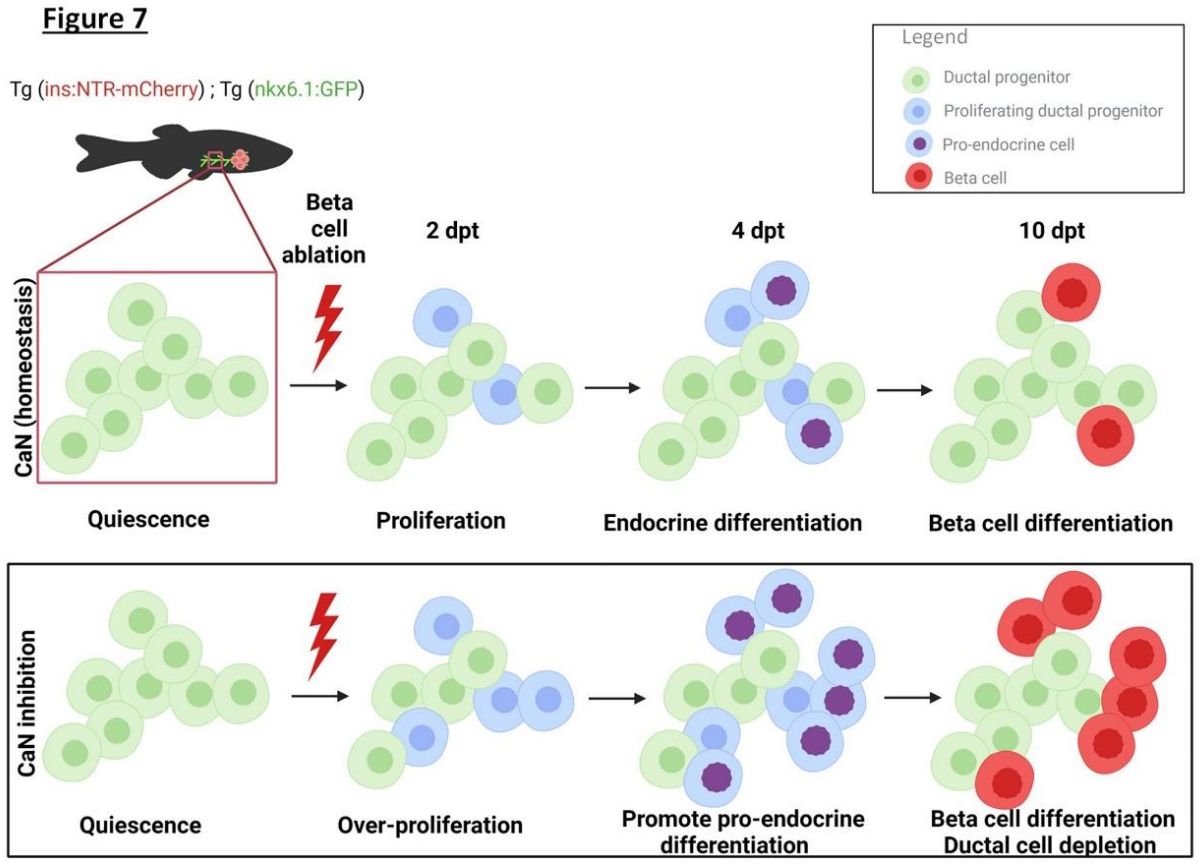


Figure 7: *Model of CaN action on ductal progenitors to regenerate β cells*

Under physiological conditions, the behavior of the ductal progenitors is determined by Notch signaling. Calcineurin is active in these progenitors and enable a proper control between proliferation and differentiation. When CaN in repressed, more ductal progenitors enter in the cell cycle (2dpt) and switch to a mode of proliferation leading to differentiation of the two daughter cells (4dpt), as more pro-endocrine cells are formed. The result is a exhaustion of the progenitors and a premature β cell differentiation (10dpt).

More precisely, a previous study in mice uncover the existence of three division modes of the pancreatic progenitors during embryonic development, *i.e.* symmetric self-renewing resulting in two progenitors cells ; asymmetric resulting in a progenitor and a endocrine cell and symmetric differentiative resulting in two differentiated cells (Kim et al. 2015). The authors actually show that the type of division is defined by the timing of induction of endocrine program by NEUROG3 (Kim et al. 2015). Interestingly, NEUROG3 seems to be the link between proliferation and differentiation, and its expression is regulated by Notch signalling (Krentz et al. 2017). In zebrafish, endocrine differentiation is not induced by *Neurog3* but by *Ascl1b* and *Neurod1* (L. C. Flasse, Stern, et al. 2013). Concerning that subject, CaN inhibition accelerates the formation of neurod1+ cells. Hence CaN could possibly act via the determination of the type of division *i.e.* symmetric vs asymmetric. This model is supported by previous observation in others systems. In stem cells and neuronal and hematopoietic progenitors, premature differentiation results from a switch in the mode of cellular division, from symmetric amplifying division to asymmetric differentiating division (Ho and Wagner 2007; Huttner and Kosodo 2005). Notch determines the choice between both types of divisions (Bultje et al. 2009; Guo, Jan, and Jan 1996).

Calcineurin is known to be implicated in cellular senescence (Wu et al. 2010). Usually thought as negative regulators of development and cellular growth, DNA repair (Sousounis et al. 2020) and cellular senescence (Da et al. 2020) appear to be required in both developmental and regenerative processes. Our transcriptomic data suggest that these mechanisms are required for β cell regeneration. Therefore, it would be interesting to determine the contribution of these cellular processes in the ductal progenitors and determine if CaN acts via cellular senescence in this case.

Overall, this study brings new insights on β cell regeneration and highlights the ductal progenitor cell cycle as a cornerstone in the process. Some studies report an increase of proliferation of some ductal cell population in diabetic patients (Md Moin, Butler, and Butler 2017; Qadir et al. 2020), implying a regenerative response. However, these ductal cells cannot efficiently reform the β cell mass, suggesting a dormant mechanism of regeneration. As such, the balance between proliferation and induction of endocrine differentiation could be a key to improve β cell neogenesis. However, as CaN is also important for β cell function, this approach would require to be transient to induce neogenesis. Therefore, it should be combined with methods to induce β cell proliferation to ultimately reconstitute the β cell mass. Overall, this study brings a better understanding on the regulation of the balance between ductal progenitors

proliferation and endocrine differentiation. These results should provide new hints to help improve regenerative competences in mammals.

Figure 1 supplemental

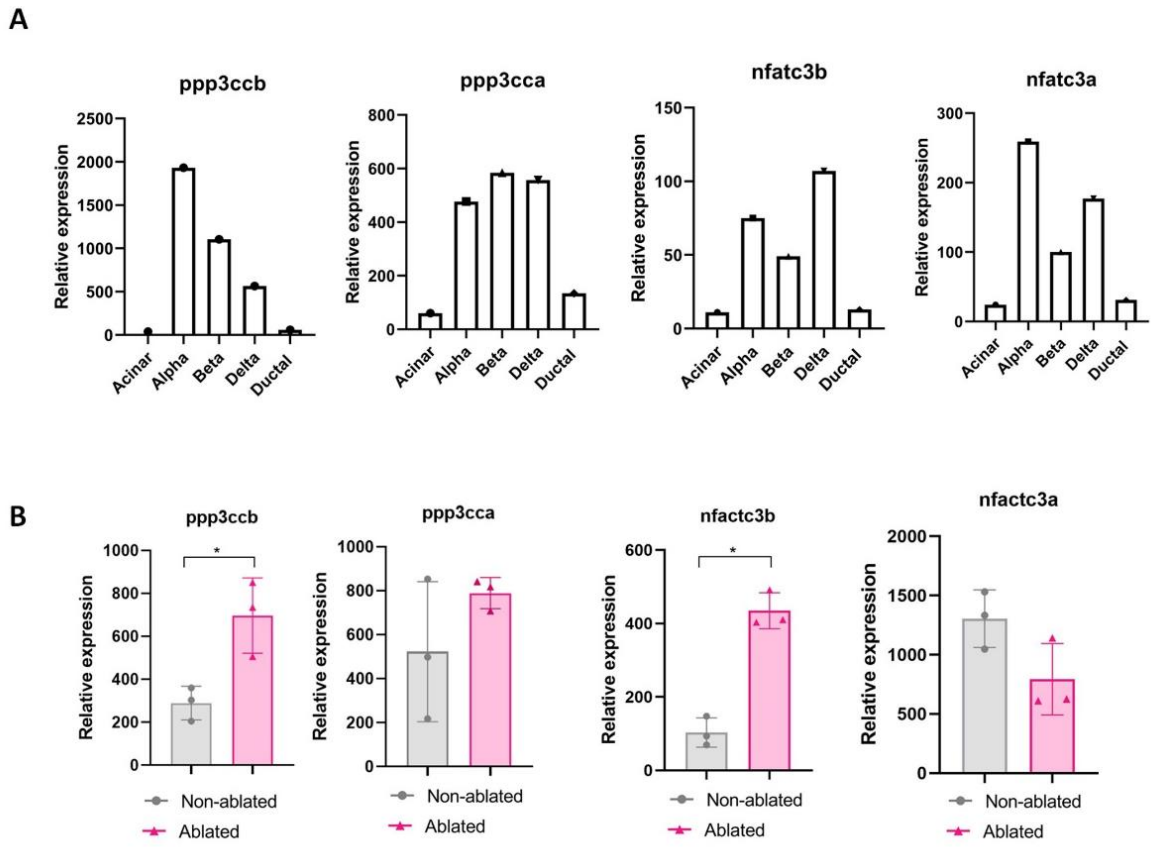


Figure 2 supplemental

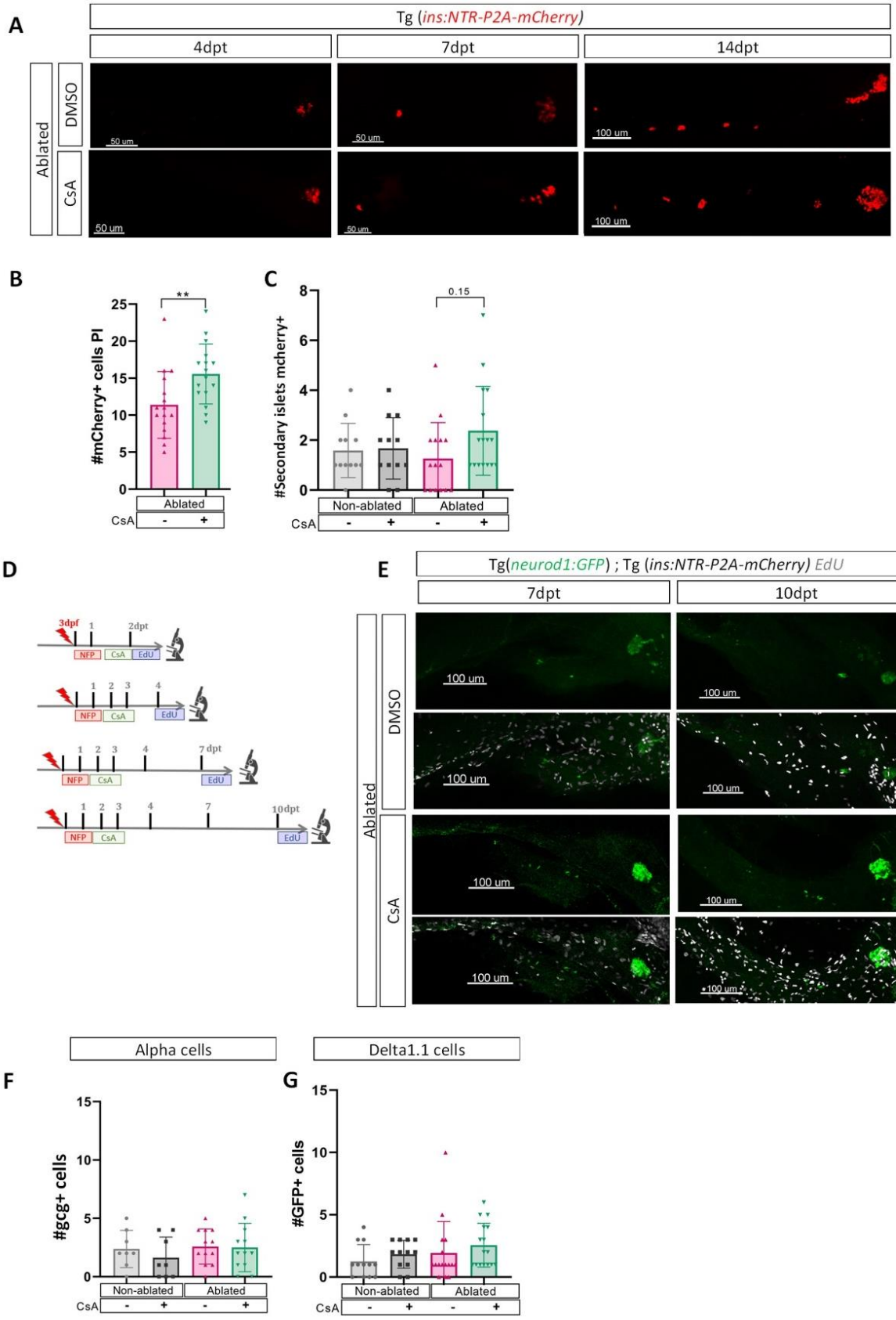


Figure 4 supplemental

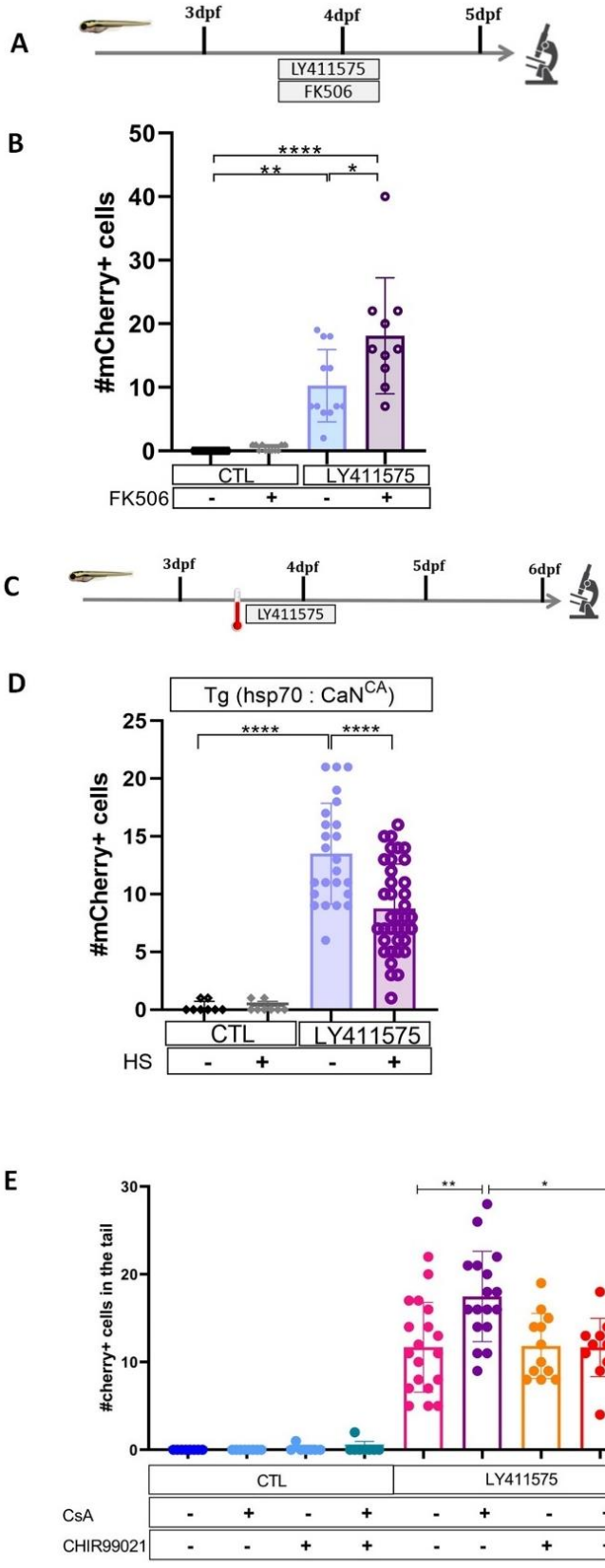


Figure 5 supplemental

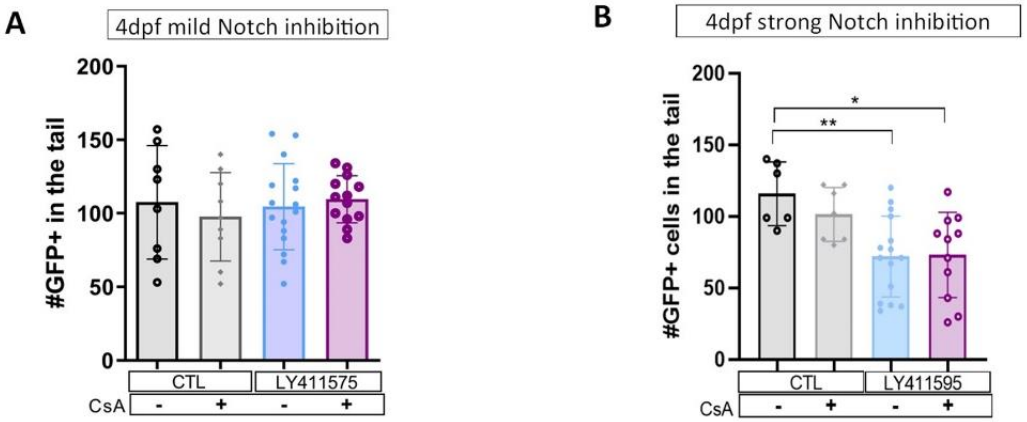


Figure 6 supplemental

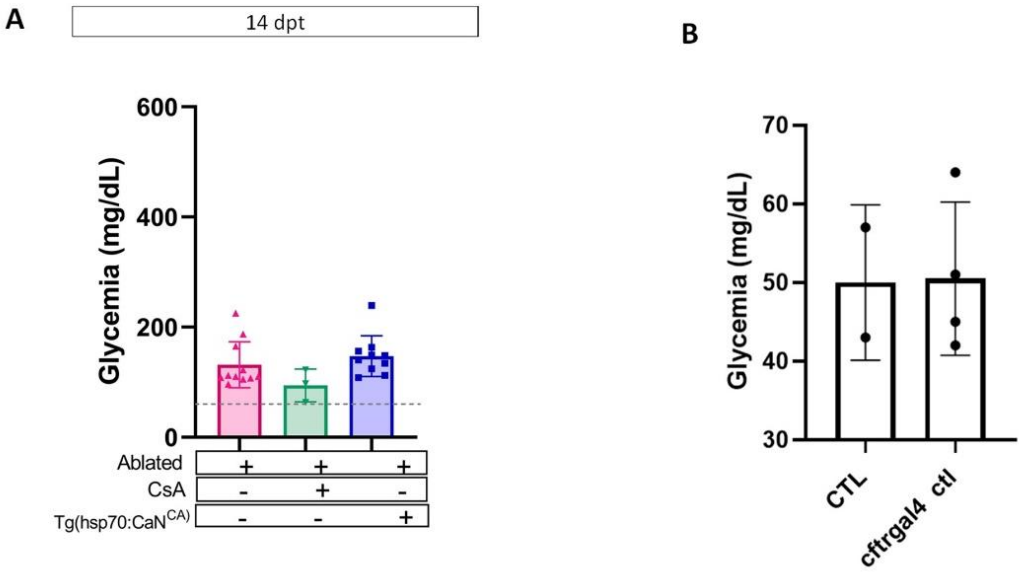


Figure 1 supplemental : *Transcriptomic profiling of ductal cells during β cell regeneration and validation in larvae*

A) Expression of *ppp3cca; ppp3ccb ; nfatc3a ; nfatc3b* in acinar, α, β, δ or ductal cells population from the zebrafish pancreas (Tarifeño-Saldivia et al. 2017).

B) Calcineurin (*ppp3ccb and ppp3cca)* and NFATc3 (*nfatc3a and nfatc3b)* expression in ductal cells from zebrafish in non-ablated and ablated conditions.

Figure 2 supplemental : *Calcineurin inhibition with CsA increases the ductal regenerative response*

A) Whole mount fluorescent immunohistochemistry (mCherry) of the pancreas of *Tg(ins:NTR-P2A-mCherry)* larvae at 4-7 and 14dpt. 3D projection (stack) of ablated larvae treated with DMSO or CsA representative samples. The principal islet (PI) and the pancreatic tail are shown. Scale 50 or 100µM.

B-C) Barplot representing the number of mCherry+ β cells in the principal islet (PI) **(B)** and the number of secondary islets of mCherry+ β cells in the pancreatic tail **(C)** of Tg(ins:NTR-P2A-mCherry) ; Tg(nkx6.1:GFP) larvae at 10dpt. Data are presented as mean values ± SD. T-test or Two-way ANOVA with Tukey's multiple comparison test. **p-value<0.005

D) Experimental design for EdU assay in larvae. After NFP treatment for 3 to 4dpf, larvae were exposed to EdU before fixation for analysis.

E) Whole mount fluorescent immunohistochemistry (GFP and EdU) of the pancreas of Tg(ins:NTR-P2A-mCherry) ; Tg(neurod1:GFP) larvae at 2-4-7 and 10 dpt. 3D projection (stack) of non-ablated and ablated larvae treated with DMSO or CsA representative samples. The principal islet (PI) and the pancreatic tail are shown. Scale 50µM.

F-G) Barplot representing the number of gcg+ α cells **(F)**; the number of GFP+ sst1.1 δ cells **(G);** of Tg(*ins:NTR-P2A-mCherry*) ; Tg(*sst1.1:GFP*) larvae at 10dpt. Gcg was detected by IHC. The gray spheres represent non-ablated condition ; the pink triangles represent the ablated condition ; the black squares CsA condition and inverted green triangles ablated + CsA condition. Data are presented as mean values ± SD. Two-way ANOVA with Tukey's multiple comparison test. **p-value<0.005

Figure 4 supplemental : *CaN repression potentializes the effect of Notch inhibition on β cell formation*

A) Experimental design for Notch inhibition test in non-ablated condition. Larvae were treated concomitantly with LY411575 (Notch inhibitor) and FK506 from 3 to 4dpf and were fixed and analyzed at 5dpf.

B) Barplot representing the mean number of mCherry+ β cells in the pancreatic tail of *Tg(ins:NTR-P2A-mCherry) ; Tg(nkx6.1:GFP)* larvae at 5dpf. The black dots represent the control; gray FK506 treatment ;blue LY411575; and purple combination of LY411575 and FK506. Data are presented as mean values ± SD. Two-way ANOVA with Tukey multiple comparison test, *p-value<0.05,**p-value<0.005, ****p-value<0.00005.

C) Experimental design for Notch inhibition test in non-ablated condition. Larvae were heat-shocked and then directly treated with LY411575 (Notch inhibitor) from 3 to 4dpf and were fixed and analyzed at 6dpf.

D) Barplot representing the mean number of mCherry+ β cells in the pancreatic tail of *Tg(hsp70:CaN^{CA})*; *Tg(ins:NTR-P2A-mCherry) ; Tg(nkx6.1:GFP)* larvae at 5dpf. The black dots represent the control; gray heat-shock ;blue LY411575; and purple combination of LY411575 and heat-shock. Data are presented as mean values ± SD. Two-way ANOVA with Tukey multiple comparison test, ****p-value<0.00005.

E) Barplot representing the mean number of mCherry+ β cells in the pancreatic tail of *Tg(ins:NTR-P2A-mCherry) ; Tg(nkx6.1:GFP)* larvae at 6dpf after Notch inhibition (LY411575), CaN inhibition (CsA) and NFATc inhibition (CHIR99021). Data are presented as mean values ± SD. Two-way ANOVA with Tukey multiple comparison test, *p-value<0.05,**p-value<0.005, ****p-value<0.00005.

Figure 5 supplemental: *CaN repression increases the proportion of duct proliferating cells*

A-B) Barplot representing the number of GFP+ ductal cells which in pancreatic tail of *Tg(ins:NTR-P2A-mCherry) ; Tg(nkx6.1:GFP)* larvae at 4dpf for the Notch test. **(A)** Mild Notch inhibition with LY411575 5µM and **(B)** stronger Notch inhibition with LY411575 15µM. The black dots represent the control; gray CsA treatment ;blue LY411575; and purple combination of LY411575 and CsA. Data are presented as mean values ± SD. T-test. Two-way ANOVA test with Tukey's multiple comparisons test, *p-value<0.05 ; ****p-value<0.00005 ; ns = non-significant.

Figure 6 supplemental: *CaN regulation is important in juveniles/adults and necessary for correct glycemia recovery*

A) Barplot representing the glycemia (mg/dL) of *Tg(ins:NTR-P2A-mCherry) ;* months old adult zebrafish at 14dpt**.** The pink triangles represent the ablated condition ; the inverted green triangles ablated + CsA condition; the blue squares *Tg(hsp70:CaN^{CA})* after heat shocks. The grey line represents the mean glycemia of controls (non-ablated) fish. Data are presented as mean values ± SD.
B) Barplot representing the glycemia (mg/dL) of *Tg(ins:NTR-P2A-mCherry) ; and Tg(UAS:CaN^{CA}); Tg(cftr:gal4)* in non-ablated adult zebrafish. Data are presented as mean values ± SD.

Material and Methods

Key resources table

Reagent type or resource	Designation	Source or reference	Identifier	Additional information
Genetic reagent (Danio rerio)	*TgBAC(nkx6.1:eGFP)ulg004*	PMID: 26329351	ZFIN: ZDB-ALT-160205-1	
Genetic reagent (Danio rerio)	*Tg(ins:NTR-P2A-mCherry)ulg034*	PMID: 29663654	ZFIN: ZDB-ALT-171122-9	
Genetic reagent (Danio rerio)	*Tg(cftr:gal4)*	PMID: 25592226	ZFIN : ZDB-FISH-150901-25442	
Genetic reagent (Danio rerio)	*Tg(tp1:VenusPest)*	PMID: 22492351	ZFIN: ZDB-FISH-150901-8023	
Genetic reagent	*Tg(hsp70:eGFP-P2A-ppp3ccaCA) ulg068*	This paper		See Zebrafish husbandry and generation of the

(Danio rerio)				*Tg(hsp70:eGFP-P2A-ppp3ccaCA) zebrafish line*
Genetic reagent (Danio rerio)	*Tg(UAS:eGFP-P2A-ppp3ccaCA) ulg069*	This paper		See Zebrafish husbandry and generation of the *Tg(UAS:eGFP-P2A-ppp3ccaCA) zebrafish line*
Antibody	Anti-GFP (chicken polyclonal)	Aves Labs	GFP-1020	1:1000
Antibody	anti-mCherry/dsRed (Living Colors Polyclonal)	Clontech	632496	1:500
Antibody	Anti-glucagon (mouse polyclonal)	Sigma	G2654	1:300
Antibody	Goat anti-Chicken IgY (H+L), Alexa Fluor™ 488	Invitrogen	A-11039	1:750
Antibody	Goat anti-dsred 568	Invitrogen		1:750
Antibody	Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 633	Invitrogen		1:750
Chemical compound	Nifurpirinol (NFP)	Sigma-Aldrich	32439	
Chemical compound	Metronidazole (MTZ)	Sigma-Aldrich	M1547	
Chemical compound	Cyclosporine A (CsA)	Selleckchem	S2286	
Chemical compound	LY411575	Sigma-Aldrich	SML0506	
Chemical compound	CHIR990211	Sellekchem	CT99021	
Commercial assay or kit	Gateway™ LR Clonase™ II Enzyme mix	Invitrogen	11791020	
Commercial assay or kit	Gateway™ BP Clonase™ II Enzyme mix	Invitrogen	11789020	
Sequence based reagent	IM369	This paper	PCR primer	gaagaaaaccccggtcctatgtcgacgaaagagccgaaag

Sequence based reagent	IM380	This paper	PCR primer	ccttacacattcccgtcagtgc
Sequence based reagent	IM371	This paper	PCR primer	CGGCTCTTTCGTCGACATAGGACCGGGGTTTTCTTCCACG
Sequence based reagent	O226	This paper	PCR primer	GCCACCATGGTGAGCAAGGGCGAGGA
Sequence based reagent	IM370	This paper	PCR primer	ttattagatcttatttctgatcacctcctt
Sequence based reagent	IM459	This paper	PCR primer	cacacgaattcgccgccaccATGGTGAGCAAGGGCGAG
Sequence based reagent	IM460	This paper	PCR primer	ggatcggtcgagatccttacGATCTTATTTCTGATCACCTCCTTACG
Sequence based reagent	IM457	This paper	PCR primer	GTAAGGATCTCGACCGATCCTG
Sequence based reagent	IM458	This paper	PCR primer	GGTGGCGGCGAATTCGTG
Commercial assay or kit	Nextera® XT DNA Library kit	Illumina	FC-131-1024	
Commercial assay or kit	Click-iT™ EdU Cell Proliferation Kit for Imaging, Alexa Fluor™ 647 dye	Invitrogen	C10340	
Software, algorithm	Imaris	Bitplane (http://www.bitplane.com/imaris/imaris)	RRID:SCR_007370	Version 9.5
Software, algorithm	GraphPad Prism	GraphPad Prism (https://graphpad.com)	RRID:SCR_015807	Version 8
Software, algorithm	DESeq2	DESeq2 (https://bioconductor.org/packages/release/bioc/html/DESeq2.html)	RRID:SCR_015687	

Software, algorithm	WebGestalt	WebGestalt (http://www.webgestalt.org/)	RRID:SCR_006786	

Zebrafish husbandry and generation of the Tg(hsp70:eGFP-P2A-ppp3ccaCA)ulg068 and Tg(UAS:eGFP-P2A-ppp2ccaCA)ulg069 zebrafish lines

Tg BAC(nkx6.1:eGFP)ulg004 (A.P. Ghaye et al. 2015) *; Tg(ins:NTR-P2A-mCherry)ulg034* (Bergemann et al. 2018) *; Tg(cftr:gal4)* and *Tg(tp1:VenusPest)* were used. Zebrafish were raised in standard conditions at 28°C. All experiments were carried out in compliance with the European Union and Belgian law and with the approval of the ULiège Ethical Committee for experiments with laboratory animals (approval number : 2075).

The *hsp70:GFP-P2A-ppp3ccaCA* transgene has been generated by cloning a PCR fragment containing the Gateway vector pCR8/GW/TOPO. Firstly, we amplified the full length of ppp3ccaCA with primers IM369/IM380 and amplified *GFP-P2A* with overlapping regions with IM371/O226. The overlapping PCR used the primers O226/IM380. Then to obtain a truncated *ppp3ccaCA* lacking the calmodulin biding and the autoinhibitory domain, resulting in a constantly active form of calcineurin, we amplified the last fragments with IM370/O226 and cloned into PCR8 vector. The promoter was assembled by LR recombination with pE5-hsp70 into pDestTol2p2A from the Tol2kit (Kwan et al. 2007). *Tg (hsp70:GFP-P2A-ppp3ccaCA)* fish have been generated using the Tol2 mediated transgenesis (Kawakami 2007). *The Tg(UAS:GFP-P2A-ppp3ccacA*) has been generated by ligation (KLD kit, NEB) of PCR fragments *GFP-P2A-ppp3ccacA (*IM459/IM460) with the UAS sequences (IM457/IM458) in plasmid from (Distel 2009) and then inserted into pDestTol2p2A from the Tol2kit. Final constructions has been injected with transposase into wild type (WT) AB embryos.

B-cell ablation

Adults fish for RNA-sequencing experiment were treated with freshly prepared metronidazole (MTZ) (Sigma M1547) at 10mM with 0.2% DMSO in fish water. Control treatments consisted of fish water containing 0.2% DMSO. Fish were treated for 18 hours in the dark. Nifurpirinol (NFP) (32439, Sigma-Aldrich) stock solution was dissolved at 2.5 mM in DMSO. b-cell ablation in *Tg(nkx6.1:eGFP); Tg(ins:NTR-P2A-mCherry)* larvae was induced by treatment with 2.5 µM NFP in E3. Control treatments consisted of E3 containing 0.2% DMSO. Larvae were treated for 18 hours in the dark.

Drug treatments

Cyclosporine A (Selleckchem, S2286), CHIR99021 and LY411575 (Sigma-Aldrich, SML0506) stock solution were dissolved at 10mM in DMSO. Larvae treatment were respectively performed at 0.1 µM and 5µM in E3. Control treatments consisted of E3 containing the same amount of DMSO than drug treatment. Larvae were treated for 18 hours in the dark.

5-ethynyl-2'-deoxyuridine (EdU) incorporation assay

Zebrafish larvae were incubated in 4 mM EdU dissolved in E3 water for 8 hours, they were then directly euthanised in tricaine and fixed in 4% PFA. EdU was detected according to the protocol of Click-iT™ EdU Cell Proliferation Kit for Imaging, Alexa Fluor™ 647 (ThermoFisher C10340) after whole mount immunodetection.

Heat Shock

Successive heat shocks of 30 minutes and 12 hours apart were performed at 39°C for larvae and 37°C for juveniles and adults zebrafish.

Whole mount immunodetection

Larvae were euthanized in tricaine and fixed in 4% PFA at 4 °C for IHC. The digestive tract of juveniles was dissected prior immunodetection and kept in methanol for at least 18 hours. After depigmentation with 3% H2O2/1% KOH, larvae were permeabilised 30 min in PBS/ Triton X-100 and incubated for two hours in blocking buffer (4% goat serum/1% BSA/PBS/0.1% Triton X-100). Primary and secondary antibodies were incubated at 4 °C overnight.

	Fixation duration	Depigmentation duration	Permeabilization solution	Permeabilization duration
5-10 dpf	18 hours	15 min	PBTr 0.05%	30 min
13-17 dpf	36 hours	20 min	PBTr 2%	30 min
2months (digestive tract)	18 hours	15 min	/	/

Primary antibodies: Living Colors Polyclonal anti-mCherry/dsRed (rabbit, 1:500, Clontech 632496), anti-GFP (chicken, 1:1000),

Secondary antibodies: Alexa Fluor-488, -568, -633 (goat, 1:750, Molecular Probes).

Flow cytometry and FACS

The whole pancreas from 3-4 fish of *Tg(nkx6.1:eGFP); Tg(ins:NTR-P2A-mCherry)* adult fish (6–10 months old, males and females) were dissected, collected and washed in HBSS without Ca2+/Mg2+. Live cell dissociation was performed in Tryple Select 1x solution (GIBCO) supplemented with 100 U/mL collagenase IV (Life Technologies 17104-019) and 40 µg/mL proteinase K (Invitrogen, 25530031) for 10 min at 28 °C.

The GFP+ cells were selected on FACS Aria III and sorted under purity mode and after exclusion of the doublets. The purity of the sorted cells was confirmed by epifluorescence microscopy (~95 %). Cells (about 1000-5000/fish depending on the cell type) were immediately lysed with 0.5% Triton X-100 containing 2U/µl RNAse inhibitor and stored at –80 °C.

mRNA sequencing of FACSed cells and bioinformatic analyses

cDNAs were prepared from lysed cells according to SMART-Seq2.0 (Picelli et al., 2014) for low input RNA sequencing and libraries were prepared with Nextera® DNA Library kit (Illumina). Independent biological replicates of each cell type sequenced using Illumina NextSeq500 and obtained ~20 million 100 bp paired-end reads. Reads were mapped and aligned

to the zebrafish genome GRCz11 from Ensembl gene annotation version using STAR version 2.6.1 (Dobin et al., 2013). Gene expression levels were calculated with featureCounts (http://bioinf.wehi.edu.au/featureCounts/) and differential expression determined with DESeq2 (Love, Huber, & Anders, 2014). Expression values are given as normalized read counts. Poorly expressed genes with mean normalized expression counts <10 were excluded from the subsequent analyses. DESeq2 uses Wald test for significance with posterior adjustment of P values (Padj) using Benjamini and Hochberg multiple testing. The differentially expressed (DE) genes identified with a Padj cutoff of 0.05 were submitted for GO analysis using WebGestalt tool (Liao, Wang, Jaehnig, Shi, &Zhang, 2019).

Confocal microscopy and image analysis

Images were acquired using Leica SP5 confocal microscope. We used ImageJ to count the cells and Imaris to do the pictures.

Glycemia measurement

Glycemia measurement were performed as described in (Bergemann et al. 2018).

Data availability

The sequences that support the findings are of this study have been deposit in Geo with the accessions codes GSE212124, https://www.ncbi.nlm.nih.gov/geo/info/linking.html. The authors declare that all other data supporting the findings of this study are available within the paper and its supplementary information files.

Authors contribution

L.M. conducted experiments, acquired and analysed data, analysed transcriptomic data, wrote the manuscript. **D.B.** conducted RNA-seq experiment and revised the manuscript **A.L.** implemented bioinformatic programs and analysed transcriptomic data. **C.R., C.G. and C.D.** conducted experiments. **L.F.** revised the manuscript. **B.P.** provided resources and advised on research studies. **M.L.V.** provided resources, advised on research studies and revised the manuscript **I.M.** supervised the whole work, acquired funding, designed the research studies, verified data and assisted in writing the manuscript.

Acknowledgments

The authors thank the GIGA technology platforms GIGA-Genomics and GIGA-Imaging. The authors also thanks Jérémie Zappia for critically reading the manuscript, Jordane Bourdouxhe and Marie Alice Dupont for their help with the constructions.

Duality of interest

No potential conflicts of interest.

References

Aguayo-Mazzucato, Cristina, and Susan Bonner-Weir. 2018. "Pancreatic β Cell Regeneration as a Possible Therapy for Diabetes." *Cell Metabolism* 27(1): 57–67. https://doi.org/10.1016/j.cmet.2017.08.007.

Aguirre, A. et al. 2014. "In Vivo Activation of a Conserved MicroRNA Program Induces Robust Mammalian Heart Regeneration." *Cell stem cell*: 7.

Al-Hasani, Keith et al. 2013. "Adult Duct-Lining Cells Can Reprogram into β-like Cells Able to Counter Repeated Cycles of Toxin-Induced Diabetes." *Developmental Cell* 26(1): 86–100.

Andersson, Olov et al. 2012. "Adenosine Signaling Promotes Regeneration of Pancreatic β- Cells in Vivo." *Cell Metabolism*.

Ben-Othman, Nouha et al. 2017. "Long-Term GABA Administration Induces Alpha Cell-Mediated Beta-like Cell Neogenesis." *Cell* 168(1–2): 73-85.e11.

Bergemann, David et al. 2018. "Nifurpirinol: A More Potent and Reliable Substrate Compared to Metronidazole for Nitroreductase-Mediated Cell Ablations." *Wound Repair and Regeneration* 26(2): 238–44.

Berghmans, Stéphane et al. 2005. "Tp53 Mutant Zebrafish Develop Malignant Peripheral Nerve Sheath Tumors." *Proceedings of the National Academy of Sciences of the United States of America* 102(2): 407–12.

Biemar, Frédéric et al. 2001. "Pancreas Development in Zebrafish: Early Dispersed Appearance of Endocrine Hormone Expressing Cells and Their Convergence to Form the Definitive Islet." *Developmental Biology* 230(2): 189–203.

Binot, A. C. et al. 2010. "Nkx6.1 and Nkx6.2 Regulate α- and β-Cell Formation in Zebrafish by Acting on Pancreatic Endocrine Progenitor Cells." *Developmental Biology* 340(2): 397–407. http://dx.doi.org/10.1016/j.ydbio.2010.01.025.

Bonner-Weir, Susan et al. 2000. "In Vitro Cultivation of Human Islets from Expanded Ductal Tissue." *Proceedings of the National Academy of Sciences of the United States of America* 97(14): 7999–8004.

Bonner-Weir, Susan, Leslie A. Baxter, George T. Schuppin, and Fannie E. Smith. 1993. "A Second Pathway for Regeneration of Adult Exocrine and Endocrine Pancreas: A Possible Recapitulation of Embryonic Development." *Diabetes* 42(12): 1715–20.

Bonner-Weir, Susan, Brooke A. Sullivan, and Gordon C. Weir. 2015. "Human Islet Morphology Revisited: Human and Rodent Islets Are Not So Different After All." *Journal of Histochemistry and Cytochemistry* 63(8): 604–12.

Bouwens, Luc, and Ilse Rooman. 2005. "Regulation of Pancreatic Beta-Cell Mass." *Physiological Reviews* 85(4): 1255–70.

Bultje, Ronald S. et al. 2009. "Mammalian Par3 Regulates Progenitor Cell Asymmetric Division via Notch Signaling in the Developing Neocortex." *Neuron* 63(2): 189–202. http://dx.doi.org/10.1016/j.neuron.2009.07.004.

Butler, Alexandra E et al. 2003. "Humans With Type 2 Diabetes." *Diabetes* 52(January): 102–10. http://www.ncbi.nlm.nih.gov/pubmed/12502499.

Butler, Peter C., Juris J. Meier, Alexandra E. Butler, and Anil Bhushan. 2007. "The Replication of β Cells in Normal Physiology, in Disease and for Therapy." *Nature Clinical Practice Endocrinology and Metabolism* 3(11): 758–68.

Cabrera, Over et al. 2006. "The Unique Cytoarchitecture of Human Pancreatic Islets Has Implications for Islet Cell Function." *Proceedings of the National Academy of Sciences of the United States of America* 103(7): 2334–39.

Cao, Zigang et al. 2021. "Calcineurin Controls Proximodistal Blastema Polarity in Zebrafish Fin Regeneration." *Proceedings of the National Academy of Sciences of the United States of America* 118(2): 2–9.

Carril Pardo, Claudio et al. 2022. "A Delta-Cell Subpopulation with a pro-Beta-Cell Identity Contributes to Efficient Age-Independent Recovery in a Zebrafish Model of Diabetes." *eLife*.

Van De Casteele, M. et al. 2013. "Neurogenin 3+ Cells Contribute to B-Cell Neogenesis and Proliferation in Injured Adult Mouse Pancreas." *Cell Death and Disease* 4(3).

Charbord, Jérémie et al. 2021. "In Vivo Screen Identifies a SIK Inhibitor That Induces β Cell Proliferation through a Transient UPR." *Nature Metabolism* 3(5): 682–700. http://dx.doi.org/10.1038/s42255-021-00391-x.

Charni, Meital, Ronit Aloni-Grinstein, Alina Molchadsky, and Varda Rotter. 2017. "P53 on the Crossroad between Regeneration and Cancer." *Cell Death and Differentiation* 24(1): 8–14. http://dx.doi.org/10.1038/cdd.2016.117.

Chera, Simona et al. 2014a. "Diabetes Recovery by Age-Dependant Conversion of Pancreatic Delta-Cells into Insulin Producers." *Nature* 514(7523): 503–7.

———. 2014b. "Diabetes Recovery By Age-Dependent Conversion of Pancreatic δ-Cells." *Nature*.

Choe, Chong Pyo et al. 2021. "Transgenic Fluorescent Zebrafish Lines That Have Revolutionized Biomedical Research." *Laboratory Animal Research* 37(1): 1–29. https://doi.org/10.1186/s42826-021-00103-2.

Choi, Tae Young, Nikolay Ninov, Didier Y.R. Stainier, and Donghun Shin. 2014. "Extensive Conversion of Hepatic Biliary Epithelial Cells to

Hepatocytes after near Total Loss of Hepatocytes in Zebrafish." *Gastroenterology* 146(3): 776–88.

Cinti, Francesca et al. 2016. "Evidence of β-Cell Dedifferentiation in Human Type 2 Diabetes." *Journal of Clinical Endocrinology and Metabolism* 101(3): 1044–54.

Cohrs, Christian M. et al. 2020. "Dysfunction of Persisting β Cells Is a Key Feature of Early Type 2 Diabetes Pathogenesis." *Cell Reports* 31(1).

Corradi, Laura et al. 2022. "Hypothalamic Galanin-Producing Neurons Regulate Stress in Zebrafish through a Peptidergic, Self-Inhibitory Loop." *Current Biology* 32(7): 1497-1510.e5. https://doi.org/10.1016/j.cub.2022.02.011.

Courtney, Monica et al. 2013. "The Inactivation of Arx in Pancreatic α-Cells Triggers Their Neogenesis and Conversion into Functional β-Like Cells." *PLoS Genetics* 9(10): 1–18.

Creamer, Trevor P. 2020. "Calcineurin." *Cell communication and Signalling* 4: 1–12.

Crippa, Stefania et al. 2016. "Comparative Transcriptome Profiling of the Injured Zebrafish and Mouse Hearts Identifies MiRNA-Dependent Repair Pathways." *Cardiovascular research* 110(1): 73–84.

Curado, Silvia et al. 2007. "Conditional Targeted Cell Ablation in Zebrafish: A New Tool for Regeneration Studies." *Developmental Dynamics* 236(February): 1025–35.

D'Amour, Kevin A. et al. 2006. "Production of Pancreatic Hormone-Expressing Endocrine Cells from Human Embryonic Stem Cells." *Nature Biotechnology* 24(11): 1392–1401.

Da, Sabela et al. 2020. "Cell Senescence Contributes to Tissue Regeneration in Zebrafish." (July 2019): 1–5.

Dai, Chunhua et al. 2017. "Age-Dependent Human β Cell Proliferation Induced by Glucagon-like Peptide 1 and Calcineurin Signaling." *Journal of Clinical Investigation* 127(10): 3835–44.

Delaspre, Fabien et al. 2015. "Centroacinar Cells Are Progenitors That Contribute to Endocrine Pancreas Regeneration." *Diabetes* 64(October): 3499–3509.

Delgadillo-Silva, Luis Fernando et al. 2019. "Modelling Pancreatic β-Cell Inflammation in Zebrafish Identifies the Natural Product Wedelolactone for Human Islet Protection." *DMM Disease Models and Mechanisms* 12(1): 1–12.

Van Deursen, Jan M. 2014. "The Role of Senescent Cells in Ageing." *Nature* 509(7501): 439–46. http://dx.doi.org/10.1038/nature13193.

DiMeglio, Linda A., Carmella Evans-Molina, and Richard A. Oram. 2018. "Type 1 Diabetes." *The Lancet* 391(10138): 2449–62. http://dx.doi.org/10.1016/S0140-6736(18)31320-5.

Dor, Yuval, Juliana Brown, Olga I Martinez, and Douglas a Melton. 2004. "Adult Pancreatic Beta-Cells Are Formed by Self-Duplication Rather than Stem-Cell Differentiation." *Nature* 429(model 3): 41–46.

Druelle, Noémie et al. 2017. "Ectopic Expression of Pax4 in Pancreatic δ Cells Results in β-like Cell Neogenesis." *Journal of Cell Biology* 216(12): 4299–4311.

Duggirala, Ravindranath et al. 1999. "Linkage of Type 2 Diabetes Mellitus and of Age at Onset to a Genetic Location on Chromosome 10q in Mexican Americans." *American Journal of Human Genetics* 64(4): 1127–40.

Dupuis, Josée et al. 2010. "New Genetic Loci Implicated in Fasting Glucose Homeostasis and Their Impact on Type 2 Diabetes Risk." *Nature Genetic*. http://tocs.ub.uni-mainz.de/pdfs/100559395.pdf%7D, URL = %7Bhttp://www.bsz-bw.de/cgi-bin/ekz.cgi?SWB09525289 ;

Edlund, Helena. 2002. "Pancreatic Organogenesis - Developmental Mechanisms and Implications for Therapy." *Nature Reviews Genetics* 3(7): 524–32.

Elliott, Sarah A., and Alejandro Sánchez Alvarado. 2018. 1774 Methods in Molecular Biology *Planarians and the History of Animal Regeneration: Paradigm Shifts and Key Concepts in Biology*.

Elsaeidi, Fairouz et al. 2018. "Notch Suppression Collaborates with Ascl1 and Lin28 to Unleash a Regenerative Response in Fish Retina, but Not in Mice." *Journal of Neuroscience* 38(9): 2246–61.

Fausett, Blake V., Jessica D. Gumerson, and Daniel Goldman. 2008. "The Proneural Basic Helix-Loop-Helix Gene Ascl1a Is Required for Retina Regeneration." *Journal of Neuroscience* 28(5): 1109–17.

Flasse, Lydie C., Justine L. Pirson, et al. 2013. "Ascl1b and Neurod1, Instead of Neurog3, Control Pancreatic Endocrine Cell Fate in Zebrafish." *BMC Biology* 11: 1–18.

Flasse, Lydie C., David G. Stern, et al. 2013. "The BHLH Transcription Factor Ascl1a Is Essential for the Specification of the Intestinal Secretory Cells and Mediates Notch Signaling in the Zebrafish Intestine." *Developmental Biology* 376(2): 187–97. http://dx.doi.org/10.1016/j.ydbio.2013.01.011.

Flasse, Lydie, Coline Schewin, and Anne Grapin-Botton. 2021. 143 Current Topics in Developmental Biology *Pancreas Morphogenesis: Branching in and Then Out*. 1st ed. Elsevier Inc. http://dx.doi.org/10.1016/bs.ctdb.2020.10.006.

Galicia-Garcia, Unai et al. 2020. "Pathophysiology of Type 2 Diabetes Mellitus." *International Journal of Molecular Sciences* 21(17): 1–34.

Gemberling, Matthew, Travis J. Bailey, David R. Hyde, and Kenneth D. Poss. 2013. "The Zebrafish as a Model for Complex Tissue Regeneration." *Trends in Genetics* 29(11): 611–20. http://dx.doi.org/10.1016/j.tig.2013.07.003.

Ghaye, A.P. et al. 2015. "Progenitor Potential of Nkx6.1-Expressing Cells throughout Zebrafish Life and during Beta Cell Regeneration." *BMC Biology* 13(1).

Ghaye, Aurélie P. et al. 2015. "Progenitor Potential of Nkx6.1-Expressing Cells throughout Zebrafish Life and during Beta Cell Regeneration." *BMC Biology* 13: 70.

Di Giovanni, Simone et al. 2006. "The Tumor Suppressor Protein P53 Is Required for Neurite Outgrowth and Axon Regeneration." *EMBO Journal* 25(17): 4084–96.

Goldshmit, Y et al. 2015. "Decreased Anti-Regenerative Effects after Spinal Cord Injury in Spry4-/- Mice." *Neuroscience* 287: 104–12.

Goldshmit, Yona et al. 2012. "Fgf-Dependent Glial Cell Bridges Facilitate Spinal Cord Regeneration in Zebrafish." *The Journal of neuroscience : the official journal of the Society for Neuroscience* 32(22): 7477–92.

Goshima, Takahiro et al. 2019. "Calcineurin Regulates Cyclin D1 Stability through Dephosphorylation at T286." *Scientific Reports* 9(1): 1–11.

Gribben, Christopher et al. 2021. "Ductal Ngn3-Expressing Progenitors Contribute to Adult β Cell Neogenesis in the Pancreas." *Cell Stem Cell* 28(11): 2000-2008.e4. https://doi.org/10.1016/j.stem.2021.08.003.

De Groef, Sofie et al. 2015. "Surgical Injury to the Mouse Pancreas through Ligation of the Pancreatic Duct as a Model for Endocrine and Exocrine Reprogramming and Proliferation." *Journal of Visualized Experiments* 2015(102): 16–19.

Guo, Ming, Lily Yeh Jan, and Yuh Nung Jan. 1996. "Control of Daughter Cell Fates during Asymmetric Division: Interaction of Numb and Notch." *Neuron* 17(1): 27–41.

Han, Lei et al. 2022. "Distinctive Clinical and Pathologic Features of Immature Teratomas Arising from Induced Pluripotent Stem Cell-Derived Beta Cell Injection in a Diabetes Patient." *Stem Cells and Development* 31(5–6): 97–101.

He, Jianbo et al. 2022. "DNA Methylation Maintenance at the P53 Locus Initiates Biliary-Mediated Liver Regeneration." *npj Regenerative Medicine* 7(1): 1–14.

Heit, Jeremy J. et al. 2006. "Calcineurin/NFAT Signalling Regulates Pancreatic β-Cell Growth and Function." *Nature* 443(7109): 345–49.

Helker, Christian S.M. et al. 2019. "A Whole Organism Small Molecule Screen Identifies Novel Regulators of Pancreatic Endocrine Development." *Development (Cambridge)* 146(14).

Herrera, P. L. et al. 1994. "Ablation of Islet Endocrine Cells by Targeted Expression of Hormone- Promoter-Driven Toxigenes." *Proceedings of the National Academy of Sciences of the United States of America* 91(26): 12999–3.

Ho, Anthony D., and Wolfgang Wagner. 2007. "The Beauty of Asymmetry: Asymmetric Divisions and Self-Renewal in the Haematopoietic System." *Current Opinion in Hematology* 14(4): 330–36.

Howe, Kerstin et al. 2013. "The Zebrafish Reference Genome Sequence and Its Relationship to the Human Genome." *Nature* 496(7446): 498–503.

Huttner, Wieland B., and Yoichi Kosodo. 2005. "Symmetric versus Asymmetric Cell Division during Neurogenesis in the Developing Vertebrate Central Nervous System." *Current Opinion in Cell Biology* 17(6): 648–57.

Ilonen, Jorma et al. 2013. "Patterns of β-Cell Autoantibody Appearance and Genetic Associations during the First Years of Life." *Diabetes* 62(10): 3636–40.

Inada, Akari et al. 2008. "Carbonic Anhydrase II-Positive Pancreatic Cells Are Progenitors for Both Endocrine and Exocrine Pancreas after Birth." *Proceedings of the National Academy of Sciences of the United States of America* 105(23): 19915–19.

Karampelias, Christos et al. 2021. "Reinforcing One-Carbon Metabolism via Folic Acid/Folr1 Promotes β-Cell Differentiation." *Nature Communications* 12(1): 1–13. http://dx.doi.org/10.1038/s41467-021-23673-0.

———. 2022. "MNK2 Deficiency Potentiates β-Cell Regeneration via Translational Regulation." *Nature Chemical Biology* 18(9): 942–53.

Katsarou, Anastasia et al. 2017. "Type 1 Diabetes Mellitus." *Nature Reviews Disease Primers* 3: 17016.

Kawakami, Koichi. 2007. "Tol2: A Versatile Gene Transfer Vector in Vertebrates." *Genome biology* 8 Suppl 1: S7.

Kim, Yung Hae et al. 2015. "Cell Cycle–Dependent Differentiation Dynamics Balances Growth and Endocrine Differentiation in the Pancreas." *PLoS Biology* 13(3).

Kimmel, Charles B. 1989. "Genetics and Early Development of Zebrafish." *Trends in Genetics* 5(C): 283–88.

Kinkel, Mary D, and Victoria E Prince. 2009. "On the Diabetic Menu: Zebrafish as a Model for Pancreas Development and Function." *Bioassays* 31(2): 139–52.

Ko, Sungjin et al. 2016. "Bromodomain and Extraterminal (BET) Proteins Regulate Biliary-Driven Liver Regeneration." *Journal of Hepatology* 64(2): 316–25.

Kopp, J L et al. 2011. “Sox9+ Ductal Cells Are Multipotent Progenitors throughout Development but Do Not Produce New Endocrine Cells in the Normal or Injured Adult Pancreas.” *Development* 138: 653–65.

Krentz, Nicole A J et al. 2017. “Phosphorylation of NEUROG3 Links Endocrine Differentiation to the Cell Cycle in Pancreatic Article Phosphorylation of NEUROG3 Links Endocrine Differentiation to the Cell Cycle in Pancreatic Progenitors.” *Developmental Cell* 41(2): 129-142.e6. http://dx.doi.org/10.1016/j.devcel.2017.02.006.

Krishnamurthy, Janakiraman et al. 2006. “P16INK4a Induces an Age-Dependent Decline in Islet Regenerative Potential.” *Nature* 443(7110): 453–57.

Kroehne, Volker et al. 2011. “Regeneration of the Adult Zebrafish Brain from Neurogenic Radial Glia-Type Progenitors.” *Development* 138(22): 4831–41.

Kroon, Evert et al. 2008. “Pancreatic Endoderm Derived from Human Embryonic Stem Cells Generates Glucose-Responsive Insulin-Secreting Cells in Vivo.” *Nature Biotechnology* 26(4): 443–52.

Kujawski, Satu et al. 2014. “Calcineurin Regulates Coordinated Outgrowth of Zebrafish Regenerating Fins.” *Developmental Cell* 28(5): 573–87.

Kulkarni, Abhishek A. et al. 2018. “An in Vivo Zebrafish Model for Interrogating ROS-Mediated Pancreatic β-Cell Injury, Response, and Prevention.” *Oxidative Medicine and Cellular Longevity* 2018.

Kume, Kazunori et al. 2011. “Calcineurin Ensures a Link between the DNA Replication Checkpoint and Microtubule-Dependent Polarized Growth.” *Nature Cell Biology* 13(3): 234–42. http://dx.doi.org/10.1038/ncb2166.

Kwan, Kristen M et al. 2007. “The Tol2kit: A Multisite Gateway-Based Construction Kit for Tol2 Transposon Transgenesis Constructs.” *Developmental dynamics : an official publication of the American Association of Anatomists* 236(11): 3088–99.

Lacroix, Matthieu et al. 2020. “Metabolic Functions of the Tumor Suppressor P53: Implications in Normal Physiology, Metabolic Disorders, and Cancer.” *Molecular Metabolism* 33(October 2019): 2–22. https://doi.org/10.1016/j.molmet.2019.10.002.

Lai, Siting et al. 2021. “Chemical Screening Reveals Ronidazole Is a Superior Prodrug to Metronidazole for Nitroreductase-Induced Cell Ablation System in Zebrafish Larvae.” *Journal of Genetics and Genomics* 48(12): 1081–90. https://doi.org/10.1016/j.jgg.2021.07.015.

Lee, Peter J., and Georgios I. Papachristou. 2019. “New Insights into Acute Pancreatitis.” *Nature Reviews Gastroenterology and Hepatology* 16(8): 479–96. http://dx.doi.org/10.1038/s41575-019-0158-2.

Leech, Cassandra M. et al. 2020. “The Coordinate Actions of Calcineurin and Hog1 Mediate the Stress Response through Multiple Nodes of the Cell Cycle Network.” *PLoS Genetics* 16(4): 1–27. http://dx.doi.org/10.1371/journal.pgen.1008600.

Li, Wan Chun et al. 2010. “Activation of Pancreatic-Duct-Derived Progenitor Cells during Pancreas Regeneration in Adult Rats.” *Journal of Cell Science* 123(16): 2792–2802.

Liu, Haisong et al. 2021. “Chemical Combinations Potentiate Human Pluripotent Stem Cell-Derived 3D Pancreatic Progenitor Clusters toward Functional β Cells.” *Nature Communications* 12(1): 3–12. http://dx.doi.org/10.1038/s41467-021-23525-x.

Liu, Jun et al. 1991. “Calcineurin Is a Common Target of Cyclophilin-Cyclosporin A and FKBP-FK506 Complexes.” *Cell* 66(4): 807–15.

Liu, Ka Cheuk et al. 2017. “Inhibition of Cdk5 Promotes β-Cell Differentiation from Ductal Progenitors.” *Diabetes* 67(1): 58–70.

Lu, Jing et al. 2016. “ IGFBP 1 Increases B-cell Regeneration by Promoting A- to B-cell Transdifferentiation .” *The EMBO Journal* 35(18): 2026–44.

Lu, Wei Yu et al. 2015. “Hepatic Progenitor Cells of Biliary Origin with Liver Repopulation Capacity.” *Nature Cell Biology* 17(8): 973–83.

Maddocks, Oliver D.K. et al. 2013. “Serine Starvation Induces Stress and P53-Dependent Metabolic Remodelling in Cancer Cells.” *Nature* 493(7433): 542–46.

Maden, Malcolm. 2018. “The Evolution of Regeneration – Where Does That Leave Mammals?” *International Journal of Developmental Biology* 62(6–8): 369–72.

Magenheim, Judith et al. 2023. “Matters Arising: Insufficient Evidence That Pancreatic β Cells Are Derived from Adult Ductal Neurog3-Expressing Progenitors.” *Cell Stem Cell* 30(4): 488-497.e3. https://doi.org/10.1016/j.stem.2023.03.003.

Mammucari, Cristina et al. 2005. “Integration of Notch 1 and Calcineurin/NFAT Signaling Pathways in Keratinocyte Growth and Differentiation Control.” *Developmental Cell* 8(5): 665–76.

Manfroid, Isabelle et al. 2012. “Zebrafish Sox9b Is Crucial for Hepatopancreatic Duct Development and Pancreatic Endocrine Cell Regeneration.” *Dev. Biol.* 366(2): 268–78.

Mannioui, Abdelkrim, and Bernard Zalc. 2019. “Conditional Demyelination and Remyelination in a Transgenic Xenopus Laevis.” *Methods in Molecular Biology* 1936: 239–48.

Marín-Peñalver, Juan José, Iciar Martín-Timón, Cristina Sevillano-Collantes, and Francisco Javier del Cañizo-Gómez. 2016. “Update on the Treatment of Type 2 Diabetes Mellitus.” *World Journal of Diabetes* 7(17): 354.

Marinaga, Marcia. 1990. “Swimming into the Development Mainstream.” *Science* 250.

Marques, Ines J., Eleonora Lupi, and Nadia Mercader. 2019. “Model Systems for Regeneration: Zebrafish.” *Development (Cambridge)* 146(18).

Masaki, Takahiro, and Midori Shimada. 2022. “Decoding the Phosphatase Code: Regulation of Cell Proliferation by Calcineurin.” *International Journal of Molecular Sciences* 23(3).

Mashkaryan, Violeta et al. 2020. “Type 1 Interleukin-4 Signaling Obliterates Mouse Astroglia in Vivo but Not in Vitro.” *Frontiers in Cell and Developmental Biology* 8(February): 1–16.

Massoz, Laura, Marie Alice Dupont, and Isabelle Manfroid. 2021. “Zebra-Fishing for Regenerative Awakening in Mammals.” *Biomedicines* 9(1): 1–18.

Mathias, Jonathan R., Zhanying Zhang, Meera T. Saxena, and Jeff S. Mumm. 2014. “Enhanced Cell-Specific Ablation in Zebrafish Using a Triple Mutant of Escherichia Coli Nitroreductase.” *Zebrafish* 11(2): 85–97.

McDonald, Georgina L.K., Mengdi Wang, Chrissy L. Hammond, and Dylan J.M. Bergen. 2021. “Pharmacological Manipulation of Early Zebrafish Skeletal Development Shows an Important Role for Smad9 in Control of Skeletal Progenitor Populations.” *Biomolecules* 11(2): 1–20.

McIntyre, H. David et al. 2019. “Gestational Diabetes Mellitus.” *Nature Reviews Disease Primers* 5(1). http://dx.doi.org/10.1038/s41572-019-0098-8.

McMillan, Stephanie C. et al. 2018. “A Regulatory Pathway Involving Retinoic Acid and Calcineurin Demarcates and Maintains Joint Cells and Osteoblasts in Regenerating Fin.” *Development (Cambridge)* 145(11).

Md Moin, Abu Saleh, Peter C. Butler, and Alexandra E. Butler. 2017. “Increased Proliferation of the Pancreatic Duct Gland Compartment in Type 1 Diabetes.” *Journal of Clinical Endocrinology and Metabolism* 102(1): 200–209.

Meier, J. J. et al. 2005. “Sustained Beta Cell Apoptosis in Patients with Long-Standing Type 1 Diabetes: Indirect Evidence for Islet Regeneration?” *Diabetologia* 48(11): 2221–28.

Millman, Jeffrey R. et al. 2016. “Generation of Stem Cell-Derived β-Cells from Patients with Type 1 Diabetes.” *Nature Communications* 7(May).

Moin, Abu Saleh Md, and Alexandra E. Butler. 2019. “Alterations in Beta Cell Identity in Type 1 and Type 2 Diabetes.” *Current Diabetes Reports* 19(9).

Moss, Jennifer B. et al. 2009. “Regeneration of the Pancreas in Adult Zebrafish.” *Diabetes* 58(August): 1844–51.

Nauck, Michael A., Daniel R. Quast, Jakob Wefers, and Juris J. Meier. 2021. “GLP-1 Receptor Agonists in the Treatment of Type 2 Diabetes – State-of-the-Art.” *Molecular Metabolism* 46(October 2020): 101102. https://doi.org/10.1016/j.molmet.2020.101102.

Ni, Qianqian et al. 2019. “Advances in Immunotherapy of Type I Diabetes.” *Advanced Drug Delivery Reviews* 139: 83–91. https://doi.org/10.1016/j.addr.2018.12.003.

Ninov, Nikolay et al. 2013. “Metabolic Regulation of Cellular Plasticity in the Pancreas.” *Current Biology* 23(13): 1242–50.

Ninov, Nikolay, Maxim Borius, and Didier Y.R. Stainier. 2012. “Different Levels of Notch Signaling Regulate Quiescence, Renewal and Differentiation in Pancreatic Endocrine Progenitors.” *Development* 139(9): 1557–67.

Nir, Tomer, Douglas A Melton, and Yuval Dor. 2007. “Nir, Dor 2007 Recovery from Diabetes in Mice by ββcell Regeneration.Pdf.” 117(9): 2553–61.

Norris, Jill M., Randi K. Johnson, and Lars C. Stene. 2020. “Type 1 Diabetes—Early Life Origins and Changing Epidemiology.” *The Lancet Diabetes and Endocrinology* 8(3): 226–38. http://dx.doi.org/10.1016/S2213-8587(19)30412-7.

Offield, Martin F et al. 1996. “PDX-1 Is Required for Pancreatic Outgrowth and Differentiation of the Rostral Duodenum.” *Development* 122.

Oh-hora, Masatsugu, and Anjana rao. 2009. “The Calcium/NFAT Pathway: Role in Development and Function of Regulatory T Cells.” *Microbes and Infection* 11(5): 612–19. http://dx.doi.org/10.1016/j.micinf.2009.04.008.

Oyadomari, S., E. Araki, and M. Mori. 2002. “Endoplasmic Reticulum Stress-Mediated Apoptosis in Pancreatic β-Cells.” *Apoptosis* 7(4): 335–45.

Pagliuca, Felicia W. et al. 2014. “Generation of Functional Human Pancreatic β Cells in Vitro.” *Cell* 159(2): 428–39.

Pagola, A. Martin- et al. 2008. “Insulin Expression and Proliferation in Ductal Cells in the Transplanted Pancreas of Patients with Type 1 Diabetes and Recurrence of Autoimmunity.” *Diabetologia* 51(10): 1803–13.

Pan, Fong Cheng et al. 2013. “Spatiotemporal Patterns of Multipotentiality in Ptf1aexpressing Cells during Pancreas Organogenesis and Injuryinduced Facultative Restoration.” *Development (Cambridge)* 140(4): 751–64.

Pan, Fong Cheng, and Chris Wright. 2011. “Pancreas Organogenesis: From Bud to Plexus to Gland.” *Developmental Dynamics* 240(3): 530–65.

Papadimitriou, Christos et al. 2018. “3D Culture Method for Alzheimer’s Disease Modeling Reveals Interleukin-4 Rescues Aβ42-Induced Loss of Human Neural Stem Cell Plasticity.” *Developmental Cell* 46(1): 85-101.e8. https://doi.org/10.1016/j.devcel.2018.06.005.

Parsons, Michael J. et al. 2009. “Notch-Responsive Cells Initiate the Secondary Transition in Larval Zebrafish Pancreas.” *Mechanisms of Development* 126(10): 898–912. http://dx.doi.org/10.1016/j.mod.2009.07.002.

Pelletier, Joffrey et al. 2020. “ Nucleotide Depletion Reveals the Impaired Ribosome Biogenesis Checkpoint as a Barrier against DNA Damage .” *The EMBO Journal* 39(13): 1–19.

Perez-Frances, Marta et al. 2021. “Pancreatic Ppy-Expressing γ-Cells Display Mixed Phenotypic Traits and the Adaptive Plasticity to Engage Insulin Production.” *Nature Communications* 12(1): 1–16. http://dx.doi.org/10.1038/s41467-021-24788-0.

Pisharath, Harsan et al. 2007. “Targeted Ablation of Beta Cells in the Embryonic Zebrafish Pancreas Using E.Coli Nitroreductase.” *Mech Dev.*

Pisharath, Harshan. 2007. “Validation of Nitroreductase, a Prodrug-Activating Enzyme, Mediated Cell Death in Embryonic Zebrafish (Danio Rerio).” *Comparative Medicine* 57(3): 241–46.

Pociot, Flemming, and Åke Lernmark. 2016. “Genetic Risk Factors for Type 1 Diabetes.” *The Lancet* 387(10035): 2331–39.

Poss, Kenneth D., Lindsay G. Wilson, and Mark T. Keating. 2002. “Heart Regeneration in Zebrafish.” *Science* 298(5601): 2188–90.

Qadir, Mirza Muhammad Fahd et al. 2018. “P2RY1/ALK3-Expressing Cells within the Adult Human Exocrine Pancreas Are BMP-7 Expandable and Exhibit Progenitor-like Characteristics.” *Cell Reports* 22(9): 2408–20.

———. 2020. “Single-Cell Resolution Analysis of the Human Pancreatic Ductal Progenitor Cell Niche.” *Proceedings of the National Academy of Sciences of the United States of America* 117(20): 10876–87.

Ramachandran R., Fausett B.V., Goldman D. 2010. “Ascl1A Regulates Muller Glia Dedifferentiation and Retina Regeneration via a Lin-28 Dependent, Let7 MiRNA Signaling Pathway.” *Nature Cell Biology* 7(12): 1959–67.

Rankin, Matthew M., and Jake A. Kushner. 2009. “Adaptive β-Cell Proliferation Is Severely Restricted with Advanced Age.” *Diabetes* 58(6): 1365–72.

Rezania, Alireza et al. 2014. “Reversal of Diabetes with Insulin-Producing Cells Derived in Vitro from Human Pluripotent Stem Cells.” *Nature Biotechnology*.

Rix, Iben et al. 2015. *Glucagon Physiology*. MDText.com, Inc., South Dartmouth (MA). http://europepmc.org/books/NBK279127.

Roden, Michael, and Gerald I. Shulman. 2019. “The Integrative Biology of Type 2 Diabetes.” *Nature* 576(7785): 51–60. http://dx.doi.org/10.1038/s41586-019-1797-8.

Rorsman, Patrik, and Mark O. Huising. 2018. “The Somatostatin-Secreting Pancreatic δ-Cell in Health and Disease.” *Nature Reviews Endocrinology* 14(7): 404–14.

Rovira, Meritxell et al. 2010. “Isolation and Characterization of Centroacinar/Terminal Ductal Progenitor Cells in Adult Mouse Pancreas.” *Proceedings of the National Academy of Sciences of the United States of America* 107(1): 75–80.

Roy, Jagoree, and Martha S. Cyert. 2020. “Identifying New Substrates and Functions for an Old Enzyme: Calcineurin.” *Cold Spring Harbor Perspectives in Biology* 12(3).

Schwitzgebel, V. M. et al. 2000. “Expression of Neurogenin3 Reveals an Islet Cell Precursor Population in the Pancreas.” *Development* 127(16): 3533–42.

Scully, Tony. 2012. “DIABETES IN Numbers.” *Nature* (2011): 0–1.

Senk, Anita, and Valentin Djonov. 2021. “Collagen Fibers Provide Guidance Cues for Capillary Regrowth during Regenerative Angiogenesis in Zebrafish.” *Scientific Reports* 11(1): 1–17. https://doi.org/10.1038/s41598-021-98852-6.

Serreze, David V., and Edward H. Leiter. 1994. “Genetic and Pathogenic Basis of Autoimmune Diabetes in NOD Mice.” *Current Opinion in Immunology* 6(6): 900–906.

Seymour, Philip A. et al. 2007. “SOX9 Is Required for Maintenance of the Pancreatic Progenitor Cell Pool.” *Proceedings of the National Academy of Sciences of the United States of America* 104(6): 1865–70.

Shapiro, A. M.James et al. 2021. “Insulin Expression and C-Peptide in Type 1 Diabetes Subjects Implanted with Stem Cell-Derived Pancreatic Endoderm Cells in an Encapsulation Device.” *Cell Reports Medicine* 2(12): 100466. https://doi.org/10.1016/j.xcrm.2021.100466.

Shapiro, A.M. James et al. 2000. “ISLET TRANSPLANTATION IN SEVEN PATIENTS WITH TYPE 1 DIABETES I S L ET T R A N S P L A N TAT I O N I N PAT I E N T S W I T H D I A B ET E S U S I N G G LU C O C O R T I C O I D - F R E E I M M U N O S U P P R E S S I O N.” *Journal of Medicine*.

———. 2006. “International Trial of the Edmonton Protocol for Islet Transplantation.” *New England Journal of Medicine* 355(13): 1318–30.

Sharrock, Abigail et al. 2020. “NTR 2.0: A Rationally-Engineered Prodrug Converting Enzyme with Substantially Enhanced Efficacy for Targeted Cell Ablation.” : 1–26.

Shiraki, Nobuaki et al. 2014. “Methionine Metabolism Regulates Maintenance and Differentiation of Human Pluripotent Stem Cells.” *Cell Metabolism* 19(5): 780–94. http://dx.doi.org/10.1016/j.cmet.2014.03.017.

Shoffner, Adam et al. 2020a. “Tp53 Suppression Promotes Cardiomyocyte Proliferation during Zebrafish Heart Regeneration.” *Cell Reports*

32(9): 108089. https://doi.org/10.1016/j.celrep.2020.108089.

———. 2020b. “Tp53 Suppression Promotes Cardiomyocyte Proliferation during Zebrafish Heart Regeneration.” *Cell Reports*.

Sims, Emily K. et al. 2021. “100 Years of Insulin: Celebrating the Past, Present and Future of Diabetes Therapy.” *Nature Medicine* 27(7): 1154–64. http://dx.doi.org/10.1038/s41591-021-01418-2.

Singh, Sumeet Pal et al. 2017. “Different Developmental Histories of Beta-Cells Generate Functional and Proliferative Heterogeneity during Islet Growth.” *Nature Communications* 8(1): 1–15. http://dx.doi.org/10.1038/s41467-017-00461-3.

———. 2022. “A Single-Cell Atlas of de Novo β-Cell Regeneration Reveals the Contribution of Hybrid β/δ-Cells to Diabetes Recovery in Zebrafish.” *Development (Cambridge, England)* 149(2).

Solar, Myriam et al. 2009a. “Pancreatic Exocrine Duct Cells Give Rise to Insulin-Producing β Cells during Embryogenesis but Not after Birth.” *Developmental Cell* 17(6): 849–60.

———. 2009b. “Pancreatic Exocrine Duct Cells Give Rise to Insulin-Producing β Cells during Embryogenesis but Not after Birth.” *Developmental Cell* 17: 849–60.

Sousounis, Konstantinos et al. 2020. “Eya2 Promotes Cell Cycle Progression by Regulating DNA Damage Response during Vertebrate Limb Regeneration.”

Stanger, Ben Z., and Matthias Hebrok. 2013. “Control of Cell Identity in Pancreas Development and Regeneration.” *Gastroenterology* 1(3): 233–45.

Steiner, Donald J., Abraham Kim, Kevin Miller, and Manami Hara. 2010. “Pancreatic Islet Plasticity: Interspecies Comparison of Islet Architecture and Composition.” *Islets* 2(3): 135–45.

Stepniak, Ewa et al. 2006. “C-Jun/AP-1 Controls Liver Regeneration by Repressing P53/P21 and P38 MAPK Activity.” *Genes and Development* 20(16): 2306–14.

Streisinger, George et al. 1981. “Production of Homozygous Diploid Zebra Fish (Brachydanio Rerio).” *Nature* 112(1): 25–37.

Sun, Hong et al. 2022. “IDF Diabetes Atlas: Global, Regional and Country-Level Diabetes Prevalence Estimates for 2021 and Projections for 2045.” *Diabetes Research and Clinical Practice* 183: 109119. https://doi.org/10.1016/j.diabres.2021.109119.

Tarifeño-Saldivia, Estefania et al. 2017. “Transcriptome Analysis of Pancreatic Cells across Distant Species Highlights Novel Important Regulator Genes.” *BMC Biology* 15(1): 1–19.

Teta, Monica et al. 2005. “Very Slow Turnover of β-Cells in Aged Adult Mice.” *Diabetes* 54(9): 2557–67.

Theodoropoulou, Marily, and Günter K. Stalla. 2013. “Somatostatin Receptors: From Signaling to Clinical Practice.” *Frontiers in Neuroendocrinology* 34(3): 228–52. http://dx.doi.org/10.1016/j.yfrne.2013.07.005.

Thorel, Fabrizio, Virginie Nepote, et al. 2010. “Conversion of Adult Pancreatic Alpha-Cells to Beta-Cells after Extreme Beta Cell Loss.” *Nature* 464(7292): 1149–54. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2877635&tool=pmcentrez&rendertype=abstract.

Thorel, Fabrizio, Virginie Népote, et al. 2010. “Conversion of Adult Pancreatic Alpha Cells to Beta Cells after Extreme Beta Cell Loss.” *Nature* 464(7292): 1149–54.

Tornini, Valerie A. et al. 2016. “Live Monitoring of Blastemal Cell Contributions During Appendage Regeneration.” *Current Biology*.

Tsuji, Naoki et al. 2014. “Whole Organism High Content Screening Identifies Stimulators of Pancreatic Beta-Cell Proliferation.” *PLoS ONE* 9(8).

Ueki, Yumi et al. 2015. “Transgenic Expression of the Proneural Transcription Factor Ascl1 in Müller Glia Stimulates Retinal Regeneration in Young Mice.” *Proceedings of the National Academy of Sciences of the United States of America* 112(44): 13717–22.

Velazco-Cruz, Leonardo et al. 2019. “Acquisition of Dynamic Function in Human Stem Cell-Derived β Cells.” *Stem Cell Reports* 12(2): 351–65. https://doi.org/10.1016/j.stemcr.2018.12.012.

Venegoni, Mauro. 2002. “Tacrolimus.” *Occhio Clinico* 8(5): 29.

Veres, Adrian et al. 2019. “Charting Cellular Identity during Human in Vitro β-Cell Differentiation.” *Nature* 569(7756): 368–73. http://dx.doi.org/10.1038/s41586-019-1168-5.

Voight, Benjamin F et al. 2011. “Twelve Type 2 Diabetes Susceptibility Loci Identified through Large-Scale Association Analysis.” *Nature Genetic* 42(7): 579–89.

Vousden, Karen H., and Kevin M. Ryan. 2009. “P53 and Metabolism.” *Nature Reviews Cancer* 9(10): 691–700.

Wang, R N, G Klöppel, and L Bouwens. 1995. “Duct- to Islet-Cell Differentiation and Islet Growth in the Pancreas of Duct-Ligated Adult Rats.” *Diabetologia* 38(12): 1405–11.

Weiss, Michael, Donald F Steiner, and Louis H Philipson. 2014. *Insulin Biosynthesis, Secretion, Structure, and Structure-Activity Relationships*. MDText.com, Inc., South Dartmouth (MA). http://europepmc.org/books/NBK279029.

Wu, Xunwei et al. 2010. “Calcineurin and ATF3: Opposite Roles in Squamous Skin Cancer.” *Nature*.

Xu, Jin et al. 2018. “Inhibition of TBK1/IKKε Promotes Regeneration of Pancreatic β-Cells.” *Scientific Reports* 8(1): 1–14.

Xu, Xiaobo et al. 2008. “Beta Cells Can Be Generated from Endogenous Progenitors in Injured Adult Mouse Pancreas.” *Cell* 132: 197–207.

Xue, Angli et al. 2018. “Genome-Wide Association Analyses Identify 143 Risk Variants and Putative Regulatory Mechanisms for Type 2 Diabetes.” *Nature Communications* 9(1).

Ye, Lihua et al. 2015. “Glucagon Is Essential for Alpha Cell Transdifferentiation and Beta Cell Neogenesis.” *Development (Cambridge)* 142(8): 1407–17.

Yoneda, S. et al. 2013. “Predominance of β-Cell Neogenesis Rather than Replication in Humans with an Impaired Glucose Tolerance and Newly Diagnosed Diabetes.” *Journal of Clinical Endocrinology and Metabolism* 98(5): 2053–61.

Zecchin, Elisabetta et al. 2004. “Evolutionary Conserved Role of Ptf1a in the Specification of Exocrine Pancreatic Fates.” *Developmental Biology* 268(1): 174–84.

Zhao, Huan et al. 2021. “Pre-Existing Beta Cells but Not Progenitors Contribute to New Beta Cells in the Adult Pancreas.” *Nature Metabolism* 3(3): 352–65.

Ziegler, Annette G. et al. 2013. “Seroconversion to Multiple Islet Autoantibodies and Risk of Progression to Diabetes in Children Anette.” *Physiology & behavior*.

2.2. p53/PFTα

Our transcriptomic data underline other putative candidate signalling pathways in the ductal cells during β cell regeneration. The cell cycle signatures from the upregulated genes comprised particularly numerous antiproliferative and repair pathways evocative of cell cycle arrest such as DNA damage repair pathways, the p53 pathway and cellular senescence (Massoz et al., 2023 in review, eLife). Among them, the p53 pathway encompassed genes involved in cell cycle checkpoints (*chek1/2, gtse1*) and response to DNA damage (*gadd45ab*). The absence of apoptosis signatures in the ductal cells suggested that p53 do not reflect ductal cell death but instead adaptation and repair consequent to robust activation of duct cell proliferation and cellular stress. Based on these observations, we hypothesized that a tight control of cell cycle progression plays a role in β cell regeneration from ductal progenitors. In order to study p53 role in β cell regeneration, we used the p53 inhibitor, the pifithrin-α (PFT-α).

Firstly, we determined the effect of (PFT-α) on the regenerative response from the ducts. We performed β cell ablation on 3 dpf *Tg(ins:NTR-P2A-mCherry); Tg(nkx6.1:GFP*) larvae with NFP, as performed previously, before PFT-α (10µM) treatment from 1 to 3 dpt. Assessment of the regenerative response at 14 dpt revealed that PFT-α decreased the regenerative response (**Figure 22A)**. PFTα indeed blocked the excess of β cell formed after ablation, whereas it did not alter basal β cell differentiation in non-ablated larvae. This indicates that p53 could be essential for the regenerative response but not for β cell formation in normally growing larvae. Next, we asked whether p53 regulates the differentiation of β cells elicited by Notch downregulation. Surprisingly, while p53 inhibition repressed the ductal regenerative response (**Figure 22A)**, PFTa did not influence β cell formation in LY411575-treated, non-ablated, larvae (**Figure 22B)**.

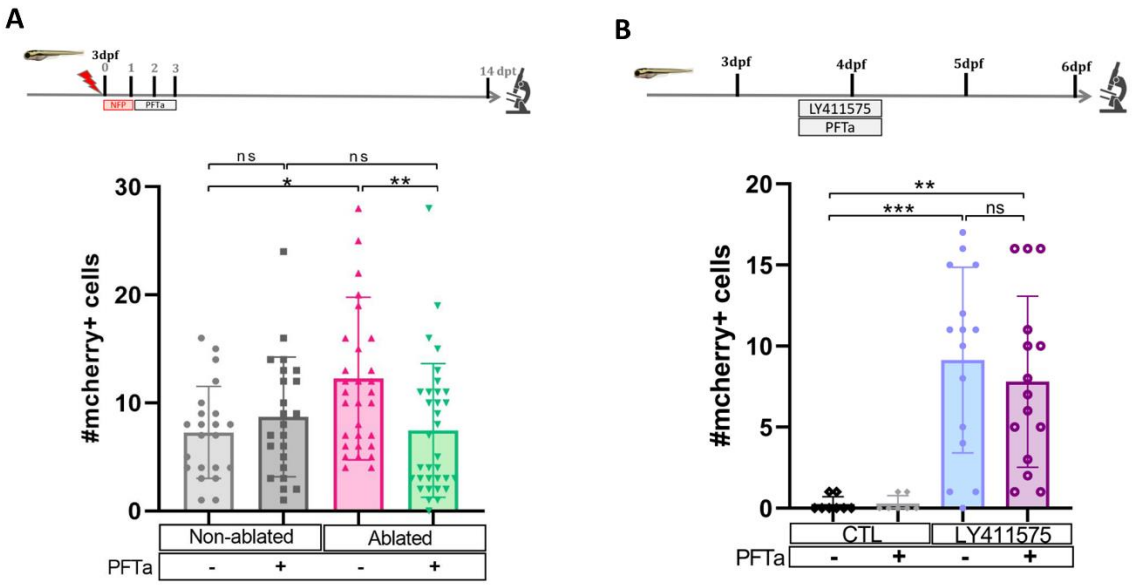


Figure 22 : *PFTα decreases the ductal regenerative response but do not affect β cell formation under Notch repression*

A) Barplot representing the number of number of mCherry+ β cells in the pancreatic tail of *Tg(ins:NTR-P2A-mCherry) ; Tg(nkx6.1:GFP)* larvae at 14dpt. The gray spheres represent non-ablated condition ; the pink triangles represent the ablated condition ; the black squares PFTα condition and inverted green triangles ablated + PFTα condition. Data are presented as mean values ± SD. Two-way ANOVA with Tukey's multiple comparison test, *p-value<0.05, **p-value<0.005 .

B) Barplot representing the mean number of mCherry+ β cells in the pancreatic tail of *Tg(ins:NTR-P2A-mCherry) ; Tg(nkx6.1:GFP)* larvae at 6dpf. The black dots represent the control; gray PFTα treatment ;blue LY411575; and purple combination of LY411575 and PFTα. Data are presented as mean values ± SD. Two-way ANOVA with Tukey multiple comparison test, **p-value<0.005, ***p-value<0.0005.

As p53 is a well-known master player in proliferation, being involved in cell cycle arrest in response to stress, we next wanted to determine its involvement in progenitor proliferation during β cell regeneration. EdU incorporation in the ducts was examined in *Tg(ins:NTR-P2A-mCherry); Tg(nkx6.1:GFP)* larvae after ablation and PFTα treatment (Figure 5F). PFTα completely blocked the stimulation of progenitor proliferation induced by β cell ablation (**Figure 23**). These results strongly suggest that the effect of PFTα on β cell regeneration is mediated by their action in ductal progenitor proliferation.

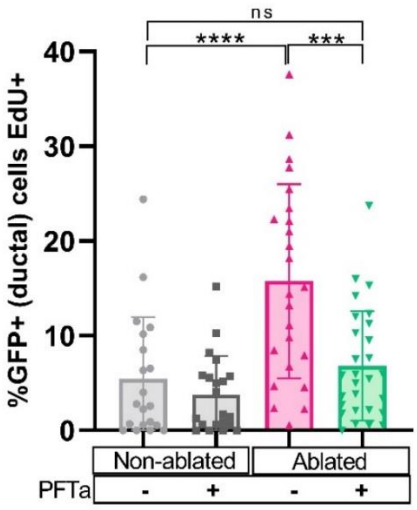


Figure 23 : *PFTα decreases the ductal cell proliferation during β cell regeneration*

Barplot representing the percentage of GFP+ ductal cells which incorporated EdU+ in pancreatic tail of *Tg(ins:NTR-P2A-mCherry) ; Tg(nkx6.1:GFP)* larvae at 5dpf. The gray spheres represent non-ablated condition ; the pink triangles the ablated condition ; the black squares PFTα condition and inverted green triangles ablated + PFTα condition. Data are presented as mean values ± SD. Two-way ANOVA test with Tukey's multiple comparisons test, ***p-value<0.0005 ; ****p-value<0.00005 ; ns = non-significant.

Then, we wanted to confirm these results in another model of p53 inhibition, in the tp53^{M214K} loss of function mutant line (Berghmans et al. 2005). Of note, the mutation did not affect β cell ablation (Marie Dupont, personal communication). After β cell ablation with NFP, tp53^{M214K} *Tg(ins:NTR-P2A-mCherry); Tg(nkx6.1:GFP)* larvae were exposed to EdU. However, assessment of EdU in the ductal cells did not show any difference of proliferation between wild type and mutant larvae (**Figure 24**).

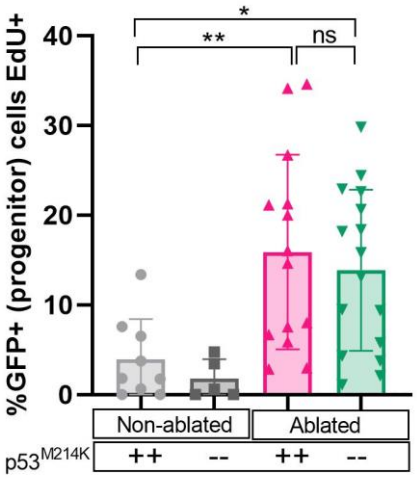


Figure 24 : tp53^{M214K} *do not affect the ductal cell proliferation during β cell regeneration*

Barplot representing the percentage of GFP+ ductal cells that incorporated EdU+ in pancreatic tail of tp53M214K homozygous mutant or WT in *Tg(ins:NTR-P2A-mCherry) ; Tg(nkx6.1:GFP)* larvae at 5dpf. The gray spheres represent non-ablated condition ; the pink triangles the ablated condition ; the black squares tp53M214K condition and inverted green triangles tp53M214K ablated condition. Data are presented as mean values ± SD. Two-way ANOVA test with Tukey's multiple comparisons test, *p-value<0.05 ; **p-value<0.005 ; ns = non-significant.

Overall, these results show that PFTα strongly diminished the proliferative response in the ducts and subsequent β cell formation triggered by β cell ablation. Interestingly, PFTα treatment has only an effect during regeneration and not during normal development or under Notch signalling repression. Nevertheless, we could not reproduce these results in the $tp53^{M214K}$ mutant line, rising the questions whether PFTα effect is mediated by p53 inhibition in this case, or if all p53 functions are repressed in the $tp53^{M214K}$ mutant line. Notably, p21 activation and the apoptosis are blocked in this mutant (Berghmans et al. 2005). However, there is a possibility that others function of p53 could be conserved. Our transcriptomic data indeed suggest that the apoptosis is not activated, underlying that p53 could have other function in the ducts during regeneration. As a consequence, it would be interesting to test another mutant line as the full KO and study more deeply the effects of PFTα in this system.

3. The origins and function of bi-hormonal sst1.1+/ins+ cells

The intriguing observation that β cells co-express the insulin and the somatostatin hormones after ablation, led us to characterized more deeply the regenerated β cells. An exploratory transcriptomic study of regenerated β cells showed that these cells express the *sst1.1* hormone, which has not yet been described. The study of this new δ cells population and its contribution in β cell regeneration was published in a paper entitled " A δ-cell subpopulation with a pro-β-cell identity contributes to efficient age-independent recovery in a zebrafish model of diabetes" in eLife (Carril Pardo et al. 2022).

I contributed to this manuscript by exploring the origins of the bi-hormonal cells *ins+/sst1.1+* during β cell regeneration. Time lapse experiment performed on transgenic *Tg(sst1.1:GFP); Tg(ins:NTR-mCherry)* larvae strongly suggest that sst1.1+ δ cells in the primary islet switch on insulin production after β cell ablation. While genetic lineage tracing would have been an ideal approach to follow the sst1.1+ δ cells, unfortunately, despite our efforts, we were unable to design an efficient *sst1.1:cre*^{ERT2} transgenic line to carry out this experiment. However, an independent study (Singh et al. 2022) led to the same conclusions without genetic lineage tracing, highlighting the significance of sst1.1+ δ cells in β cell regeneration.

Additionally, we wondered if the bi-hormonal cells were also involved in β cell regeneration occurring in the pancreatic tail, where the ductal network is highly represented. A first set of FACS experiments showed that the vast majority of regenerated β cells co-express *sst1.1* and *ins* in the adult zebrafish, similarly as in the primary islet of young larvae. As such, the bi-hormonal cells are also important for regeneration in the pancreatic tail. Moreover, we showed in an experimental setting enabling assessment of β cell regeneration from the ducts in the zebrafish larvae (detailed in Massoz et al. 2023), that bi-hormonal cells arise from the ducts. Overall, we showed that sst1.1+ δ cells rapidly convert into ins+ bi-hormonal cells and that these cell arise as well from the ducts.

eLife

RESEARCH ARTICLE

A δ-cell subpopulation with a pro-β-cell identity contributes to efficient age-independent recovery in a zebrafish model of diabetes

Claudio Andrés Carril Pardo[1†], Laura Massoz[1†], Marie A Dupont[1†], David Bergemann[1], Jordane Bourdouxhe[1], Arnaud Lavergne[1,2], Estefania Tarifeño-Saldivia[1,3], Christian SM Helker[4], Didier YR Stainier[4], Bernard Peers[1], Marianne M Voz[1], Isabelle Manfroid[1]*

[1]Zebrafish Development and Disease Models laboratory, GIGA-Stem Cells, University of Liège, Liège, Belgium; [2]GIGA-Genomics core facility, University of Liège, Liège, Belgium; [3]Gene Expression and Regulation Laboratory, Department of Biochemistry and Molecular Biology, University of Concepción, Concepción, Chile; [4]Department of Developmental Genetics, Max Planck Institute for Heart and Lung Research, Bad Nauheim, Germany

Abstract Restoring damaged β-cells in diabetic patients by harnessing the plasticity of other pancreatic cells raises the questions of the efficiency of the process and of the functionality of the new *Insulin*-expressing cells. To overcome the weak regenerative capacity of mammals, we used regeneration-prone zebrafish to study β-cells arising following destruction. We show that most new *insulin* cells differ from the original β-cells as they coexpress Somatostatin and Insulin. These bihormonal cells are abundant, functional and able to normalize glycemia. Their formation in response to β-cell destruction is fast, efficient, and age-independent. Bihormonal cells are transcriptionally close to a subset of δ-cells that we identified in control islets and that are characterized by the expression of *somatostatin 1.1* (*sst1.1*) and by genes essential for glucose-induced Insulin secretion in β-cells such as *pdx1*, *slc2a2* and *gck*. We observed in vivo the conversion of monohormonal *sst1.1*-expressing cells to *sst1.1+ ins +* bihormonal cells following β-cell destruction. Our findings support the conclusion that *sst1.1* δ-cells possess a pro-β identity enabling them to contribute to the neogenesis of Insulin-producing cells during regeneration. This work unveils that abundant and functional bihormonal cells benefit to diabetes recovery in zebrafish.

Editor's evaluation

Recently, there has been a growing appreciation for the existence of cellular plasticity in the adult islet. This study probes this phenomenon by exploiting the zebrafish experimental model, which has a much higher adult regeneration capacity than in mammals. A particular novel finding from the study is the identification of a subpopulation of islet delta cells that are similar to beta cells at the transcriptional level and can convert into an insulin/somatostatin co-expressing cell population upon beta cell ablation. The findings will be of particular interest to researchers interested in islet cell biology and pancreatic endocrine cell reprogramming; it will be interesting to explore whether similar delta cell subpopulations exist in mammalian islets to serve as an alternative source of insulin producing cells.

***For correspondence:** Isabelle.Manfroid@uliege.be

[†]These authors contributed equally to this work

Competing interest: See page 24

Funding: See page 24

Received: 15 February 2021
Preprinted: 24 June 2021
Accepted: 20 January 2022
Published: 21 January 2022

Reviewing Editor: Lori Sussel, University of Colorado Anschutz Medical Campus, United States

Introduction

Insulin-producing β-cells reside in pancreatic islets where they are intermingled with other endocrine cells such as α-cells, secreting glucagon (Gcg), and δ-cells secreting somatostatin (Sst). Elevation of extracellular glucose concentration triggers glucose uptake by β-cells through the glucose transporter GLUT2 (*Slc2a2*). Glucose is then metabolized to generate ATP which will trigger the closure of the K_{ATP} channel formed by Kir6.2 (*Kcnj11*) and SUR1 (*Abcc8*), membrane depolarization, Ca^{2+} influx and release through exocytosis of insulin secretory granules into the blood. In mature β-cells, this process is further amplified by other molecules such as amino acids, fatty acids, hormones (incretins GLP-1, GIP), and neural factors (dopamine, adrenaline…) via the cAMP messenger. Dysfunction of these processes leads to impaired insulin secretion, chronic hyperglycemia, and diabetes. In Type 2 diabetes, chronic glucolipotoxic stress ultimately provokes β-cell failure and death. In Type 1 diabetes, on the other hand, the destruction of β-cells is mediated by autoimmune attack.

Human adult β-cells are quiescent and barely possess the capacity to compensate their destruction through increased proliferation. Alternative mechanisms inferred from studies in mice revealed the striking plasticity of other pancreatic endocrine cell types towards the β-cell phenotype. For example, Ins+ Gcg + bihormonal cells form after acute β-cell destruction mediated by transgenic expression of the diphteria toxin receptor (DTR) in adult mice (**Thorel et al., 2010**). These cells derive from a small fraction of α-cells that switch on the β-cell markers Pdx1, Nkx6.1 and Ins through direct conversion, leading to restoration of about 10% of the β-cell mass after 10 months. As this process is quite slow and inefficient, adult DTR mice do not survive without injection of insulin during the first months after ablation. In contrast, at juvenile stages, β-cell neogenesis occurs from transdifferentiation of δ-cells (**Chera et al., 2014**). In this case, δ-cells dedifferentiate, lose *Sst* expression, replicate and redifferentiate into β-cells. About 23% of the initial β-cell mass has recovered 4 months after ablation emphasising faster and more efficient improvement of glycemia than in adult mice. Very recently, a rare population of pancreatic polypeptide (Ppy)-expressing γ-cells has also been shown to display plasticity and to activate *Ins* expression in response to β-cell injury (**Perez-Frances et al., 2021**). Hence, various pancreatic islet cells possess a remarkable plasticity yet the regeneration potential is generally limited in adult mammals.

In contrast to the limited regeneration capacity of adult mammals, zebrafish are notorious for their potent, spontaneous and rapid regeneration of β-cells from larval to adult stages (**Curado et al., 2007**; **Delaspre et al., 2015**; **Ghaye et al., 2015**; **Moss et al., 2009**; **Ninov et al., 2013**; **Pisharath and Parsons, 2009**; **Ye et al., 2015**). In zebrafish, α-cells transdifferentiate into Ins-expressing cells after β-cell destruction (**Ye et al., 2015**). On the other hand, unlike mouse models in which regeneration via progenitors or precursors is debated, β-cell neogenesis is well recognised in zebrafish to involve regenerative processes from progenitor-like cells present in the ducts (**Delaspre et al., 2015**; **Ghaye et al., 2015**; **Ninov et al., 2013**). β-cell destruction is accomplished in zebrafish using a chemo-genetic system based on the transgenic expression of the bacterial nitroreductase (NTR) under the control of the *ins* promoter where cell death is induced by a nitroaromatic prodrug (**Bergemann et al., 2018**; **Curado et al., 2007**; **Pisharath and Parsons, 2009**). In adults, after a huge rise of glycemia within 3 days, the pancreas is replenished with new β-cells in 2–3 weeks which correlates to a return to normoglycemia.

De novo formation of β-cells in order to repair damaged islets constitutes a promising therapeutic perspective for diabetic patients. However, new β-cells could show differences in their number and identity impacting on their activity. For example, the presence in mice of Gcg+ Ins + cells, although apparently functional, should be considered cautiously as inappropriate differentiation of β-cells and impaired maturation or identity are common shortcoming in diabetes (**Moin and Butler, 2019** for review).

Using the larval and adult zebrafish as regeneration models, we investigated the identity of regenerated β-cells and discovered that most new *ins*-expressing cells are Ins + Sst1.1+ bihormonal cells. We identified a specific δ-cell subpopulation distinct from the previously identified zebrafish *sst2* δ-cells that is characterized by the expression of *sst1.1* and of several important β-cell features. The transcriptomic profile of bihormonal cells is also very close to the *sst1.1* δ-cells, making them resemble β/δ hybrids. By in vivo imaging of larvae, we observed the appearance of *ins*-expressing bihormonal cells from monohormonal *sst1.1* δ-cells early after β-cell ablation. We also provide evidence that pancreatic ducts contribute to the pool of bihormonal cells. Furthermore, bihormonal cells are abundant in the

regenerated pancreas and able to normalize glycemia after a glucose challenge. Our findings show the importance of bihormonal cells in the spontaneous recovery of diabetic zebrafish.

Results

Most regenerated β-cells in adult zebrafish coexpress Ins and Sst

To characterize the new β-cells after regeneration, we used 6- to 10-month-old *Tg(ins:NTR-P2A-mCherry)* (**Bergemann et al., 2018**) adult fish to first ablate β-cells. Basal blood glucose was monitored to evaluate ablation (3 days post treatment, dpt) and regeneration (20 dpt). As expected, fasting basal blood glucose dramatically raised at three dpt compared to CTL fish which reflected efficient ablation (**Figure 1A** and **Figure 1—source data 1**). After 20 days, glycemia was impressively improved though still slightly above control values. A preliminary RNAseq experiment on mCherry+ cells isolated from the main islet of *Tg(ins:NTR-P2A-mCherry)* adult fish 2 months after ablation revealed strong expression of the *sst1.1* gene in regenerated β-cells just below *ins* (**Figure 1—source data 3**), thereby suggesting that regenerated β-cells are bihormonal. As blood glucose is nearly normalized after 20 days, we characterized these cells at this time point. Immunofluorescence on regenerated 20 dpt islets showed many Ins + cells that also displayed Sst immunolabeling (**Figure 1B**). In contrast, control islets showed robust staining of the endogenous Ins and Sst hormones without appreciable overlap, thus demarcating monohormonal β- and δ-cells (**Figure 1B**). We next created a *Tg(sst1.1:eGFP)* reporter line driving GFP in *sst1.1*-expressing cells. This transgene was not active in β-cells of control islets (**Figure 1—figure supplement 1**). Similar to what was observed with the endogenous Sst and Ins proteins, regenerated 20 dpt islets of *Tg(sst1.1:eGFP); Tg(ins:NTR-P2A-mCherry)* fish contained many cells coexpressing GFP with mCherry, while GFP and mCherry-labeled distinct cells in control islets (**Figure 1C**). Strikingly, double positive cells could already be detected 3 days after ablation, although they displayed low levels of mCherry.

We next quantified *ins*+ β-cells, *sst1.1*+ cells and double *ins* + *sst1.1*+ cells by measuring the number of mCherry+, GFP+, and GFP+ mCherry + cells, respectively, in *Tg(sst1.1:eGFP); Tg(ins:NTR-P2A-mCherry)* adult fish. The main islet was obtained by dissection and the different cell populations were analyzed by FACS (**Figure 1D–G**, **Figure 1—figure supplement 2** and **Figure 1—source data 2**). At 3 and 20 dpt, we observed a drastic loss of mCherry+ (GFP-) β-cells with a drop to 3.2% of the initial β-cell mass at 3 dpt (**Figure 1E**). In contrast, a large population of double GFP+ mCherry + cells appeared that represented 43% of the initial β-cell mass (**Figure 1F**). These cells still persisted at 20 dpt and they made up at this stage 98% of the *ins*-expressing cells. At 20 dpt, mCherry+ GFP- β-cells still constituted a very minor population. (**Figure 1E**). After ablation, the amount of GFP+ mCherry cells also decreased (**Figure 1G**).

In conclusion, these results indicate that *ins* + *sst1.1*+ bihormonal cells rapidly appear in the main islet after β-cell ablation in adult fish and persist steadily for at least 20 days. They constitute the vast majority of the new *ins*-expressing cells following ablation.

Genesis of bihormonal cells also occurs during regeneration in larval stages and is independent of the ablation model

As in mouse the process of bihormonal cells (in that case Gcg+ Ins +) formation after β-cell ablation is specific to adult stages (**Thorel et al., 2010**; **Chera et al., 2014**), we next asked whether Sst1.1+ Ins + bihormonal cells also appear in zebrafish larvae. We therefore performed the ablation in *Tg(sst1.1:eGFP); Tg(ins:NTR-P2A-mCherry)* at 3 days post fertilization (dpf) and assessed the expression of *ins*:mCherry and *sst1.1*:GFP. Like in adults, bihormonal cells were detected 3 days after ablation (3 dpt, 6 dpf) (**Figure 2A–B**). We confirmed by in situ hybridization detecting the endogenous mRNAs that these bihormonal cells express *sst1.1* together with *ins* (**Figure 2C**). This experiment also revealed that they do not coexpress *sst2* (**Figure 2C**).

Then we questioned if the bihormonal cells can also be induced using another system of β-cell destruction. We chose the Diphteria Toxin chain alpha (DTA) suicide transgene which has previously been used to efficiently ablate β-cells (**Ninov et al., 2013**). Ablation was achieved in *Tg(ins:lox-mCherry-lox-DTA); Tg(ins:CRE-ERT2)* larvae by performing a 4-OHT treatment at 7 dpf and the larvae

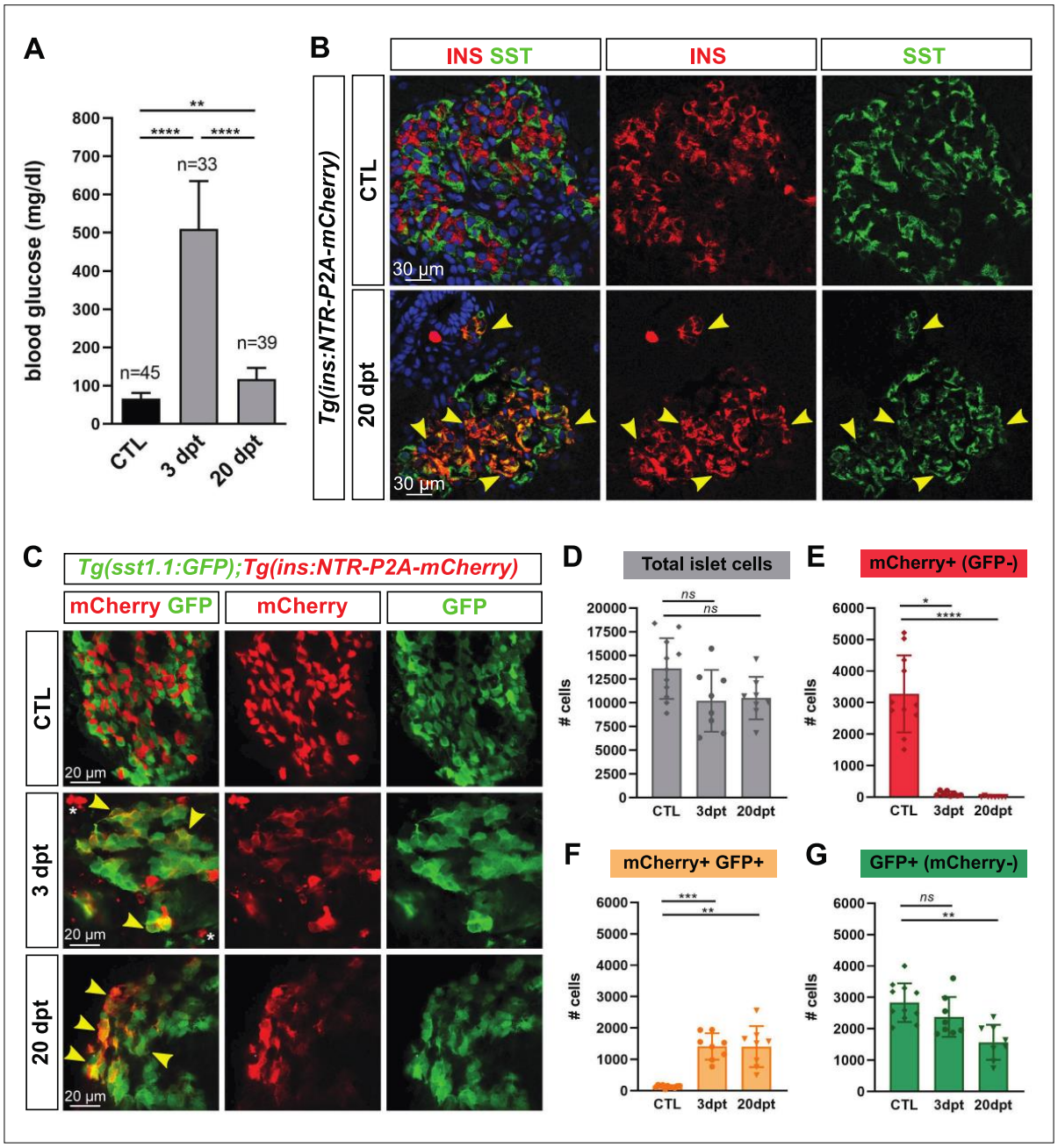


Figure 1. Most new ins + cells after ablation and regeneration in zebrafish are Ins + Sst1.1+ bihormonal cells. (**A**) Blood glucose level (mg/ml) of adult *Tg(ins:NTR-P2A-mCherry)* control fish (CTL, 66 ± 15 mg/dl), 3 days (510 ± 126 mg/dl) and 20 days post treatment (dpt) (117 ± 29 mg/dl) with the NFP prodrug to trigger β-cell ablation. The huge rise of glycemia at three dpt confirms the efficiency of ablation. One-way ANOVA Kruskal-Wallis test (with Dunn's multiple comparisons); Mean ± SD; **p < *0.005*, ****p < *0.0001*. (See ***Figure 1—source data 1***). (**B**) Immunolabeling of β- and δ-cells with anti-INS (red) and anti-SST (green), respectively, on paraffin sections through the main islet of *Tg(ins:NTR-P2A-mCherry)* adult fish in control condition (CTL) and at 20 dpt. In CTL islet, no appreciable overlap between the two markers can be detected while broad colabeling is observed

Figure 1 continued on next page

Figure 1 continued

at 20 dpt and represented by many yellow cells (arrowheads). (**C**) Whole mount immunodetection of β- and *sst1.1*+ cells in the main islet of adult *Tg(sst1.1:GFP);Tg(ins:NTR-P2A-mCherry)* fish by labeling with anti-GFP marking *sst1.1*-expressing cells and anti-mCherry for β-cells. Both cell types show no or very few overlapping in CTL fish. At 3 and 20 dpt, many double GFP+ mCherry + cells are observed (yellow cells, arrowheads). Bright mCherry+ β-cell debris are detectable at 3 dpt (white asterisk). (**D–G**) Quantification of the GFP+, mCherry+ (β-cells) and double GFP+ mCherry + cells detected by FACS in the main islets of *Tg(sst1.1:GFP);Tg(ins:NTR-P2A-mCherry)* CTL fish and following β-cell ablation (3 and 20 dpt), based on fluorescence analysis shown in ***Figure 1—figure supplement 2***. (**D**) Total islet cell number in CTL, 3 dpt, and 20 dpt islets. (**E**) CTL islets contain 3277 ± 1220 mCherry+ (GFP-) β-cells. At 3 dpt, ablated β-cells represent 105 ± 70 cells and were even more scarce at 20 dpt (14 cells). (**F**) Double GFP+ mCherry + bihormonal cells represent 135 ± 45 cells in CTL islets, 1411 ± 421 cells at 3 dpt and 1409 ± 655 cells at 20 dpt. (**G**) GFP+ (mCherry-) cells represent 2833 ± 615 cells in CTL islets. One-way ANOVA Kruskal-Wallis test (with Dunn's multiple comparison); *ns*, not significant, *p < 0.05, **p < 0.005, ***p < 0.0005, ****p < 0.0001; Mean ± SD (See ***Figure 1—source data 2***).

The online version of this article includes the following source data and figure supplement(s) for figure 1:

Source data 1. Blood glucose values.

Source data 2. Cell quantification values.

Source data 3. Top 20 genes expressed in regenerated β-cells.

Figure supplement 1. *Tg(sst1.1:GFP)* is active in sst1.1+ cells and not in β-cells.

Figure supplement 2. Analysis of sst1.1:GFP and *ins:NTR-P2A-mCherry* fluorescent cells by flow cytometry.

were then analyzed at 16 dpf (***Figure 2—figure supplement 1***). Similar to our observations with the NTR system, Ins and Sst immunofluorescence revealed many coexpressing cells.

In conclusion, these data demonstrate that there is no specific competent stage for the formation of Ins + Sst1.1+ bihormonal cells in zebrafish. In addition, this process does not depend on the method of ablation.

Most bihormonal cells do not derive from pre-existing β-cells

To explore the possibility that bihormonal cells derive from pre-existing β-cells spared by the ablation, β-cells were traced before ablation using *Tg(ins:CRE-ERT2); Tg(ubb:loxP-CFP-loxP-zsYellow); Tg(sst1.1:GFP); Tg(ins:NTR-P2A-mCherry)* fish. As bihormonal cells were also observed at six dpf, we used larvae to tackle their origin by CRE-mediated recombination (***Hans et al., 2009***; ***Mosimann et al., 2011***). We treated the larvae with 4-OHT at six dpf to label the β-cells and performed the ablation the next day (***Figure 2D***). We found that, 7 days after ablation, only 10% of the bihormonal cells were positive for the zsYellow lineage tracer (***Figure 2E–E' , and H***). To ensure that this low level was not due to an inefficient tracing, we checked non-ablated larvae and found that 94% of the β-cells were labeled with zsYellow (***Figure 2F–G***). In addition, the *sst1.1*:GFP+ cells were not labeled (***Figure 2F***). These data demonstrate good efficiency and specificity of the tracing. Based on these observations, we can conclude that some bihormonal cells originate from pre-existing β-cells but the majority arises from non-β origin(s).

ins+ sst1.1+ bihormonal cells share similarities with β- and δ-cells, and possess the basic machinery for glucose responsiveness

In order to characterize the *ins + sst1.1+* bihormonal cells after regeneration, we analyzed their transcriptomic profile. To this end, double GFP+ mCherry + cells were isolated by FACS from the main islet of *Tg(sst1.1:eGFP); Tg(ins:NTR-P2A-mCherry)* adult fish at 20 dpt. Control β-cells (mCherry+ GFP-) were obtained from age-matched, non-ablated, transgenic fish. We compared their RNAseq profiles and identified 887 DE genes with a higher expression in bihormonal cells and 705 DE genes higher in β-cells (Padj <0.05 and above twofold differential expression) (***Figure 3A–B*** and ***Figure 3—source data 1***). In accordance with the weak mCherry fluorescence harbored by GFP+ mCherry + cells as compared to native β-cells, the expression of *ins* in bihormonal cells was fivefold below its typical level in β-cells (***Figure 3C***). Also, as expected, the δ-cell hormone *sst1.1* was sharply overexpressed in bihormonal cells (209-fold) compared to its basal level in β-cells, and was even the top hormone just above *ins* (***Figure 3C***). The other pancreatic hormones known in zebrafish, *sst1.2*, *sst2*, *gcga*, *gcgb*, and *ghrl*, were detected at much weaker levels in both ins + populations (***Figure 3C***). Accordingly, Gcg protein was undetectable in bihormonal cells by immunofluorescence (***Figure 3—figure***

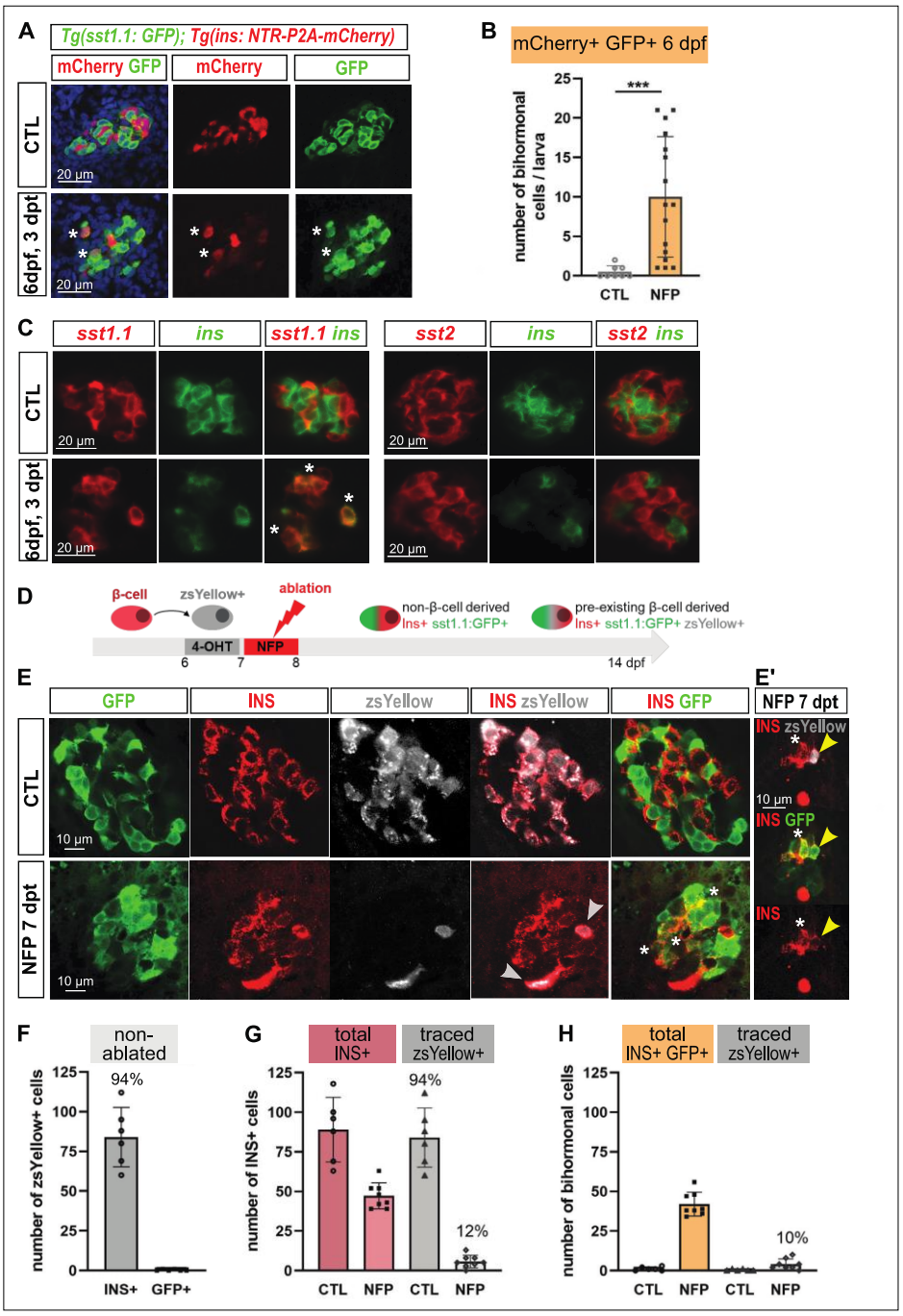


Figure 2. Bihormonal cell formation is age- and ablation model-independent and mostly do not derive from escaping β-cells. (**A**) Whole mount immunodetection in six dpf *Tg(sst1.1:GFP); Tg(ins:NTR-P2A-mCherry)* larvae showing β-cells (mCherry, red), *sst1.1*-expressing cells (GFP, green) and double positive bihormonal cells (asterisks) in the main islet in control (CTL) and 3 days after NFP-mediated ablation (3 dpt). Representative confocal images (single optical planes). dpf: days post-fertilization. (**B**) Quantification of bihormonal cells co-labeled by mCherry and GFP based on confocal

Figure 2 continued on next page

Figure 2 continued

images of 6 dpf larvae. Unpaired two-tailed t-test (with Welch correction); ***p < *0.001*; Mean ± SD. (**C**) Whole mount fluorescent in situ hybridization performed on 6 dpf *Tg(ins:NTR-P2A-mCherry)* larvae with an *ins* antisense RNA probe (green) combined with either a *sst1.1* or a *sst2* probe (red). NFP-mediated ablation was performed from 3 to 4 dpf. Representative confocal images of the main islet (single optical planes). (**D–G**) β-cell tracing with *Tg(ins:CRE-ERT2); Tg(ubb:loxP-CFP-loxP-zsYellow); Tg(sst1.1:GFP); Tg(ins:NTR-P2A-mCherry)* larvae. (**D**) Experimental design: CRE recombination was performed by treatment with 4-OHT treatment at six dpf to induce the expression of the lineage tracer zsYellow (gray) in β-cells (INS, red). β-cell ablation (NFP) was then performed at seven dpf and the lineage tracer was analysed in the main islet at 14 dpf (7 dpt). (**E-E'**) Confocal images showing immunodetection of GFP (green), zsYellow (gray), and INS (red) antibodies. After ablation, traced β-cells are evidenced by double zsYellow+ Ins + staining (gray arrowheads) and bihormonal cells by double Ins + GFP + staining (white asterisks). (**E'**) Close-up showing two bihormonal cells, one zsYellow+ (derived from a pre-existing β-cell) (yellow arrowhead) and one zsYellow- (asterisk). (**F–H**) Quantification (CTL, n = 6; NFP, n = 8) based on the confocal images. (**F**) In CTL non-ablated islets, ZsYellow marked efficiently the Ins+ β-cells (84 ± 19 zsYellow+ Ins + cells out of 89 ± 20 total Ins+ β-cells, representing 94% of the total β-cells). ZsYellow was not detected in sst1.1:GFP+ cells, showing a good specificity. (**G**) 7 days after ablation (NFP), 47.3 ± 8 Ins + cells were detected and 5.8 ± 4 of them (12%) expressed zsYellow. (**H**) 42 ± 7.5 Ins + cells are also GFP+ bihormonal and 10% of them (4 ± 3 cells) are labeled with zsYellow. Mean ± SD.

The online version of this article includes the following figure supplement(s) for figure 2:

Figure supplement 1. Bihormonal cell formation following β-cell ablation with Diphteria Toxin A.

supplement 1). Collectively, these data confirm that bihormonal cells coexpress high levels of two main hormones, *ins* and *sst1.1*, at both the mRNA and protein levels.

To further characterize these bihormonal cells, we assessed the expression of transcription factors important for β-cell development and identity in zebrafish and mouse/human (see list in ***Figure 3—source data 5***). We first checked the expression of the pan-endocrine genes *neurod1*, *pax6b* and *isl1* and found similar expression (***Figure 3C***). We also examined the expression of *pdx1*, a transcription factor essential for *ins* expression in β-cells. *pdx1* was equally expressed in both native β-cells and post-regeneration GFP+ mCherry + cells. We next evaluated the β-cell identity of bihormonal cells by interrogating the expression of zebrafish β-cell markers. We defined these markers as genes enriched in β-cells (> 4 fold) versus the other main pancreatic cell types (α-, *sst2* δ-cells, acinar and ductal cells) based on previous RNAseq data (***Tarifeño-Saldivia et al., 2017***; ***Figure 3—source data 2***). This list of β-cell genes includes *nkx6.2*, a previously identified β-cell marker in zebrafish (***Binot et al., 2010***; ***Tarifeño-Saldivia et al., 2017***) which is the equivalent of *Nkx6.1* in mouse/human β-cells (***Figure 3—source data 5***). More than half of the 62 'β-cell genes' were expressed at similar levels in both bona fide β-cells and post-regeneration bihormonal cells. In contrast, 27 β-cell genes showed either over- or underexpression (***Figure 3D***). In particular, 18 β-cell genes were underexpressed in bihormonal cells like, for example, *nkx6.2* which was not expressed at all (***Figure 3E***). We also looked at markers of dedifferentiation and found that the zebrafish pancreatic progenitor markers *nkx6.1*, *sox9b,* and *ascl1b*, were barely expressed in bihormonal cells, like in control β-cells.

When considering key genes for β-cell function and maturation, that is glucose sensing, uptake, Ins maturation and secretion, many were expressed at comparable levels in both cell types, such as notably *slc2a2*, *pcsk1*, *abcc8,* and *snap25a* (***Figure 3E***). *ucn3l*, a marker of mature β-cells in mammals (***Blum et al., 2012***) and zebrafish (***Singh et al., 2017***), was overexpressed in bihormonal cells.

Gene Ontology (GO) analysis of the genes overexpressed in bihormonal cells compared to β-cells showed that the top significant biological processes were related to adhesion and neuronal synapses with many genes that are known in β-cells to be important for Insulin processing and exocytosis (***Figure 3F–G*** and ***Figure 3—source data 3***). Other processes included intracellular Calcium and cAMP signaling (***Figure 3F–G*** and ***Figure 3—source data 3***). These data strongly suggest that bihormonal cells, like β-cells, are excitable cells with the capacity to secrete Insulin in response to glucose.

Altogether, these data indicate that bihormonal cells possess the molecular bases of functional mature β-cells such as a glucose-responsiveness and hormone secretion machinery. However, although many β-cell genes are similarly expressed between bihormonal and β-cells, bihormonal cells display a divergent identity such as lack of the zebrafish β-cell marker *nkx6.2* and strong expression of *sst1.1*.

Bihormonal cells constitute the main source of insulin in regenerated zebrafish and restore blood glucose homeostasis

The basal glycemia of regenerated fish is nearly normalized after 20 days, strongly suggesting that bihormonal cells – that represent 98% of the Ins-producing cells – contribute to blood glucose control.

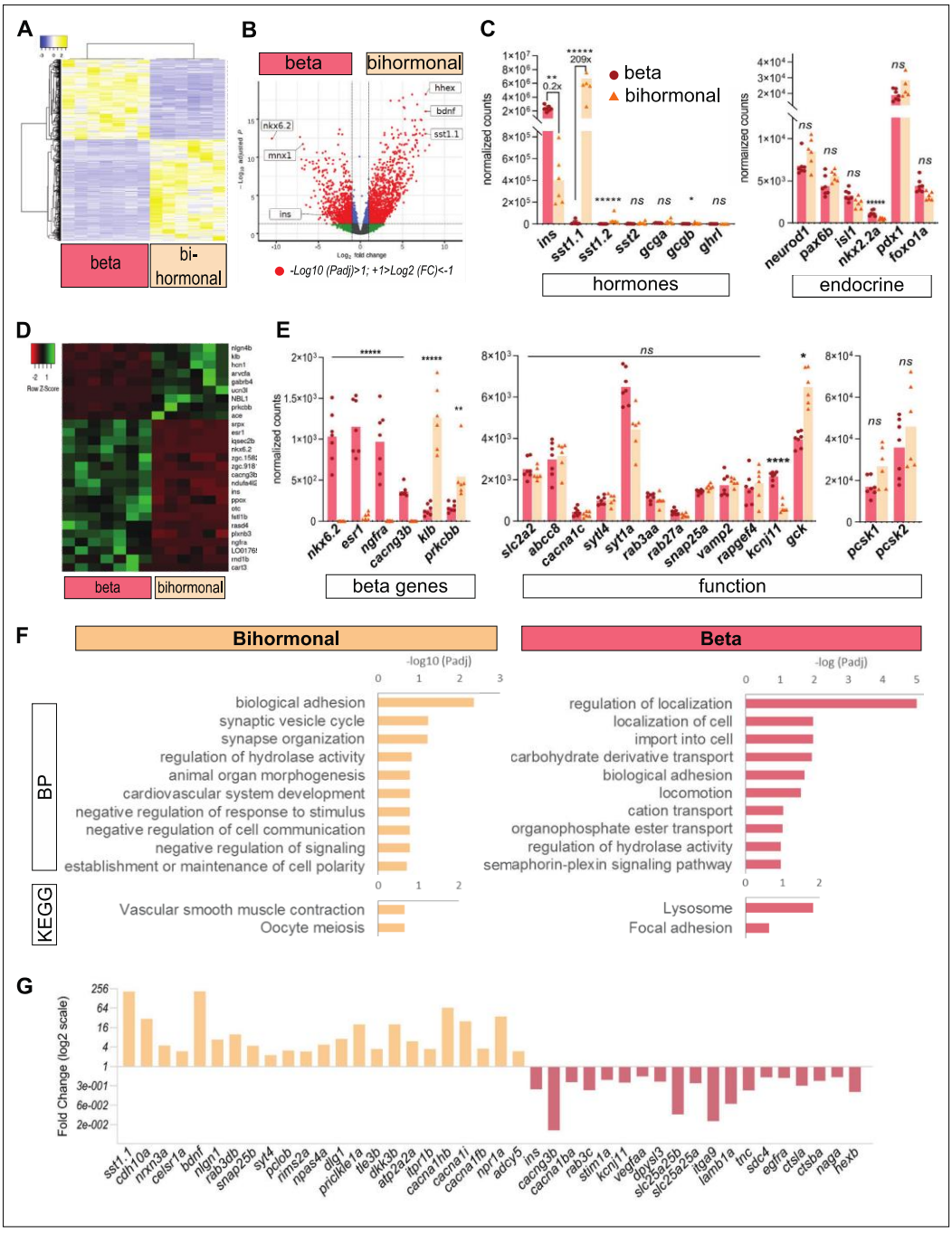


Figure 3. Transcriptomic comparison of bihormonal cells and β-cells. (**A**) Heatmap representation of the transcriptomes of 20 dpt bihormonal (six replicates) and β-cells (seven replicates) (significant DE genes). (**B**) Volcano plot showing the distribution of genes in β-cells without ablation and bihormonal cells. The x-axis represents the $\log_2$ of fold change (FC) and the y-axis the $\log_{10}$ of adjusted P value (Padj) provided by DESeq. The red dots highlight the significantly DE genes (Padj <0.05). A full list of significant DE genes is provided in ***Figure 3—source data 1***. (**C**) Expression values (mean

Figure 3 continued on next page

Figure 3 continued

normalized reads) as provided by DESeq of the main hormones and endocrine genes in β-cell and bihormonal cell transcriptomes. *sst1.1* and *ins* are the two highest expressed hormones. Padj are calculated by DESeq. *ns:* no significant DE between the two conditions, *0.05< P* < 0.005, 0.005< P** < 0.0005, P***** < 0.000005.* (**D**) Heatmap plot showing the direction and amplitude of changes in expression of the β-cell markers between normal β-cells and bihormonal cells (significant DEG only). The 62 β-cell markers are provided in ***Figure 3—source data 2***. (**E**) Expression values (mean normalized reads) as provided by DESeq of selected β-cell markers and genes important for β-cell function in β-cells and bihormonal cells. Padj are calculated by DESeq. *ns:* no significant DE between the two conditions, 0.05<* < 0.005, 0.005<** < 0.0005, 0.00005<**** < 0.000005, ***** < 0.000005. (**F**) Enriched Gene Ontology (GO) terms. Top 10 or Padj (FDR) < 0.25 Biological Processes (BP) and KEGG pathways are shown. The plots represent the enrichment ratio of Biological Processes and KEGG pathways identified with WebGestalt (***Liao et al., 2019***) using the genes over- and underexpressed in bihormonal cells compared to β-cells obtained with a twofold differential expression and Padj <0.05. All overrepresented Biological Processes and Pathways (< FDR 0.25) are listed in ***Figure 3—source data 3*** (bihormonal cells) and ***Figure 3—source data 4*** (β-cells). (**G**) Over- and underexpression of selected significantly DE genes from the BP and KEGG pathways identified in β-cells and bihormonal cells (Fold Change, log2 scale).

The online version of this article includes the following source data and figure supplement(s) for figure 3:

Source data 1. Differentially expressed genes between beta cells and bihormonal cells.

Source data 2. List of the beta cell markers from ***Tarifeño-Saldivia et al., 2017***.

Source data 3. Gene Ontology analysis of genes overexpressed in bihormonal cells.

Source data 4. Gene Ontology analysis of genes overexpressed in beta cells.

Source data 5. Table of the main transcription factors considered in this study, their expression and comparison between zebrafish and mouse/human.

Figure supplement 1. Gcg is not detected in bihormonal cells.

To exclude the possibility that glycemia is regulated by a population of genuine monohormonal β-cells regenerated outside the main islet, we analyzed the pancreatic tail. Indeed, zebrafish possess smaller secondary islets scattered in the pancreatic tail in addition to the large main islet located in the head. Similar to the main islets, regenerated 20 dpt secondary islets harbored many bihormonal cells and very scarce monohormonal β-cells (***Figure 4A–B*** and ***Figure 4—source data 1***). Thus, bihormonal cells constitute the predominant source of Ins throughout the whole pancreas.

To assess the functionality of adult bihormonal cells, we performed a glucose tolerance test and blood glucose levels were followed after an intraperitoneal injection of D-Glucose. Regenerated fish 20 days after β-cell ablation displayed completely normal glucose tolerance (***Figure 4C*** and ***Figure 4—source data 2***). Together, all these data support the conclusion that the bihormonal cells are responsible for the normalization of glycemia and glucose tolerance in regenerated zebrafish.

sst1.1 δ-cells are distinct from *sst2* δ-cells and display similarities with β-cells

Given the expression of *sst1.1* in bihormonal cells, we sought to characterize the *sst1.1*-expressing cells in normal islets without ablation. Previous transcriptomic studies of pancreatic cells detected three *Sst* genes in normal adult pancreatic islets, *sst1.1*, *sst1.2*, and *sst2* (***Spanjaard et al., 2018***; ***Tarifeño-Saldivia et al., 2017***). However, so far, only the *sst2* δ-cells, which also express *sst1.2*, have been fully characterized (***Tarifeño-Saldivia et al., 2017***). We thus isolated the *sst1.1*-expressing GFP+ cells from control non-ablated islets of *Tg(sst1.1:eGFP); Tg(ins:NTR-P2A-mCherry)* adult fish to determine their transcriptome. Close examination of these *sst1.1:*GFP+ cells by flow cytometry actually distinguished two subpopulations recognised by different levels of GFP fluorescence, GFP^{low} and GFP^{high} (***Figure 5—figure supplement 1A***). The GFP^{high} population represented 35% of all GFP cells. The presence of cells with high and low GFP were also observed in situ by immunofluorescence on fixed whole pancreas (***Figure 5A***).

The transcriptomic profile of the two GFP populations was obtained (***Figure 5—figure supplement 1B***). Principal Component Analysis (PCA) unveiled that GFP^{high} cells are very similar to bihormonal cells (***Figure 5B***). In addition, they are also more similar to β-cells than GFP^{low} cells. Clustering analysis of the two GFP populations, the bihormonal cells and the other endocrine cells (a, b, and *sst2* δ-cells ***Tarifeño-Saldivia et al., 2017***) also showed that the GFP^{high} cells cluster together with bihormonal cells and apart from the GFP^{low} cells (***Figure 5C***). Indeed, GFP^{low} cells were closer to *sst2* δ-cells than to the other endocrine subtypes. Comparison of the two GFP populations identified 975 and 1206 DE genes overexpressed in GFP^{high} and GFP^{low}, respectively (FC >2, Padj <0.05) (***Figure 5D*** and ***Figure 5—source data 1***). *sst1.1* was by far the predominant *Sst* gene expressed in GFP^{high} cells

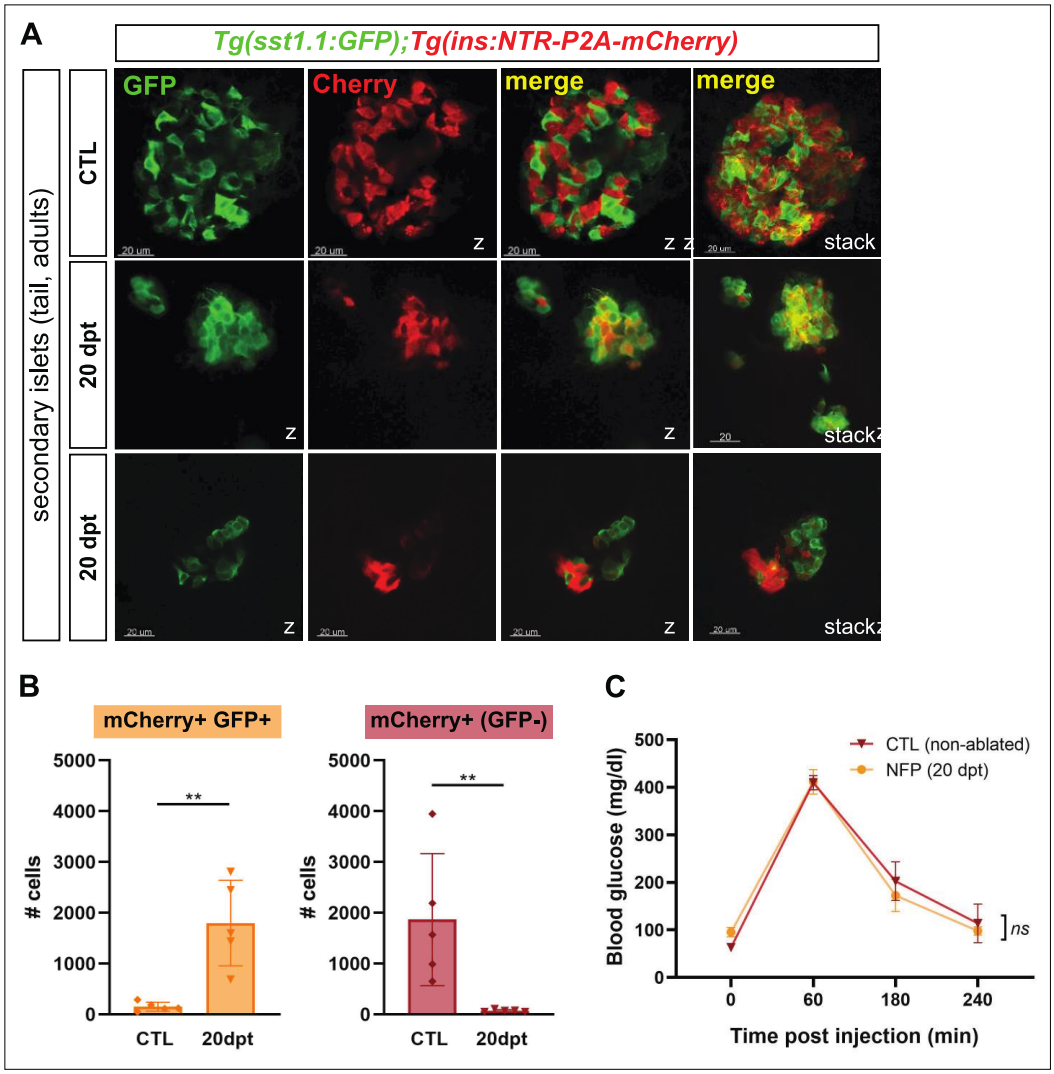


Figure 4. Bihormonal cells are the main source of Insulin in the whole pancreas after regeneration and regulate blood glucose homeostasis. (**A**) Whole mount immunofluorescence (GFP and mCherry) on the pancreas of *Tg(sst1.1:eGFP); Tg(ins:NTR-P2A-mCherry)* adult zebrafish showing secondary islets in the pancreatic tail. One representative CTL and two independent 20 dpt samples are shown. Coexpressing cells appear in yellow due to overlapping GFP and mCherry staining. Confocal optical section (Z-planes) and 3D projections (stacks) are shown. (**B**) Quantification of monohormonal mCherry+ β-cells and GFP+ mCherry + bihormonal cells detected by FACS in the tail of CTL fish and after 20 days regeneration (20 dpt). Mann-Whitney test. p** = *0.0079* in both graphs. Mean ± SD. (See also ***Figure 4—source data 1***). (**C**) Intraperitoneal glucose tolerance test performed in adult zebrafish. Blood glucose was measured over time in control (non-ablated, DMSO) and NFP-treated (ablated) fish after intraperitoneal injection of 0.5 mg/µl of D-Glucose. 4≤ N ≤ 9 per time point for CTL and NFP. Two-way ANOVA test with Sidak's multiple comparison test. Mean ± SEM; *ns*: not significant.

The online version of this article includes the following source data for figure 4:

Source data 1. Cell quantification in the pancreatic tail.

Source data 2. Blood glucose values (glucose tolerance test).

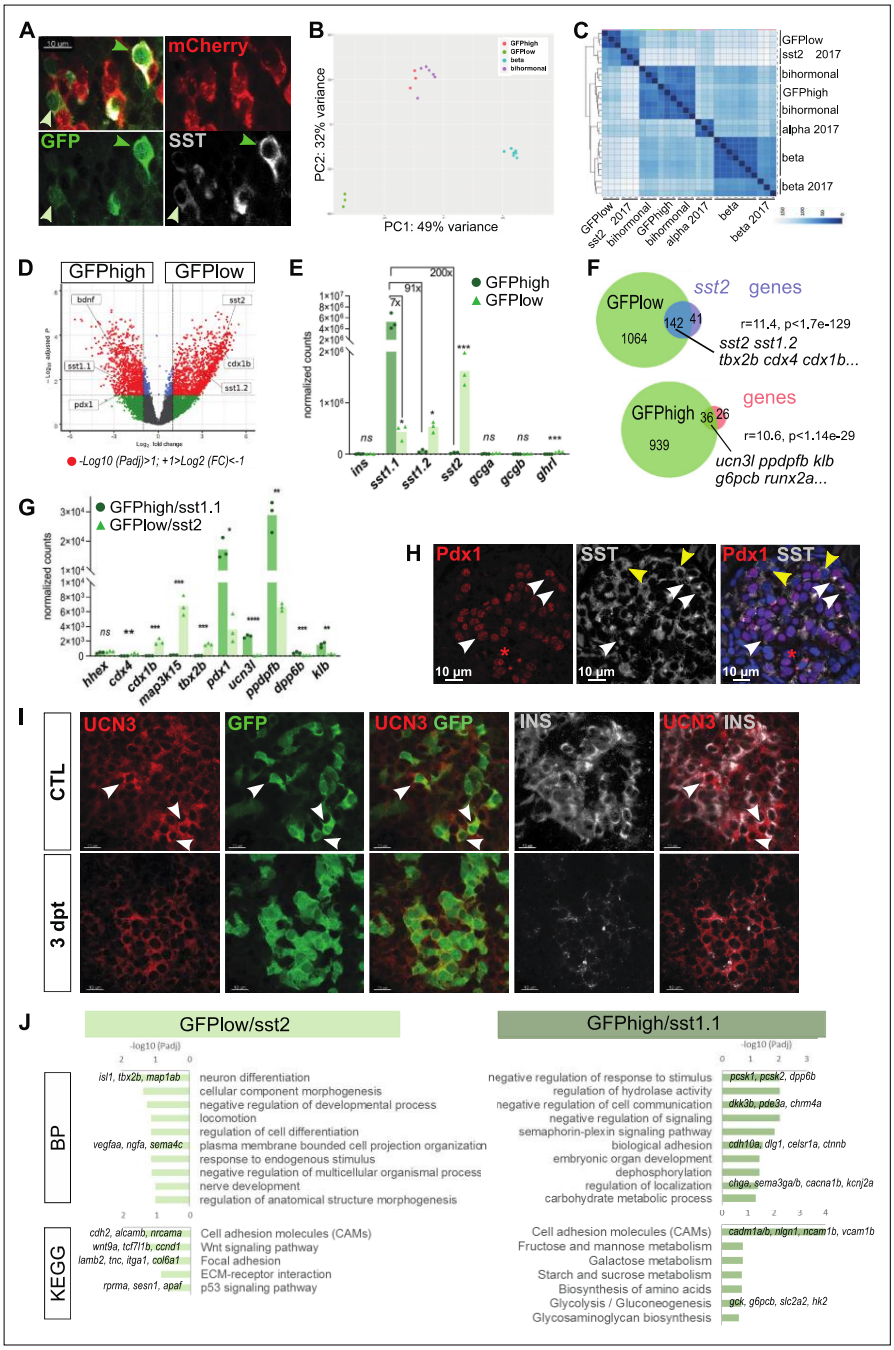


Figure 5. sst1.1 δ-cells (GFP^{high}) constitute a δ-cell subpopulation distinct from sst2 δ-cells (GFP^{low}) that presents similarities with β-cells. (**A**) Whole mount immunodetection on t *Tg(sst1.1:eGFP); Tg(ins:NTR-P2A-mCherry)* main islets of GFP (green), mCherry (red) and Sst (gray) revealing two levels of GFP expression (green light and dark arrowheads) that parallel the expression level of Sst. These cells are mCherry negative. (**B**) PCA plot showing the

Figure 5 continued on next page

Figure 5 continued

separation between *sst1.1*:GFP^{high} (n = 3), sst1.1:GFP^{low} (n = 3), bihormonal (n = 6) and β-cells (n = 7) based on their transcriptomic profile. 49% of the variance is explained in PC1. PCA analysis failed to separate bihormonal and *sst1.1*:GFP^{high} cells while separated well β-cells from the sst1.1:GFP^{low} cells. The *sst1.1*:GFP^{high}/bihormonal cluster located between β-cells and sst1.1:GFP^{low} cells shows that β-cells are more similar to *sst1.1*:GFP^{high}/bihormonal cells. (**C**) Heatmap plot showing the clustering of the sst1.1:GFP^{high} and sst1.1:GFP^{low} populations, the bihormonal cells, the β-cells of the present study and the previously published data for β-, α-, and *sst2* δ-cells (n = 3) (***Tarifeño-Saldivia et al., 2017***). In addition to revealing the expected clustering between both RNAseq data from β-cells (***Tarifeño-Saldivia et al., 2017***) and this study, this plot also shows the clustering of the GFP^{low} cells together with *sst2* δ-cells. (**D**) Volcano plot showing the distribution of genes expressed in GFP^{high} and GFP^{low} populations. The x-axis represents the $\log_2$ of fold change (FC) and the y-axis the $\log_{10}$ of adjusted p value (Padj) provided by DESeq. The list of all DE genes is provided in ***Figure 5—source data 1***. (**E**) Expression of the main pancreatic hormones in GFP^{high} and GFP^{low} populations (mean normalized reads). Expression is expressed as normalized counts and Padj are calculated by DESeq. *ns:* no significant DE between the two conditions, 0.05<* < 0.005, 0.0005<*** < 0.00005. (**F**) Venn diagram showing the overlap between genes overexpressed in GFP^{low} cells (versus GFP^{high}) and *sst2* δ-cell markers previously identified, and between genes overexpressed in GFP^{high} cells (versus GFP^{low} cells) and β-cell genes (***Figure 5—source data 2***). Representation factor and p value calculated by Fisher's exact test. (**G**) Expression of selected β- and *sst2* δ-cell genes in each replicate of GFP^{high} and GFP^{low} cells. GFP^{high} cells distinctly express high levels of *sst1.1* and will be referred to as GFP^{high}/*sst1.1* δ-cells, and GFP^{low} to GFP^{low}/*sst2* δ-cells. 0.05<* < 0.005, 0.005<** < 0.0005, 0.0005<*** < 0.00005, **** < 0.00001 (**H**) Confocal images showing immunodetection of Pdx1 (anti-Pdx1, red) and Sst (anti-SST, gray) on paraffin section through the main islet of a non-ablated adult fish showing double Pdx1+ Sst + cells (white arrowheads) and Pdx1- Sst+ cells (yellow arrowheads). Red asterisks highlight Pdx1 single positive cells β-cells. (**I**) Confocal images showing whole mount immunodetection of Ucn3 (red), GFP (green) and Ins (gray) in CTL and three dpt main islets from *Tg(sst1.1:eGFP); Tg(ins:NTR-P2A-mCherry)* adult fish. In CTL islets, strong Ucn3 labeling is detected in β-cells as well as in some *sst1.1*:GFP cells (white arrowheads). After β-cell ablation, Ucn3 is principally expressed in GFP+ cells that also harbor faint Ins staining. (**J**) Biological Processes (BP) and KEGG pathways overrepresented in GFP^{high}/*sst1.1* δ-cells (UP) compared to GFP^{low} cells (DOWN) (Padj<0.25). Gene Ontology (GO) terms were identified by WebGestalt (***Liao et al., 2019***) using the list of DE genes between GFP^{high}/*sst1.1* δ-cells and GFP^{low}/*sst2* δ-cells obtained with at least twofold differential expression and Padj <0.05 provided by DESeq. The list of all BP and KEGG pathways below FDR 0.25 is given in ***Figure 5—source data 4***, ***Figure 5—source data 5***.

The online version of this article includes the following source data and figure supplement(s) for figure 5:

Source data 1. Differential gene expression between sst1.1:GFPhigh and GFPlow cells.

Source data 2. List of the beta cell markers expressed in sst1.1GFPhigh cells and of the sst2 delta cell markers expressed in GFPlow cells.

Source data 3. List of the sst1.1:GFPhigh markers defined in this study and updated beta cell markers.

Source data 4. Gene Ontology analysis of genes overexpressed in sst1.1:GFPlow cells.

Source data 5. Gene Ontology analysis of genes overexpressed in sst1.1:GFPhigh (sst1.1 delta) cells.

Figure supplement 1. *sst1.1*:GFP expression delineates two distinct δ-cell subpopulations.

(***Figure 5E***). On the opposite, *sst2* was predominant in GFP^{low} cells though these cells also expressed *sst1.2* and *sst1.1* at lower levels. In addition, while both populations expressed the universal δ-cell marker *hhex*, other previously identified markers of zebrafish *sst2* δ-cells such as *cdx4, tbx2b*, and *map3k15* (***Tarifeño-Saldivia et al., 2017***) were specific to GFP^{low} cells (***Figure 5F–G***). Indeed, more than 75% of the *sst2* δ-cell genes (enriched >4 fold based on previous data ***Tarifeño-Saldivia et al., 2017***) were also enriched in GFP^{low} cells (***Figure 5F*** and ***Figure 5—source data 2***). Ectopic activity of the *sst1.1:GFP* transgene in the *sst2* δ-cells was confirmed by ISH showing *sst2* probe signal exclusively in the weakest GFP+ cells (***Figure 5—figure supplement 1C***). These data show that the GFP^{low} population contains *sst2* δ-cells, while the GFP^{high} population consists of a pure and distinct δ-cell population characterized by strong *sst1.1* expression. These δ-cells will be named *sst1.1* δ-cells hereafter.

Focusing on the *sst1.1* δ-cells, we noticed high expression of *pdx1* (***Figure 5G***). In addition to being expressed in all β-cells, *Pdx1* in mammals is also expressed in a subset of δ-cells (***Piran et al., 2014***; ***Segerstolpe et al., 2016***). In zebrafish, *pdx1* is expressed in β-cells but not in *sst2* δ-cells (***Tarifeño-Saldivia et al., 2017***). In agreement with the transcriptome of *sst1.1* δ-cells, Pdx1 immunolabeling was confirmed in a subset of Sst + cells on paraffin section through the adult main islet (***Figure 5H***). Next, we investigated the expression of the 62 zebrafish 'β-cell genes'. Strikingly, most of them (36/62), such

as *ucn3l*, were found enriched in *sst1.1* δ-cells (***Figure 5F–G*** and ***Figure 5—source data 2***) while none was preferentially expressed in the GFP^{low} cells. By immunofluorescence, Ucn3 decorated β-cells in control islets and, additionally, an even more intense staining was detected in a subset of GFP^{high} cells. After ablation, the anti-Ucn3 also marked bihormonal cells, confirming our RNAseq data (***Figure 5I***). Based on these new transcriptomic datasets, we defined the genes selectively enriched (> 4 fold) in *sst1.1* δ-cells versus the other endocrine cell types already available (*sst2*δ, β and α) and identified 152 specific *sst1.1* δ-cell markers, among which *bdnf, cdh10a, sox11b,* and *dkk3b* (***Figure 5—source data 3***). An updated list of 60 markers enriched in β-cells versus *sst1.1* δ-cells, α and *sst2* δ-cells altogether could also be defined. Our RNAseq data also revealed that *dkk3b* and *ucn3l*, previously attributed to β-cells, were even more enriched in *sst1.1* δ-cells.

Top GO terms overrepresented in GFP^{low}/*sst2* δ-cells (***Figure 5J*** and ***Figure 5—source data 4***) were related to neuron differentiation, adhesion and Wnt signaling. Top most significant GO terms and pathways in *sst1.1* δ-cells (***Figure 5J*** and ***Figure 5—source data 5***) included 'biological adhesion' and proprotein convertases important in the secretory pathway such as *pcsk1* and *pcsk2*. Together with *gck, g6pcb, slc2a2,* and *hk2* associated with 'metabolism of carbohydrates', these signatures suggest some competence of *sst1.1* δ-cells for glucose-responsiveness and hormone secretion.

Overall, these data unveil that *sst1.1* δ-cells represent a distinct δ-cell population possessing basic features of β-cells and sensors to integrate Ins signaling, glucose metabolism and carry hormone secretory activity.

Monohormonal *sst1.1*-expressing cells transcriptionally activate the *ins* gene following β-cell ablation

The transcriptomic profile of *sst1.1* δ-cells suggests that they represent a promising candidate as cellular origin of bihormonal cells. In line with a conversion of *sst1.1* δ-cells to bihormonal cells, the number of monohormonal GFP^{high} cells was reduced after ablation in adult fish compared to CTL (from 979 cells to 315 at 20 dpt) (***Figure 6A***, ***Figure 6—source data 1***). To test the hypothesis of a direct conversion of *sst1.1* δ-cells, we followed the appearance of bihormonal cells by in vivo time lapse imaging of the main islet in *Tg(sst1.1:eGFP); Tg(ins:NTR-P2A-mCherry)* larvae after ablation from 3 to 4 dpf. ***Figure 6B–B'*** show mCherry fluorescence progressively appearing in monohormonal *sst1.1*:GFP+ cells presenting strong GFP fluorescence, most likely *sst1.1* δ-cells. These results indicate the activation of the *ins* promoter of the *ins:mCherry* transgene in *sst1.1*:eGFP cells and suggest that at least some *sst1.1* δ-cells directly convert into bihormonal cells immediately after ablation.

Bihormonal cells have a transcriptomic profile very similar to *sst1.1* δ–cells but with distinct cell cycle signatures

As the PCA and clustering analyses shown ***Figure 5B–C*** revealed that bihormonal and monohormonal GFP^{high}/*sst1.1* δ-cells are transcriptionally similar, we next directly performed a pairwise comparison of their transcriptome. This analysis revealed a few DE genes, with 293 over- and 180 underexpressed genes in bihormonal cells versus *sst1.1* δ-cells (FC twofold, Padj <0.05) (***Figure 6C*** and ***Figure 6—source data 2***), indicating that the identity of bihormonal cells is very close to *sst1.1* δ-cells. The *ins* gene was the top overexpressed gene in bihormonal cells (54-fold) (***Figure 6D***). Among the 293 over-expressed genes in bihormonal cells, 9 were β-cell markers such as *ins* and *fstl1a* and, among the 180 underexpressed genes, 8 were *sst1.1* δ-cell markers. Both *sst1.1* and *hhex* were equally expressed, further underscoring that bihormonal cells and *sst1.1* δ-cells have a close identity.

GO analyses of the genes overexpressed in bihormonal cells identified 'ribosome', 'proteasome', 'p53 signaling pathway', and 'cell cycle' pathways as top enriched pathways (***Figure 6E*** and ***Figure 6—source data 3*** and ***Figure 6—source data 4***). To corroborate the cell cycle signature, we examined Proliferating Cell Nuclear Antigen (PCNA) in the main islet of *Tg(ins:NTR-P2A-mCherry)* adult fish. In CTL islets, PCNA immunodetection was almost absent. In contrast, it was widely observed in mCherry+ cells at 20 dpt (***Figure 6F***). As mCherry+ cells are also bihormonal, it can be concluded that PCNA is expressed in bihormonal cells. We also examined Pdx1 as a proxy for β, *sst1.1* δ and bihormonal cells (***Figure 6—figure supplement 1A***). The proportion of PCNA+ Pdx1+ cell was strongly increased in 3 and 20 dpt islets compared to CTL. To assess more specifically DNA replication, we performed a 2-day incorporation of the established marker of DNA synthesis EdU in *Tg(sst1.1:eGFP); Tg(ins:NTR-P2A-mCherry)* larvae (***Figure 6—figure supplement 1B***). Larval *sst1.1*:GFP+ cells and

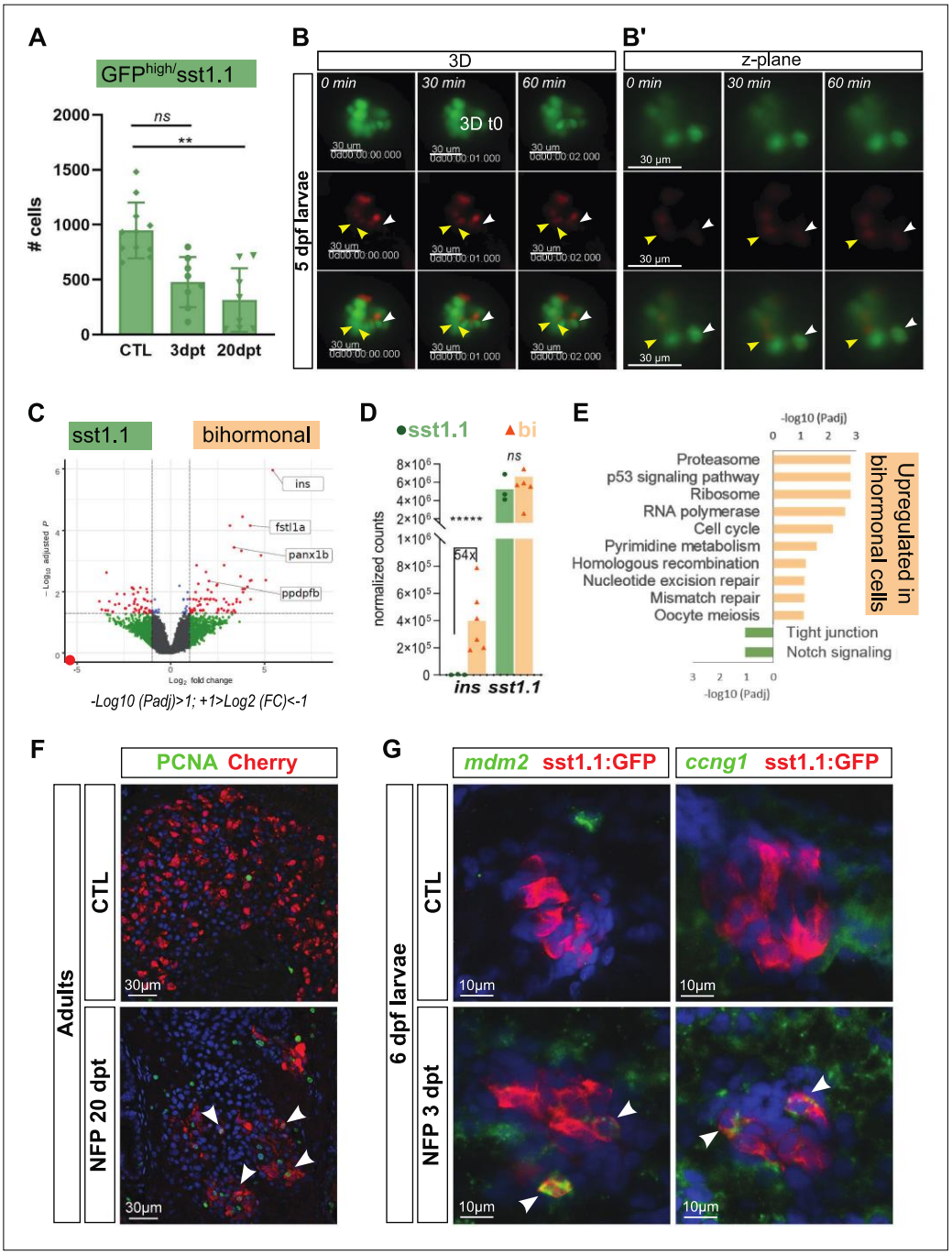


Figure 6. *sst1.1* δ-cells convert to Sst1.1+ Ins + bihormonal cells after β-cell destruction and activate cell cycle genes and p53. (**A**) Quantification by flow cytometry of GFP^{high}/*sst1.1* δ-cells before ablation (CTL) and at 3 and 20 dpt showing depletion of *sst1.1* δ-cells during regeneration. Cells were isolated from dissected main islets of adult *Tg(sst1.1:eGFP); Tg(ins:NTR-P2A-mCherry)*. Mean ± SD; Kruskal-Wallis test; *ns*: not significant, **p < *0.005* (see also ***Figure 6—source data 1***). (**B**) In vivo time lapse of the main islet of a four dpf *Tg(sst1.1:eGFP); Tg(ins:NTR-P2A-mCherry)* larva following β-cell

Figure 6 continued on next page

Figure 6 continued

ablation from 3 to 4 dpf. 3D representation (**B**) and one z-plane (**B'**) of the same islet are shown. The arrowheads point at two GFP+ cells (green) that start to express *ins*:mCherry (red) fluorescence between *t0* and *t1* (visible in the same z-plane). The white arrowhead points to a strongly fluorescent sst1.1:GFP^{high} cell. Images were acquired every 30 min starting from four dpf (96 hpf). (**C**) Volcano plot showing the significant DE genes over- or underexpressed in 20 dpt bihormonal cells versus CTL GFP^{high}/*sst1.1* δ-cells (FC >2 < , Padj <0.05). The full list of significant DE genes calculated by DESeq is provided in ***Figure 6—source data 2***. (**D**) Expression in normalized counts of the *sst1.1* and *ins* genes in CTL GFP^{high}/*sst1.1* δ-cells and bihormonal cells (bi). Padj are calculated by DESeq. *ns:* no significant DE between the two conditions, ***** < 0.000005. (**E**) Top significant KEGG pathways identified among the genes upregulated (in orange) and downregulated (in green) in bihormonal cells compared to CTL GFP^{high}/*sst1.1* δ-cells. The list of GO terms below FDR 0.25 is given in ***Figure 6—source data 3***, ***Figure 6—source data 4***. (**F**) Immunofluorescence of PCNA and mCherry on paraffin sections through the main islet of *Tg(ins:NTR-P2A-mCherry)* adult zebrafish, CTL and regenerated (20 dpt after NFP-mediated ablation), showing PCNA+ nuclei in mCherry+ cells in regenerated islets (confocal images, white arrowheads). (**G**) Expression of p53 target genes *mdm2* and *ccng1* mRNA (green) revealed by whole mount in situ hybridization on 6 dpf CTL and ablated *Tg(ins:NTR-P2A-mCherry); Tg(sst1.1:GFP)* larvae (main islet). Ablation was performed at 3 dpf. Immunodetection of GFP (in red) was revealed following in situ hybridization. White arrowheads point to sst1.1:GFP+ cells expressing *mdm2* and *ccng1* after ablation.

The online version of this article includes the following source data and figure supplement(s) for figure 6:

Source data 1. sst1.1 delta cell (GFPhigh) quantification.

Source data 2. Differentially expressed genes between sst1.1 delta and bihormonal cells.

Source data 3. Gene Ontology analysis of genes overexpessed in bihormonal cells.

Source data 4. Gene Ontology analysis of genes overexpressed in sst1.1 delta cells.

Figure supplement 1. Analysis of proliferation in the main islet of adults and larvae during regeneration.

Figure supplement 2. Effect on bihormonal cells of different candidate signals linked to the destruction of β-cells.

ins:mCherry+ β-cells displayed basal DNA replication (CTL). In NFP-treated larvae, the few monohormonal β-cells detected 3 days post-ablation rarely incorporated EdU showing that most escaping β-cells do not proliferate after ablation. In contrast, monohormonal GFP+ EdU + cells were observed in similar proportion between control and ablated larvae. Importantly, a significant fraction of bihormonal cells induced by the ablation showed DNA replication (***Figure 6—figure supplement 1B***).

To assess p53 activity, important for cell cycle checkpoints, we also used larvae to analyze the expression of p53 target genes by in situ hybridization. *mdm2* and *ccng1* were found induced in a subset of *sst1.1*:GFP+ cells at 3 dpt (***Figure 6G***), confirming the activation of the p53 pathway in response to the destruction of β-cells.

Given the activation of the p53 pathway following β-cell ablation, and as p53 is generally activated in response to cellular stress, we investigated the role of common stresses caused by β-cell death like hyperglycemia, oxidative stress and impaired Insulin signaling, in bihormonal cell formation. In particular, we asked whether these signals could induce by themselves the formation of bihormonal cells. However, none of these stresses was sufficient to trigger the formation of bihormonal cells (***Figure 6—figure supplement 2***).

Together, these results demonstrate that bihormonal cells in regenerating islets express genes involved in cell cycle progression and checkpoints. In line with these findings, our data also show that bihormonal cells and possibly *sst1.1* δ-cells engage in proliferation in response to the ablation of β-cells.

Bihormonal cells also arise from pancreatic ducts

In zebrafish, the secondary islets originate from pancreatic duct-associated progenitors in a process initiated during normal larval development (***Parsons et al., 2009***; ***Wang et al., 2011***). Ducts also contribute to β-cell regeneration in the adult zebrafish, providing new β-cells to the main and secondary islets (***Delaspre et al., 2015***; ***Ghaye et al., 2015***). The striking observation that the vast majority of new *ins*-expressing cells are bihormonal in the entire pancreas raises the hypothesis that duct-derived Ins + cells also express Sst1.1. To explore this possibility, we used larvae, a well-established model to study β-cell regeneration from the ducts (***Ninov et al., 2013***). In this model, destruction of β-cells not only induces their regeneration in the main islet but also activates duct-associated progenitors to produce more β-cells. We first determined the time course of duct-derived β and *sst1.1* δ-cell formation during normal development and established that they start to differentiate between 7 and 10 dpf (***Figure 7—figure supplement 1***). Next, we used the *Tg(nkx6.1:eGFP); Tg(ins:NTR-P2A-mCherry)* line, where *nkx6.1* is a marker of pancreatic ducts and of duct-associated progenitors (***Ghaye et al.,***

2015), to perform the ablation of β-cells at 3 dpf, that is before the normal differentiation of β and *sst1.1* δ-cells in the tail. Thus, potential Ins + Sst1.1+ bihormonal cells appearing in the tail after ablation are expected to originate from the ducts and not from secondary β or *sst1.1* δ-cells. At 17 dpf, mCherry and Sst immunodetection was analyzed (***Figure 7A–B***). Double positive bihormonal cells were found in the ductal nkx6.1:GFP+ domain in the tail of regenerating larvae while they were almost absent in CTL ducts (***Figure 7B–B'–C*** and ***Figure 7—source data 1***). These findings support that duct cells give rise to bihormonal cells during regeneration and that they contribute to the overall bihormonal cell mass.

Bihormonal cells persist long after β-cell ablation

Finally, we questioned the persistence of bihormonal cells long after ablation and analyzed the main islet from *Tg(sst1.1:eGFP); Tg(ins:NTR-P2A-mCherry)* adult fish 4 months after ablation. Surprisingly, most Ins + cells still coexpressed GFP as well as high levels of Ucn3 at this stage (***Figure 8A***), similarly to 20 dpt bihormonal cells. Bihormonal cells still constituted the vast majority of *ins*-expressing cells in the main islet compared to monohormonal β-cells (***Figure 8B–D***). This also suggests that they do not represent a transient intermediary population that would ultimately resolve into *ins*-only β-cells.

Discussion

Pancreatic endocrine cell plasticity and impaired identity has emerged as an important cellular adaptive behavior in response to β-cell stress and death in human and in mammalian diabetic models. Here, we show that, in zebrafish, a large and predominant population of Ins + Sst1.1+ bihormonal cells arise after β-cell destruction, confers glucose responsiveness and restores blood glucose homeostasis. Moreover, contrasting with the age-dependent and limited β-cell neogenesis of mouse models (***Chera et al., 2014***; ***Perez-Frances et al., 2021***; ***Thorel et al., 2010***), bihormonal cell formation in zebrafish is fast and efficient and occurs all along life.

Our study provides an in-depth characterization of the zebrafish *sst1.1* δ-cell subpopulation. The existence of two distinct δ-cell subpopulations corroborates a recent report of two clusters of δ-cells detected by single cell RNAseq, one expressing *sst2/sst1.2* and the other *sst1.1* (***Spanjaard et al., 2018***). Although our β-cell lineage tracing experiment in larvae indicates that a subset of bihormonal cells derive from pre-existing β-cells, the majority have a non-β origin. Here, we present evidences that bihormonal cells originate from *sst1.1* δ-cells and duct cells. In contrast to *sst2* δ-cells which have previously been excluded as a source of new Ins-expressing cells (***Ye et al., 2015***), our results strongly suggest that *sst1.1* δ-cells rapidly adapt to the loss of β-cells and activate *ins* expression. First, pre-existing *sst1.1* δ-cells already express many genes essential for β-cells such as *pdx1, ucn3l* and the glucose transporter *slc2a2* (Glut2). Second, *sst1.1* δ-cells and bihormonal cells have a very close transcriptomic profile meaning that only minor changes in *sst1.1* δ-cells would generate bihormonal cells. Third, *sst1.1* δ-cells express the basic molecular machinery for glucose-sensing, glucose- and calcium-dependent stimulation of Insulin secretion and blood glucose control. Fourth, the appearance of bihormonal cells during regeneration concurs with a reduction of the *sst1.1* δ-cell mass. Finally, in vivo imaging revealed the activation of *ins* expression in *sst1.1* δcells early after ablation. All these observations support the conclusion that *sst1.1* δ-cells constitute a distinct zebrafish δ-cell population expressing β-cell features enabling them to rapidly reprogram to bihormonal cells by activating *ins* expression and engender functional surrogate β-cells. Importantly, during the preparation of our manuscript, Singh et al. also identified *sst1.1+ ins* + δ/β hybrid cells in zebrafish by scRNAseq (***Singh et al., 2022***). They also proposed the *sst1.1* δ-cells as possible cellular origin after β-cell ablation, thereby consolidating our findings. A difference between our two studies, however, is that they detected some hybrid Sst1.1+ Ins + cells in control islets while we could not clearly identify them, probably due to different technical approaches.

The fact that the bihormonal cell population is somewhat larger than the *sst1.1* δ-cell population (compare 979 GFP^{high}/*sst1.1* δ-cells in CTL fish in ***Figure 6A*** with ~1400 bihormonal cells post-ablation in ***Figure 1F***) suggests the implication of mechanisms complementary to direct conversion. Indeed, beside *sst1.1* δ-cells as cellular origin of bihormonal cells, our findings also point to alternative sources, (i) a β-cell origin from pre-existing cells spared by the ablation and (ii) a ductal origin, at least in larvae. Our results show that a small but significant fraction of bihormonal cells arises from β-cells.

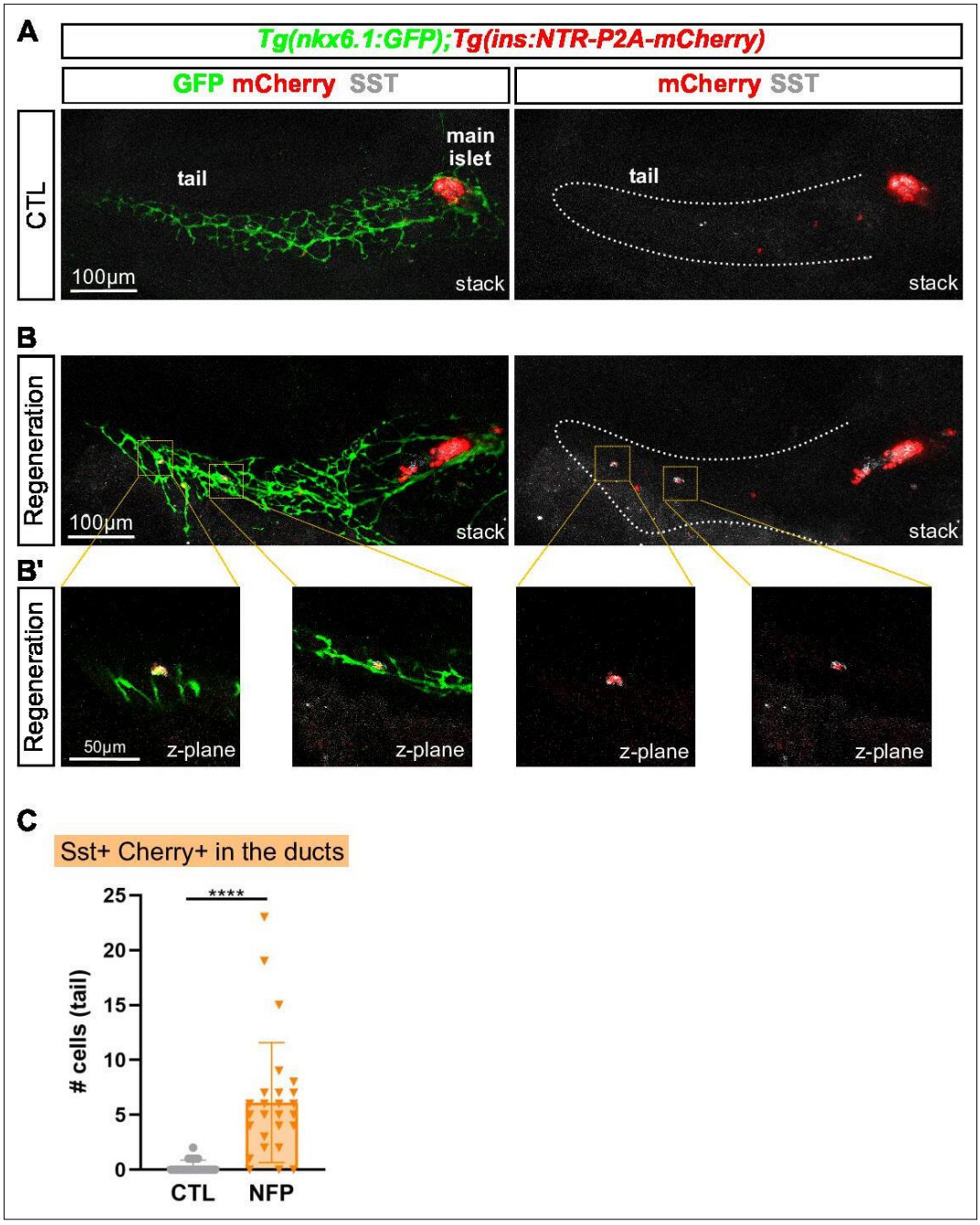


Figure 7. Bihormonal cells can also arise in the pancreatic ducts. (**A–B**) Whole mount immunodetection of GFP that highlights the ducts (green), mCherry (red) for β-cells and Sst (gray) on the entire pancreas of *Tg(nkx6.1:eGFP); Tg(ins:NTR-P2A-mCherry)* larvae at 17 dpf. (**A**) CTL larvae showing the main islet in the head and a few monohormonal endocrine cells (mCherry+ or Sst+) in the ductal GFP+ domain in the tail. The pancreatic tail is delineated by white dashed lines. (**B**) After treatment with NFP from 3 to 4 dpf, regenerating larvae display scattered bihormonal cells (red and gray) in

Figure 7 continued on next page

Figure 7 continued

the tail along the ducts. Stacks represent 3D projections of confocal images of the whole pancreas. (**B'**) Close-ups of two individual bihormonal cells in the tail (z-planes showing one unique optical section). (**C**) Quantification of Sst + mCherry + bihormonal cells based on confocal images. Mann-Whitney test, ****$p < 0.0001$. (See also ***Figure 7—source data 1***).

The online version of this article includes the following source data and figure supplement(s) for figure 7:

Source data 1. Bihormonal cell quantification in the tail (larvae).

Figure supplement 1. Time course of normal β and sst1.1 δ-cells differentiation from intrapancreatic ducts in the tail of *Tgnkx6.1:eGFP; Tg(ins:NTR-P2A-mCherry)* control larvae.

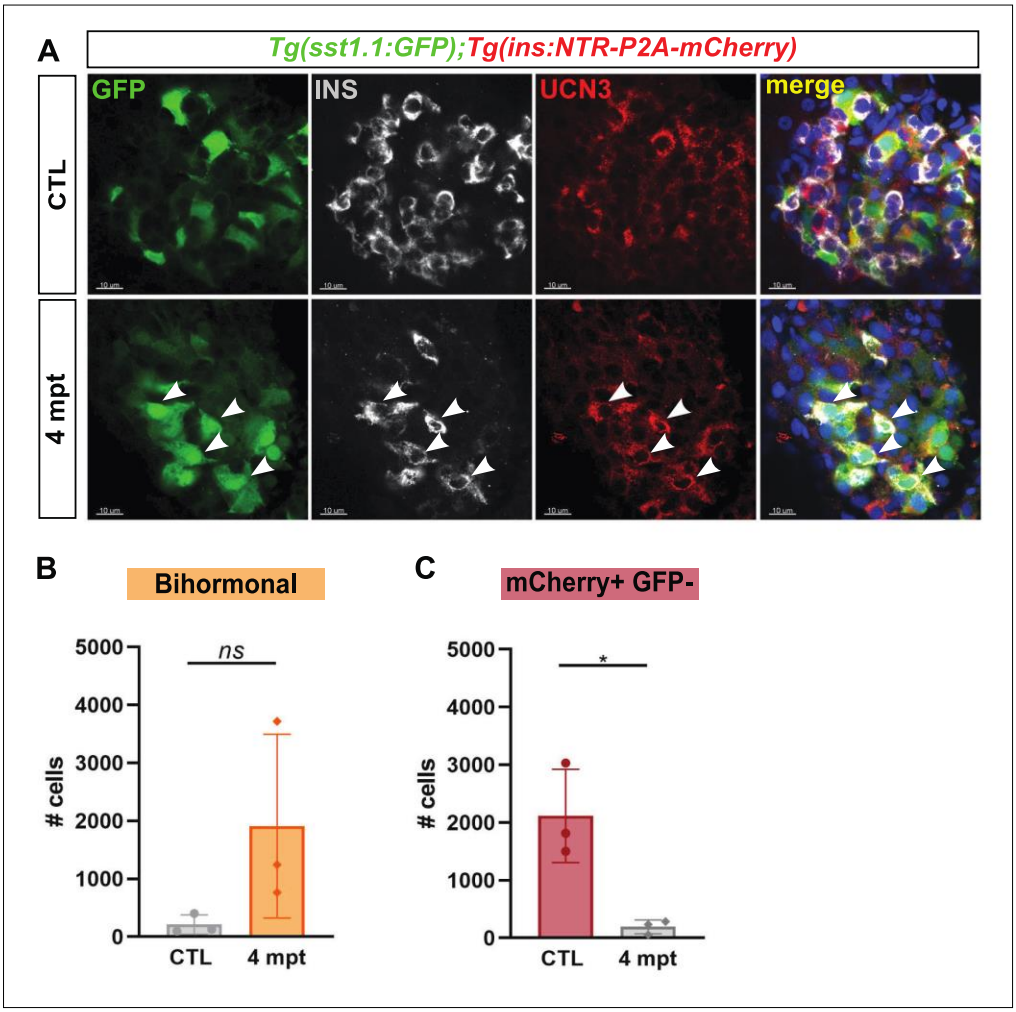


Figure 8. Protracted bihormonal cells 4 months after β-cell ablation. (**A**) Whole mount immunodetection of Ucn3 (red), GFP (green), Ins (grey) on the main islet of *Tg(sst1.1:eGFP); Tg(ins:NTR-P2A-mCherry)* adult fish revealing persistent bihormonal GFP+ Ins + cells still 4 months after ablation. These cells still also express Ucn3 (white arrowheads). (**B–D**) Quantification by flow cytometry of islet cell populations in CTL and 4 months after ablation. (**B**) mCherry+ GFP + bihormonal cells. (**C**) mCherry+ GFP- monohormonal β-cells. Means ± SD; Unpaired t-test with Welch's correction; *$p < 0.05$.

We also show that bihormonal cells form in the pancreatic ducts. As ducts are present in the tail as well as in the head, these results suggest a ductal contribution to the global bihormonal cell mass, that is the main and secondary islets. Whether regenerating duct-derived bihormonal cells differentiate via a monohormonal *sst1.1* δ-cell transitional state remains to be determined. Moreover, the ducts could help repopulate the *sst1.1* δ-cells after conversion. Besides neogenesis, our results suggest that proliferation contributes to the formation and/or maintenance of the pool of bihormonal cells and *sst1.1* δ-cells. Notably, we observed evidences of proliferation at an early stage after β-cell ablation, 3 dpt, as illustrated by replicating EdU + bihormonal cells in larvae and broad PCNA expression in adults. Interestingly, the activation of p53 indicates a tight control on proliferation in bihormonal cells. At 20 dpt, the p53 pathway represents the second most enriched signature in bihormonal cells, while PCNA is still widely expressed. To understand this observation, it would be interesting to tackle the dynamics of cell cycle and to perform a detailed analysis of different markers of cell cycle progression and checkpoints in the different cell populations during regeneration.

The identification of bihormonal cells in zebrafish brings the question of the molecular mechanisms underlying this β/δ hybrid identity. In mammals, *Pdx1* is essential for β-cell function notably through activation of *Ins* and of the glucose-sensing machinery genes *Slc2a2* and *Gck* (**Ahlgren et al., 1998**; **Waeber et al., 1996**; **Watada et al., 1996**). *Pdx1* is also crucial to promote and maintain β-cell identity as it activates β-cell genes and represses the α-cell program (**Ahlgren et al., 1998**; **Gao et al., 2014**). Interestingly, *Pdx1*, also known as STF1 (Somatostatin Transcription Factor 1), is expressed in a subset of mouse/human δ-cells (**Piran et al., 2014**; **Segerstolpe et al., 2016**) and stimulates *Sst* expression (**Leonard et al., 1993**). In both murine α and γ-cells, the efficiency of reprogramming to *Insulin*-expressing cells is potentiated by forced expression of Pdx1 (**Cigliola et al., 2018**; **Perez-Frances et al., 2021**). Thus, the expression of *pdx1* could underlie the intrinsic competence of *sst1.1* δ-cells (or mammalian δ-cells) to induce *ins*. However, *pdx1* expression alone is obviously not sufficient to guarantee *ins* expression, and other mechanisms consequent to β-cell loss must operate in synergy, such as metabolic changes and epigenetic regulations. In contrast to *pdx1*, *nkx6.2* and *mnx1*, two genes essential for β-cell development in zebrafish (**Binot et al., 2010**; **Dalgin et al., 2011**), are totally absent in bihormonal cells (**Figure 3** and **Figure 3—source data 1**). In mammals, the homologue of *nkx6.2* in β-cells is *Nkx6.1* (see species-specific expression in **Figure 3—source data 5**). Both *Nkx6.1* and *Mnx1* genes in mouse are important to repress non-β endocrine lineage programs (**Pan et al., 2015**; **Schaffer et al., 2013**). Together, the robust expression of *pdx1* and the lack of *mnx1* and *nkx6.2* are potential key players in the hybrid β/δ phenotype.

Normal glycemia is nearly recovered after 20 days and regenerated animals display perfectly normal glucose tolerance despite the very low abundance of genuine monohormonal β-cells. Bihormonal cells formed after β-cell destruction are abundant – nearly half the initial β-cell mass – and constitute the vast majority of *ins*-expressing cells throughout the whole pancreas and hence the main source of Ins. Their capacity to regulate blood glucose levels is corroborated by their transcriptomic profile showing the expression of the machinery required for glucose responsiveness and insulin secretion as illustrated by the glucose transporter Glut2 (*slc2a2)*, the prohormone convertase *pcsk1*, the K_{ATP} subunit SUR1 (*abcc8)* and several components of the secretory pathway. All these findings are further supported by the observation by Singh et al that β/δ hybrid cells gain glucose responsiveness during regeneration as assessed by in vivo Calcium imaging (**Singh et al., 2022**). Altogether, we propose that, despite the fact that bihormonal cells are not identical to β-cells, they are the functional units that control glucose homeostasis in regenerated fish, compensate for the absence of monohormonal β-cells and reverse diabetes.

Materials and methods

Key resources table

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
Genetic reagent (*Danio rerio*)	*TgBAC(nkx6.1:eGFP)*^{ulg004}	PMID:26329351	ZFIN: ZDB-ALT-160205–1	
Genetic reagent (*Danio rerio*)	*Tg(ins:NTR-P2A-mCherry)*^{ulg034}	PMID:29663654	ZFIN: ZDB-ALT-171122–9	

Continued on next page

Continued

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
Genetic reagent (*Danio rerio*)	*Tg(sst1.1:eGFP)^{ulg054}*	This paper		See Zebrafish husbandry and generation of the *Tg(sst1.1:eGFP)^{ulg054}* *zebrafish line*
Antibody	Anti-GFP (chicken polyclonal)	Aves Labs	GFP-1020	(1:500)
Antibody	Anti-Insulin (guinea pig polyclonal)	Dako	A0564	(1:500)
Antibody	anti-mCherry/ dsRed (Living Colors Polyclonal)	Clontech	632,496	(1:500)
Antibody	anti-Pan-RCFP (Living Colors Polyclonal)	Clontech	632,475	(1:500)
Antibody	anti-Somatostatin (rat polyclonal)	Invitrogen	MA5-16987	(1:300)
Antibody	anti-Somatostatin (rabbit polyclonal)	Dako	A0566	(1:300)
Antibody	anti-Glucagon (mouse monoclonal)	Sigma	G2654	(1:300)
Antibody	anti-Urocortin 3 (rabbit polyclonal)	Phoenix Pharmaceuticals	H-019–29	(1:300)
Antibody	Anti-Pdx1 (guinea pig polyclonal)	From Chris Wright		(1:200)
Antibody	PCNA	Sigma-Aldrich	P8825	(1:500)
Antibody	Goat anti-Rat IgG (H + L) Cross-Adsorbed, Alexa Fluor 488	Invitrogen	A11006	(1:750)
Antibody	Goat anti-Chicken IgY (H + L), Alexa Fluor 488	Invitrogen	A-11039	(1:750)
Antibody	Goat anti-Chicken IgY (H + L), Alexa Fluor 568	Invitrogen	A-11041	(1:750)
Antibody	Goat anti-Mouse IgG (H + L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	Invitrogen	A-11001	(1:750)
Recombinant DNA reagent	p3E-CRE^{ERT2}	This paper		plasmid
Recombinant DNA reagent	p5E-MCS	Tol2kit	228	plasmid
Recombinant DNA reagent	p3E-eGFP	Tol2kit	366	plasmid
Recombinant DNA reagent	pDestTol2p2A	Tol2kit	394	plasmid
Recombinant DNA reagent	pDONRP2R-P3			plasmid
Sequence-based reagent	O99	This article	PCR primer	GGGGACAGCTTT CTTGTACAAAGTGG CTGCTAACCAT GTTCATGCCTTC
Recombinant DNA reagent	*Tg(ubb:loxP-CFP-loxP-zsYellow)*	PMID:21623370	ZDB-TGCONSTRCT-111115–6	

Continued on next page

Continued

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
Sequence-based reagent	O100	This article	PCR primer	GGGGACAACTTTG TATAATAAAGTTGTC AAGCTGTGGCA GGGAAACCC
Sequence-based reagent	IM217	This article	PCR primer	ttttattaaagtgtttat ttggtctcagag
Sequence-based reagent	IM256	This article	PCR primer	AAGAGCACTT CAGATGTCTTCCC
Sequence-based reagent	O097	This article	PCR primer	GTATCTATAGTT GAACATGA AAGCAT
Sequence-based reagent	O098	This article	PCR primer	GGTCACACTG ACACAAA CAC ACA
Sequence-based reagent	pCR8/GW/TOPO	Invitrogen	K250020	
Commercial assay or kit	Gateway LR Clonase II Enzyme mix	Invitrogen	11791020	
Commercial assay or kit	Gateway BP Clonase II Enzyme mix	Invitrogen	11789020	
Commercial assay or kit	Nextera XT DNA Library kit	Illumina	FC-131–1024	
Commercial assay or kit	Click-iT EdU Cell Proliferation Kit for Imaging, Alexa Fluor 647 dye	Invitrogen	C10340	
Chemical compound, drug	4-Hydroxytamoxifen	Sigma-Aldrich	H7904	
Chemical compound, drug	Nifurpirinol	Sigma-Aldrich	32,439	
Software, algorithm	Flowing Software 2	https://bioscience.fi/services/cell-imaging/flowing-software/	RRID:SCR_015781	Version 2.5.1
Software, algorithm	Imaris	Bitplane (http://www.bitplane.com/imaris/imaris)	RRID:SCR_007370	Version 9.5
Software, algorithm	GraphPad Prism	GraphPad Prism (https://graphpad.com)	RRID:SCR_015807	Version 8
Software, algorithm	DESeq2	DESeq2 (https://bioconductor.org/packages/release/bioc/html/DESeq2.html)	RRID:SCR_015687	
Software, algorithm	WebGestalt	WebGestalt (http://www.webgestalt.org/)	RRID:SCR_006786	

Zebrafish husbandry and generation of the Tg(sst1.1:eGFP)^{ulg054} zebrafish line

Zebrafish wild-type AB were used in all the experiments. *TgBAC(nkx6.1:eGFP)^{ulg004}* (**Ghaye et al., 2015**) and *Tg(ins:NTR-P2A-mCherry)^{ulg034}* (**Bergemann et al., 2018**) were used. Zebrafish were raised in standard conditions at 28 °C. All experiments were carried out in compliance with the European Union and Belgian law and with the approval of the ULiège Ethical Committee for experiments with laboratory animals (approval numbers 14–1662, 16–1872; 19–2083, 21–2353).

To generate the *Tg(sst1.1:eGFP)^{ulg054}* zebrafish line, the *sst1.1:eGFP* transgene has been generated by cloning a 770 pb PCR fragment containing the *sst1.1* regulatory regions just upstream the ATG of the *sst1.1* ORF (ENSDARG00000040799.4) amplified with primers IM217 and IM256 into the Gateway

vector pCR8/GW/TOPO. The promoter was assembled by LR recombination with p5E-MCS and p3E-eGFP into pDestTol2p2A from the Tol2kit (**Kwan et al., 2007**). *Tg(sst1.1:eGFP)*^{ulg054} fish have been generated using the Tol2 mediated transgenesis (**Kawakami, 2007**). Adult *Tg(sst1.1:eGFP)*^{ulg054} fish (abbreviated *Tg(sst1.1:eGFP)*) were crossed with *Tg(ins:NTR-P2A-mCherry)*^{ulg034} to generate a double transgenic line. The insbglob:loxP-mCherry-nls-loxP-DTA construct was created by cloning a loxP-mCherry-nls loxP cassette downstream of the *ins* promoter beta-globin intron (**Ninov et al., 2013**). Subsequently, a DTA gene was cloned downstream of the last loxP site via ligation independent cloning (InFusion, Clontech). The *Tg(ins.bglob:loxP-NLS-mCherry-loxP-DTA)*^{bns525} line (abbreviated *Tg(ins:lox-mCherry-lox-DTA)*) was generated using the Tol2 system (**Kawakami, 2007**). *The Tg(ins:CRE-ERT2)* has been generated by LR recombination combining p5E-MCS (**Kwan et al., 2007**), pME-ins and p3E-CRE^{ERT2} vectors into pDestTol2p2A from the Tol2kit. pME-ins was obtained by cloning into the pCR8/GW/TOPO a PCR fragment of 897 pb using the primers O097 et O098 and which contains 744 bp of the insulin promoter, the exon 1 (47 bp), the intron 1 (99 bp) and the 7 bp of exon two just upstream of the ATG. p3E-CRE^{ERT2} was obtained by BP cloning into the pDONRP2R-P3 the 2200 bp PCR fragment using the primers O99 and O100 and as template the pCRE^{ERT2} kindly received from P. Chambon (**Feil et al., 1997**).

β-Cell ablation

Nifurpirinol (NFP) (32439, Sigma-Aldrich) stock solution was dissolved at 2.5 mM in DMSO. 4-Hydroxytamoxifen (4-OHT, H7904, Sigma-Aldrich) was dissolved in DMSO as a concentrated solution of 10 mM and kept as single-use aliquots at –80 °C. β-cell ablation in *Tg(sst1.1:eGFP); Tg(ins:NTR-P2A-mCherry)* larvae was induced by treatment with 4 µM NFP in E3 egg water. Adult fish were treated in fish water with 2.5 µM NFP. Control treatments consisted of E3 containing 0.16% DMSO. Larvae and adults were treated for 18 hr in the dark.

To induce β-cell ablation with *Tg(ins:lox-mCherry-lox-DTA); Tg(ins:CRE-ERT2)* line, larvae were treated at 7 dpf with 5 µM 4-OHT at in the dark during 2 × 2 hr with replacement with fresh 4-OHT. Larvae were then washed several times with E3 egg water to eliminate 4-OHT and allowed to regenerate.

β-Cell tracing in larvae

β-cell labeling was performed in *Tg(ins:CRE-ERT2); Tg(ubb:loxP-CFP-loxP-zsYellow); Tg(sst1.1:GFP); Tg(ins:NTR-P2A-mCherry)* larvae at 6 dpf by 2 × 2 hr 5 µM 4-OHT before several washes in E3 egg water. At 7 dpf, β-cells were ablated with NFP and larvae were allowed to regenerate until 14 dpf before fixation.

Intraperitoneal glucose tolerance test and blood glucose measurements

Adult fish were fasted for 24 hr then euthanized with tricaine and the glycemia was immediately measured using the Accu-Chek Aviva glucometer (Roche Diagnostics) with blood collected at the tail.

D-Glucose was dissolved in PBS at 0.5 mg/µl. After anesthesia, adult fish were injected intraperitoneally at 1 mg/g fish weight with tricaïne as described in **Eames et al., 2010**.

5-Ethynyl-2′-deoxyuridine (EdU) incorporation assay

Zebrafish larvae were incubated in 4 mM EdU dissolved in fish E3 water for 2 days, with replacement of the solution after 24 hr, the were euthanized in tricaine and fixed in 4% PFA. EdU was detected according to the protocol of Click-iT EdU Cell Proliferation Kit for Imaging, Alexa Fluor 647 (ThermoFisher C10340) and processed for whole mount immunodetection.

Immunodetection of paraffin Sections

Samples were fixed and processed for immunofluorescence as previously described (**Ghaye et al., 2015**).

Whole-mount immunodetection

Larvae were euthanized in tricaine and fixed in 4% PFA at 4 °C for 24 hr before IHC. After depigmentation with 3% H2O2/1% KOH during 15 min, larvae were permeabilized 30 min in PBS/0.5% Triton X-100 and incubated for 2 hr in blocking buffer (4% goat serum/1% BSA/PBS/0.1% Triton X-100). Primary and

secondary antibodies were incubated at 4 °C overnight. Adult fish (6–10 months) were euthanized and fixed for 48 hr. Digestive tracts were dissected, dehydrated and stored in 100% methanol at –20 °C. Before IHC, the samples were permeabilized in methanol at room temperature for 30 min, placed 1 hr at –80 °C then back at room temperature. After rehydration in PBS/0.05% Triton X-100, depigmentation was performed for 15 min followed by incubation in blocking buffer containing 4% goat serum /1% BSA/PBS/0.01% Triton X-100. The primary antibodies were incubated for 48 hr on adult samples and overnight on larvae, followed by overnight incubation with the secondary antibodies overnight at 4 °C. Primary antibodies: Anti-Insulin (guinea pig, 1:500, Dako A0564), Living Colors Polyclonal anti-mCherry/dsRed (rabbit, 1:500, Clontech 632496), Living Colors Polyclonal anti-Pan-RCFP (rabbit, 1:500, Clontech 632475), anti-GFP (chicken, 1:1000, Aves lab GFP-1020), anti-Somatostatin (rat, 1:300, Invitrogen MA5-16987), anti-Somatostatin (rabbit, 1:300, Dako, A0566), anti-Glucagon (mouse, 1:300, Sigma G2654), anti-Urocortin 3 (rabbit, 1:300, Phoenix Pharmaceuticals H-019–29), anti-Pdx1 (guinea pig, 1:200, kind gift from Chris Wright, Vanderbilt University), anti-PCNA (clone PC10 Sigma P8825). Secondary antibodies: Alexa Fluor-488,–568, –633 (goat, 1:750, Molecular Probes).

Whole-mount in situ hybridization on embryos

The *sst1.1* and *sst2* probes were described in ***Devos et al., 2002***. The *ins* probe has been described in ***Milewski et al., 1998***. Fluorescent in situ hybridization were performed as described in ***Tarifeño-Saldivia et al., 2017*** on 3 or 6 days post fertilization embryos (dpf). The antisense RNA probes were revealed using tyramide-Cy3 followed by immunodetection of GFP.

Images of immunodetection and in situ hybridization were acquired with a Leica SP5 or a Zeiss LSM880 confocal microscope, and processed with Imaris 9.5 (Bitplane) for visualization.

In vivo imaging

In vivo imaging was performed with a Lightsheet Zeiss Z1 microscope using a 20 x water immersion objective and 488 nm and 561 nm lasers. *Tg(sst1.1:eGFP); Tg(ins:NTR-P2A-mCherry)* larvae were treated from 1 dpf with 1-phenyl 2-thiourea (0.003% (w:v)) to inhibit pigment synthesis. After ablation with NFP from 3 to 4 dpf, larvae were anesthetized, embedded in 0.25% low melting agarose containing and mounted into FEP capillaries. Images were acquired every 30 min and were maintained during the whole experiment at 28° and with 100 ml/L tricaine. Images were converted with Imaris 9.5 (Bitplane) for visualization.

Flow cytometry and FACS

The zebrafish pancreas contains one main big islet in the head and several smaller secondary islets in the tail. The main islets from 2 to 4 pancreata of *Tg(sst1.1:eGFP); Tg(ins:NTR-P2A-mCherry)* adult fish (6–10 months old, males and females) were dissected under epifluorescence to eliminate a maximum of non-fluorescent surrounding exocrine tissue, collected and washed in HBSS without Ca^{2+}/Mg^{2+}. Live cell dissociation was performed in Tryple Select 1 x solution (GIBCO) supplemented with 100 U/mL collagenase IV (Life Technologies 17104–019) and 40 µg/mL proteinase K (Invitrogen, 25530031) for 10 min at 28 °C, and stopped with 15% FBS. The GFP+ cells, mCherry+ cell and double GFP+ mCherry + cells were selected according to gates as shown in ***Figure 1—figure supplement 1*** (dashed lines) on FACS Aria III and sorted under purity mode and after exclusion of the doublets. The purity of the sorted cells was confirmed by epifluorescence microscopy (~95 %). Cells (about 1000–5000/fish depending on the cell type) were immediately lysed with 0.5% Triton X-100 containing 2 U/µl RNAse inhibitor and stored at –80 °C. Similar strategy was followed for cell quantification in secondary islets present in the pancreatic tail. The pancreas was dissected excluding the anterior most part containing the main islet and whole posterior tissues were dissociated and analyzed.

Cell quantification in adults by flow cytometry

The percentage of mCherry+, GFP+ and double mCherry+ GFP + fluorescent cells in the dissociated islets was inferred from flow cytometry experiments in each quadrant delimiting negative and positive fluorescence. FACS plots were generated by FlowJo 10.6.2 and quantifications were performed using Flowing Software 2.5.1.

mRNA sequencing of FACSed cells and bioinformatic analyses

cDNAs were prepared from lysed cells according to SMART-Seq2.0 (***Picelli et al., 2014***) for low input RNA sequencing and libraries were prepared with Nextera DNA Library kit (Illumina). Independent

biological replicates of each cell type sequenced using Illumina HiSeq2500 and obtained ~20 million 75 bp single-end reads (seven replicates for β-cells, 6 for 20 dpt bihormonal cells, 3 for sst1.1GFP^{high}, 3 for sst1.1GFP^{low}). Reads were mapped and aligned to the zebrafish genome GRCz11 from Ensembl gene annotation version 92 using STAR (**Dobin et al., 2013**). Gene expression levels were calculated with featureCounts (http://bioinf.wehi.edu.au/featureCounts/) and differential expression determined with DESeq2 (**Love et al., 2014**). Expression values are given as normalized read counts. Poorly expressed genes with mean normalized expression counts <10 were excluded from the subsequent analyses. DESeq2 uses Wald test for significance with posterior adjustment of P values (Padj) using Benjamini and Hochberg multiple testing. The differentially expressed (DE) genes identified with a Padj cutoff of 0.05 and fold change above two were submitted for GO analysis using WebGestalt tool (**Liao et al., 2019**).

The genes enriched in β-cells and *sst2δ*-cells above fourfold were identified using sequences obtained previously (**Tarifeño-Saldivia et al., 2017**) with prior mapping on the more recent GRCz11 v92 assembly of the zebrafish genome; they thus slightly differ from the gene list previously published (provided in **Figure 3—source data 2**). Then, new enrichment was updated to take into account the new transcriptomic data obtained for *sst1.1δ*-cells from *Tg(sst1.1:eGFP)* and the new β-cells from *Tg(ins:NTR-P2A-mCherry)* (presented in **Figure 3—source data 3**).

Statistical Analyses

Graphs and statistical analyses were performed using GraphPad Prism 8. Data are represented as Mean ± SD except in **Figure 4C** where Mean ± SEM are shown. The statistical tests are described in the legend of the Figures.

Acknowledgements

The authors thank the GIGA technology platforms GIGA-Zebrafish, GIGA-Genomics and GIGA-Imaging. The authors also thanks Chris Wright for providing the Pdx1 antibody.

Additional information

Competing interests

Didier YR Stainier: Senior editor, eLife. The other authors declare that no competing interests exist.

Funding

Funder	Grant reference number	Author
Chilean National Agency for Research and Development (ANID), Becas Chile	Scholarship	Claudio Andrés Carril Pardo
Belgian National Fund for Scientific Research	FRIA PhD fellowship	Laura Massoz David Bergemann Arnaud Lavergne
National Belgian Funds for Scientific Research	EoS Program	Marie A Dupont Jordane Bourdouxhe
European Regional Development Fund	Biomed Hub Technology Support	Arnaud Lavergne
National Belgian Funds for Scientific Research		Bernard Peers Isabelle Manfroid Marianne M Voz
Belgian National Fund for Scientific Research	30826052	Marie A Dupont Jordane Bourdouxhe

Funder	Grant reference number	Author
Chilean National Agency for Research and Development (ANID), Becas Chile	72170660	Claudio Andrés Carril Pardo
European Regional Development Fund (Biomed Hub Technology Support)	2.2.1/996	Arnaud Lavergne

The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

Author contributions

Claudio Andrés Carril Pardo, Conceptualization, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – review and editing; Laura Massoz, Marie A Dupont, Conceptualization, Formal analysis, Investigation, Methodology, Validation, Visualization; David Bergemann, Formal analysis, Investigation, Writing – review and editing; Jordane Bourdouxhe, Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Validation, Visualization, Writing – review and editing; Arnaud Lavergne, Formal analysis, Resources, Software; Estefania Tarifeño-Saldivia, Formal analysis, Software, Writing – review and editing; Christian SM Helker, Methodology, Resources; Didier YR Stainier, Funding acquisition, Resources; Bernard Peers, Funding acquisition, Resources, Supervision, Writing – review and editing; Marianne M Voz, Funding acquisition, Investigation, Methodology, Resources, Writing – review and editing; Isabelle Manfroid, Conceptualization, Formal analysis, Funding acquisition, Project administration, Resources, Supervision, Validation, Visualization, Writing - original draft, Writing – review and editing

Author ORCIDs

Didier YR Stainier http://orcid.org/0000-0002-0382-0026
Isabelle Manfroid http://orcid.org/0000-0003-3445-3764

Ethics

All experiments were carried out in compliance with the European Union and Belgian law and with the approval of the ULiège Ethical Committee for experiments with laboratory animals (approval numbers 14-1662, 16-1872, 19-2083, 21-2353).

Decision letter and Author response

Decision letter https://doi.org/10.7554/eLife.67576.sa1
Author response https://doi.org/10.7554/eLife.67576.sa2

Additional files

Supplementary files

- Transparent reporting form

Data availability

RNA sequencing data have been deposited at NCBI GEO.

The following dataset was generated:

Author(s)	Year	Dataset title	Dataset URL	Database and Identifier
Carril Pardo CA, Massoz L, Dupont MA, Bergemann D, Bourdouxhe J, Lavergne A, Tarifeño-Saldivia E, Helker CSM, Stainier DYR, Peers P, Voz ML, Manfroid I	2022	δ-cell conversion to insulin+ bihormonal cells in zebrafish	https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE167187	NCBI Gene Expression Omnibus, GSE167187

The following previously published dataset was used:

Author(s)	Year	Dataset title	Dataset URL	Database and Identifier
Tarifeño-Saldivia E	2017	RNAseq from the pancreatic acinar, alpha, beta and delta cells from zebrafish	https://www.ebi.ac.uk/ena/browser/view/PRJEB10140	EBI, PRJEB10140

References

Ahlgren U, Jonsson J, Jonsson L, Simu K, Edlund H. 1998. beta-cell-specific inactivation of the mouse Ipf1/Pdx1 gene results in loss of the beta-cell phenotype and maturity onset diabetes. *Genes & Development* **12**:1763–1768. DOI: https://doi.org/10.1101/gad.12.12.1763, PMID: 9637677

Bergemann D, Massoz L, Bourdouxhe J, Carril Pardo CA, Voz ML, Peers B, Manfroid I. 2018. Nifurpirinol: A more potent and reliable substrate compared to metronidazole for nitroreductase-mediated cell ablations. *Wound Repair and Regeneration* **26**:238–244. DOI: https://doi.org/10.1111/wrr.12633, PMID: 29663654

Binot AC, Manfroid I, Flasse L, Winandy M, Motte P, Martial JA, Peers B, Voz ML. 2010. Nkx6.1 and nkx6.2 regulate alpha- and beta-cell formation in zebrafish by acting on pancreatic endocrine progenitor cells. *Developmental Biology* **340**:397–407. DOI: https://doi.org/10.1016/j.ydbio.2010.01.025, PMID: 20122912

Blum B, Hrvatin S, Schuetz C, Bonal C, Rezania A, Melton DA. 2012. Functional beta-cell maturation is marked by an increased glucose threshold and by expression of urocortin 3. *Nature Biotechnology* **30**:261–264. DOI: https://doi.org/10.1038/nbt.2141, PMID: 22371083

Chera S, Baronnier D, Ghila L, Cigliola V, Jensen JN, Gu G, Furuyama K, Thorel F, Gribble FM, Reimann F, Herrera PL. 2014. Diabetes recovery by age-dependent conversion of pancreatic δ-cells into insulin producers. *Nature* **514**:503–507. DOI: https://doi.org/10.1038/nature13633, PMID: 25141178

Cigliola V, Ghila L, Thorel F, van Gurp L, Baronnier D, Oropeza D, Gupta S, Miyatsuka T, Kaneto H, Magnuson MA, Osipovich AB, Sander M, Wright CEV, Thomas MK, Furuyama K, Chera S, Herrera PL. 2018. Pancreatic islet-autonomous insulin and smoothened-mediated signalling modulate identity changes of glucagon+ α-cells. *Nature Cell Biology* **20**:1267–1277. DOI: https://doi.org/10.1038/s41556-018-0216-y, PMID: 30361701

Curado S, Anderson RM, Jungblut B, Mumm J, Schroeter E, Stainier DYR. 2007. Conditional targeted cell ablation in zebrafish: a new tool for regeneration studies. *Developmental Dynamics* **236**:1025–1035. DOI: https://doi.org/10.1002/dvdy.21100, PMID: 17326133

Dalgin G, Ward AB, Hao LT, Beattie CE, Nechiporuk A, Prince VE. 2011. Zebrafish mnx1 controls cell fate choice in the developing endocrine pancreas. *Development* **138**:4597–4608. DOI: https://doi.org/10.1242/dev.067736, PMID: 21989909

Delaspre F, Beer RL, Rovira M, Huang W, Wang G, Gee S, Vitery MDC, Wheelan SJ, Parsons MJ. 2015. Centroacinar Cells Are Progenitors That Contribute to Endocrine Pancreas Regeneration. *Diabetes* **64**:3499–3509. DOI: https://doi.org/10.2337/db15-0153, PMID: 26153247

Devos N, Deflorian G, Biemar F, Bortolussi M, Martial JA, Peers B, Argenton F. 2002. Differential expression of two somatostatin genes during zebrafish embryonic development. *Mechanisms of Development* **115**:133–137. DOI: https://doi.org/10.1016/S0925-4773(02)00082-5, PMID: 12049777

Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, Batut P, Chaisson M, Gingeras TR. 2013. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* **29**:15–21. DOI: https://doi.org/10.1093/bioinformatics/bts635, PMID: 23104886

Eames SC, Philipson LH, Prince VE, Kinkel MD. 2010. Blood Sugar Measurement in Zebrafish Reveals Dynamics of Glucose Homeostasis. *Zebrafish* **7**:205–213. DOI: https://doi.org/10.1089/zeb.2009.0640, PMID: 20515318

Feil R, Wagner J, Metzger D, Chambon P. 1997. Regulation of Cre Recombinase Activity by Mutated Estrogen Receptor Ligand-Binding Domains. *Biochemical and Biophysical Research Communications* **237**:752–757. DOI: https://doi.org/10.1006/bbrc.1997.7124, PMID: 9299439

Gao T, McKenna B, Li C, Reichert M, Nguyen J, Singh T, Yang C, Pannikar A, Doliba N, Zhang T, Stoffers DA, Edlund H, Matschinsky F, Stein R, Stanger BZ. 2014. Pdx1 Maintains β Cell Identity and Function by Repressing an α Cell Program. *Cell Metabolism* **19**:259–271. DOI: https://doi.org/10.1016/j.cmet.2013.12.002, PMID: 24506867

Ghaye AP, Bergemann D, Tarifeño-Saldivia E, Flasse LC, Von Berg V, Peers B, Voz ML, Manfroid I. 2015. Progenitor potential of nkx6.1-expressing cells throughout zebrafish life and during beta cell regeneration. *BMC Biology* **13**:70. DOI: https://doi.org/10.1186/s12915-015-0179-4, PMID: 26329351

Hans S, Kaslin J, Freudenreich D, Brand M. 2009. Temporally-controlled site-specific recombination in zebrafish. *PLOS ONE* **4**:e4640. DOI: https://doi.org/10.1371/journal.pone.0004640, PMID: 19247481

Kawakami K. 2007. Tol2: a versatile gene transfer vector in vertebrates. *Genome Biology* **8 Suppl 1**:S7. DOI: https://doi.org/10.1186/gb-2007-8-s1-s7, PMID: 18047699

Kwan KM, Fujimoto E, Grabher C, Mangum BD, Hardy ME, Campbell DS, Parant JM, Yost HJ, Kanki JP, Chien C-B. 2007. The Tol2kit: a multisite gateway-based construction kit for Tol2 transposon transgenesis constructs. *Developmental Dynamics* **236**:3088–3099. DOI: https://doi.org/10.1002/dvdy.21343, PMID: 17937395

Leonard J, Peers B, Johnson T, Ferreri K, Lee S, Montminy MR. 1993. Characterization of somatostatin transactivating factor-1, a novel homeobox factor that stimulates somatostatin expression in pancreatic islet cells. *Molecular Endocrinology* **7**:1275–1283. DOI: https://doi.org/10.1210/mend.7.10.7505393, PMID: 7505393

Liao Y, Wang J, Jaehnig EJ, Shi Z, Zhang B. 2019. WebGestalt 2019: gene set analysis toolkit with revamped UIs and APIs. *Nucleic Acids Research* **47**:W199–W205. DOI: https://doi.org/10.1093/nar/gkz401, PMID: 31114916

Love MI, Huber W, Anders S. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology* **15**:550. DOI: https://doi.org/10.1186/s13059-014-0550-8, PMID: 25516281

Milewski WM, Duguay SJ, Chan SJ, Steiner DF. 1998. Conservation of PDX-1 structure, function, and expression in zebrafish. *Endocrinology* **139**:1440–1449. DOI: https://doi.org/10.1210/endo.139.3.5768, PMID: 9492081

Moin ASM, Butler AE. 2019. Alterations in Beta Cell Identity in Type 1 and Type 2 Diabetes. *Current Diabetes Reports* **19**:83. DOI: https://doi.org/10.1007/s11892-019-1194-6, PMID: 31401713

Mosimann C, Kaufman CK, Li P, Pugach EK, Tamplin OJ, Zon LI. 2011. Ubiquitous transgene expression and Cre-based recombination driven by the ubiquitin promoter in zebrafish. *Development* **138**:169–177. DOI: https://doi.org/10.1242/dev.059345, PMID: 21138979

Moss JB, Koustubhan P, Greenman M, Parsons MJ, Walter I, Moss LG. 2009. Regeneration of the pancreas in adult zebrafish. *Diabetes* **58**:1844–1851. DOI: https://doi.org/10.2337/db08-0628, PMID: 19491207

Ninov N, Hesselson D, Gut P, Zhou A, Fidelin K, Stainier DYR. 2013. Metabolic regulation of cellular plasticity in the pancreas. *Current Biology* **23**:1242–1250. DOI: https://doi.org/10.1016/j.cub.2013.05.037, PMID: 23791726

Pan FC, Brissova M, Powers AC, Pfaff S, Wright CVE. 2015. Inactivating the permanent neonatal diabetes gene Mnx1 switches insulin-producing β-cells to a δ-like fate and reveals a facultative proliferative capacity in aged β-cells. *Development* **142**:3637–3648. DOI: https://doi.org/10.1242/dev.126011, PMID: 26534984

Parsons MJ, Pisharath H, Yusuff S, Moore JC, Siekmann AF, Lawson N, Leach SD. 2009. Notch-responsive cells initiate the secondary transition in larval zebrafish pancreas. *Mechanisms of Development* **126**:898–912. DOI: https://doi.org/10.1016/j.mod.2009.07.002, PMID: 19595765

Perez-Frances M, van Gurp L, Abate MV, Cigliola V, Furuyama K, Bru-Tari E, Oropeza D, Carreaux T, Fujitani Y, Thorel F, Herrera PL. 2021. Pancreatic Ppy-expressing γ-cells display mixed phenotypic traits and the adaptive plasticity to engage insulin production. *Nature Communications* **12**:4458. DOI: https://doi.org/10.1038/s41467-021-24788-0, PMID: 34294685

Picelli S, Faridani OR, Björklund AK, Winberg G, Sagasser S, Sandberg R. 2014. Full-length RNA-seq from single cells using Smart-seq2. *Nature Protocols* **9**:171–181. DOI: https://doi.org/10.1038/nprot.2014.006, PMID: 24385147

Piran R, Lee SH, Li CR, Charbono A, Bradley LM, Levine F. 2014. Pharmacological induction of pancreatic islet cell transdifferentiation: relevance to type I diabetes. *Cell Death & Disease* **5**:e1357. DOI: https://doi.org/10.1038/cddis.2014.311, PMID: 25077543

Pisharath H, Parsons MJ. 2009. Nitroreductase-mediated cell ablation in transgenic zebrafish embryos. *Methods in Molecular Biology* **546**:133–143. DOI: https://doi.org/10.1007/978-1-60327-977-2_9

Schaffer AE, Taylor BL, Benthuysen JR, Liu J, Thorel F, Yuan W, Jiao Y, Kaestner KH, Herrera PL, Magnuson MA, May CL, Sander M. 2013. Nkx6.1 controls a gene regulatory network required for establishing and maintaining pancreatic Beta cell identity. *PLOS Genetics* **9**:e1003274. DOI: https://doi.org/10.1371/journal.pgen.1003274, PMID: 23382704

Segerstolpe Å, Palasantza A, Eliasson P, Andersson E-M, Andréasson A-C, Sun X, Picelli S, Sabirsh A, Clausen M, Bjursell MK, Smith DM, Kasper M, Ämmälä C, Sandberg R. 2016. Single-Cell Transcriptome Profiling of Human Pancreatic Islets in Health and Type 2 Diabetes. *Cell Metabolism* **24**:593–607. DOI: https://doi.org/10.1016/j.cmet.2016.08.020, PMID: 27667667

Singh SP, Janjuha S, Hartmann T, Kayisoglu Ö, Konantz J, Birke S, Murawala P, Alfar EA, Murata K, Eugster A, Tsuji N, Morrissey ER, Brand M, Ninov N. 2017. Different developmental histories of beta-cells generate functional and proliferative heterogeneity during islet growth. *Nature Communications* **8**:664. DOI: https://doi.org/10.1038/s41467-017-00461-3, PMID: 28939870

Singh SP, Chawla P, Hnatiuk A, Kamel M, Silva LD, Spanjaard B, Eski SE, Janjuha S, Olivares-Chauvet P, Kayisoglu O, Rost F, Bläsche J, Kränkel A, Petzold A, Kurth T, Reinhardt S, Junker JP, Ninov N. 2022. A single-cell atlas of de novo β-cell regeneration reveals the contribution of hybrid β/δ-cells to diabetes recovery in zebrafish. *Development (Cambridge, England)* **149**:dev199853. DOI: https://doi.org/10.1242/dev.199853, PMID: 35088828

Spanjaard B, Hu B, Mitic N, Olivares-Chauvet P, Janjuha S, Ninov N, Junker JP. 2018. Simultaneous lineage tracing and cell-type identification using CRISPR-Cas9-induced genetic scars. *Nature Biotechnology* **36**:469–473. DOI: https://doi.org/10.1038/nbt.4124, PMID: 29644996

Tarifeño-Saldivia E, Lavergne A, Bernard A, Padamata K, Bergemann D, Voz ML, Manfroid I, Peers B. 2017. Transcriptome analysis of pancreatic cells across distant species highlights novel important regulator genes. *BMC Biology* **15**:21. DOI: https://doi.org/10.1186/s12915-017-0362-x, PMID: 28327131

Thorel F, Népote V, Avril I, Kohno K, Desgraz R, Chera S, Herrera PL. 2010. Conversion of adult pancreatic alpha-cells to beta-cells after extreme beta-cell loss. *Nature* **464**:1149–1154. DOI: https://doi.org/10.1038/nature08894, PMID: 20364121

Waeber G, Thompson N, Nicod P, Bonny C. 1996. Transcriptional activation of the GLUT2 gene by the IPF-1/STF-1/IDX-1 homeobox factor. *Molecular Endocrinology* **10**:1327–1334. DOI: https://doi.org/10.1210/mend.10.11.8923459, PMID: 8923459

Wang Y, Rovira M, Yusuff S, Parsons MJ. 2011. Genetic inducible fate mapping in larval zebrafish reveals origins of adult insulin-producing β-cells. *Development* **138**:609–617. DOI: https://doi.org/10.1242/dev.059097, PMID: 21208992

Watada H, Kajimoto Y, Miyagawa J, Hanafusa T, Hamaguchi K, Matsuoka T, Yamamoto K, Matsuzawa Y, Kawamori R, Yamasaki Y. 1996. PDX-1 induces insulin and glucokinase gene expressions in alphaTC1 clone 6 cells in the presence of betacellulin. *Diabetes* **45**:1826–1831. DOI: https://doi.org/10.2337/diabetes.45.12.1826

Ye L, Robertson MA, Hesselson D, Stainier DYR, Anderson RM. 2015. Glucagon is essential for alpha cell transdifferentiation and beta cell neogenesis. *Development* **142**:1407–1417. DOI: https://doi.org/10.1242/dev.117911, PMID: 25852199

IV. Discussion and Perspectives

The main interest of this study was deciphering mechanisms of β cell regeneration occurring spontaneously in zebrafish, with particular emphasis on the contribution of the ductal progenitors. To this end, we followed several directions. Firstly, we improved the NTR-mediated ablation system by using nifurpirinol (NFP) instead of metronidazole (MTZ) prodrug. We showed that the utilization of NFP significantly improved the quality and the robustness of ablation at non-toxic doses. Overall, these results enabled the establishment of an experimental pipeline for functional tests in the zebrafish larvae.

Secondly, we determined the importance of several candidates highlighted by transcriptomic profiling of ductal cells after β cell ablation in zebrafish. The major part of this thesis consisted of unravelling the function of the protein phosphatase calcineurin (CaN) in β cell regeneration. We showed that CaN inhibition increased ductal cell proliferation and as a consequence, accelerated the formation of endocrine progenitors and subsequent β cells. Interestingly, this phenomenon is coupled with an exhaustion of the pool of ductal progenitors. On the opposite, CaN overactivation decreased β cell regeneration resulting in a delayed glycemia recovery. Additionally, our results showed that CaN acts with Notch pathway, a master regulator of the specification of ductal progenitors. Overall, CaN appears as a guardian of the pool of progenitor, preventing an excessive proliferation to ensure a proper regeneration.

Another interesting candidate revealed by the same transcriptomic study, was the tumour suppressor p53. To determine the function of p53, we used a well-known inhibitor, the pifithrin α (PFTα). We showed that PFTα decreased ductal cell proliferation and β cell formation only in a regenerative context, suggesting that p53 is important for β cell regeneration. However, these results could not be reproduced in a p53 mutant and require more investigations.

Surprisingly, we observed that regenerated β cells co-express the insulin and the somatostatin1.1 hormones in zebrafish. We identified a novel population of delta cells expressing *sst1.1*, these cells can rapidly convert into insulin producing cells, resulting in bi-hormonal cells able to efficiently regulate the glycemia. Interestingly, transcriptomic profiling and preliminary experiments suggest that p53 is also important in these bi-hormonal cells. Importantly, we showed that these cells can also arise from the ductal progenitors.

1. Tissue injury and selective ablation models : at the core of regeneration

In order to decipher regenerative mechanisms, an essential and critical step is to provoke tissue injury or selective ablation of the specific cell type of interest to trigger regeneration. Several techniques have been developed from surgical to chemical or genetically induced cell destruction, which collectively contributed to significant advancements in the field. In zebrafish, a powerful regenerative organism, the NTR mediated ablation with the MTZ pro-drug is widely used (Curado et al. 2007; Harsan Pisharath et al. 2007). However, this system had certain drawbacks such as toxicity related to pro-drug treatment. The dose of MTZ needed (10mM) is indeed slightly toxic for the fish, since non-specific apoptosis could be detected (Mathias et al. 2014). A triple mutant of the NTR showed 2x improvement towards MTZ utilization for cell ablation (Mathias et al. 2014). In our study, we used that system to induce β cell ablation thanks to the transgenic line *Tg(ins:NTR-mCherry)*, in which the NTR was modified according to (Mathias et al. 2014), combined with MTZ treatments. However, the severity of ablation was not reproducible between individuals, which could lead to misinterpretation of data. For example, in the liver, regeneration from the ducts takes place only when hepatocyte ablation is complete or when hepatocyte proliferation is repressed (W. Y. Lu et al. 2015), underlying that the ablation system and its severity need to be tightly controlled.

By replacing the MTZ by another nitro-aromatic compound, the NFP, not only β cells ablation was more efficient, but the efficiency of ablation was more reproducible between individuals (Bergemann et al. 2018). Of note, some cell types such as neurons that require higher dose of MTZ were successfully ablated with NFP. Importantly, the concentration of NFP used is 2x below the general toxicity dose, enabling the combination with other chemical compounds for functional studies.

Since the publication of these data, several studies used the NFP to perform ablation of various cell types as osteoblasts (McDonald et al. 2021), neurons (Corradi et al. 2022) or capillary (Senk and Djonov 2021), underlying the efficiency of NFP. Interestingly, the use of NFP is under investigation in *Xenopus laevis* to trigger oligodendrocyte ablation (Mannioui and Zalc 2019). Another group aimed at replacing the MTZ with others pro-drugs to ablate spinal cord neurons and found ronidazole, which can efficiently induce ablation in the NTR system (Lai et al. 2021). Both ronidazole and NFP showed better efficiency for spinal cord neurons ablation compared to MTZ (Lai et al. 2021). Another way to improve this ablation system is to use another form of the NTR enzyme. Sharrock and colleagues developed a mutated variant of the NTR, which

they called NTR 2.0, that showed 100 times increased activity for MTZ ablation (Sharrock et al. 2020). As a consequence, the NTR 2.0 enable the use of lower concentrations of MTZ and prolonged treatment duration without toxicity. Nevertheless, the NTR 2.0 did not show an increased activity towards the pro-drug NFP, showing substrate specific activity (Sharrock et al. 2020). As such, the development of NTR variants specific to NFP could enable independent ablation of different cell types expressing distinct variants of the NTR enzyme (Sharrock et al. 2020).

The NTR system has been widely used to study β cell regeneration and enable to identify several cellular mechanisms that takes place to regenerate β cells (*i.e.* β cell proliferation, transdifferentiation or differentiation from progenitors). Acute β cell ablation is particularly valuable to mirror long stage diabetes, especially type 1 diabetes when the presence of β cells can no longer be detected. On the other hand, other models can be beneficial to replicate different aspects of diabetes as chronic β cell loss or inflammation. To perform chronic ablation, the NTR system as well as the DTA system that constitutively ablate β cells can be used (Ninov et al. 2013). Additionally, zebrafish models that induce inflammation in β cells with the transgenic line *Tg(ins:il1b)* enabled the identification of wedelolactone, which is able to protect the islet from chronic inflammation (Delgadillo-Silva et al. 2019).

When it comes to regenerative studies, the choice of the ablation/injury model is obviously a key point. However, every model comes with its own limitations. Hence, their constant improvement and the development of new pertinent models enable to study more deeply and precisely regenerative mechanisms.

2. Molecular mechanisms controlling β cell regeneration from pancreatic progenitor

Drug and genetic screenings were previously performed in zebrafish larvae to identify modulators of the endocrine islet (Andersson et al. 2012; Karampelias et al. 2021; K. C. Liu et al. 2017). This identified chemicals compounds or secreted factors impacting β cell regeneration from several pancreatic cellular sources. For example, cdk5 repression (K. C. Liu et al. 2017) as well as folinic acid/Folr1 (Karampelias et al. 2021) promote β cell regeneration from the pancreatic ducts. Even though these studies bring interesting elements of molecular mechanisms, they only focused on the principal islet of larvae. Hence, it persists a necessity for a comprehensive understanding of the signalling pathways involved in this process at adult stage. To gain insights in molecular mechanisms occurring in ductal cells during regeneration,

we established the transcriptomic profile of these cells after β cell ablation in the adult zebrafish. These data showed that the regulated genes encompass most of the genes and pathways identified in previous studies (*igfbp1a* (J. Lu et al. 2016), mTor, Notch (Ninov et al. 2013), carbon metabolism (Karampelias et al. 2021), etc.), confirming their observations. Still in accordance with previous studies (A.P. Ghaye et al. 2015), the transcriptomic analyses during regeneration revealed that DNA replication is the most enriched signature. This attests that ductal cells undergo a strong proliferative response after β cells ablation. Intriguingly, our data uncover the unanticipated upregulation of many genes implicated in DNA repair and cell cycle arrest, suggesting that highly proliferating ductal cells activate counteracting mechanisms. Our findings strongly suggest that these mechanisms preserve robust regeneration via the safeguard of efficient progenitor proliferation and the maintenance of the pool of progenitors. Based on functional assays in zebrafish larvae, we not only confirmed the activation of the proliferation of ductal cells soon after β cell ablation, but also that the rate of progenitor proliferation is carefully controlled by CaN in order to achieve proper and timely regeneration of β cells. While CaN prevents premature β cell formation and regeneration, our results obtained with PFTα suggest that p53 is necessary for progenitor proliferation and β cell regeneration.

Diverse functions of CaN in cell proliferation are already well described in the literature. For example, stressing the cell provokes an entry of calcium and subsequent CaN activation resulting in cell cycle arrest (Leech et al. 2020). Nevertheless, depending the cell type, CaN can be either necessary or deleterious to cell proliferation (Goshima et al. 2019; Masaki and Shimada 2022). The intriguing dual role of CaN is well illustrated in β cell regeneration. While CaN should be repressed to enable ductal progenitor amplification and subsequent endocrine differentiation, CaN is then necessary for β cell function and for their replication (Dai et al. 2017; Heit et al. 2006).

CaN has been reported as a key regulator of proliferation dynamics during fin regeneration (Tornini et al. 2016). In the regenerating fin, low CaN activity is found in the proximal region of the blastema characterized by a high rate of proliferation and regeneration and its activity increases distally to lower proliferation (Cao et al. 2021). It was suggested that CaN controls blastemal cell progeny divisions (Tornini et al. 2016). In differentiating keratinocytes, CaN cooperates with Notch signalling to regulate p21/cdkn1a (which is upregulated in the ducts at 3 dpt), cell cycle withdrawal and differentiation (Mammucari et al. 2005). In other progenitors, as neuronal, hematopoietic progenitors and in stem cells, premature differentiation results from a switch in the mode of cellular division, from symmetric amplifying division to asymmetric

differentiating division (Ho and Wagner 2007; Huttner and Kosodo 2005). Notch determines the choice between both types of divisions (Bultje et al. 2009; Guo, Jan, and Jan 1996). Furthermore, CaN is known to control polarized growth in fission yeasts (Kume et al. 2011), a process underlying asymmetric division not only in yeast but in vertebrate tissues as well. The importance of progenitor cell polarity and symmetric endocrinogenic divisions has been shown during embryonic development of the murine pancreas (Kim et al. 2015). Hence, a possible mechanism whereby Notch and CaN together maintain an appropriate number of progenitors and guarantee efficient β cell regeneration could be via the regulation of cell polarity and differentiating divisions.

In contrast to CaN, our results with the p53 inhibitor PFTα suggest that the tumour suppressor p53 is necessary for progenitor proliferation and β cell regeneration. This pro-regenerative function of p53 seems distinct from the one previously described in the zebrafish heart (Shoffner et al. 2020b). In that case, p53 is downregulated following damage, relieving proliferation suppression in differentiated cardiomyocytes and enabling their re-entry in cell cycle. Contrasting with this conventional role, inhibiting p53 after β cell ablation instead leads to a global reduction of ductal cell proliferation. In line with this, recent studies showed that DNA repair and senescence, usually thought to be negative for growth, are in fact necessary for developmental processes and regeneration in other systems (Da et al. 2020; Sousounis et al. 2020). Our transcriptomic data showed enrichment of DNA repair signatures and downregulated ribosome biogenesis, which are two hallmarks of p53 activity. Together, they provide the bases for a mechanism whereby a subset of ductal cells experience stress, activate p53 to promote their repair and/or adaptation. Our results suggest that failure of this mechanism compromises β cell regeneration. More experiments are required to firstly demonstrate that this effect observed is truly due to p53 repression. Secondly, it would be interesting to address the importance of the cellular senescence and DNA repair pathways in β cell regeneration. This study should determine if CaN and p53 fall into these cellular mechanisms to enable proper ductal cell proliferation.

In addition to replication, the analysis of the ductal transcriptome points to metabolic changes. p53 is an increasingly recognized master regulator of metabolism that coordinates cell cycle with metabolic adaptation and response to stress (Lacroix et al. 2020). Signatures linked to energy production (fatty acid degradation), nucleotide synthesis and carbon metabolism suggest that acute β cell ablation causes anabolic and catabolic adaptations. It is therefore intriguing to investigate the importance of p53 in the metabolism of the ductal cell during regeneration using

metabolomic techniques. Such experiments were performed in zebrafish to unravel the importance of the One-Carbon metabolism, fuelled by folinic acid, to promote β cell regeneration from ductal progenitors in zebrafish (Karampelias et al. 2021).

A compelling question within the field of regeneration is to which extent regenerative mechanisms mimic developmental processes. Notably, the activation of pancreatic progenitors residing within the ducts (Delaspre et al. 2015; Ghaye et al. 2015), and the involvement of pathways such as Notch signalling or mTor (Ninov et al. 2013) for β cell neogenesis is evident in both developmental and regenerative contexts. This underscores that β cell regeneration is orchestrated by developmental mechanisms. Intriguingly, the signalling pathways identified in our study exhibit specificity to the regenerative context, as they do not impact normal larval development. Collectively, these findings suggest that β cell regeneration is governed by developmental processes that can be modulated by signals unique to the regenerative context.

Taken together, our study underlines proliferation of ductal progenitors as a cornerstone in β cell regeneration. Interestingly, several studies reported ductal cell proliferation in diabetic patients (Md Moin, Butler, and Butler 2017; Pagola et al. 2008), which can be translated as an attempt to form new β cells. As such, an important axis would be now to determine if CaN could be exploited to activate β cell regeneration in mammals. Even though *in vitro* or *ex vivo* experiments could bring relatively quickly interesting knowledge, this system does not enable to study regeneration and could not reflect the complexity of an *in vivo* model. However, since the cellular mechanism of regeneration depends on the type of injury model in mouse (Aguayo-Mazzucato and Bonner-Weir 2018), the choice of the model is a crucial aspect. To tackle the function of CaN in the ductal cells, the ideal model should at least show an activation of ductal cell proliferation. The model from Collombat group (Al-Hasani et al. 2013) could be an interesting possibility since by inducing alpha cell transdifferentiation in the STZ-model, it stimulated ductal cell proliferation and endocrine differentiation. Nonetheless, it is important to keep in mind the different role of CaN in the β cells versus ductal cells. CaN is indeed necessary for β cell function (Heit et al. 2006), and its inhibitors FK506 and CsA both decreased β cell regeneration by their proliferation in the DTA mice model with only 70% ablation (Nir, Melton, and Dor 2007). Therefore, it becomes evident that CaN inhibition needs to be only transient, in a model of complete β cell ablation, to trigger their regeneration from the ductal progenitors.

3. The δ1.1 as a new source for β cell regeneration : towards a global view of regenerative mechanisms

In this study, we showed that regenerated β cells co-express the *ins* and *sst1.1* genes. These cells appear quickly, *i.e.* 3 days after β cell ablation, in the islets and stay long after ablation (Carril Pardo et al. 2022). Our live imaging experiments strongly suggest that pre-existing *sst1.1*+ cells start to express *insulin* in response to β cell destruction. Moreover, transcriptomic study of these *sst1.1* δ cells highlights that they are similar to β cells and already possess the cellular machinery to produce hormones. These characteristics could explained how the conversion can be that quick. Unfortunately, we could not formally confirm the origin of these bi-hormonal by cell lineage tracing. However, another study draw the same conclusions (Singh et al. 2022), confirming the conversion of the δ 1.1 cells. Future studies will aim at deciphering the molecular mechanisms under bi-hormonal cell formation. Preliminary results highlight that CaN (data not shown) and p53 (Marie Dupont, personal communication) are not involved in the first steps of this process.

Importantly, we showed that bi-hormonal cells also arise later from ductal progenitors. Differentiation from progenitors appears to be a slower process. Overall, our results suggest that in a first instance, δ1.1 cells within the islets rapidly convert to insulin producing cells to respond to β cell destruction. In a second time, progenitors give rise to newly formed endocrine cells. This mechanism is similar to one described in mice (Al-Hasani et al. 2013), where massive α cell transdifferentiation towards β cells results in a depletion of α cells. In response to this need, ductal cells proliferate and give rise to new α cells (Al-Hasani et al. 2013). An important characteristic of progenitor cells is indeed their ability to proliferate in order to keep the pool of progenitors. On the opposite, mature endocrine cells are usually quiescent, as illustrated by β cells (Teta et al. 2005). Overall, these data suggest that both δ1.1 and ductal progenitor contribution are not mutually exclusive but instead work together to handle a critical situation, meaning β cell destruction. Still in line with this, we showed that most regenerated β cells in the pancreatic ducts are bi-hormonal in the adult zebrafish, enabling a dynamic regulation of the glycemia. Nevertheless, we showed that after ablation, overactivation of CaN leads to inhibition of regeneration from the ducts in the larvae causing a delay of glycemia recovery. As a conclusion, while most regenerated β cells are bi-hormonal, the contribution of the ducts is still crucial since repression of ductal regeneration leads to hyperglycaemia.

These results emphasize the need to understand how β cell regeneration is coordinated within the endocrine islets and the ducts through lineage tracing experiments. Two intriguing studies have shed some light on this coordination in mice (Gribben et al. 2021; Magenheim et al. 2023). On the opposite to all previous studies using genetic lineage tracing (Zhao et al. 2021), Gribben and colleagues demonstrated that in physiological conditions, ductal cells contribute to the generation of new endocrine cells in the adult mice (Gribben et al. 2021). Remarkably, the driver they used, *Hnf1b*-CreERT2, also labelled some isolated δ cells within the islets and in the ducts. Furthermore, with the *Neurog3*-CreER driver, they found that Neurog3+ cells within the ducts expressed somatostatin and eventually delaminated within the islets where they found insulin expression. These results were further supported by sg-cell RNA-sequencing data of Neurog3-traced cells, found in β and δ cell populations. Gribben et al., concluded that Neurog3+ cells in the ductal tree first activate the expression of somatostatin and then insulin (Gribben et al. 2021). However, this conclusion was challenged by Magenheim and colleagues (Magenheim et al. 2023). Since both drivers used in the study also labelled δ cells, it suggests that newly generated β cells might originate from δ cells rather than the ducts. Even though more experiments are necessary to show the contribution of the ducts, both studies corroborate the presence of Neurog3+ cells expressing somatostatin within the ducts (Gribben et al. 2021; Magenheim et al. 2023), a promising population to characterize. Furthermore, future investigations in the zebrafish model could provide crucial insights into the coordination of β cell regeneration, since both origins of regeneration are clearly demonstrated. Notably, single cell RNA-sequencing of the ducts and the endocrine islets, not only from the principal islet but comprising the pancreatic tail during regeneration could be ideal to tackle firstly the heterogeneity of ductal cells and the connections between the ducts and δ1.1 conversion.

The observation of poly-hormonal cells in diabetic patients suggest that endocrine cells retain plasticity to produce insulin in humans as well. The contribution to endocrine cell conversion is also demonstrated in mice model of pancreas injury (Chera et al. 2014b; Perez-Frances et al. 2021; Thorel, Népote, et al. 2010). However, the presence of the specific subtype of δ1.1 cells in zebrafish raises questions about their existence in mammals, particularly in humans. Notably, the presence of δ1.1 cells and the absence of PP-cells in zebrafish necessitate further exploration through cross-species comparisons, extending previous investigations (Tarifeño-Saldivia et al. 2017) to include the study of δ1.1 cells. Moreover, it would be interesting to compare zebrafish δ1.1 cells and mice δ Neurog3+ cells (Gribben et al. 2021; Magenheim et al. 2023) and characterise these populations.

4. Concluding remarks

The reminiscence of multipotent pancreatic progenitor in the adult represents a hope for diabetes therapy. Observation of β cells near the ductal tree and detection of C-peptide in long term diabetic patients led the possibility of the persistence of progenitor cells during the adulthood. However, cell lineage tracing studies performed in mouse model of pancreas injuries led to different results, nourishing the debate. While cell lineage tracing is an impactful tool to determine the origin and the descendance of a cell, the technique is limited by the need of a specific promotor. When it comes to ductal cells, their high heterogeneity increases the difficulty of the choice of the driver. In addition, often the transgene is not expressed in all cells, resulting in mosaic expression. Coupled with the poor regenerative capacities of mammals, this could explain why cell lineage tracing studies could not clearly identify a pancreatic progenitor (Kopp et al. 2011; Zhao et al. 2021). Nonetheless, the development of technologies as single cell sequencing enabled the identification of a ductal sub-population that behave as multipotent pancreatic progenitor (Qadir et al. 2020), keeping the debate open. On the opposite to mammals, it is well established that pancreatic progenitors remains within the ductal network in the adult zebrafish (A.P. Ghaye et al. 2015). As such, that is enthralling to understand how regeneration occurs in zebrafish.

In the present thesis, we bring new insights into β cell regeneration mechanisms and we highlighted that progenitor proliferation is critical in this process. Furthermore, we showed that the protein phosphatase calcineurin fine tunes the balance between progenitor proliferation and endocrine differentiation. Since ductal cell proliferation was observed in diabetic patients, balancing towards endocrine differentiation could be a key for diabetes therapy. Future studies should determine if CaN function can be translated in mammals.

We also emphasize that most regenerated β cells co-express the insulin and the somatostatin 1.1 hormones, and called them bi-hormonal cells (Carril Pardo et al. 2022). Our results strongly suggested that δ1.1 cells can rapidly convert to bi-hormonal cells and that later, newly generated cells arise from the ductal tree, whose can either express insulin alone or are bi-hormonal. Further studies are required to unravel bi-hormonal cell formation and to establish a global view of β cell regeneration from these different cellular sources. Altogether, this thesis bring important new insights into β cell regeneration in zebrafish. This knowledge could improve regenerative competences in mammals and be a basis for new diabetic therapies.

V. Material and Methods

1. Fish husbandry and transgenic lines

Zebrafish (*Danio rerio*) were raised in compliance with standard protocols at the GIGA-zebrafish facility. The Techniplast system was used to ensure the temperature, pH, and salinity of water were properly maintained. The fish were fed with Zebrafeed and living food, with paramecia provided for larvae and *Artemia nauplii* for juveniles and adults. All experimental procedures were approved by the ULiège Ethical Committee.

List of the transgenic lines used in this study :

- *Tg(ins:NTR*-P2A-mCherry)*^{ulg034} (Bergemann et al. 2018) referred as *Tg(ins:NTR-mCherry)*
- *TgBAC(nkx6.1:eGFP)* (Aurélie P. Ghaye et al. 2015) referred as *Tg(nkx6.1:GFP)*
- *Tg(TP1bglob:VenusPest)S940* (Ninov, Borius, and Stainier 2012) referred as *Tg(tp1:VenusPest)*
- *Tg(neurod1:GFP)*
- *Tg(sst1.1:eGFP)* (Carril Pardo et al. 2022)
- *Tg(cftr:gal4)*
- *Tg(hsp70:ppp3cca*^{CA}*-P2A-eGFP)*
- *Tg(UAS:ppp3cca*^{CA}*-P2A-eGFP)*
- *tp53*^{M214K} mutant line (Berghmans et al. 2005)

Generation of transgenic lines is detailed in the associated papers.

2. β cell ablation

The transgenic line *Tg(ins:NTR*-P2A-mCherry)*^{ulg034} was used to drive the expression of the nitroreductase in β cells. Two different pro-drugs, the metronidazole (MTZ) and the nifurpirinol (NFP) were used to trigger ablation. MTZ (Sigma M1547) was prepared freshly at 10mM in fish water supplemented with 0.2% DMSO. NFP (Sigma 32439) was dissolved in DMSO at 2.5mM to make the stock solution, and was kept at -20°C. NFP treatments depended of the stage and are described below. All treatments were performed in the dark for 18 hours.

Larvae were treated with NFP at 4µM, or at 2.5 µM when other treatments with signalling pathway inhibitors were performed after. To ablate β cells in adult, a first treatment with Baktopur (BKT, active molecule is NFP) (Sera) concentrated at 25X was performed for 30 minutes in the dark, before NFP treatment at 2.5 µM.

3. Treatment targeting signalling pathways

Larvae were treated with different compounds targeting signalling pathways (see Table X for more details). The drugs were dissolved in fish water (E3 for larvae and water from the system for juveniles and adults). Larvae were treated in 6-well plate and the juveniles/adults in small tank. Control treatments consisted of fish water containing the same amount of DMSO than drug treatment. All treatment last for 18 hours in the dark, expect for the NAC. NAC was added in the fish water and was renew every day.

Drug	Target	Stock concentration	Treatment concentration	Reference
Cyclosporin A	calcineurin	10mM (DMSO)	1µM	Selleckchem S2286
FK506	calcineurin	10µg/mL (DMSO)	0.1µg/mL	Sigma, F4659
LY411575	Notch pathway	10mM (DMSO)	1-5-10 µM	Sigma SML0506
CHIR99021	GSK-3	10mM (DMSO)	10µM	Sellekchem CT99021
Pifithrin α	p53	10mM (DMSO)		Sigma P4236

Table 1 : Inhibitors of signalling pathways used in this study

4. EdU incorporation assay

EdU was dissolved in DMSO at 400mM for the stock solution that was kept at -20°C. Zebrafish larvae were incubated in 4 mM EdU dissolved in E3 water for 8 hours in the dark, before euthanasia and fixation. EdU was detected according to the protocol of Click-iT™ EdU Cell Proliferation Kit for Imaging, Alexa Fluor™ 647 (ThermoFisher C10340) after whole mount immunodetection.

5. Heat shock

Successive heat shocks of 30 minutes and 12 hours apart were performed in fish water at 39°C for larvae and 37°C for juveniles and adults zebrafish.

6. Glucose measurements

Fasted adult zebrafish were euthanized and a drop of blood from the tail is directly collected to measure the glycemia with the Accu-Check Aviva glucometer (Roche Diagnostics). The maximum measure with this system is 600mg/dL.

7. Fish euthanasia and fixation

Zebrafish were euthanized by their immersion in a concentred tricaine solution (MS222, at 200-300 mg/L), before fixation in paraformaldehyde (PFA) (Thermo Scientific, 28908) at 4%. Adult fish were injected in intraperitoneal with PFA and the abdomen was open before immersion in PFA 4% for 24 hours at 4°C. Juveniles fish were kept two days, and larvae 24 hours in the PFA at 4°C.

8. Whole mount immunohistochemistry (IHC)

After washing the fixative agent with PBTr0.3%, pigments from larvae were removed using 3% H2O2/1% KOH in PBS1X, followed by washes with PBTr0.3%. The duration of the depigmentation depended on the larvae stage, see Table X. Permeabilization of membranes was carried out with PBS 1X/ Triton X-100 for 30 minutes at RT, before washed with PBTr0.3%. Non-specific binding sites were blocked with 2 hours incubation in blocking buffer (4% goat

serum/1% BSA/PBS/0.3% Triton X-100), before incubation of primary antibodies for at least 24 hours at 4°C. After extensive washes, larvae were again incubated with blocking buffer, followed by incubation of secondary antibodies for 18 hours, in the dark at 4°C. Samples were mounted on slides in Prolong antifade after extensive washes.

As for the juveniles fish, the digestive tract was dissected and kept in methanol for at least 18 hours at -20°C, prior immunodetection. After rehydration of the tissues, depigmentation with 3% H2O2/1% KOH, they were incubated for two hours in blocking buffer (4% goat serum/1% BSA/PBS/0.1% Triton X-100). Primary antibodies were incubated at 4 °C for two days and secondary antibodies for 18 hours.

	Fixation duration	**Depigmentation duration**	**Permeabilization solution (30 min)**	**Permeabilization duration**
5-10 dpf	18 hours	15 min	PBTr 0.05%	30 min
13-17 dpf	36 hours	20 min	PBTr 2%	30 min
2months (digestive tract)	18 hours	15 min	/	/

Table 2 : IHC conditions from zebrafish at different stages

Anti-GFP (chicken polyclonal)	Aves Labs	GFP-1020	1:1000
anti-mCherry/dsRed (Living Colors Polyclonal)	Clontech	632496	1:500
Anti-glucagon (mouse polyclonal)	Sigma	G2654	1:300
Goat anti-Chicken IgY (H+L), Alexa Fluor™ 488	Invitrogen	A-11039	1:750
Goat anti-dsred 568	Invitrogen		1:750
Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary antibody, Alexa Fluor 633	Invitrogen		1:750

Table 3: Antibodies

9. Image acquisition and analysis

The GIGA Imaging facility was used to acquire images. Fixed samples were imaged using a Leica SP5 confocal microscope with LAS AF software. Qualitative analysis and image capturing were performed using Imaris 8.1.2 (Bitplane). For quantitative analysis, manual cell counting was done using ImageJ (Fiji) software.

In vivo imaging was conducted using a Light Sheet Zeiss Z1 microscope, equipped with a 20X water immersion objective. Zebrafish larvae were treated with 1-phenyl 2-thiourea (PTU) from 1dpf to inhibit pigment synthesis. Following β cell ablation with NFP, anesthetized larvae were embedded in 0.25% low melting agarose in E3 and placed into FEP tubes. The tubes were then immersed in water at 28°C within an immersive chamber. Images were captured at 30-minute intervals and analysed using Imaris 8.1.2 software (Bitplane).

10. Statistical analysis

Graphs and subsequent statistical analysis were realised using GraphPad 8 software. Statistics are described in the legend of every figures.

11. Pancreas dissection

Pancreatic tissues were collected from euthanized adult zebrafish. The abdominal cavity was incised to provide access to the digestive tract, which was then gently uncoiled without breaking it. Using forceps, the pancreatic tissues were carefully dissected and collected in cold dissection buffer (HBSS from Lonza, 10mM Hepes, 2mM EDTA, and 0.16µl/mL RNasin from Promega).

12. Cell dissociation and FACS

The protocols of cell dissociation were adapted from (Aurélie P. Ghaye et al. 2015) and (Tarifeño-Saldivia et al. 2017). Protocol to dissociate ductal cells for RNA-sequencing is detailed in (Massoz et al., 2023). FACS analysis and transcriptomic QC analysis can be found in David Bergemann's thesis, 2018: "Pancreatic β cell regeneration in zebrafish : investigation of the ductal contribution and involved molecular mechanisms." Protocol to dissociate the main islet is detailed in (Carril Pardo et al. 2022).

VI. Supplemental Material

biomedicines

MDPI

Review

Zebra-Fishing for Regenerative Awakening in Mammals

Laura Massoz †, Marie Alice Dupont † and Isabelle Manfroid *

Zebrafish Development and Disease Models Laboratory, GIGA-Stem Cells, University of Liège, B-4000 Liège, Belgium; laura.massoz@doct.uliege.be (L.M.); marie.dupont@uliege.be (M.A.D.)
* Correspondence: isabelle.manfroid@uliege.be; Tel.: +32-4-366-3374
† These authors contributed equally to this work.

Abstract: Regeneration is defined as the ability to regrow an organ or a tissue destroyed by degeneration or injury. Many human degenerative diseases and pathologies, currently incurable, could be cured if functional tissues or cells could be restored. Unfortunately, humans and more generally mammals have limited regenerative capabilities, capacities that are even further declining with age, contrary to simpler organisms. Initially thought to be lost during evolution, several studies have revealed that regenerative mechanisms are still present in mammals but are latent and thus they could be stimulated. To do so there is a pressing need to identify the fundamental mechanisms of regeneration in species able to efficiently regenerate. Thanks to its ability to regenerate most of its organs and tissues, the zebrafish has become a powerful model organism in regenerative biology and has recently engendered a number of studies attesting the validity of awakening the regenerative potential in mammals. In this review we highlight studies, particularly in the liver, pancreas, retina, heart, brain and spinal cord, which have identified conserved regenerative molecular events that proved to be beneficial to restore murine and even human cells and which helped clarify the real clinical translation potential of zebrafish research to mammals.

Keywords: zebrafish; regeneration; mammal; liver; pancreas; heart; retina; brain; spinal cord

Citation: Massoz, L.; Dupont, M.A.; Manfroid, I. Zebra-Fishing for Regenerative Awakening in Mammals. *Biomedicines* **2021**, *9*, 65. https://doi.org/10.3390/biomedicines9010065

Received: 23 December 2020
Accepted: 5 January 2021
Published: 12 January 2021

1. Introduction

Humankind has been fascinated with regeneration abilities since the times of the Ancient Greece. In Greek mythology, one of the labors of Hercules was to kill the Hydra, which is able to regrow two heads when one is ablated. In another myth, Prometheus' liver is renewed every night. However, it was only in the late seventeenth century that scholars paid formal attention to regeneration. Abraham Trembley became a pioneer in this field with his work on fresh water polyps. He described that after cutting a polyp in pieces, each of them was able to regrow an entire organism. He named the polyp "hydra" for its regenerative capacities. After that, regenerative biology had a major influence in the history of biological sciences as it contributed to legitimize biology as an experimental discipline rather than a descriptive science [1].

In recent decades, new technologies such as imaging, genetic engineering and stem cells have enabled the development of regenerative biology, which laid the foundation of a new branch of medicine, i.e., regenerative medicine. Many human diseases and pathologies such as diabetes, Alzheimer's disease, blindness, heart failure or spine injuries, today incurable, could be cured if functional tissues or cells could be restored by regeneration. However, humans, and more generally mammals, possess limited regenerative capabilities, capacities that even further decline with age. In contrast, invertebrates and phylogenetically primitive vertebrates are able to regenerate full tissues after injury. Even though species with strong regenerative capacities are non-uniformly widespread across the phylogenetic tree, simpler organisms generally perform better in this respect [2]. For this reason, it has been assumed that regenerative potential has been lost during evolution. However, in the last years, several studies have revealed that regenerative mechanisms are still present

in mammals but are latent or dormant, and thus it would be possible to stimulate them. This is why elucidating the regenerative mechanisms in competent species is important to permanently cure patients.

Classical models of regeneration are found in invertebrate and vertebrate phylum such as the *hydra*, *planarian*, *drosophila*, *zebrafish*, *axolotl* and *newt*. First exploited to study embryonic development, the zebrafish became in the last 40 years a powerful model organism for deciphering regenerative mechanisms [3]. In 2013, the keyword "regeneration" was the 20th most frequently used in publications using zebrafish [4]. Its success is also due to its fast and external development, the abundant number of eggs and its transparency in the first development stages, making easier the observation of organs and live imaging. Recent technologies, such as CRISPR/Cas9 and TALENs to generate mutant or transgenic lines [5] and high throughput drug screenings [6], have facilitated the study of regeneration in zebrafish. Moreover, its genome is well characterized: 71.4% of the human genes possess at least one or two orthologs in zebrafish and 82% of disease-linked genes listed in the Online Mendelian Inheritance in Man (OMIM) database can be related to at least one zebrafish orthologue [7]. As it has been shown that most of the studied mechanisms in zebrafish implicate the same factors as in mammals, the genetic cascades implicated in a given regenerative process in zebrafish are most likely to be conserved in mammals, rendering possible their manipulation to stimulate regeneration in mammals.

Here we focused on several organs (the heart, liver, pancreas and central nervous system) where research performed in zebrafish clearly helped promote regeneration in murine and human models. These zebrafish studies were selected based on direct evidence of experimental validation in mammalian models (in the same study or in citing references). The overview of these studies also contributes to understanding why the response to tissue damage differs between organs and species and how mechanisms detrimental to regeneration could be overcome.

2. Awakening the Regenerative Capacity in Different Organs

2.1. The Heart

Unsurprisingly, healthy cardiac function is essential for survival and heart failure remains one of the leading cause of death worldwide [8]. In mammals, even if cardiomyocyte self-renewal does occur, the annual turnover is low, decreasing from 1% to 0.3% between 20 and 75 years old [9], and it is not sufficient to repair injured hearts. Instead, after a myocardial infarction, the damaged myocardium is replaced by fibrotic scar tissue, which tampers cardiac function, ultimately leading to fatal heart failure [10]. By contrast, following a 20% ventricular ablation by resection or cryoinjury, the zebrafish fully regenerates a functional myocardium within a few months without scarring, even at the adult stage (Table 1) [10–15]. Although this regenerative capacity is also observed in neonatal mice, in contrast to zebrafish, it is lost after the first week of postnatal life [16].

Using cardiomyocyte lineage tracing systems in adult fish and neonatal mice, regenerated cardiomyocytes were shown to derive from dedifferentiation of pre-existing mature cardiomyocytes followed by proliferation and redifferentiation, rather than from progenitor or stem cells [16,17]. The ploidy of cardiomyocytes is one of the major differences between adult zebrafish and mice. Adult zebrafish cardiomyocytes are mainly diploid and mononucleated with a high proliferative potential during regeneration [18], whereas the non-regenerative myocardium of adult rodents and humans is largely composed of polyploid mono- or binucleated cardiomyocytes [19–21]. Polyploidy has been proposed to account for the decreased regenerative potential of these species [22].

The RhoGEF Ect2 is required for cytokinesis initiation [23] and its expression in murine cardiomyocytes decreases during the first week of postnatal life [18] correlating with the binucleation event and the loss of regenerative ability [16,24]. However, its expression remains high in zebrafish [18]. Using a transgenic line inhibiting *ect2*, Gonzalez-Rosa et al. managed to induce cardiomyocyte polyploidization in zebrafish. After heart injury, they observed that an excess of 50% of polyploid cardiomyocytes dampens the proliferation

of remaining cells, and thus the regeneration of the organ, while it induces a persistent scarring. This highlighted an inverse correlation between the percentage of polyploid cells and the regeneration ability of the heart [18]. The mobilization of diploid instead of polyploid cardiomyocytes in mammals, by maintaining the expression of *ect2*, would offer a therapeutic alternative to stimulate the proliferation of cardiac cells and heart regeneration.

Table 1. Overview of injury models presented in the review.

Organ	Model Organism	Injury Model	Type of Injury	Mechanism of Regeneration	Characteristics	References
Heart	Zebrafish	Ventricular resection	Surgical	Proliferation from pre-existing myocytes	-	[11]
		Cryoinjury	Surgical	Proliferation from pre-existing myocytes	Clinically relevant to mammalian infarcts with massive cell death	[13]
	Mouse	Ventricular resection	Surgical	Proliferation from pre-existing myocytes	Fully regenerates a functional myocardium in 1–2 months without scarring	[11,12]
		Myocardial infarction	Surgical	Proliferation from pre-existing myocytes	Left anterior descending coronary artery occluded with a nylon suture	[25]
Liver	Zebrafish	Partial hepatectomy	Surgical	Hepatocyte-driven	Clinically relevant	[26]
		APAP overdose	Chemical	BEC-driven regeneration	Paracetamol overdose	[26]
		Nitroreductase (NTR)-mediated ablation	Genetic/Chemical	Hepatocyte-driven BEC-driven regeneration	*Tg(fabp10a:CFP-NTR)*	[27–29]
	Mouse	CDE diet	Chemical	BEC-driven regeneration	Ethionine, a toxic analog of methionine, in association with choline deficiency, leads to hepatocyte death and liver inflammation	[30]
		Ctnnb1 hepatocyte KO	Genetic	BEC-driven regeneration	Represses hepatocyte proliferation—in combination with an injury model	[29]
		Mdm2 deletion (hepatocyte-specific)	Genetic	BEC-driven regeneration	AhCreMdm2flox/floxInducible, repress hepatocyte proliferation	[30]
Beta cell/Pancreas	Zebrafish	NTR-mediated ablation	Genetic/Chemical	Beta cell proliferation;alpha cell transdifferentiation; Neogenesis from ductal progenitors	*Tg(ins:CFP-NTR)* In cells expressing NTR, reduces non-toxic pro-drug into cytotoxic products causing targeted cell apoptosis	[31–34]
	Mouse	Pancreatic Duct Ligation (PDL)	Surgical	Neogenesis from ductal progenitors	Induces acinar cell death and acute inflammation without destruction of beta cells	[35–37]
		Streptozotocin (STZ)	Chemical	Beta cell proliferation; Neogenesis from ductal progenitors	Toxic glucose analogue that enters into beta cells via the GLUT2 transporter causing their death	[31,38]
		Diphtheria Toxin Analogue (DTA)	Genetic/Chemical	Alpha cell transdifferentiation (adult only) Delta cell transdifferentiation (neonatal only)	*Tg(RIP:DTR)* The toxin enters in cells expressing the DTR and inhibits protein synthesis, leading to cell apoptosis. Here targeted in beta cells with the Rat Insulin Promoter (RIP).	[39,40]
Spinal Cord	Zebrafish	Spinal cord transection	Surgical	Glial bridge	Complete cutting of the vertebral column	[41]
	Mouse	Laminectomy and spinal cord hemisection	Surgical	-	Hemisection leading to complete paralysis of the ipsilateral limb	[42]
Brain	Zebrafish	Stab-lesion assay	Surgical	Regeneration from radial cells	Injury in the telencephalon parenchyma	[43]
		B42 mediated injury	Surgical/Chemical	Regeneration from radial cells	Alzheimer's-disease-like	[44]
	Mouse	AD-like model	Genetic	No regeneration	*APP/PS1dE9* transgenic	[45]
Retina	Zebrafish	Needle poke	Surgical	From Muller cells	-	[46]
		Optic nerve lesion	Surgical	From Muller cells	-	[47]
	Mouse	NMDA	Chemical	From Muller cells	-	[46,48]
		Excessive light	Surgical	From Muller cells	-	[46]
		AD-like model	Genetic	No regeneration	APP/PS1dE9 transgenic	[45]

In order to identify other mechanisms underlying heart regeneration in adult zebrafish and potentially conserved but dormant mechanisms in mammals, Aguirre and colleagues focused on the microRNAs (miRNAs) differentially regulated after amputation of the ventricular apex in adult zebrafish [25]. They identified miR99/100 and let-7a/c that are downregulated during regeneration. These miRs are known to be implicated in proliferation, chromatin remodeling and morphogenesis, including cardiomyogenesis. Downregulation of miR99/100 during the cardiac regenerative process in zebrafish allows a significant de-repression of their targets *fntb* and *smarca5* in cardiomyocytes, associated with increased cell cycle entry. By contrast, in adult mouse and in human heart tissue, the expression of miR-99/100 stays high after injury, inhibiting the expression of *Fntb* and *Smarca5*. Silencing of miR99/100 or let-7a/c in isolated primary murine adult cardiomyocytes or in murine organotypic slices induced cardiomyocyte dedifferentiation and the acquisition of a proliferative phenotype similar to what was observed in zebrafish. Similar results were obtained in vivo by intracardiac injection of anti-miR-99/100 and anti-Let-7a/c in a murine model of myocardial infarction. More importantly, this led to an improvement of functional heart parameters after 15 days and to the reduction of fibrotic scarring and of the infarct size compared to scrambled controls. Of note, the dedifferentiation observed after miR99/100 downregulation in mammalian cardiomyocytes is limited to the mononucleated cells. Either the polyploid cardimoyocytes are able to convert to a mononucleated state or the mononucleated cardiomyocyte population is more responsive to the regenerative pathway [25]. It would be interesting to answer this question in order to find the best strategy to induce cardiomyocyte proliferation in mammals. In conclusion, the limited cardiac regeneration in mice is at least due to the failure to modulate the miR99/100 and let-7a/c/FNTB and SMARCA5 axis and anti-miR delivery can reactivate this dormant pathway in mammals [25,49].

In the same way, comparison of gene and miRNA profiling of injured zebrafish and mouse adult hearts identified miR-26a [50]. miR-26a represses expression of *ezh2*, a key component of the polycomb repressive complex involved in the methylation of histone H3K27 that is implicated in cardiomyocyte proliferation and in the maintenance of cardiac identity in mice. After ventricular resection in zebrafish, *ezh2* expression is induced due to the downregulation of miR-26a whereas, in the murine heart, miR-26a expression remains high after injury and maintains inhibition of *Ezh2*. Knock-down of miR26a in neonatal mice via injection of anti-miR-26a oligonugleotides increased expression of *Ezh2* and augmented the number of proliferating cardiomyocytes [50].

Together, miR-99/100 and miR-26a are downregulated during the regenerative process in zebrafish whereas their expression is high in adult mice [25,50]. As their expression can be inhibited by antagomir therapy in the mammals, miRNAs could constitute clinical targets to stimulate cardiac regeneration [25,49,50].

Other transcriptomic analyses from adult zebrafish have shown that leptin B (*lepB*), a paralog of mammalian leptin, is induced in the regenerating tail fin and heart [51]. By epigenetic profiling, Kang et al. have identified a short sequence upstream the *lepB* promoter, called *lepb-linked enhancer* (LEN), which acquires H3K27ac marks and open chromatin marks during regeneration [51]. Moreover, the authors showed that LEN can direct regeneration-activated gene expression not only from *lepB* but also from different promoters such as *cmlc2* (cardiomyocytes) or *α-cry* (lens). They exploited this LEN sequence to overexpress *neuregulin 1* (*nrg1*), known to be implicated in cardiomyocyte proliferation and regeneration [52], in adult zebrafish via transgenesis using a promoter combining LEN and the *lepB* minimal promoter. After ventricular resection, these fish strongly activated *nrg1* expression at the injured area and exacerbated cardiomyocyte proliferation. In contrast, control fish did not induce expression of *nrg1* under the *lepB* minimal promoter only without the LEN sequence, showing that LEN can modulate heart regeneration. Even if the LEN sequence is poorly conserved in mammals, LEN-hsp68::lacZ transgenic mice where the zebrafish LEN was fused to the murine *hsp68* promoter revealed injury-dependent LEN activity in wounds after heart resection or even digit amputation in neonates [51]. This

result shows that mammalian gene regulatory networks have the potential to activate zebrafish LEN enhancer and to enable injury-induced expression in mice [53], suggesting that similar constructs could be designed to stimulate timely regeneration of different organs in mammals. It remains to determine whether overexpression of *Nrg1*, or of other positive regulators, under the LEN promoter could give similar results.

2.2. The Liver

Despite their poor regenerative capabilities, mammals are able to efficiently regrow their liver. After partial hepatectomy or mild injury, liver regeneration is mainly achieved by proliferation of pre-existing hepatocytes. However, this process is impaired after acute injury or in hepatic chronic diseases such as liver cirrhosis, viral hepatitis and liver cancer. These diseases are characterized by inflammation, fibrosis and exhaustion of the proliferative potential and finally the death of the hepatocytes. In these situations, activation and expansion of biliary ductular cells, the so-called "ductular response", takes place. Oval cells have been observed in mammals next to these ducts [54] and it has been hypothesized that they could represent liver progenitors deriving from ducts able to restore hepatocytes. Many rodent models of chronic liver injury have been developed to study this process (Table 1). One category of models involves hepatotoxins such as ethionine, CCl4 or N-acetyl-p-aminophenol (APAP), (also called paracetamol or acetaminophen), which are repeatedly injected or delivered in association with specific pro-inflammatory diets, causing chronic death of hepatocytes. The second category of models consists of mutant models (*Mdm2*, *Ctnnb1*, *Itgb1*, *p21/Cdkn1a*) with impaired hepatocyte proliferation. Models to study liver regeneration from the ducts usually combine chronic hepatocyte injury and repression of replication. Previously the subject of controversy, mainly owing to the diversity of the models, there is now strong evidence that biliary epithelial cells (BECs), also known as cholangiocytes, are able to dedifferentiate into liver progenitor cells (LPCs), or oval cells, when hepatocyte-driven regeneration is compromised [30]. These LPCs are bipotent progenitors able to redifferentiate into BECs or hepatocytes. It is of utmost clinical importance to identify the molecular regulation of this process, still not yet fully understood, to improve liver regrowth in chronic hepatic disease patients. This particular topic has been recently reviewed [55,56].

The zebrafish can efficiently replenish its liver with new hepatocytes through both hepatocyte-driven (i.e., replication) or BEC-driven regeneration, providing a valuable model to decipher the mechanisms of both types of liver regeneration. A chemical screening performed in the zebrafish *Tg(fabp10a:CFP-NTR)* line, based on nitroreductase (NTR)-mediated near-total ablation of hepatocytes, pinpointed that the bromodomain and extra-terminal proteins (BET) are required for BEC-driven regeneration [28]. BETs recognize lysine acetylation in histones and other transcription factors, thereby positively or negatively regulating transcription. They mediate different steps of BEC-driven regeneration in zebrafish, BEC dedifferentiation into LPC, proliferation of LPC and redifferentiation into new hepatocytes and their maturation [28]. In addition, BET proteins also promote hepatocyte-driven liver regeneration in a zebrafish liver injury model of paracetamol (APAP) overdose (Table 1) [26]. Importantly, the requirement for BET proteins in both types of liver regeneration is conserved in mice. In the choline-deficient ethionine-supplemented CDE-diet mouse model of chronic liver injury that induces BEC-driven regeneration, BET proteins are required for activation of LPC [28]. In mice after partial hepatectomy, BET proteins are required for hepatocyte proliferation [26]. These data are of high clinical relevance as the same BET inhibitor, JQ1, has been used in a clinical trial for cancer therapy, including liver cancer. The authors stressed that, even though such drugs could be beneficial in this specific context, they would also inhibit liver regeneration, thereby limiting their therapeutic use [26]. Given the importance of BET proteins as epigenetic regulators, a second chemical screen with a library of compounds targeting epigenetic factors has been conducted with the same zebrafish NTR-mediated liver ablation model [29]. This screening identified the histone deacetylase HDAC1 as a potential regulator of BEC-driven

regeneration. HDAC1 was already known to be involved in liver regeneration in mouse models of hepatocyte-driven regeneration [57]. Ko et al. 2019 showed that *hdac1* regulates LPC differentiation into hepatocytes and BECs during BEC-driven regeneration in zebrafish [29]. More exactly, the loss of *hdac1* impairs LPC differentiation into hepatocytes by increasing the expression of *sox9b*, and into BECS via the increased expression of *cdk8*, a negative regulator of Notch signaling. Administration of the HDAC1 inhibitor MS-275 to a mouse model of chronic liver injury combining hepatocyte-specific loss of *ctnnb1* (β-catenin) and a choline-deficient, methionine-supplemented diet impairs differentiation of LPCs into hepatocytes [29]. Interestingly, *HDAC1* is expressed in liver tissues from patients with cirrhosis, suggesting a conserved role of *Hdac1* from zebrafish to human in LPC differentiation [29].

Another approach to decipher regenerative molecular mechanisms is the identification of candidates by RNA sequencing. Using this approach and the zebrafish NTR-mediated ablation model, the Bone Morphogenetic Protein (BMP) pathway was found to be modulated during liver regeneration [27] and BMP inhibition impaired BEC-driven regeneration. Based on these findings, BMP2 treatment was shown to increase the differentiation of a murine liver progenitor cell line into hepatocytes in vitro. To conclude, screenings conducted in zebrafish enabled us to identify mechanisms of both hepatocyte- and BEC-driven liver regeneration, which seems conserved in mammals.

2.3. The Pancreas

Diabetes is a leading health issue worldwide with an incidence of 1 out of 11 people, and causes 1.5 million of deaths per year according to the WHO. The disease is characterized by a dysfunction of blood glucose regulation and various consequent life threatening health conditions. In type 1 diabetes (T1D) or in late stages of type 2 diabetes (T2D), the insulin-producing beta cell mass is dramatically decreased, resulting in a lack of insulin. Besides therapeutic strategies to preserve the beta cell mass and its function and to improve insulin treatments, beta cell regeneration constitutes a promising alternative to replenish the pancreas with functional beta cells. This process is extensively studied in mice, using a model of pancreas injuries. In rodents, the main models of beta cell regeneration consist of injections of a toxic glucose analogue streptozotocin (STZ), expression of the diphtheria toxin A (DTA) suicide transgene, and the pancreatic duct ligation (PDL) model which is characterized by high levels of inflammation in the pancreas but no destruction of beta cells per se (Table 1). Using these models, mice revealed a certain plasticity of mammalian pancreatic cells despite the poor regenerative capacity of mammals. Besides replication of remaining beta cells [58], neogenesis can proceed from alpha cells [40], delta cells [39] or acinar cells [59]. Duct-associated pancreatic progenitors have also been proposed [37,38] even though this source is under controversy [35,36]. These studies underline the importance of the injury model and of age in regeneration efficiency and cellular origin of new beta cells.

To get new insights into beta cell regeneration and to overcome the limited regeneration ability of rodent models, researchers have exploited the zebrafish model. One of the strategies was to identify pharmacological compounds able to enhance beta cell proliferation. Several groups performed medium or high-throughput drug screenings using zebrafish larvae and the inducible NTR-mediated ablation model [31,32,60]. Two independent studies discovered that drugs stimulating the production of cAMP promote beta cell regeneration by proliferation. One class of compounds activates the adenosine/cAMP pathway and promotes beta cell proliferation after beta cell ablation [31], of which the more potent is the *50-N-ethylcarboxamidoadenosine* (NECA), an adenosine agonist activating GPCR signaling. The other study identified the TBK1 and IKKε inhibitor *(E)-3-(3-phenylbenzo[c]isoxazol-5-yl) acrylic acid* (PIAA), which appeared to activate the cAMP-PKA-mTOR pathway leading to increased beta cell proliferation after ablation [32]. Importantly, both drugs, NECA and PIAA, also increased beta cell proliferation in mammalian ex vivo models, NECA being validated in mouse islets and PIAA in both

rat and human islets. NECA and PIAA were also able to enhance beta cell regeneration in vivo in STZ-treated mice. Moreover, these drugs led to functional improvement by lowering glycemia in mice [31,32]. By coupling the advantage of assessing the effect of various compounds on a given phenotype (here beta cell regeneration) with toxicological assays, the zebrafish allows not only the pinpointing of the adenosine pathway but also the identification of, among the numerous cAMP modulators, the non-toxic compounds most promising for further clinical studies. Validations in human models are particularly critical in the context of beta cell replication as adult human beta cells are extremely resistant to cell cycle re-entry compared to mice [61]. In addition, beta cell replication is inversely correlated with functional maturation, thus such a strategy should be used with caution.

Besides beta cell proliferation, other pancreatic cells can give rise to new beta cells. The glucagon-producing alpha cells are able to transdifferentiate into beta cells in various mouse models [40], though the regeneration is very slow and low, as well as in the zebrafish NTR model [34]. After a transcriptomic profiling by microarray of zebrafish islets isolated during regeneration following NTR-mediated ablation, secreted proteins were selected as candidate enhancers of beta cell regeneration [33]. One of them, the insulin-like growth factor (Igf) binding-protein 1 (*igfbp1*), was shown to increase transdifferentiation of alpha cells into beta cells when overexpressed by transgenesis, leading to potentiation of beta cell regeneration and accelerated restoration of normoglycemia. Furthermore, IGFBP1 could also promote alpha cell transdifferentiation in mouse and human islets ex vivo. Since *igfbp1* is known to be repressed by insulin, the study also showed that patients with insulin resistance have a lower level of IGFBP1 in their blood while those with a high level of IGFBP1 have a lower risk to develop T2D [33]. In T1D or in late stages of T2D, when the beta cell mass is reduced, the level of IGFBP1 is elevated due to the lack of insulin [62]. These observations demonstrate that IGFBP1 could be a potential biomarker for insulin resistance/diabetes in addition to be a good candidate for beta cell regeneration in (pre)clinical studies.

Another axis of regeneration is to harness pancreatic progenitors. As endocrine cells arise from the pancreatic ductal tree during the development, it has been hypothesized that progenitors could still be associated to the pancreatic ducts in adults. Although beta cell neogenesis from ducts in the adult is under controversy in mammals [35–38,63], it is well established in the zebrafish [64–66]. A drug screening performed without regeneration but in conditions boosting beta cell formation from the ducts in zebrafish larvae, pinpointed two inhibitors of CDK5, roscovitine and DRF, as enhancers of beta cell differentiation from ductal-associated progenitors [67]. Inhibition of *cdk5* has then been shown to stimulate regeneration after beta cell ablation. This finding has been validated in mouse embryonic pancreatic explants, in human iPSCs and in vivo in the PDL mouse model, though glycemia and glucose tolerance were not improved. To summarize, with one unique zebrafish model (the NTR-mediated ablation) it was possible to explore beta cell regeneration from different cellular origins and to identify pharmacological compounds and signaling pathways able to promote beta cell regeneration in mammals.

2.4. The Central Nervous System (CNS)

The central nervous system (CNS) is composed of two main cell types: neurons and glial cells. Neuronal cells are the basic functional units of the CNS capable of sensing and transmitting information via electrochemical pulses. The main roles of glial cells are to maintain homeostasis and to support and protect neurons. The earliest glial cells formed during embryonic development are the radial cells. These cells act as neuronal progenitors and thus give rise to neurons and intermediate progenitors. However, their neurogenic capacity decreases while they differentiate into star-shaped astrocytes. In the adult mammalian brain, the neurogenic capacity of the glia is restricted to few specific regions, called neurogenic niches, where the astroglia can still give rise to a few new neurons. Some of the astroglial cells in these neurogenic niches are considered as neural stem cells. Although it is possible to observe star-shaped cells in zebrafish, there are

no clearly defined astroglial cells in this species, and glial cells in zebrafish retain their radial identity through life. Thus these radial/astroglial cells have important neurogenic capacities. These cells can give rise to new neurons not only in the neurogenic niches but more broadly in the CNS [68], and they constitute the basis of regeneration in the CNS, i.e., the spinal cord, the brain and the retina.

2.4.1. The Spinal Cord

Spinal cord injury in mammals is followed by formation of a dense and heterogenous network composed of hypertrophic stellate astrocyte gliosis, fibroblasts and inflammatory immune cells, called the glial scar. This scar establishes a mechanical and impenetrable barrier impeding the regeneration of severed axons and repair of neuronal circuits [69–71]. In zebrafish, complete transection of the spinal cord (Table 1) results in tissue discontinuity and loss of glial and axonal connections. Then, glial cells proliferate and migrate to the injured area and acquire a bipolar and elongated morphology, forming a glial bridge. This allows, by 5 weeks, the regeneration of axons from viable neurons across the lesion site and their reconnection to the central canal, and fish recover their normal swimming behavior [72]. Notably, unlike mammals, this regeneration process is not accompanied by formation of a scar [72–74]. The formation of this bridge results from differential regulations compared to mammals allowing the presence of a permissive pro-regenerative microenvironment in zebrafish. One of these key regulations is a dynamic transient inflammatory response in zebrafish. Indeed, 2–3 days after spinal cord injury, the initially proinflammatory environment switches to an anti-inflammatory one with notably the presence of M2 macrophages, whereas, in mammals, pro-inflammatory macrophages persist at the wound site for a long time after injury [74].

A key regulator of the formation of the glial bridge is Fibroblast Growth Factor (FGF) signaling. Indeed, in zebrafish, the expression of several FGF ligands (*Fgf2*, *3*, *8*) and their downstream targets (*spry4*, *pea3* and *erm*) are increased at the injured site [72,75]. Using several models of gain or loss of function, Goldshmit and colleagues have examined the role of FGF during spinal cord regeneration in a series of studies from zebrafish to in vitro and in vivo mammalian models [42,72,76]. They first established in zebrafish that the FGF signaling is necessary for the formation of the glial bridge and for axonal regeneration. Next, they showed that in vitro treatment of primate primary astrocytes with recombinant human Fgf2 (hFgf2) recapitulated some of the characteristics of zebrafish glia cells during spinal cord regeneration such as acquisition of a bipolar elongated shape [72]. In mice, hFgf2 injection after spinal cord hemisection promotes formation of a glial bridge rather than a scar, allowing the growth of neurites and axonal regeneration through the lesion site. Mice injected with hFgf2 also displayed reduced inflammation, less macrophage and microglia activation and reduced leukocyte infiltration [42]. Moreover, these mice showed an improved functional recovery compared to control animals. These results are consistent with previous studies showing that acidic FGF and FGF2 are implicated in locomotor recovery in rodents [77,78]. Interestingly, similar observations were made with endogenous increase of FGF signaling in *spry4*$^{-/-}$ mutant mice, *spry4* being a feedback inhibitor of this pathway [76].

One promising cellular therapy following a spinal cord injury is the transplantation of stem cells directly into the injured site. Dental pulp cells (DPC) are composed of many types of stem cells and their transplantation induced an enhanced improvement of the functional recovery in a rodent spinal cord injury model compared to bone marrow-derived stromal cells transplantation [79]. These results are even more promising when human DPC are pretreated with FGF2 for several consecutive serial passages and then directly transplanted into the injury site with, notably, an improvement of axonal regeneration and of the locomotor recovery of the hind limbs by improving the survival rate of DPC at the lesion site [80].

Altogether, these results demonstrate a conserved pro-regenerative role for the FGF signaling in the formation of the glial bridge and, hence, in axonal regeneration in the spinal cord.

2.4.2. The Brain

Aging, brain injury or neurodegenerative conditions such as Alzheimer's disease (AD) and Parkinson's disease cause a major loss of neural cells. After brain injury, glial cells present in the neurogenic niches have the potential to proliferate. However, a reactive gliosis also occurs, producing a glial scar that inhibits this proliferation and hampers neurogenesis. The zebrafish brain has an incredible capacity of regeneration that can be partially explained by its numerous neurogenic niches and by the capacity of radial cells in the parenchyma to form new neurons. Depending on the type of injury and its localization and severity, different mechanisms of regeneration can be activated. This topic has been recently reviewed [68,81,82]. In this section, we focus on two different models of zebrafish brain injury/neurodegeneration that lead to mechanistic translation in mammalian models. In the zebrafish stab lesion assay (Table 1), the parenchyma of the telencephalon is surgically injured but leaves the neurogenic niches intact, allowing radial cells to proliferate, migrate and generate new neurons [83]. Kizil et al. 2012b showed that the expression of the zinc finger transcription factor Gata3 is induced in radial cells in response to injury [43] where its activity is necessary to properly activate their proliferation, neurogenesis and to promote migration of the newborn neurons, specifically in an injury context [43]. Human/mouse astrocytes fail to induce *Gata3* in response to injury. To mimic injury conditions, scratches have been performed in 2D and 3D cultures of human astrocytes. However, though *Gata3* delivery increased the number of neuronal progenitors, they could not achieve neurogenesis [84]. These results show that *Gata3* enhances the neurogenic potential of human astrocytes but is not sufficient.

To study the mechanisms of brain plasticity in response to neurodegeneration, a zebrafish model of Alzheimer's-disease-like (AD) has been developed [44] (Table 1). A hallmark of AD is the accumulation of β-amyloid Ab42 aggregates in the brain. Injection of Ab42 peptides coupled with a cell peptide transporter (transportan) into the zebrafish brain lead to neurodegeneration [44]. In contrast to mammals, neurodegeneration triggers radial/astroglia cell proliferation and neurogenesis in zebrafish. Transcriptomic profiling of this zebrafish model showed that *gata3* does not seem to be involved but pinpointed immune signaling pathways upregulated in response to Ab42-mediated neurodegeneration. This uncovered the specific upregulation of the anti-inflammatory interleukin-4 (IL4)/STAT6 pathway and its beneficial action on glial cell proliferation in the AD-like model. In contrast, this pathway is not activated in mammals. Furthermore, IL4 overexpression in healthy zebrafish could increase brain progenitor proliferation and neurogenesis [44]. Papadimitriou et al. 2018 later developed a 3D-culture model of human astrocytes and neural stem cells and examined the effect of IL4 as these cells naturally express the IL4 receptor. Interestingly, the inhibitory effect of Ab42 peptide on proliferation capacities could be rescued by treatment with IL4 [85]. However, in an in vivo mouse model of AD, the expression of the IL4 receptor could not be detected in astrocytes [45] and its artificial delivery led to astrocyte death [45]. The authors hypothesized that the mammalian brain evolved to avoid hyper-proliferation by establishing a non-permissive environment for cells expressing the IL4 receptor.

To summarize, thanks to two different zebrafish models of brain regeneration, mediators of brain plasticity with favorable potential in mammalian models were identified though the complexity of the mammalian brain, which has evolved rigid barriers to repress regeneration in order to, presumably, avoid tumorigenesis.

2.4.3. The Retina

Photoreceptor death characterizes retinal degeneration and eye diseases like diabetic retinopathy, retinitis pigmentosa or glaucoma, leading to loss of vision and even to com-

plete and untreatable blindness. A promising strategy to restore sight would be to activate endogenous regeneration of photoreceptors within the retina. Exploiting regenerative capacities of amphibians and fish, researchers revealed several cellular sources to regrow new photoreceptors. Among them, we can cite the retinal pigment epithelium (in amphibians but not in fish), the ciliary margin (the region which contains the retinal stem cells in fish and amphibians) and the Müller glia [86]. The Müller cells (MCs) constitute the major glial cells spread through the entire retina and are conserved from fish to mammals. Their function is to maintain retinal homeostasis and structure. During retinal development, the MCs are the latest cells to arise from retinal multipotent progenitors. MCs share molecular signatures with late retinal progenitor cells [87], leading to the hypothesis that MCs could be progenitors with a glial function. In response to retinal injury, MCs undergo reactive gliosis, i.e., change in morphology, dedifferentiation and proliferation [88,89]. However, this proliferation is rapidly inhibited in mammals, resulting in scar formation and preventing regeneration. On the other hand, zebrafish MCs can differentiate into new retinal neurons after replication [47] and restore vision. Assuming that this regenerative capacity is present in mammals but dormant, researchers focused on key factors specifically expressed or induced in zebrafish but not in mammals. The most tangible example is the case of achaete scute-like family bHLH transcription factor 1a (ASCL1a). In response to surgical injury, *ascl1a* expression is induced in the zebrafish retina and is necessary for MC proliferation and thus regeneration [90], while *Ascl1* is not expressed in the mammalian retina. In order to test if *Ascl1* expression can stimulate the neurogenic potential of mammalian MCs, *Ascl1* has been overexpressed in ex vivo explants of mice MCs, which enabled their dedifferentiation into retinal progenitors [91]. Furthermore, while *Ascl1* expression driven in vivo by transgenesis in mice retina did not affect the uninjured retina, it could activate regeneration after injury induced by N-methyl D-aspartate (NMDA) or excessive light [92] (Table 1). However, only juvenile mice were able to generate new retinal neurons, showing that *Ascl1* is important but not sufficient to induce retinal regeneration in adult mammals [92]. Epigenetic regulations were proposed to underlie the age-dependent regenerative capacities as the *Ascl1* target genes are accessible in juvenile MCs but less accessible in adult MCs [92]. Supporting this hypothesis, *Ascl1* overexpression in MCs combined with an eraser of epigenetic marks, the histone deacetylase inhibitor trichostatin-A, could stimulate photoreceptor regeneration after injury in adult mice [48]. Importantly, the regenerated cells responded to light [48], demonstrating functional recovery. Nevertheless, this mechanism of regeneration did not involve MC proliferation [48] and rather suggested direct transdifferentiation of MCs into retinal neurons, which could possibly lead to MC depletion. This could be overcome by the combined overexpression of *Ascl1* and *Lin28*. Lin28a is an RNA binding protein expressed in response to retinal injury in zebrafish [93] but not in mice [46]. *lin28a* expression is also necessary for retinal regeneration in zebrafish [93]. Combined *ascl1a* and *lin28a* overexpression mimics a regenerative response in the zebrafish retina without injury [46]. While *ascl1a* or *lin28a* expression alone does not impact the retinal phenotype, their combination induces MC proliferation and differentiation into several types of retinal neurons [46]. In the NMDA mice model of retinal injury, *Ascl1* and *Lin28* co-overexpression enhances MC proliferation in young mice [46] compared to *Ascl1* overexpression alone [92]. These studies taking advantage of the regenerative capacities of zebrafish revealed that Ascl1 and Lin28 are pieces to unlock the regeneration potential of mammalian MCs.

3. Conclusions

A question often asked to biomedical researchers using the zebrafish as a model organism is how a fish can help patients. Many studies point out that the zebrafish anatomy and physiology share many features with mammals and this is exemplified by the rapid expansion of zebrafish disease models. This review seeks to bring an answer to how zebrafish could benefit regenerative medicine by emphasizing the transposable potential of the zebrafish regenerative abilities. All the studies highlighted here share a common workflow

(Figure 1) such as drug and genetic screenings to enable the identification of regulators of regeneration first in zebrafish [28,31–33,43]. An important trait of these studies is the versatility of a few zebrafish injury models that allow us to tackle different regenerative processes. It is for example the case of the pancreas and the liver where the zebrafish NTR model is almost exclusively employed in contrast to the various mice models of injury that are used to cover regeneration from different cellular sources (Table 1) [31,33,67]. The NTR model is also exploited to study regeneration in the heart and the brain and is continuously improving [94–97]. Although this relatively simple model provides valuable clues about regeneration, zebrafish models recapitulating more closely the disease will determine how zebrafish regenerate in such settings, therefore further increasing our understanding of regeneration and the success of transposition to mammals. A critical step of the workflow is to choose the most relevant mammalian model of injury to further explore the zebrafish discoveries (Table 1). Another key aspect is to ensure that modulating the mechanisms identified in zebrafish can improve the function of regenerated cells in vivo in mammal models.

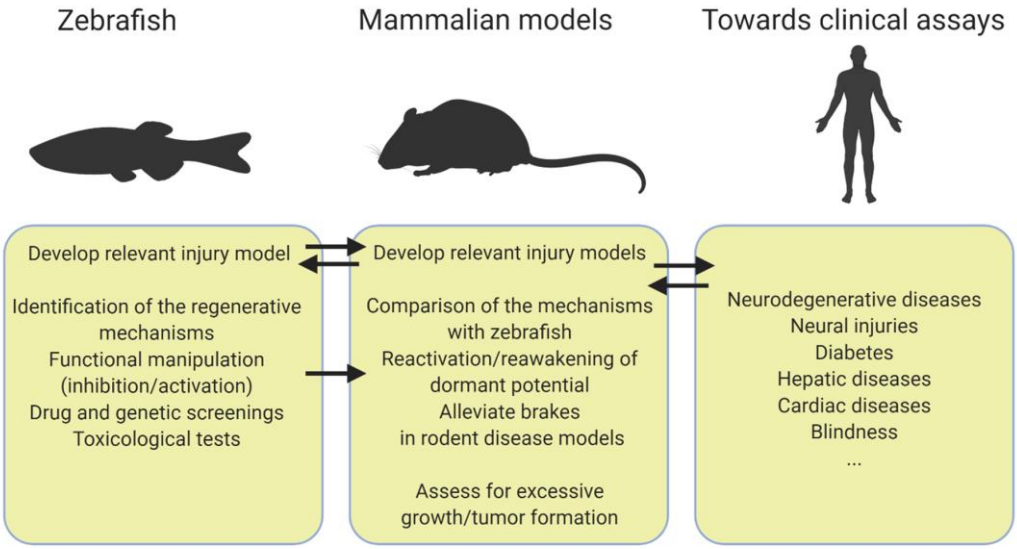


Figure 1. Workflow from zebrafish to mammals. Created with Biorender.com.

Altogether, these studies support the idea that regenerative mechanisms are relatively well conserved even in species with low regenerative capacities, but they are repressed. It can be assumed that mammals have evolved in a way to safeguard against hyper-proliferation to prevent tissue overgrowth and tumorigenesis while maintaining functionality. In this respect, polyploidization is a common feature of many mammalian tissues during aging, homeostasis and cancer. Polyploidy has also emerged to play a role in heart regeneration [18]. Similar to cardiomyocytes, many cell types in mammals such as hepatocytes also become polyploid after birth. Although the significance for liver regeneration is poorly understood, it is likely to play a role [98,99]. It would be interesting to assess the effect of polyploidy in hepatocyte regeneration in zebrafish.

Two other major types of obstacles involve the immune system/inflammation and epigenetic regulations (Figure 2). Repressing a prolonged inflammatory response improves regenerative responses in the mammalian brain or spinal cord. The immune system/inflammatory response differs between organisms able to regenerate and those which cannot [100]. In organisms unable to regenerate, the immune response is generally sustained. On the other hand, the zebrafish immune response is transient, as observed in the

heart [101,102] and the spinal cord [74]. This environment favors a proper regenerative response without scarring. Another obstacle to regeneration is the epigenetic repression or the loss of enhancers of pro-regenerative genes [103] as it is the case in the heart, the liver and the retina (Figure 2).

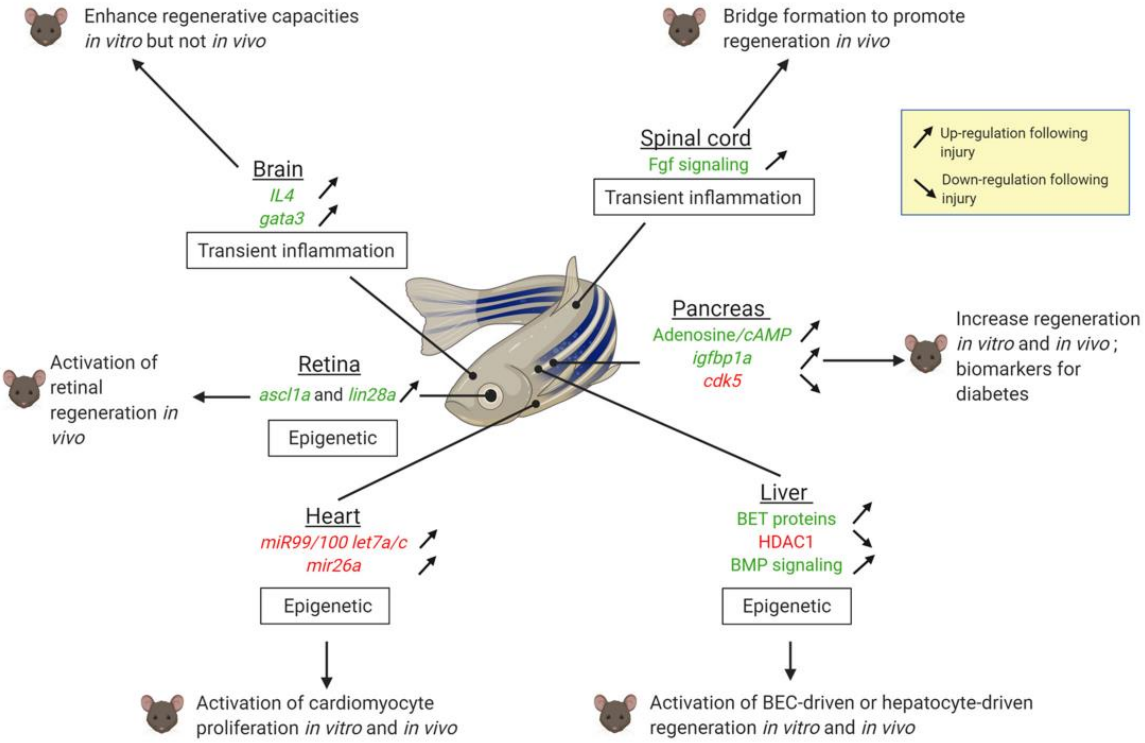


Figure 2. Summary of regenerative mechanisms identified in zebrafish which are able to awake the regenerative potential in mammals in the brain, the spine, the retina, the pancreas, the liver and the heart. The up-headed (vs. back-headed) arrows mean that the expression is upregulated (vs. downregulated) in zebrafish after injury. Factors highlighted in green exert positive effect in regeneration, those in red impair regeneration. Created with Biorender.com.

In addition to help decipher the mechanisms of regeneration, the studies performed in zebrafish also illustrate its great amenability to preclinical drug testing. To promote tissue repair, transplantation-free (or cell-free) therapies rely on administration of soluble factors, vesicles or microRNA that can be first tested in zebrafish for their efficiency and toxicity.

Even if the path is still long before we are able to overcome these obstacles and to offer beneficial treatments to patients, the zebrafish is a powerful model to help elucidate universal mechanisms of regeneration and to give clues about how and why more complex vertebrates erected barriers dampening this potential. The versatility of zebrafish enables the development of innovative models of regeneration and of novel technologies such as scRNAseq associated with CRISPR/Cas9 barcode editing for fine cell lineage tracing [104] and a growing number of genetic and metabolic reporter tools enabling non-toxic and non-invasive in vivo imaging to follow organ reconstruction and functional recovery. Associated with its regenerative capacity, all these assets confer the zebrafish with undeniable advantages over other preclinical models that will certainly accelerate research in regenerative medicine.

Funding: This work was supported by the National Belgian Funds for Scientific Research FNRS-FRS (I.M.), the FRIA PhD fellowship program (L.M.) and the Belgian FNRS under EOS Project No. 30826052 (M.A.D.).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Acknowledgments: We thank Bernard Peers, Marianne Voz and Jérémie Zappia for critically reading the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

AD	Alzheimer's disease
APAP	N-acetyl-p-aminophenol
ASCL1	Achaete Scute-Like family bHLH transcription factor 1
BECs	Biliary Epithelial Cells
BET	Bromodomain and Extra-Terminal proteins
BMP	Bone Morphogenetic Protein
CDE	Choline-Deficient Ethionine-supplemented
CNS	Central Nervous System
DTA	Diphtheria Toxin Subunit A
DTR	Diphtheria Toxin Receptor
FGF	Fibroblast Growth Factor
HDAC1	Histone Deacetylase 1
Igf	insulin-like growth factor
igfbp1	Igf binding-protein 1
IL4	interleukin-4
LEN	lepb-linked enhancer
LPCs	Liver Progenitors Cells
MCs	Müller cells
miRNAs	micro RNAs
NECA	50-N-ethylcarboxamidoadenosine
NMDA	N-methyl D-Aspartate
NTR	Nitroreductase
PDL	Pancreatic Duct Ligation
PIAA	(E)-3-(3-phenylbenzo[c]isoxazol-5-yl) acrylic acid
STZ	StrepToZotocin
T1D	Type 1 Diabetes
T2D	Type 2 Diabetes

References

1. Elliott, S.A.; Sánchez Alvarado, A. Planarians and the History of Animal Regeneration: Paradigm Shifts and Key Concepts in Biology. In *Planarian Regeneration. Methods in Molecular Biology*; Humana Press: New York, NY, USA, 2018; Volume 1774, ISBN 9781493978021.
2. Maden, M. The evolution of regeneration—Where does that leave mammals? *Int. J. Dev. Biol.* **2018**, *62*, 369–372. [PubMed]
3. Gemberling, M.; Bailey, T.J.; Hyde, D.R.; Poss, K.D. The zebrafish as a model for complex tissue regeneration. *Trends Genet.* **2013**, *29*, 611–620. [CrossRef] [PubMed]
4. Kinth, P.; Mahesh, G.; Panwar, Y. Mapping of zebrafish research: A global outlook. *Zebrafish* **2013**, *10*, 510–517. [CrossRef] [PubMed]
5. Mehta, A.S.; Singh, A. Insights into regeneration tool box: An animal model approach. *Dev. Biol.* **2019**, *453*, 111–129. [CrossRef]
6. Marques, I.J.; Lupi, E.; Mercader, N. Model systems for regeneration: Zebrafish. *Development* **2019**, *146*, dev167692. [CrossRef]
7. Howe, K.; Clark, M.D.; Torroja, C.F.; Torrance, J.; Muffato, M.; Collins, J.E.; Humphray, S.; Mclaren, K.; Matthews, L.; Mclaren, S.; et al. The zebrafish reference genome sequence and its relationship to the human genome. *Nature* **2014**, *496*, 498–503. [CrossRef]
8. Roth, G.A.; Johnson, C.; Abajobir, A.; Abd-Allah, F.; Abera, S.F.; Abyu, G.; Ahmed, M.; Aksut, B.; Alam, T.; Alam, K.; et al. Global, Regional, and National Burden of Cardiovascular Diseases for 10 Causes, 1990 to 2015. *J. Am. Coll. Cardiol.* **2017**, *70*, 1–25. [CrossRef]

9. Bergmann, O.; Bhardwaj, R.D.; Bernard, S.; Zdunek, S.; Barnabé-Heider, F.; Walsh, S.; Zupicich, J.; Alkass, K.; Buchholz, B.A.; Druid, H.; et al. Evidence for cardiomyocyte renewal in humans. *Science* **2009**, *324*, 98–102. [CrossRef]
10. Hashimoto, H.; Olson, E.N.; Bassel-Duby, R. Therapeutic approaches for cardiac regeneration and repair. *Nat. Rev. Cardiol.* **2018**, *15*, 585–600. [CrossRef]
11. Poss, K.D.; Wilson, L.G.; Keating, M.T. Heart Regeneration in Zebrafish. *Science* **2002**, *298*, 2188–2190. [CrossRef]
12. Raya, A.; Koth, C.M.; Büscher, D.; Kawakami, Y.; Itoh, T.; Raya, R.M.; Sternik, G.; Tsai, H.-J.; Rodríguez-Esteban, C.; Izpisúa-Belmonte, J.C. Activation of Notch signaling pathway precedes heart regeneration in zebrafish. *Proc. Natl. Acad. Sci. USA* **2003**, *100* (Suppl. 1), 11889–11895. [CrossRef] [PubMed]
13. Chablais, F.; Veit, J.; Rainer, G.; Jaźwińska, A. The zebrafish heart regenerates after cryoinjury-induced myocardial infarction. *BMC Dev. Biol.* **2011**, *11*, 21. [CrossRef] [PubMed]
14. González-Rosa, J.M.; Martín, V.; Peralta, M.; Torres, M.; Mercader, N. Extensive scar formation and regression during heart regeneration after cryoinjury in zebrafish. *Development* **2011**, *138*, 1663–1674. [CrossRef] [PubMed]
15. Rubin, N.; Harrison, M.R.; Krainock, M.; Kim, R.; Lien, C.-L. Recent advancements in understanding endogenous heart regeneration-insights from adult zebrafish and neonatal mice. *Semin. Cell Dev. Biol.* **2016**, *58*, 34–40. [CrossRef]
16. Porrello, E.R.; Mahmoud, A.I.; Simpson, E.; Hill, J.A.; Richardson, J.A.; Olson, E.N.; Sadek, H.A. Transient regenerative potential of the neonatal mouse heart. *Science* **2011**, *331*, 1078–1080. [CrossRef]
17. Jopling, C.; Sleep, E.; Raya, M.; Martí, M.; Raya, A.; Izpisúa Belmonte, J.C. Zebrafish heart regeneration occurs by cardiomyocyte dedifferentiation and proliferation. *Nature* **2010**, *464*, 606–609. [CrossRef]
18. González-Rosa, J.M.; Sharpe, M.; Field, D.; Soonpaa, M.H.; Field, L.J.; Burns, C.E.; Burns, C.G. Myocardial Polyploidization Creates a Barrier to Heart Regeneration in Zebrafish. *Dev. Cell* **2018**, *44*, 433–446.e7. [CrossRef]
19. Li, F.; Wang, X.; Capasso, J.M.; Gerdes, A.M. Rapid Transition of Cardiac Myocytes from Hyperplasia to Hypertrophy During Postnatal Development. *J. Mol. Cell. Cardiol.* **1996**, *28*, 1737–1746. [CrossRef]
20. Soonpaa, M.H.; Kim, K.K.; Pajak, L.; Franklin, M.; Field, L.J. Cardiomyocyte DNA synthesis and binucleation during murine development. *Am. J. Physiol.* **1996**, *271*, H2183–H2189. [CrossRef]
21. Mollova, M.; Bersell, K.; Walsh, S.; Savla, J.; Das, L.T.; Park, S.-Y.; Silberstein, L.E.; Dos Remedios, C.G.; Graham, D.; Colan, S.; et al. Cardiomyocyte proliferation contributes to heart growth in young humans. *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 1446–1451. [CrossRef]
22. Vivien, C.J.; Hudson, J.E.; Porrello, E.R. Evolution, comparative biology and ontogeny of vertebrate heart regeneration. *NPJ Regen. Med.* **2016**, *1*, 16012. [CrossRef]
23. Green, R.A.; Paluch, E.; Oegema, K. Cytokinesis in Animal Cells. *Annu. Rev. Cell Dev. Biol.* **2012**, *28*, 29–58. [CrossRef]
24. Porrello, E.R.; Mahmoud, A.I.; Simpson, E.; Johnson, B.A.; Grinsfelder, D.; Canseco, D.; Mammen, P.P.; Rothermel, B.A.; Olson, E.N.; Sadek, H.A. Regulation of neonatal and adult mammalian heart regeneration by the miR-15 family. *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 187–192. [CrossRef]
25. Aguirre, A.; Montserrat, N.; Zacchigna, S.; Nivet, E.; Hishida, T.; Krause, M.N.; Kurian, L.; Ocampo, A.; Vazquez-Ferrer, E.; Rodriguez-Esteban, C.; et al. In vivo activation of a conserved microRNA program induces robust mammalian heart regeneration. *Cell Stem Cell* **2014**, *15*, 589–604. [CrossRef]
26. Russell, J.O.; Ko, S.; Saggi, H.S.; Singh, S.; Poddar, M.; Shin, D.; Monga, S.P. Bromodomain and Extraterminal (BET) Proteins Regulate Hepatocyte Proliferation in Hepatocyte-Driven Liver Regeneration. *Am. J. Pathol.* **2018**, *188*, 1389–1405. [CrossRef]
27. Choi, T.; Khaliq, M.; Tsurusaki, S.; Ninov, N.; Stainier, D.; Tanaka, M.; Shin, D. Bmp Signaling Governs Biliary-Driven Liver Regeneration in Zebrafish via Tbx2b and Id2a. *Hepatology* **2017**, *66*, 1616–1630. [CrossRef]
28. Ko, S.; Choi, T.; Russell, J.O.; So, J.; Shin, D. Bromodomain and extraterminal (BET) proteins regulate biliary-driven liver regeneration. *J. Hepatol.* **2016**, *64*, 316–325. [CrossRef]
29. Ko, S.O.; Russell, J.; Tian, J.; Gao, C.; Kobayashi, M.; Feng, R.; Yuan, X.; Shao, C.; Ding, H.; Poddar, M.; et al. Hdac1 Regulates Differentiation of Bipotent Liver Progenitor Cells During Regeneration via Sox9b and Cdk8. *Gastroenterology* **2019**, *156*, 187–202.e4. [CrossRef]
30. Lu, W.Y.; Bird, T.G.; Boulter, L.; Tsuchiya, A.; Cole, A.M.; Hay, T.; Guest, R.V.; Wojtacha, D.; Man, T.Y.; Mackinnon, A.; et al. Hepatic progenitor cells of biliary origin with liver repopulation capacity. *Nat. Cell Biol.* **2015**, *17*, 973–983. [CrossRef]
31. Andersson, O.; Adams, B.A.; Yoo, D.; Ellis, G.C.; Gut, P.; Anderson, R.M.; German, M.S.; Stainier, D.Y.R. Adenosine Signaling Promotes Regeneration of Pancreatic b Cells In Vivo. *Cell Metab.* **2012**, 885–894. [CrossRef]
32. Xu, J.; Jia, Y.F.; Tapadar, S.; Weaver, J.D.; Raji, I.O.; Pithadia, D.J.; Javeed, N.; García, A.J.; Choi, D.S.; Matveyenko, A.V.; et al. Inhibition of TBK1/IKKε Promotes Regeneration of Pancreatic β-cells. *Sci. Rep.* **2018**, *8*, 1–14. [CrossRef]
33. Lu, J.; Liu, K.; Schulz, N.; Karampelias, C.; Charbord, J.; Hilding, A.; Rautio, L.; Bertolino, P.; Östenson, C.; Brismar, K.; et al. IGFBP 1 increases β-cell regeneration by promoting α- to β-cell transdifferentiation. *EMBO J.* **2016**, *35*, 2026–2044. [CrossRef]
34. Ye, L.; Robertson, M.A.; Hesselson, D.; Stainier, D.Y.R.; Anderson, R.M. Glucagon is essential for alpha cell transdifferentiation and beta cell neogenesis. *Development* **2015**, *142*, 1407–1417. [CrossRef]
35. Kopp, J.L.; Dubois, C.L.; Schaffer, A.E.; Hao, E.; Shih, H.P.; Seymour, P.A.; Ma, J.; Sander, M. Sox9+ ductal cells are multipotent progenitors throughout development but do not produce new endocrine cells in the normal or injured adult pancreas. *Development* **2011**, *138*, 653–665. [CrossRef]

36. Solar, M.; Cardalda, C.; Houbracken, I.; Martín, M.; Maestro, M.A.; De Medts, N.; Xu, X.; Grau, V.; Heimberg, H.; Bouwens, L.; et al. Pancreatic Exocrine Duct Cells Give Rise to Insulin-Producing β Cells during Embryogenesis but Not after Birth. *Dev. Cell* **2009**, *17*, 849–860. [CrossRef]
37. Xu, X.; D'Hoker, J.; Stangé, G.; Bonné, S.; De Leu, N.; Xiao, X.; Van De Casteele, M.; Mellitzer, G.; Ling, Z.; Pipeleers, D.; et al. β Cells Can Be Generated from Endogenous Progenitors in Injured Adult Mouse Pancreas. *Cell* **2008**, *132*, 197–207. [CrossRef]
38. Al-Hasani, K.; Pfeifer, A.; Courtney, M.; Ben-Othman, N.; Gjernes, E.; Vieira, A.; Druelle, N.; Avolio, F.; Ravassard, P.; Leuckx, G.; et al. Adult duct-lining cells can reprogram into β-like cells able to counter repeated cycles of toxin-induced diabetes. *Dev. Cell* **2013**, *26*, 86–100. [CrossRef]
39. Chera, S. Diabetes Recovery By Age-Dependent Conversion of Pancreatic δ-Cells. *Nature* **2014**, *514*, 503–507. [CrossRef]
40. Thorel, F.; Nepote, V.; Avril, I.; Kohno, K.; Desgraz, R.; Chera, S.; Herrera, P.L.; Népote, V.; Kohno, K.; Desgraz, R. Conversion of Adult Pancreatic alpha-cells to Beta-cells after Extreme Beta Cell Loss. *Nature* **2010**, *464*, 1149–1154. [CrossRef]
41. Becker, T.; Wullimann, M.F.; Becker, C.G.; Bernhardt, R.R.; Schachner, M. Axonal regrowth after spinal cord transection in adult zebrafish. *J. Comp. Neurol.* **1997**, *377*, 577–595. [CrossRef]
42. Goldshmit, Y.; Frisca, F.; Pinto, A.R.; Pébay, A.; Tang, J.-K.K.Y.; Siegel, A.L.; Kaslin, J.; Currie, P.D. Fgf2 improves functional recovery-decreasing gliosis and increasing radial glia and neural progenitor cells after spinal cord injury. *Brain Behav.* **2014**, *4*, 187–200. [CrossRef] [PubMed]
43. Kizil, C.; Kyritsis, N.; Dudczig, S.; Kroehne, V.; Freudenreich, D.; Kaslin, J.; Brand, M. Regenerative Neurogenesis from Neural Progenitor Cells Requires Injury-Induced Expression of Gata3. *Dev. Cell* **2012**, *23*, 1230–1237. [CrossRef] [PubMed]
44. Bhattarai, P.; Thomas, A.K.; Cosacak, M.I.; Papadimitriou, C.; Mashkaryan, V.; Froc, C.; Reinhardt, S.; Kurth, T.; Dahl, A.; Zhang, Y.; et al. IL4/STAT6 Signaling Activates Neural Stem Cell Proliferation and Neurogenesis upon Amyloid-β42 Aggregation in Adult Zebrafish Brain. *Cell Rep.* **2016**, *17*, 941–948. [CrossRef] [PubMed]
45. Mashkaryan, V.; Siddiqui, T.; Popova, S.; Cosacak, M.I.; Bhattarai, P.; Brandt, K.; Govindarajan, N.; Petzold, A.; Reinhardt, S.; Dahl, A.; et al. Type 1 Interleukin-4 Signaling Obliterates Mouse Astroglia in vivo but Not in vitro. *Front. Cell Dev. Biol.* **2020**, *8*, 114. [CrossRef] [PubMed]
46. Elsaeidi, F.; Macpherson, P.; Mills, E.A.; Jui, J.; Flannery, J.G.; Goldman, D. Notch suppression collaborates with Ascl1 and Lin28 to unleash a regenerative response in fish retina, but not in mice. *J. Neurosci.* **2018**, *38*, 2246–2261. [CrossRef] [PubMed]
47. Fausett, B.V.; Goldman, D. A role for alpha1 tubulin-expressing Müller glia in regeneration of the injured zebrafish retina. *J. Neurosci.* **2006**, *26*, 6303–6313. [CrossRef]
48. Jorstad, N.L.; Wilken, M.S.; Grimes, W.N.; Wohl, S.G.; Vandenbosch, L.S.; Yoshimatsu, T.; Wong, R.O.; Rieke, F.; Reh, T.A. Stimulation of functional neuronal regeneration from Müller glia in adult mice. *Nature* **2017**, *548*, 103–107. [CrossRef]
49. Hodgkinson, C.P.; Dzau, V.J. Conserved MicroRNA Program as Key to Mammalian Cardiac Regeneration. *Circ. Res.* **2015**, *116*, 1109–1111. [CrossRef]
50. Crippa, S.; Nemir, M.; Ounzain, S.; Ibberson, M.; Berthonneche, C.; Sarre, A.; Boisset, G.; Maison, D.; Harshman, K.; Xenarios, I.; et al. Comparative transcriptome profiling of the injured zebrafish and mouse hearts identifies miRNA-dependent repair pathways. *Cardiovasc. Res.* **2016**, *110*, 73–84. [CrossRef]
51. Kang, J.; Hu, J.; Karra, R.; Dickson, A.L.; Tornini, V.A.; Nachtrab, G.; Gemberling, M.; Goldman, J.A.; Black, B.L.; Poss, K.D. Modulation of tissue repair by regeneration enhancer elements. *Nature* **2016**, *532*, 201–206. [CrossRef]
52. Bersell, K.; Arab, S.; Haring, B.; Kühn, B. Neuregulin1/ErbB4 signaling induces cardiomyocyte proliferation and repair of heart injury. *Cell* **2009**, *138*, 257–270. [CrossRef] [PubMed]
53. Begeman, I.J.; Kang, J. Transcriptional Programs and Regeneration Enhancers Underlying Heart Regeneration. *J. Cardiovasc. Dev. Dis.* **2018**, *6*, 2. [CrossRef] [PubMed]
54. Xiao, J.C.; Ruck, P.; Adam, A.; Wang, T.X.; Kaiserling, E. Small epithelial cells in human liver cirrhosis exhibit features of hepatic stem-like cells: Immunohistochemical, electron microscopic and immunoelectron microscopic findings. *Histopathology* **2003**, *42*, 141–149. [CrossRef] [PubMed]
55. So, J.; Kim, A.; Lee, S.H.; Shin, D. Liver progenitor cell-driven liver regeneration. *Exp. Mol. Med.* **2020**, *52*, 1230–1238. [CrossRef]
56. Ko, S.; Russell, J.O.; Molina, L.M.; Monga, S.P. Liver Progenitors and Adult Cell Plasticity in Hepatic Injury and Repair: Knowns and Unknowns. *Annu. Rev. Pathol. Mech. Dis.* **2020**, *15*, 23–50. [CrossRef]
57. Xia, J.; Zhou, Y.; Ji, H.; Wang, Y.; Wu, Q.; Bao, J.; Ye, F.; Shi, Y.; Bu, H. Loss of histone deacetylases 1 and 2 in hepatocytes impairs murine liver regeneration through Ki67 depletion. *Hepatology* **2013**, *58*, 2089–2098. [CrossRef]
58. Dor, Y.; Brown, J.; Martinez, O.I.; Melton, D.A. Adult pancreatic beta-cells are formed by self-duplication rather than stem-cell differentiation. *Nature* **2004**, *429*, 41–46. [CrossRef]
59. Pan, F.C.; Bankaitis, E.D.; Boyer, D.; Xu, X.; Van de Casteele, M.; Magnuson, M.A.; Heimberg, H.; Wright, C.V.E. Spatiotemporal patterns of multipotentiality in Ptf1aexpressing cells during pancreas organogenesis and injuryinduced facultative restoration. *Development* **2013**, *140*, 751–764. [CrossRef]
60. Tsuji, N.; Ninov, N.; Delawary, M.; Osman, S.; Roh, A.S.; Gut, P.; Stainier, D.Y.R. Whole organism high content screening identifies stimulators of pancreatic beta-cell proliferation. *PLoS ONE* **2014**, *9*, e104112. [CrossRef]
61. Basile, G.; Kulkarni, R.N.; Morgan, N.G. How, When, and Where Do Human β-Cells Regenerate? *Curr. Diabetes Rep.* **2019**, *19*, 48. [CrossRef]

62. Sharma, A.; Purohit, S.; Sharma, S.; Bai, S.; Zhi, W.; Ponny, S.R.; Hopkins, D.; Steed, L.; Bode, B.; Anderson, S.W.; et al. IGF-binding proteins in type-1 diabetes are more severely altered in the presence of complications. *Front. Endocrinol. (Lausanne)* **2016**, *7*, 2. [CrossRef] [PubMed]
63. Inada, A.; Nienaber, C.; Katsuta, H.; Fujitani, Y.; Levine, J.; Morita, R.; Sharma, A.; Bonner-Weir, S. Carbonic anhydrase II-positive pancreatic cells are progenitors for both endocrine and exocrine pancreas after birth. *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 19915–19919. [CrossRef] [PubMed]
64. Ninov, N.; Hesselson, D.; Gut, P.; Zhou, A.; Fidelin, K.; Stainier, D.Y.R. Metabolic regulation of cellular plasticity in the pancreas. *Curr. Biol.* **2013**, *23*, 1242–1250. [CrossRef] [PubMed]
65. Ghaye, A.P.; Bergemann, D.; Tarifeño-Saldivia, E.; Flasse, L.C.; Von Berg, V.; Peers, B.; Voz, M.L.; Manfroid, I. Progenitor potential of nkx6.1-expressing cells throughout zebrafish life and during beta cell regeneration. *BMC Biol.* **2015**, *13*, 70. [CrossRef]
66. Delaspre, F.; Beer, R.L.; Rovira, M.; Huang, W.; Wang, G.; Gee, S.; Del Carmen Vitery, M.; Wheelan, S.J.; Parsons, M.J. Centroacinar cells are progenitors that contribute to endocrine pancreas regeneration. *Diabetes* **2015**, *64*, 3499–3509. [CrossRef]
67. Liu, K.C.; Leuckx, G.; Sakano, D.; Seymour, P.A.; Mattsson, C.L.; Rautio, L.; Staels, W.; Verdonck, Y.; Serup, P.; Kume, S.; et al. Inhibition of Cdk5 promotes β-cell differentiation from ductal progenitors. *Diabetes* **2018**, *67*, 58–70. [CrossRef]
68. Jurisch-Yaksi, N.; Yaksi, E.; Kizil, C. Radial glia in the zebrafish brain: Functional, structural, and physiological comparison with the mammalian glia. *Glia* **2020**, *68*, 2451–2470. [CrossRef]
69. Asher, R.A.; Morgenstern, D.A.; Fidler, P.S.; Adcock, K.H.; Oohira, A.; Braistead, J.E.; Levine, J.M.; Margolis, R.U.; Rogers, J.H.; Fawcett, J.W. Neurocan is upregulated in injured brain and in cytokine-treated astrocytes. *J. Neurosci.* **2000**, *20*, 2427–2438. [CrossRef]
70. Stichel, C.C.; Hermanns, S.; Luhmann, H.J.; Lausberg, F.; Niermann, H.; D'Urso, D.; Servos, G.; Hartwig, H.G.; Müller, H.W. Inhibition of collagen IV deposition promotes regeneration of injured CNS axons. *Eur. J. Neurosci.* **1999**, *11*, 632–646. [CrossRef]
71. McKeon, R.J.; Schreiber, R.C.; Rudge, J.S.; Silver, J. Reduction of neurite outgrowth in a model of glial scarring following CNS injury is correlated with the expression of inhibitory molecules on reactive astrocytes. *J. Neurosci.* **1991**, *11*, 3398–3411. [CrossRef]
72. Goldshmit, Y.; Sztal, T.E.; Jusuf, P.R.; Hall, T.E.; Nguyen-Chi, M.; Currie, P.D. Fgf-dependent glial cell bridges facilitate spinal cord regeneration in zebrafish. *J. Neurosci.* **2012**, *32*, 7477–7492. [CrossRef] [PubMed]
73. Cigliola, V.; Becker, C.J.; Poss, K.D. Building bridges, not walls: Spinal cord regeneration in zebrafish. *Dis. Models Mech.* **2020**, *13*, 5. [CrossRef] [PubMed]
74. Ghosh, S.; Hui, S.P. Axonal regeneration in zebrafish spinal cord. *Regeneration* **2018**, *5*, 43–60. [CrossRef] [PubMed]
75. Reimer, M.M.; Kuscha, V.; Wyatt, C.; Sörensen, I.; Frank, R.E.; Knüwer, M.; Becker, T.; Becker, C.G. Sonic hedgehog is a polarized signal for motor neuron regeneration in adult zebrafish. *J. Neurosci.* **2009**, *29*, 15073–15082. [CrossRef]
76. Goldshmit, Y.; Frisca, F.; Kaslin, J.; Pinto, A.R.; Tang, J.-K.K.Y.; Pébay, A.; Pinkas-Kramarski, R.; Currie, P.D. Decreased anti-regenerative effects after spinal cord injury in spry4-/- mice. *Neuroscience* **2015**, *287*, 104–112. [CrossRef]
77. Tsai, M.-C.; Shen, L.-F.; Kuo, H.-S.; Cheng, H.; Chak, K.-F. Involvement of acidic fibroblast growth factor in spinal cord injury repair processes revealed by a proteomics approach. *Mol. Cell. Proteom.* **2008**, *7*, 1668–1687. [CrossRef]
78. Kasai, M.; Jikoh, T.; Fukumitsu, H.; Furukawa, S. FGF-2-responsive and spinal cord-resident cells improve locomotor function after spinal cord injury. *J. Neurotrauma* **2014**, *31*, 1584–1598. [CrossRef]
79. Sakai, K.; Yamamoto, A.; Matsubara, K.; Nakamura, S.; Naruse, M.; Yamagata, M.; Sakamoto, K.; Tauchi, R.; Wakao, N.; Imagama, S.; et al. Human dental pulp-derived stem cells promote locomotor recovery after complete transection of the rat spinal cord by multiple neuro-regenerative mechanisms. *J. Clin. Investig.* **2012**, *122*, 80–90. [CrossRef]
80. Nagashima, K.; Miwa, T.; Soumiya, H.; Ushiro, D.; Takeda-Kawaguchi, T.; Tamaoki, N.; Ishiguro, S.; Sato, Y.; Miyamoto, K.; Ohno, T.; et al. Priming with FGF2 stimulates human dental pulp cells to promote axonal regeneration and locomotor function recovery after spinal cord injury. *Sci. Rep.* **2017**, *7*, 13500. [CrossRef]
81. Zambusi, A.; Ninkovic, J. Regeneration of the central nervous system-principles from brain regeneration in adult zebrafish. *World J. Stem Cells* **2020**, *12*, 8–24. [CrossRef]
82. Lange, C.; Brand, M. Vertebrate brain regeneration—A community effort of fate-restricted precursor cell types. *Curr. Opin. Genet. Dev.* **2020**, *64*, 101–108. [CrossRef] [PubMed]
83. Kroehne, V.; Freudenreich, D.; Hans, S.; Kaslin, J.; Brand, M. Regeneration of the adult zebrafish brain from neurogenic radial glia-type progenitors. *Development* **2011**, *138*, 4831–4841. [CrossRef] [PubMed]
84. Celikkaya, H.; Cosacak, M.I.; Papadimitriou, C.; Popova, S.; Bhattarai, P.; Biswas, S.N.; Siddiqui, T.; Wistorf, S.; Nevado-Alcalde, I.; Naumann, L.; et al. GATA3 promotes the neural progenitor state but not neurogenesis in 3D traumatic injury model of primary human cortical astrocytes. *Front. Cell. Neurosci.* **2019**, *13*, 23. [CrossRef] [PubMed]
85. Papadimitriou, C.; Celikkaya, H.; Cosacak, M.I.; Mashkaryan, V.; Bray, L.; Bhattarai, P.; Brandt, K.; Hollak, H.; Chen, X.; He, S.; et al. 3D Culture Method for Alzheimer's Disease Modeling Reveals Interleukin-4 Rescues Aβ42-Induced Loss of Human Neural Stem Cell Plasticity. *Dev. Cell* **2018**, *46*, 85–101.e8. [CrossRef] [PubMed]
86. Langhe, R.; Pearson, R.A. Rebuilding the Retina: Prospects for Müller Glial-mediated Self-repair. *Curr. Eye Res.* **2020**, *45*, 349–360. [CrossRef] [PubMed]
87. Roesch, K.; Jadhav, A.P.; Trimarchi, J.M.; Stadler, M.B.; Roska, B.; Sun, B.B.; Cepko, C.L. The transcriptome of retinal Müller glial cells. *J. Comp. Neurol.* **2008**, *509*, 225–238. [CrossRef]

88. Cepko, C.; Dyer, M.A. Control of Müller glial cell proliferation and activation following retinal injury. *Nat. Neurosci.* **2000**, *3*, 873–880.
89. Hamon, A.; Roger, J.E.; Yang, X.J.; Perron, M. Müller glial cell-dependent regeneration of the neural retina: An overview across vertebrate model systems. *Dev. Dyn.* **2016**, *245*, 727–738. [CrossRef]
90. Fausett, B.V.; Gumerson, J.D.; Goldman, D. The proneural basic helix-loop-helix gene Ascl1a is required for retina regeneration. *J. Neurosci.* **2008**, *28*, 1109–1117. [CrossRef]
91. Pollak, J.; Wilken, M.S.; Ueki, Y.; Cox, K.E.; Sullivan, J.M.; Taylor, R.J.; Levine, E.M.; Reh, T.A. ASCL1 reprograms mouse Müller glia into neurogenic retinal progenitors. *Development* **2013**, *140*, 2619–2631. [CrossRef]
92. Ueki, Y.; Wilken, M.S.; Cox, K.E.; Chipman, L.; Jorstad, N.; Sternhagen, K.; Simic, M.; Ullom, K.; Nakafuku, M.; Reh, T.A. Transgenic expression of the proneural transcription factor Ascl1 in Müller glia stimulates retinal regeneration in young mice. *Proc. Natl. Acad. Sci. USA* **2015**, *112*, 13717–13722. [CrossRef] [PubMed]
93. Ramachandran, R.; Fausett, B.V.; Goldman, D. Ascl1A regulates Muller glia dedifferentiation and retina regeneration via a Lin-28 dependent, let7 miRNA signaling pathway. *Nat. Cell Biol.* **2010**, *7*, 1959–1967.
94. Pisharath, H.; Rhee, J.M.; Swanson, M.A.; Leach, S.D.; Parsons, M.J. Targeted ablation of beta cells in the embryonic zebrafish pancreas using E.coli nitroreductase. *Mech. Dev.* **2007**, *124*, 218–229. [CrossRef] [PubMed]
95. Curado, S.; Anderson, R.M.; Jungblut, B.; Mumm, J.; Schroeter, E.; Stainier, D.Y.R. Conditional targeted cell ablation in zebrafish: A new tool for regeneration studies. *Dev. Dyn.* **2007**, *236*, 1025–1035. [CrossRef] [PubMed]
96. Bergemann, D.; Massoz, L.; Bourdouxhe, J.; Carril Pardo, C.A.; Voz, M.L.; Peers, B.; Manfroid, I. Nifurpirinol: A more potent and reliable substrate compared to metronidazole for nitroreductase-mediated cell ablations. *Wound Repair Regen.* **2018**, *26*, 238–244. [CrossRef] [PubMed]
97. Sharrock, A.; Mulligan, T.; Hall, K.; Williams, E.; White, D.; Zhang, L.; Matthews, F.; Nimmagadda, S.; Washington, S.; Le, K.; et al. NTR 2.0: A rationally-engineered prodrug converting enzyme with substantially enhanced efficacy for targeted cell ablation. *bioRxiv* **2020**, 1–26. [CrossRef]
98. Wilkinson, P.D.; Alencastro, F.; Delgado, E.R.; Leek, M.P.; Weirich, M.P.; Otero, P.A.; Roy, N.; Brown, W.K.; Oertel, M.; Duncan, A.W. Polyploid Hepatocytes Facilitate Adaptation and Regeneration to Chronic Liver Injury. *Am. J. Pathol.* **2019**, *189*, 1241–1255. [CrossRef]
99. Donne, R.; Saroul-Aïnama, M.; Cordier, P.; Celton-Morizur, S.; Desdouets, C. Polyploidy in liver development, homeostasis and disease. *Nat. Rev. Gastroenterol. Hepatol.* **2020**, *17*, 391–405. [CrossRef]
100. Julier, Z.; Park, A.J.; Briquez, P.S.; Martino, M.M. Promoting tissue regeneration by modulating the immune system. *Acta Biomater.* **2017**, *53*, 13–28. [CrossRef]
101. Lai, S.L.; Marín-Juez, R.; Moura, P.L.; Kuenne, C.; Lai, J.K.H.; Tsedeke, A.T.; Guenther, S.; Looso, M.; Stainier, D.Y.R. Reciprocal analyses in zebrafish and medaka reveal that harnessing the immune response promotes cardiac regeneration. *Elife* **2017**, *6*, 1–20. [CrossRef]
102. Ryan, R.; Moyse, B.R.; Richardson, R.J. Zebrafish cardiac regeneration-looking beyond cardiomyocytes to a complex microenvironment. *Histochem. Cell Biol.* **2020**, *154*, 533–548. [CrossRef] [PubMed]
103. Suzuki, N.; Ochi, H. Regeneration enhancers: A clue to reactivation of developmental genes. *Dev. Growth Regen.* **2020**, 1–12. [CrossRef] [PubMed]
104. Zafar, H.; Lin, C.; Bar-Joseph, Z. Single-cell lineage tracing by integrating CRISPR-Cas9 mutations with transcriptomic data. *Nat. Commun.* **2020**, *11*, 305. [CrossRef] [PubMed]

VII. References

Aguayo-Mazzucato, Cristina, and Susan Bonner-Weir. 2018. "Pancreatic β Cell Regeneration as a Possible Therapy for Diabetes." *Cell Metabolism* 27(1): 57–67. https://doi.org/10.1016/j.cmet.2017.08.007.

Aguirre, A. et al. 2014. "In Vivo Activation of a Conserved MicroRNA Program Induces Robust Mammalian Heart Regeneration." *Cell stem cell*: 7.

Al-Hasani, Keith et al. 2013. "Adult Duct-Lining Cells Can Reprogram into β-like Cells Able to Counter Repeated Cycles of Toxin-Induced Diabetes." *Developmental Cell* 26(1): 86–100.

Andersson, Olov et al. 2012. "Adenosine Signaling Promotes Regeneration of Pancreatic β- Cells in Vivo." *Cell Metabolism*.

Ben-Othman, Nouha et al. 2017. "Long-Term GABA Administration Induces Alpha Cell-Mediated Beta-like Cell Neogenesis." *Cell* 168(1–2): 73-85.e11.

Bergemann, David et al. 2018. "Nifurpirinol: A More Potent and Reliable Substrate Compared to Metronidazole for Nitroreductase-Mediated Cell Ablations." *Wound Repair and Regeneration* 26(2): 238–44.

Berghmans, Stéphane et al. 2005. "Tp53 Mutant Zebrafish Develop Malignant Peripheral Nerve Sheath Tumors." *Proceedings of the National Academy of Sciences of the United States of America* 102(2): 407–12.

Biemar, Frédéric et al. 2001. "Pancreas Development in Zebrafish: Early Dispersed Appearance of Endocrine Hormone Expressing Cells and Their Convergence to Form the Definitive Islet." *Developmental Biology* 230(2): 189–203.

Binot, A. C. et al. 2010. "Nkx6.1 and Nkx6.2 Regulate α- and β-Cell Formation in Zebrafish by Acting on Pancreatic Endocrine Progenitor Cells." *Developmental Biology* 340(2): 397–407. http://dx.doi.org/10.1016/j.ydbio.2010.01.025.

Bonner-Weir, Susan et al. 2000. "In Vitro Cultivation of Human Islets from Expanded Ductal Tissue." *Proceedings of the National Academy of Sciences of the United States of America* 97(14): 7999–8004.

Bonner-Weir, Susan, Leslie A. Baxter, George T. Schuppin, and Fannie E. Smith. 1993. "A Second Pathway for Regeneration of Adult Exocrine and Endocrine Pancreas: A Possible Recapitulation of Embryonic Development." *Diabetes* 42(12): 1715–20.

Bonner-Weir, Susan, Brooke A. Sullivan, and Gordon C. Weir. 2015. "Human Islet Morphology Revisited: Human and Rodent Islets Are Not So Different After All." *Journal of Histochemistry and Cytochemistry* 63(8): 604–12.

Bouwens, Luc, and Ilse Rooman. 2005. "Regulation of Pancreatic Beta-Cell Mass." *Physiological Reviews* 85(4): 1255–70.

Bultje, Ronald S. et al. 2009. "Mammalian Par3 Regulates Progenitor Cell Asymmetric Division via Notch Signaling in the Developing Neocortex." *Neuron* 63(2): 189–202. http://dx.doi.org/10.1016/j.neuron.2009.07.004.

Butler, Alexandra E et al. 2003. "Humans With Type 2 Diabetes." *Diabetes* 52(January): 102–10. http://www.ncbi.nlm.nih.gov/pubmed/12502499.

Butler, Peter C., Juris J. Meier, Alexandra E. Butler, and Anil Bhushan. 2007. "The Replication of β Cells in Normal Physiology, in Disease and for Therapy." *Nature Clinical Practice Endocrinology and Metabolism* 3(11): 758–68.

Cabrera, Over et al. 2006. "The Unique Cytoarchitecture of Human Pancreatic Islets Has Implications for Islet Cell Function." *Proceedings of the National Academy of Sciences of the United States of America* 103(7): 2334–39.

Cao, Zigang et al. 2021. "Calcineurin Controls Proximodistal Blastema Polarity in Zebrafish Fin Regeneration." *Proceedings of the National Academy of Sciences of the United States of America* 118(2): 2–9.

Carril Pardo, Claudio et al. 2022. "A Delta-Cell Subpopulation with a pro-Beta-Cell Identity Contributes to Efficient Age-Independent Recovery in a Zebrafish Model of Diabetes." *eLife*.

Van De Casteele, M. et al. 2013. "Neurogenin 3+ Cells Contribute to B-Cell Neogenesis and Proliferation in Injured Adult Mouse Pancreas." *Cell Death and Disease* 4(3).

Charbord, Jérémie et al. 2021. “In Vivo Screen Identifies a SIK Inhibitor That Induces β Cell Proliferation through a Transient UPR.” *Nature Metabolism* 3(5): 682–700. http://dx.doi.org/10.1038/s42255-021-00391-x.

Charni, Meital, Ronit Aloni-Grinstein, Alina Molchadsky, and Varda Rotter. 2017. “P53 on the Crossroad between Regeneration and Cancer.” *Cell Death and Differentiation* 24(1): 8–14. http://dx.doi.org/10.1038/cdd.2016.117.

Chera, Simona et al. 2014a. “Diabetes Recovery by Age-Dependant Conversion of Pancreatic Delta-Cells into Insulin Producers.” *Nature* 514(7523): 503–7.

———. 2014b. “Diabetes Recovery By Age-Dependent Conversion of Pancreatic δ-Cells.” *Nature*.

Choe, Chong Pyo et al. 2021. “Transgenic Fluorescent Zebrafish Lines That Have Revolutionized Biomedical Research.” *Laboratory Animal Research* 37(1): 1–29. https://doi.org/10.1186/s42826-021-00103-2.

Choi, Tae Young, Nikolay Ninov, Didier Y.R. Stainier, and Donghun Shin. 2014. “Extensive Conversion of Hepatic Biliary Epithelial Cells to Hepatocytes after near Total Loss of Hepatocytes in Zebrafish.” *Gastroenterology* 146(3): 776–88.

Cinti, Francesca et al. 2016. “Evidence of β-Cell Dedifferentiation in Human Type 2 Diabetes.” *Journal of Clinical Endocrinology and Metabolism* 101(3): 1044–54.

Cohrs, Christian M. et al. 2020. “Dysfunction of Persisting β Cells Is a Key Feature of Early Type 2 Diabetes Pathogenesis.” *Cell Reports* 31(1).

Corradi, Laura et al. 2022. “Hypothalamic Galanin-Producing Neurons Regulate Stress in Zebrafish through a Peptidergic, Self-Inhibitory Loop.” *Current Biology* 32(7): 1497-1510.e5. https://doi.org/10.1016/j.cub.2022.02.011.

Courtney, Monica et al. 2013. “The Inactivation of Arx in Pancreatic α-Cells Triggers Their Neogenesis and Conversion into Functional β-Like Cells.” *PLoS Genetics* 9(10): 1–18.

Creamer, Trevor P. 2020. “Calcineurin.” *Cell communication and Signalling* 4: 1–12.

Crippa, Stefania et al. 2016. “Comparative Transcriptome Profiling of the Injured Zebrafish and Mouse Hearts Identifies MiRNA-Dependent Repair Pathways.” *Cardiovascular research* 110(1): 73–84.

Curado, Silvia et al. 2007. “Conditional Targeted Cell Ablation in Zebrafish: A New Tool for Regeneration Studies.” *Developmental Dynamics* 236(February): 1025–35.

D’Amour, Kevin A. et al. 2006. “Production of Pancreatic Hormone-Expressing Endocrine Cells from Human Embryonic Stem Cells.” *Nature Biotechnology* 24(11): 1392–1401.

Da, Sabela et al. 2020. “Cell Senescence Contributes to Tissue Regeneration in Zebrafish.” (July 2019): 1–5.

Dai, Chunhua et al. 2017. “Age-Dependent Human β Cell Proliferation Induced by Glucagon-like Peptide 1 and Calcineurin Signaling.” *Journal of Clinical Investigation* 127(10): 3835–44.

Delaspre, Fabien et al. 2015. “Centroacinar Cells Are Progenitors That Contribute to Endocrine Pancreas Regeneration.” *Diabetes* 64(October): 3499–3509.

Delgadillo-Silva, Luis Fernando et al. 2019. “Modelling Pancreatic β-Cell Inflammation in Zebrafish Identifies the Natural Product Wedelolactone for Human Islet Protection.” *DMM Disease Models and Mechanisms* 12(1): 1–12.

Van Deursen, Jan M. 2014. “The Role of Senescent Cells in Ageing.” *Nature* 509(7501): 439–46. http://dx.doi.org/10.1038/nature13193.

DiMeglio, Linda A., Carmella Evans-Molina, and Richard A. Oram. 2018. “Type 1 Diabetes.” *The Lancet* 391(10138): 2449–62. http://dx.doi.org/10.1016/S0140-6736(18)31320-5.

Dor, Yuval, Juliana Brown, Olga I Martinez, and Douglas a Melton. 2004. “Adult Pancreatic Beta-Cells Are Formed by Self-Duplication Rather than Stem-Cell Differentiation.” *Nature* 429(model 3): 41–46.

Druelle, Noémie et al. 2017. “Ectopic Expression of Pax4 in Pancreatic δ Cells Results in β-like Cell Neogenesis.” *Journal of Cell Biology* 216(12): 4299–4311.

Duggirala, Ravindranath et al. 1999. “Linkage of Type 2 Diabetes Mellitus and of Age at Onset to a Genetic Location on Chromosome 10q in Mexican Americans.” *American Journal of Human Genetics* 64(4): 1127–40.

Dupuis, Josée et al. 2010. “New Genetic Loci Implicated in Fasting Glucose Homeostasis and Their Impact on Type 2 Diabetes Risk.” *Nature Genetic*. http://tocs.ub.uni-mainz.de/pdfs/100559395.pdf%7D, URL = %7Bhttp://www.bsz-bw.de/cgi-bin/ekz.cgi?SWB09525289 ;

Edlund, Helena. 2002. “Pancreatic Organogenesis - Developmental Mechanisms and Implications for Therapy.” *Nature Reviews Genetics* 3(7): 524–32.

Elliott, Sarah A., and Alejandro Sánchez Alvarado. 2018. 1774 Methods in Molecular Biology *Planarians and the History of Animal Regeneration: Paradigm Shifts and Key Concepts in Biology*.

Elsaeidi, Fairouz et al. 2018. “Notch Suppression Collaborates with Ascl1 and Lin28 to Unleash a Regenerative Response in Fish Retina, but Not in Mice.” *Journal of Neuroscience* 38(9): 2246–61.

Fausett, Blake V., Jessica D. Gumerson, and Daniel Goldman. 2008. “The Proneural Basic Helix-Loop-Helix Gene Ascl1a Is Required for Retina Regeneration.” *Journal of Neuroscience* 28(5): 1109–17.

Flasse, Lydie C., Justine L. Pirson, et al. 2013. “Ascl1b and Neurod1, Instead of Neurog3, Control Pancreatic Endocrine Cell Fate in Zebrafish.” *BMC Biology* 11: 1–18.

Flasse, Lydie C., David G. Stern, et al. 2013. “The BHLH Transcription Factor Ascl1a Is Essential for the Specification of the Intestinal Secretory Cells and Mediates Notch Signaling in the Zebrafish Intestine.” *Developmental Biology* 376(2): 187–97. http://dx.doi.org/10.1016/j.ydbio.2013.01.011.

Flasse, Lydie, Coline Schewin, and Anne Grapin-Botton. 2021. 143 Current Topics in Developmental Biology *Pancreas Morphogenesis: Branching in and Then Out*. 1st ed. Elsevier Inc. http://dx.doi.org/10.1016/bs.ctdb.2020.10.006.

Galicia-Garcia, Unai et al. 2020. “Pathophysiology of Type 2 Diabetes Mellitus.” *International Journal of Molecular Sciences* 21(17): 1–34.

Gemberling, Matthew, Travis J. Bailey, David R. Hyde, and Kenneth D. Poss. 2013. “The Zebrafish as a Model for Complex Tissue Regeneration.” *Trends in Genetics* 29(11): 611–20. http://dx.doi.org/10.1016/j.tig.2013.07.003.

Ghaye, A.P. et al. 2015. “Progenitor Potential of Nkx6.1-Expressing Cells throughout Zebrafish Life and during Beta Cell Regeneration.” *BMC Biology* 13(1).

Ghaye, Aurélie P. et al. 2015. “Progenitor Potential of Nkx6.1-Expressing Cells throughout Zebrafish Life and during Beta Cell Regeneration.” *BMC Biology* 13: 70.

Di Giovanni, Simone et al. 2006. “The Tumor Suppressor Protein P53 Is Required for Neurite Outgrowth and Axon Regeneration.” *EMBO Journal* 25(17): 4084–96.

Goldshmit, Y et al. 2015. “Decreased Anti-Regenerative Effects after Spinal Cord Injury in Spry4-/- Mice.” *Neuroscience* 287: 104–12.

Goldshmit, Yona et al. 2012. “Fgf-Dependent Glial Cell Bridges Facilitate Spinal Cord Regeneration in Zebrafish.” *The Journal of neuroscience : the official journal of the Society for Neuroscience* 32(22): 7477–92.

Goshima, Takahiro et al. 2019. “Calcineurin Regulates Cyclin D1 Stability through Dephosphorylation at T286.” *Scientific Reports* 9(1): 1–11.

Gribben, Christopher et al. 2021. “Ductal Ngn3-Expressing Progenitors Contribute to Adult β Cell Neogenesis in the Pancreas.” *Cell Stem Cell* 28(11): 2000-2008.e4. https://doi.org/10.1016/j.stem.2021.08.003.

De Groef, Sofie et al. 2015. “Surgical Injury to the Mouse Pancreas through Ligation of the Pancreatic Duct as a Model for Endocrine and Exocrine Reprogramming and Proliferation.” *Journal of Visualized Experiments* 2015(102): 16–19.

Guo, Ming, Lily Yeh Jan, and Yuh Nung Jan. 1996. “Control of Daughter Cell Fates during Asymmetric Division: Interaction of Numb and Notch.” *Neuron* 17(1): 27–41.

Han, Lei et al. 2022. "Distinctive Clinical and Pathologic Features of Immature Teratomas Arising from Induced Pluripotent Stem Cell-Derived Beta Cell Injection in a Diabetes Patient." *Stem Cells and Development* 31(5–6): 97–101.

He, Jianbo et al. 2022. "DNA Methylation Maintenance at the P53 Locus Initiates Biliary-Mediated Liver Regeneration." *npj Regenerative Medicine* 7(1): 1–14.

Heit, Jeremy J. et al. 2006. "Calcineurin/NFAT Signalling Regulates Pancreatic β-Cell Growth and Function." *Nature* 443(7109): 345–49.

Helker, Christian S.M. et al. 2019. "A Whole Organism Small Molecule Screen Identifies Novel Regulators of Pancreatic Endocrine Development." *Development (Cambridge)* 146(14).

Herrera, P. L. et al. 1994. "Ablation of Islet Endocrine Cells by Targeted Expression of Hormone- Promoter-Driven Toxigenes." *Proceedings of the National Academy of Sciences of the United States of America* 91(26): 12999–3.

Ho, Anthony D., and Wolfgang Wagner. 2007. "The Beauty of Asymmetry: Asymmetric Divisions and Self-Renewal in the Haematopoietic System." *Current Opinion in Hematology* 14(4): 330–36.

Howe, Kerstin et al. 2013. "The Zebrafish Reference Genome Sequence and Its Relationship to the Human Genome." *Nature* 496(7446): 498–503.

Huttner, Wieland B., and Yoichi Kosodo. 2005. "Symmetric versus Asymmetric Cell Division during Neurogenesis in the Developing Vertebrate Central Nervous System." *Current Opinion in Cell Biology* 17(6): 648–57.

Ilonen, Jorma et al. 2013. "Patterns of β-Cell Autoantibody Appearance and Genetic Associations during the First Years of Life." *Diabetes* 62(10): 3636–40.

Inada, Akari et al. 2008. "Carbonic Anhydrase II-Positive Pancreatic Cells Are Progenitors for Both Endocrine and Exocrine Pancreas after Birth." *Proceedings of the National Academy of Sciences of the United States of America* 105(23): 19915–19.

Karampelias, Christos et al. 2021. "Reinforcing One-Carbon Metabolism via Folic Acid/Folr1 Promotes β-Cell Differentiation." *Nature Communications* 12(1): 1–13. http://dx.doi.org/10.1038/s41467-021-23673-0.

———. 2022. "MNK2 Deficiency Potentiates β-Cell Regeneration via Translational Regulation." *Nature Chemical Biology* 18(9): 942–53.

Katsarou, Anastasia et al. 2017. "Type 1 Diabetes Mellitus." *Nature Reviews Disease Primers* 3: 17016.

Kawakami, Koichi. 2007. "Tol2: A Versatile Gene Transfer Vector in Vertebrates." *Genome biology* 8 Suppl 1: S7.

Kim, Yung Hae et al. 2015. "Cell Cycle–Dependent Differentiation Dynamics Balances Growth and Endocrine Differentiation in the Pancreas." *PLoS Biology* 13(3).

Kimmel, Charles B. 1989. "Genetics and Early Development of Zebrafish." *Trends in Genetics* 5(C): 283–88.

Kinkel, Mary D, and Victoria E Prince. 2009. "On the Diabetic Menu: Zebrafish as a Model for Pancreas Development and Function." *Bioassays* 31(2): 139–52.

Ko, Sungjin et al. 2016. "Bromodomain and Extraterminal (BET) Proteins Regulate Biliary-Driven Liver Regeneration." *Journal of Hepatology* 64(2): 316–25.

Kopp, J L et al. 2011. "Sox9+ Ductal Cells Are Multipotent Progenitors throughout Development but Do Not Produce New Endocrine Cells in the Normal or Injured Adult Pancreas." *Development* 138: 653–65.

Krentz, Nicole A J et al. 2017. "Phosphorylation of NEUROG3 Links Endocrine Differentiation to the Cell Cycle in Pancreatic Article Phosphorylation of NEUROG3 Links Endocrine Differentiation to the Cell Cycle in Pancreatic Progenitors." *Developmental Cell* 41(2): 129-142.e6. http://dx.doi.org/10.1016/j.devcel.2017.02.006.

Krishnamurthy, Janakiraman et al. 2006. "P16INK4a Induces an Age-Dependent Decline in Islet Regenerative Potential." *Nature* 443(7110): 453–57.

Kroehne, Volker et al. 2011. "Regeneration of the Adult Zebrafish Brain from Neurogenic Radial Glia-Type Progenitors." *Development* 138(22): 4831–41.

Kroon, Evert et al. 2008. "Pancreatic Endoderm Derived from Human Embryonic Stem Cells Generates Glucose-Responsive Insulin-Secreting Cells in Vivo." *Nature Biotechnology* 26(4): 443–52.

Kujawski, Satu et al. 2014. "Calcineurin Regulates Coordinated Outgrowth of Zebrafish Regenerating Fins." *Developmental Cell* 28(5): 573–87.

Kulkarni, Abhishek A. et al. 2018. "An in Vivo Zebrafish Model for Interrogating ROS-Mediated Pancreatic β-Cell Injury, Response, and Prevention." *Oxidative Medicine and Cellular Longevity* 2018.

Kume, Kazunori et al. 2011. "Calcineurin Ensures a Link between the DNA Replication Checkpoint and Microtubule-Dependent Polarized Growth." *Nature Cell Biology* 13(3): 234–42. http://dx.doi.org/10.1038/ncb2166.

Kwan, Kristen M et al. 2007. "The Tol2kit: A Multisite Gateway-Based Construction Kit for Tol2 Transposon Transgenesis Constructs." *Developmental dynamics : an official publication of the American Association of Anatomists* 236(11): 3088–99.

Lacroix, Matthieu et al. 2020. "Metabolic Functions of the Tumor Suppressor P53: Implications in Normal Physiology, Metabolic Disorders, and Cancer." *Molecular Metabolism* 33(October 2019): 2–22. https://doi.org/10.1016/j.molmet.2019.10.002.

Lai, Siting et al. 2021. "Chemical Screening Reveals Ronidazole Is a Superior Prodrug to Metronidazole for Nitroreductase-Induced Cell Ablation System in Zebrafish Larvae." *Journal of Genetics and Genomics* 48(12): 1081–90. https://doi.org/10.1016/j.jgg.2021.07.015.

Lee, Peter J., and Georgios I. Papachristou. 2019. "New Insights into Acute Pancreatitis." *Nature Reviews Gastroenterology and Hepatology* 16(8): 479–96. http://dx.doi.org/10.1038/s41575-019-0158-2.

Leech, Cassandra M. et al. 2020. "The Coordinate Actions of Calcineurin and Hog1 Mediate the Stress Response through Multiple Nodes of the Cell Cycle Network." *PLoS Genetics* 16(4): 1–27. http://dx.doi.org/10.1371/journal.pgen.1008600.

Li, Wan Chun et al. 2010. "Activation of Pancreatic-Duct-Derived Progenitor Cells during Pancreas Regeneration in Adult Rats." *Journal of Cell Science* 123(16): 2792–2802.

Liu, Haisong et al. 2021. "Chemical Combinations Potentiate Human Pluripotent Stem Cell-Derived 3D Pancreatic Progenitor Clusters toward Functional β Cells." *Nature Communications* 12(1): 3–12. http://dx.doi.org/10.1038/s41467-021-23525-x.

Liu, Jun et al. 1991. "Calcineurin Is a Common Target of Cyclophilin-Cyclosporin A and FKBP-FK506 Complexes." *Cell* 66(4): 807–15.

Liu, Ka Cheuk et al. 2017. "Inhibition of Cdk5 Promotes β-Cell Differentiation from Ductal Progenitors." *Diabetes* 67(1): 58–70.

Lu, Jing et al. 2016. " IGFBP 1 Increases B-cell Regeneration by Promoting A- to B-cell Transdifferentiation ." *The EMBO Journal* 35(18): 2026–44.

Lu, Wei Yu et al. 2015. "Hepatic Progenitor Cells of Biliary Origin with Liver Repopulation Capacity." *Nature Cell Biology* 17(8): 973–83.

Maddocks, Oliver D.K. et al. 2013. "Serine Starvation Induces Stress and P53-Dependent Metabolic Remodelling in Cancer Cells." *Nature* 493(7433): 542–46.

Maden, Malcolm. 2018. "The Evolution of Regeneration – Where Does That Leave Mammals?" *International Journal of Developmental Biology* 62(6–8): 369–72.

Magenheim, Judith et al. 2023. "Matters Arising: Insufficient Evidence That Pancreatic β Cells Are Derived from Adult Ductal Neurog3-Expressing Progenitors." *Cell Stem Cell* 30(4): 488-497.e3. https://doi.org/10.1016/j.stem.2023.03.003.

Mammucari, Cristina et al. 2005. "Integration of Notch 1 and Calcineurin/NFAT Signaling Pathways in Keratinocyte Growth and Differentiation Control." *Developmental Cell* 8(5): 665–76.

Manfroid, Isabelle et al. 2012. “Zebrafish Sox9b Is Crucial for Hepatopancreatic Duct Development and Pancreatic Endocrine Cell Regeneration.” *Dev. Biol.* 366(2): 268–78.

Mannioui, Abdelkrim, and Bernard Zalc. 2019. “Conditional Demyelination and Remyelination in a Transgenic Xenopus Laevis.” *Methods in Molecular Biology* 1936: 239–48.

Marín-Peñalver, Juan José, Iciar Martín-Timón, Cristina Sevillano-Collantes, and Francisco Javier del Cañizo-Gómez. 2016. “Update on the Treatment of Type 2 Diabetes Mellitus.” *World Journal of Diabetes* 7(17): 354.

Marinaga, Marcia. 1990. “Swimming into the Development Mainstream.” *Science* 250.

Marques, Ines J., Eleonora Lupi, and Nadia Mercader. 2019. “Model Systems for Regeneration: Zebrafish.” *Development (Cambridge)* 146(18).

Masaki, Takahiro, and Midori Shimada. 2022. “Decoding the Phosphatase Code: Regulation of Cell Proliferation by Calcineurin.” *International Journal of Molecular Sciences* 23(3).

Mashkaryan, Violeta et al. 2020. “Type 1 Interleukin-4 Signaling Obliterates Mouse Astroglia in Vivo but Not in Vitro.” *Frontiers in Cell and Developmental Biology* 8(February): 1–16.

Massoz, Laura, Marie Alice Dupont, and Isabelle Manfroid. 2021. “Zebra-Fishing for Regenerative Awakening in Mammals.” *Biomedicines* 9(1): 1–18.

Mathias, Jonathan R., Zhanying Zhang, Meera T. Saxena, and Jeff S. Mumm. 2014. “Enhanced Cell-Specific Ablation in Zebrafish Using a Triple Mutant of Escherichia Coli Nitroreductase.” *Zebrafish* 11(2): 85–97.

McDonald, Georgina L.K., Mengdi Wang, Chrissy L. Hammond, and Dylan J.M. Bergen. 2021. “Pharmacological Manipulation of Early Zebrafish Skeletal Development Shows an Important Role for Smad9 in Control of Skeletal Progenitor Populations.” *Biomolecules* 11(2): 1–20.

McIntyre, H. David et al. 2019. “Gestational Diabetes Mellitus.” *Nature Reviews Disease Primers* 5(1). http://dx.doi.org/10.1038/s41572-019-0098-8.

McMillan, Stephanie C. et al. 2018. “A Regulatory Pathway Involving Retinoic Acid and Calcineurin Demarcates and Maintains Joint Cells and Osteoblasts in Regenerating Fin.” *Development (Cambridge)* 145(11).

Md Moin, Abu Saleh, Peter C. Butler, and Alexandra E. Butler. 2017. “Increased Proliferation of the Pancreatic Duct Gland Compartment in Type 1 Diabetes.” *Journal of Clinical Endocrinology and Metabolism* 102(1): 200–209.

Meier, J. J. et al. 2005. “Sustained Beta Cell Apoptosis in Patients with Long-Standing Type 1 Diabetes: Indirect Evidence for Islet Regeneration?” *Diabetologia* 48(11): 2221–28.

Millman, Jeffrey R. et al. 2016. “Generation of Stem Cell-Derived β-Cells from Patients with Type 1 Diabetes.” *Nature Communications* 7(May).

Moin, Abu Saleh Md, and Alexandra E. Butler. 2019. “Alterations in Beta Cell Identity in Type 1 and Type 2 Diabetes.” *Current Diabetes Reports* 19(9).

Moss, Jennifer B. et al. 2009. “Regeneration of the Pancreas in Adult Zebrafish.” *Diabetes* 58(August): 1844–51.

Nauck, Michael A., Daniel R. Quast, Jakob Wefers, and Juris J. Meier. 2021. “GLP-1 Receptor Agonists in the Treatment of Type 2 Diabetes – State-of-the-Art.” *Molecular Metabolism* 46(October 2020): 101102. https://doi.org/10.1016/j.molmet.2020.101102.

Ni, Qianqian et al. 2019. “Advances in Immunotherapy of Type I Diabetes.” *Advanced Drug Delivery Reviews* 139: 83–91. https://doi.org/10.1016/j.addr.2018.12.003.

Ninov, Nikolay et al. 2013. “Metabolic Regulation of Cellular Plasticity in the Pancreas.” *Current Biology* 23(13): 1242–50.

Ninov, Nikolay, Maxim Borius, and Didier Y.R. Stainier. 2012. “Different Levels of Notch Signaling Regulate Quiescence, Renewal and Differentiation in Pancreatic Endocrine Progenitors.” *Development* 139(9): 1557–67.

Nir, Tomer, Douglas A Melton, and Yuval Dor. 2007. “Nir, Dor 2007 Recovery from Diabetes in Mice by

□□cell Regeneration.Pdf.” 117(9): 2553–61.

Norris, Jill M., Randi K. Johnson, and Lars C. Stene. 2020. “Type 1 Diabetes—Early Life Origins and Changing Epidemiology.” *The Lancet Diabetes and Endocrinology* 8(3): 226–38. http://dx.doi.org/10.1016/S2213-8587(19)30412-7.

Offield, Martin F et al. 1996. “PDX-1 Is Required for Pancreatic Outgrowth and Differentiation of the Rostral Duodenum.” *Development* 122.

Oh-hora, Masatsugu, and Anjana rao. 2009. “The Calcium/NFAT Pathway: Role in Development and Function of Regulatory T Cells.” *Microbes and Infection* 11(5): 612–19. http://dx.doi.org/10.1016/j.micinf.2009.04.008.

Oyadomari, S., E. Araki, and M. Mori. 2002. “Endoplasmic Reticulum Stress-Mediated Apoptosis in Pancreatic β-Cells.” *Apoptosis* 7(4): 335–45.

Pagliuca, Felicia W. et al. 2014. “Generation of Functional Human Pancreatic β Cells in Vitro.” *Cell* 159(2): 428–39.

Pagola, A. Martin- et al. 2008. “Insulin Expression and Proliferation in Ductal Cells in the Transplanted Pancreas of Patients with Type 1 Diabetes and Recurrence of Autoimmunity.” *Diabetologia* 51(10): 1803–13.

Pan, Fong Cheng et al. 2013. “Spatiotemporal Patterns of Multipotentiality in Ptf1aexpressing Cells during Pancreas Organogenesis and Injuryinduced Facultative Restoration.” *Development (Cambridge)* 140(4): 751–64.

Pan, Fong Cheng, and Chris Wright. 2011. “Pancreas Organogenesis: From Bud to Plexus to Gland.” *Developmental Dynamics* 240(3): 530–65.

Papadimitriou, Christos et al. 2018. “3D Culture Method for Alzheimer’s Disease Modeling Reveals Interleukin-4 Rescues Aβ42-Induced Loss of Human Neural Stem Cell Plasticity.” *Developmental Cell* 46(1): 85-101.e8. https://doi.org/10.1016/j.devcel.2018.06.005.

Parsons, Michael J. et al. 2009. “Notch-Responsive Cells Initiate the Secondary Transition in Larval Zebrafish Pancreas.” *Mechanisms of Development* 126(10): 898–912. http://dx.doi.org/10.1016/j.mod.2009.07.002.

Pelletier, Joffrey et al. 2020. “ Nucleotide Depletion Reveals the Impaired Ribosome Biogenesis Checkpoint as a Barrier against DNA Damage .” *The EMBO Journal* 39(13): 1–19.

Perez-Frances, Marta et al. 2021. “Pancreatic Ppy-Expressing γ-Cells Display Mixed Phenotypic Traits and the Adaptive Plasticity to Engage Insulin Production.” *Nature Communications* 12(1): 1–16. http://dx.doi.org/10.1038/s41467-021-24788-0.

Pisharath, Harsan et al. 2007. “Targeted Ablation of Beta Cells in the Embryonic Zebrafish Pancreas Using E.Coli Nitroreductase.” *Mech Dev.*

Pisharath, Harshan. 2007. “Validation of Nitroreductase, a Prodrug-Activating Enzyme, Mediated Cell Death in Embryonic Zebrafish (Danio Rerio).” *Comparative Medicine* 57(3): 241–46.

Pociot, Flemming, and Åke Lernmark. 2016. “Genetic Risk Factors for Type 1 Diabetes.” *The Lancet* 387(10035): 2331–39.

Poss, Kenneth D., Lindsay G. Wilson, and Mark T. Keating. 2002. “Heart Regeneration in Zebrafish.” *Science* 298(5601): 2188–90.

Qadir, Mirza Muhammad Fahd et al. 2018. “P2RY1/ALK3-Expressing Cells within the Adult Human Exocrine Pancreas Are BMP-7 Expandable and Exhibit Progenitor-like Characteristics.” *Cell Reports* 22(9): 2408–20.

———. 2020. “Single-Cell Resolution Analysis of the Human Pancreatic Ductal Progenitor Cell Niche.” *Proceedings of the National Academy of Sciences of the United States of America* 117(20): 10876–87.

Ramachandran R., Fausett B.V., Goldman D. 2010. “Ascl1A Regulates Muller Glia Dedifferentiation and Retina Regeneration via a Lin-28 Dependent, Let7 MiRNA Signaling Pathway.” *Nature Cell Biology* 7(12): 1959–67.

Rankin, Matthew M., and Jake A. Kushner. 2009. “Adaptive β-Cell Proliferation Is Severely Restricted with

Advanced Age." *Diabetes* 58(6): 1365–72.

Rezania, Alireza et al. 2014. "Reversal of Diabetes with Insulin-Producing Cells Derived in Vitro from Human Pluripotent Stem Cells." *Nature Biotechnology*.

Rix, Iben et al. 2015. *Glucagon Physiology*. MDText.com, Inc., South Dartmouth (MA). http://europepmc.org/books/NBK279127.

Roden, Michael, and Gerald I. Shulman. 2019. "The Integrative Biology of Type 2 Diabetes." *Nature* 576(7785): 51–60. http://dx.doi.org/10.1038/s41586-019-1797-8.

Rorsman, Patrik, and Mark O. Huising. 2018. "The Somatostatin-Secreting Pancreatic δ-Cell in Health and Disease." *Nature Reviews Endocrinology* 14(7): 404–14.

Rovira, Meritxell et al. 2010. "Isolation and Characterization of Centroacinar/Terminal Ductal Progenitor Cells in Adult Mouse Pancreas." *Proceedings of the National Academy of Sciences of the United States of America* 107(1): 75–80.

Roy, Jagoree, and Martha S. Cyert. 2020. "Identifying New Substrates and Functions for an Old Enzyme: Calcineurin." *Cold Spring Harbor Perspectives in Biology* 12(3).

Schwitzgebel, V. M. et al. 2000. "Expression of Neurogenin3 Reveals an Islet Cell Precursor Population in the Pancreas." *Development* 127(16): 3533–42.

Scully, Tony. 2012. "DIABETES IN Numbers." *Nature* (2011): 0–1.

Senk, Anita, and Valentin Djonov. 2021. "Collagen Fibers Provide Guidance Cues for Capillary Regrowth during Regenerative Angiogenesis in Zebrafish." *Scientific Reports* 11(1): 1–17. https://doi.org/10.1038/s41598-021-98852-6.

Serreze, David V., and Edward H. Leiter. 1994. "Genetic and Pathogenic Basis of Autoimmune Diabetes in NOD Mice." *Current Opinion in Immunology* 6(6): 900–906.

Seymour, Philip A. et al. 2007. "SOX9 Is Required for Maintenance of the Pancreatic Progenitor Cell Pool." *Proceedings of the National Academy of Sciences of the United States of America* 104(6): 1865–70.

Shapiro, A. M.James et al. 2021. "Insulin Expression and C-Peptide in Type 1 Diabetes Subjects Implanted with Stem Cell-Derived Pancreatic Endoderm Cells in an Encapsulation Device." *Cell Reports Medicine* 2(12): 100466. https://doi.org/10.1016/j.xcrm.2021.100466.

Shapiro, A.M. James et al. 2000. "ISLET TRANSPLANTATION IN SEVEN PATIENTS WITH TYPE 1 DIABETES I S L ET T R A N S P L A N TAT I O N I N PAT I E N T S W I T H D I A B ET E S U S I N G G LU C O C O R T I C O I D - F R E E I M M U N O S U P P R E S S I O N." *Journal of Medicine*.

———. 2006. "International Trial of the Edmonton Protocol for Islet Transplantation." *New England Journal of Medicine* 355(13): 1318–30.

Sharrock, Abigail et al. 2020. "NTR 2.0: A Rationally-Engineered Prodrug Converting Enzyme with Substantially Enhanced Efficacy for Targeted Cell Ablation." : 1–26.

Shiraki, Nobuaki et al. 2014. "Methionine Metabolism Regulates Maintenance and Differentiation of Human Pluripotent Stem Cells." *Cell Metabolism* 19(5): 780–94. http://dx.doi.org/10.1016/j.cmet.2014.03.017.

Shoffner, Adam et al. 2020a. "Tp53 Suppression Promotes Cardiomyocyte Proliferation during Zebrafish Heart Regeneration." *Cell Reports* 32(9): 108089. https://doi.org/10.1016/j.celrep.2020.108089.

———. 2020b. "Tp53 Suppression Promotes Cardiomyocyte Proliferation during Zebrafish Heart Regeneration." *Cell Reports*.

Sims, Emily K. et al. 2021. "100 Years of Insulin: Celebrating the Past, Present and Future of Diabetes Therapy." *Nature Medicine* 27(7): 1154–64. http://dx.doi.org/10.1038/s41591-021-01418-2.

Singh, Sumeet Pal et al. 2017. "Different Developmental Histories of Beta-Cells Generate Functional and Proliferative Heterogeneity during Islet Growth." *Nature Communications* 8(1): 1–15. http://dx.doi.org/10.1038/s41467-017-00461-3.

———. 2022. "A Single-Cell Atlas of de Novo β-Cell Regeneration Reveals the Contribution of Hybrid β/δ-Cells

to Diabetes Recovery in Zebrafish." *Development (Cambridge, England)* 149(2).

Solar, Myriam et al. 2009a. "Pancreatic Exocrine Duct Cells Give Rise to Insulin-Producing β Cells during Embryogenesis but Not after Birth." *Developmental Cell* 17(6): 849–60.

———. 2009b. "Pancreatic Exocrine Duct Cells Give Rise to Insulin-Producing β Cells during Embryogenesis but Not after Birth." *Developmental Cell* 17: 849–60.

Sousounis, Konstantinos et al. 2020. "Eya2 Promotes Cell Cycle Progression by Regulating DNA Damage Response during Vertebrate Limb Regeneration."

Stanger, Ben Z., and Matthias Hebrok. 2013. "Control of Cell Identity in Pancreas Development and Regeneration." *Gastroenterology* 1(3): 233–45.

Steiner, Donald J., Abraham Kim, Kevin Miller, and Manami Hara. 2010. "Pancreatic Islet Plasticity: Interspecies Comparison of Islet Architecture and Composition." *Islets* 2(3): 135–45.

Stepniak, Ewa et al. 2006. "C-Jun/AP-1 Controls Liver Regeneration by Repressing P53/P21 and P38 MAPK Activity." *Genes and Development* 20(16): 2306–14.

Streisinger, George et al. 1981. "Production of Homozygous Diploid Zebra Fish (Brachydanio Rerio)." *Nature* 112(1): 25–37.

Sun, Hong et al. 2022. "IDF Diabetes Atlas: Global, Regional and Country-Level Diabetes Prevalence Estimates for 2021 and Projections for 2045." *Diabetes Research and Clinical Practice* 183: 109119. https://doi.org/10.1016/j.diabres.2021.109119.

Tarifeño-Saldivia, Estefania et al. 2017. "Transcriptome Analysis of Pancreatic Cells across Distant Species Highlights Novel Important Regulator Genes." *BMC Biology* 15(1): 1–19.

Teta, Monica et al. 2005. "Very Slow Turnover of β-Cells in Aged Adult Mice." *Diabetes* 54(9): 2557–67.

Theodoropoulou, Marily, and Günter K. Stalla. 2013. "Somatostatin Receptors: From Signaling to Clinical Practice." *Frontiers in Neuroendocrinology* 34(3): 228–52. http://dx.doi.org/10.1016/j.yfrne.2013.07.005.

Thorel, Fabrizio, Virginie Nepote, et al. 2010. "Conversion of Adult Pancreatic Alpha-Cells to Beta-Cells after Extreme Beta Cell Loss." *Nature* 464(7292): 1149–54. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2877635&tool=pmcentrez&rendertype=abstract.

Thorel, Fabrizio, Virginie Népote, et al. 2010. "Conversion of Adult Pancreatic Alpha Cells to Beta Cells after Extreme Beta Cell Loss." *Nature* 464(7292): 1149–54.

Tornini, Valerie A. et al. 2016. "Live Monitoring of Blastemal Cell Contributions During Appendage Regeneration." *Current Biology*.

Tsuji, Naoki et al. 2014. "Whole Organism High Content Screening Identifies Stimulators of Pancreatic Beta-Cell Proliferation." *PLoS ONE* 9(8).

Ueki, Yumi et al. 2015. "Transgenic Expression of the Proneural Transcription Factor Ascl1 in Müller Glia Stimulates Retinal Regeneration in Young Mice." *Proceedings of the National Academy of Sciences of the United States of America* 112(44): 13717–22.

Velazco-Cruz, Leonardo et al. 2019. "Acquisition of Dynamic Function in Human Stem Cell-Derived β Cells." *Stem Cell Reports* 12(2): 351–65. https://doi.org/10.1016/j.stemcr.2018.12.012.

Venegoni, Mauro. 2002. "Tacrolimus." *Occhio Clinico* 8(5): 29.

Veres, Adrian et al. 2019. "Charting Cellular Identity during Human in Vitro β-Cell Differentiation." *Nature* 569(7756): 368–73. http://dx.doi.org/10.1038/s41586-019-1168-5.

Voight, Benjamin F et al. 2011. "Twelve Type 2 Diabetes Susceptibility Loci Identified through Large-Scale Association Analysis." *Nature Genetic* 42(7): 579–89.

Vousden, Karen H., and Kevin M. Ryan. 2009. "P53 and Metabolism." *Nature Reviews Cancer* 9(10): 691–700.

Wang, R N, G Klöppel, and L Bouwens. 1995. "Duct- to Islet-Cell Differentiation and Islet Growth in the Pancreas

of Duct-Ligated Adult Rats." *Diabetologia* 38(12): 1405–11.

Weiss, Michael, Donald F Steiner, and Louis H Philipson. 2014. *Insulin Biosynthesis, Secretion, Structure, and Structure-Activity Relationships*. MDText.com, Inc., South Dartmouth (MA). http://europepmc.org/books/NBK279029.

Wu, Xunwei et al. 2010. "Calcineurin and ATF3: Opposite Roles in Squamous Skin Cancer." *Nature*.

Xu, Jin et al. 2018. "Inhibition of TBK1/IKKε Promotes Regeneration of Pancreatic β-Cells." *Scientific Reports* 8(1): 1–14.

Xu, Xiaobo et al. 2008. "Beta Cells Can Be Generated from Endogenous Progenitors in Injured Adult Mouse Pancreas." *Cell* 132: 197–207.

Xue, Angli et al. 2018. "Genome-Wide Association Analyses Identify 143 Risk Variants and Putative Regulatory Mechanisms for Type 2 Diabetes." *Nature Communications* 9(1).

Ye, Lihua et al. 2015. "Glucagon Is Essential for Alpha Cell Transdifferentiation and Beta Cell Neogenesis." *Development (Cambridge)* 142(8): 1407–17.

Yoneda, S. et al. 2013. "Predominance of β-Cell Neogenesis Rather than Replication in Humans with an Impaired Glucose Tolerance and Newly Diagnosed Diabetes." *Journal of Clinical Endocrinology and Metabolism* 98(5): 2053–61.

Zecchin, Elisabetta et al. 2004. "Evolutionary Conserved Role of Ptf1a in the Specification of Exocrine Pancreatic Fates." *Developmental Biology* 268(1): 174–84.

Zhao, Huan et al. 2021. "Pre-Existing Beta Cells but Not Progenitors Contribute to New Beta Cells in the Adult Pancreas." *Nature Metabolism* 3(3): 352–65.

Ziegler, Annette G. et al. 2013. "Seroconversion to Multiple Islet Autoantibodies and Risk of Progression to Diabetes in Children Anette." *Physiology & behavior*.

UNRAVELLING THE CONTRIBUTION OF DUCTAL PROGENITORS DURING BETA CELL REGENERATION IN ZEBRAFISH: A FOCUS ON CALCINEURIN

Diabetes is a global epidemic affecting nearly 10% of the population worldwide. The disease is coupled with a significant loss of the β cell mass, leading to dysregulation of the glycemia. Stimulating β cell regeneration holds great promise as a potential treatment for curing diabetic patients. Observations in diabetic patients and studies in mouse models of pancreatic injury showed that mammalian pancreatic cells exhibit plasticity towards insulin production. However, the regeneration process is slow and inefficient, posing challenges in understanding the underlying mechanisms. In contrast, the zebrafish, a model organism known for its remarkable tissue regeneration capabilities, can spontaneously and efficiently regenerate β cells after their destruction. This raises the intriguing question of how zebrafish are able to regenerate β cells. Therefore, the goal of our laboratory is to unravel the cellular and molecular mechanisms of β cell regeneration in zebrafish. In this thesis, we focus on investigating the contribution and the mechanisms underlying regeneration from pancreatic progenitors residing within the ductal tree.

To gain insights into the mechanisms of β cell regeneration from ductal progenitors, we conducted transcriptomic profiling of ductal cells following β cell ablation. Our data revealed intriguing candidates, including the protein phosphatase calcineurin and the tumour suppressor p53. We demonstrated that repression of calcineurin accelerates β cell regeneration, whereas overactivation of calcineurin suppresses regeneration, leading to dysregulation of blood glucose levels. Specifically, calcineurin repression enhances ductal cell proliferation and the subsequent formation of endocrine progenitors, ultimately depleting the progenitor pool. Overall, our findings indicate that calcineurin finely regulates the balance between progenitor proliferation and endocrine differentiation, ensuring proper β cell regeneration. Calcineurin emerges as a crucial guardian of the progenitor pool. Conversely, our results also highlight the importance of p53 in promoting ductal cell proliferation and subsequent β cell regeneration, as demonstrated by the inhibitory effect of the p53 inhibitor, PFTα.

Furthermore, we observed that regenerated β cells exhibit a bi-hormonal phenotype, co-expressing the sst1.1 and ins hormones. We discovered that the δ1.1 sub-population rapidly converts into insulin-producing cells. Notably, these cells also originate from the ducts, suggesting that the two cellular sources of regenerated β cells are not mutually exclusive but rather coordinately involved.

Our study uncovers novel cellular and molecular mechanisms underlying β cell regeneration in zebrafish. These findings shed light on the pivotal role of progenitor proliferation and the contribution of δ sst1.1 cells in this process. Collectively, these insights provide valuable clues that may contribute to improving β cell regeneration in mammals.