University of Liège
Faculty of Medicine
GIGA-Cancer

Metastasis Research Laboratory
Prof. Vincent CASTRONOVO

Contribution to the study of Myoferlin in Vesicle-Mediated Cancer Cellular Communication

Karim FAHMY

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Index

Introduction

1. CELLULAR COMMUNICATION

Cellular communication is a fundamental phenomenon in all-multicellular organisms. All cells in a human body must communicate with each other's to govern biological functions, to ensure all processes from the development of an egg, to the survival, and to the reproduction. In multicellular organisms, cell survival is dependent on the reception of information from their environment [1].

Cellular communication is a generic term that includes all types of inter and intra-cellular information processing. It is how a cell sends the information in the proper route, depending on the nature of the signal and of the target cells; and how a message is received and translated in the recipient cell with the known three steps of cellular response: reception, transduction and response.

McCreas described the cell-cell communication as "The music that the nucleus hears" as cell's response to the surrounding environment affects gene transcription programs. It means that cell-cell communication intervenes in morphogenesis, cell differentiation, homeostasis, cell growth and cell-cell interactions [2].

1.1. Overview on different types of vesicles in cellular communication

Cellular communication is based on sharing information that could be achieved via different signaling mechanisms thus it could be generally classified into direct and indirect; or by the signal's path into intracrine, autocrine, juxtacrine, paracrine, synaptic signaling, endocrine or microvesicles as described in the table below (table 1).

Signaling mechanism		Produced signal	Explanation
Direct	self-self communication	Intracrine	Signal is produced in the target cell and affecting itself
		Autocrine	Signal is produced by the target cell and affecting itself via a receptor
	adjacent communication	Juxtacrine	Signal targets adjacent cells that are connected by cell-cell junction
Indirect	local communication	Paracrine	Signal secreted interacts with nearby target cells
		Synaptic signaling	Neurotransmitter secreted in synapse of close cells
	long distance communication	Endocrine	Signal secreted into blood stream targeting distant target cells
	microvesicles		Signal secreted in extracellular microvesicles

Table 1: Illustration of different types of cellular communication pathways.

However in here, we will focus on vesicle-mediated cellular communication with a special interest into exocytosis and exosomes.

1.2. Focus on exocytosis vesicles and exosomes

Both exocytotic vesicles and exosomes are considered as indirect cellular communication tools. They serve for the delivery of a certain message for a nearby or distant target cells. Indeed, the origin of genesis is different but they both share some common characteristics. They are both vesicles made of phospholipids, both crafted within the cell and harboring selected components. While exocytosis is the direct secretion of soluble proteins in the extracellular milieu; exosomes are also considered as the result of the secretion of intraluminal vesicles into the extracellular milieu. They both require a fusion process with the plasma membrane to secret their cargo or themselves, for exocytotic vesicles and exosomes, respectively. This fusion process implicates an interaction with the plasma membrane that is commonly mediated by Soluble N-ethylmaleimide-sensitive-Factor-Attachment-proteins (SNARE proteins). Several ferlin proteins have been reported to mediate different membrane and vesicle fusion processes and interestingly, some of them have even been reported to interact with SNARE proteins; what brings to this protein family a particular interest.

2. FERLIN FAMILY

Ferlin is a proteins family with a main identifier of harboring multiple tandem C2 domains. This protein group shows ancient origins in eukaryotic evolution and is detected in all eukaryotic domains, including unicellular organisms [3]. This suggests an origin in a common ancestor predating eukaryotic appearance.

Up to date, six members of the mammalian ferlin family have been identified but yet they have not all been characterized. The nomenclature of “Ferlin” is in reference to their parental orthologue gene Fertilization factor 1 (Fer-1) in *Caenorhabditis elegans* [4]. The protein encoded by this gene plays an important role in spermatid motility. A mutation in this gene provokes infertility of the *C. elegans* [5]. Ferlin proteins are usually named based on “Fer-1-L” prefix for “Fer-1-Like”, followed by a number representing one single protein of the family. The six known members of the ferlin family are fer1L1 (known as Dysferlin), fer1L2 (known as Otoferlin), fer1L3 (known as Myoferlin) and the last uncharacterized members are fer1L4, fer1L5 and fer1L6.

2.1. Structure of Ferlins

All mammalian ferlin members are about 200-240 kDa proteins with alternative splicing reported for several paralogues. But in general terms, they all share some common characteristics; they are all type II transmembrane proteins with a single pass transmembrane region at the carboxy terminus (figure 1). They all harbor a series of positively charged residues within the transmembrane domain that is thought to be important for their anchoring at the inner leaflet of the plasma membrane. The cytosolic part of the proteins contains multiple C2 domains. Some members harbor specific domains like SH3, FER-I, Fer-A, Fer-B, and DysF [5]. Finally a putative extracellular domain was confirmed in one study conducted by Bernatchez [6] where myoferlin-Hemagglutinin tag (at the carboxy terminus) has been immunodetected with anti-Hemagglutinin antibody in non-permeabilized COS7 cells implying that this domain is indeed an extracellular one.

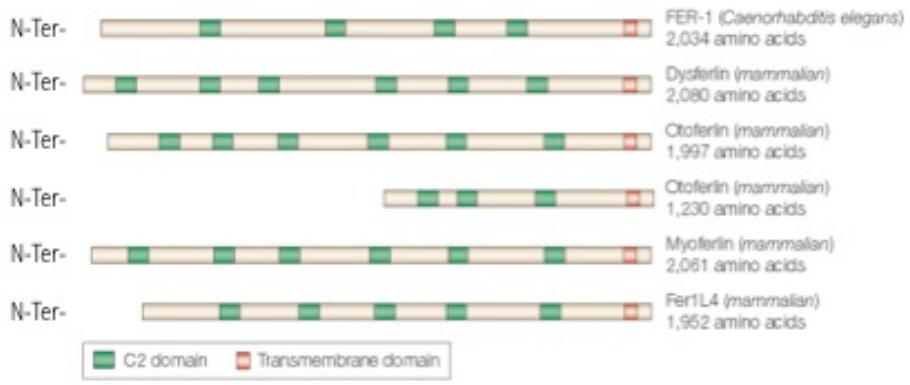

Figure 1: Ferlin protein family. Schematic structure of members of ferlin proteins showing common characteristics: type II transmembrane proteins harboring a tandem repeated C2 domains in their cytoplasmic region. P.L. McNeil et al., 2005 [7]

2.2 C2 domains

Ferlin proteins family is a unique family in terms of their possession of multiple C2 domains. In fact, there are about a hundred known C2 domain bearing proteins and they do not possess more than a couple of C2 domains with an exception of "Multiple C2 domain Transmembrane Proteins" bearing 3 C2 domains, and "Extended Synaptotagmins" (also known as Tricalbins), bearing 3 to 5 C2 domains. In general, all members of ferlin have at least five and up to seven C2 domains. The non-ferlin C2 domain bearing proteins are known to be membrane-associated proteins implicated in protein-lipid, protein-Ca^{2+} or protein-protein interactions [5].

The C2 domains in ferlin proteins are named from the amino terminal to the carboxy terminal to be C2A, then C2B and so on. C2 domains are defined to be a phospholipid binding domains involved in fusion processes in a Ca^{2+}-dependent manner. Of note, C2 domains of the well characterized protein Synaptotagmin have been shown to mediate

exocytotic vesicle fusion in a Ca^{2+}-dependent manner. Back to ferlins, crystallographic studies have shown that the C2 domains are independently folding domains composed of 8 beta-strands forming a sandwich structure. A Ca^{2+}-binding loop resides at one end of the beta-sandwich structure (figure 2) and that the Ca^{2+}-binding is mediated by a conserved aspartic acid residue [8].

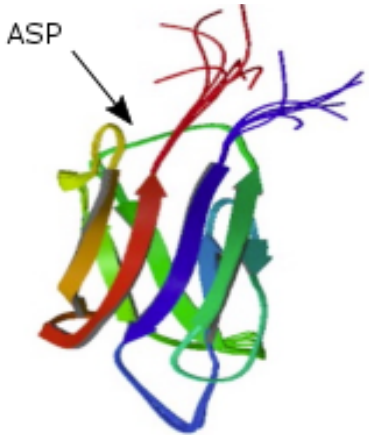

Figure 2: Crystallographic structure of C2 domain. Solution structure of the first C2 domain of myoferlin. Arrow representing Aspartic acid residue. Fahmy et al., 2015 .[9]

C2A domain, the first C2 domain from the amino-terminal, of both myoferlin and dysferlin binds to negatively charged phospholipid mixture similar to the phospholipid composition of the inner leaflet of the plasma membrane [4]. Of interest, it has been shown that only the C2A domain is able to bind a mixture of 50% phosphatidylserine and 50% phosphatidylcholine in the presence of 1mM of Ca^{2+}. However this C2A domain failed to bind a mixture of 25% phosphatidylserine in 75% phosphatidylcholine, meaning that this domain requires a certain amount of negatively charged phospholipids to bind. The requirement of negatively charged phospholipids may be influenced by the number of positively charged residues in the Ca^{2+}-binding loop in the C2A domain. This could hypothesize that myoferlin and dysferlin interact with specific regions of the plasma membrane enriched with high concentration of negatively charged phospholipids [4].

Of interest, it has been shown that neither myoferlin nor dysferlin can bind phospholipids in resting Ca^{2+} concentration, which is about 0,1 µM; however they require 1 µM and 4,5 µM of Ca^{2+} concentration, respectively. It has been shown that cytosolic Ca^{2+} concentration can reach 1 µM or even more after various stimuli including depolarization and ligand binding [10]. Meaning that myoferlin and dysferlin are implicated in specific processes inside the cell. More interesting, a point mutation in myoferlin (I67D) provokes an abolishment of the Ca^{2+}-dependent phospholipid binding. Similar results have been shown with dysferlin, where point mutation at the same amino acid position, (V67D), also provokes a decrease in Ca^{2+}-dependent phospholipid binding properties. It means that the introduction of neutral amino acid instead of an acidic residue alters Ca^{2+}-dependent phospholipid binding [4].

The same study has shown that unlike the C2A domain, the remaining C2 domains of myoferlin were unable to bind this mixture of 50% phosphatidylserine and 50% phosphatidylcholine. But this does not imply that they do not bind to other mixtures of phospholipids. In fact, their role is still unknown but like all the other C2 domains, they are probably implicated in protein-protein interactions. However, this has been criticized [11] and will be detailed later on.

In contrast, analysis of otoferlin C2 domains showed, except the C2A, a Ca^{2+}-dependent binding to liposomes composed of 15% phosphatidylserine, 55% phosphatidylcholine and 30% phosphatidylethanolamine. Otoferlin C2A domain was demonstrated to have a significantly shorter top loop and lacks Ca^{2+}-coordinating aspartates. Thus, it is predicted to be unable to bind with Ca^{2+} [3] and considered not to have a functional C2A domain.

Of interest, the similarity between C2 domains in paralogues is more important than between C2 domains within the same protein, e.g. the similarity of dysferlin-C2A domain is 74% with myoferlin-C2A while its similarity with other C2 domains of dysferlin is up to 15% [4][5].

Otoferlin lacks Ca^{2+}-sensitive C2A domain implying that in spite of the overall similarity of the protein family, each member has its specialized function. This does not imply that they can not interact together, co-help each other or even compensate each other's functions, at least partially.

2.3. Non C2 domains

Ferlins can be segregated into two major categories based on their possession or not of a DysF domain. "Type I ferlin group" possesses a DysF domain; this group includes Fer1L1, Fer1L3 and Fer1L5; while "Type II ferlin group" does not have a DysF domain and it includes Fer1L2, Fer1L4 and Fer1L6. However, the function of this DysF domain is still mysterious. Another common feature of all ferlins, is that they all possesses Fer domains (FerI, FerA or FerB), which are specific motifs of 60 to 70 residues with a well conserved secondary structure, with a yet unknown functions. However, it seems that FerA appears to occur concomitantly with DysF domains (figure 3), implying probably a functional relationship [3]. Ferlins have a highly conserved C2B-Fer1-C2C motif, C2D and 2 C-terminal C2 domains (C2E and C2F) adjacent to the transmembrane domain; while C2A and C2DE are variably expressed in different ferlins. This means that Fer1 motif may be essential for ferlin protein function as it is a well conserved domain [3].

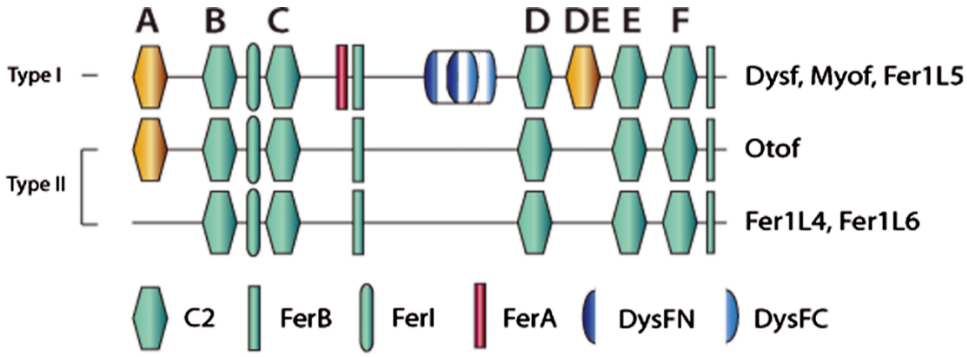

Figure 3: Ferlin protein structure. Figure showing the similarities and the differences between the different ferlin members and the two ferlin groups (I and II). Angel et al., 2012 [3].

3. FERLIN FUCTIONS

Since all ferlins share common structural characteristics, especially tandem C2 domains, their structure-activity relationship proposes a role that is dependent on their phospholipids binding capacity, like membrane fusion and fission processes.

3.1. Dysferlin function

Dysferlin is the first member of mammalian ferlin family to be discovered and characterized. Its encoding gene was first identified to be responsible for two inherited recessive autosomal diseases: the Limb Girdle Muscle Dystrophy type 2B and the Miyoshi Myopathy. Dysferlin is a 230 kDa protein containing six C2 domains. It is highly expressed in adult skeletal muscles as well as in heart where its main immunoreactivity is at the cell membrane [5]. Dysferlin plays a major role in myofusion processes during myoinitiation and myoaugmentation. Moreover, it is implicated in muscle repair, where in its absence, muscle tears are not adequately repaired and myofibers undergo necrosis. A mutation in Dysferlin gene is characterized by defective membrane repair and interestingly accumulation of intracellular vesicles in the sub-sarcolemme area [12].

3.2. Otoferlin

Otoferlin was the second member of mammalian ferlin family to be identified. It is a 226 kDa protein containing five or six C2 domains depending on the used detection algorithm. It also harbors a Ca^{2+}-independent C2A domain, which is thought to be inactive. Otoferlin is expressed in the auditory Inner Hair Cell ribbon synapse; precisely at the surface of synaptic exocytosis vesicles. Otoferlin mediates exocytosis of synaptic vesicles by binding to exocytosis component syntaxin-1 and Synaptosomal-associated protein of 25 kDa (SNAP25) in a Ca^{2+}-dependent manner [13]. Otoferlin mutation causes an autosomal recessive form of non-syndromic deafness; and in consequence otoferlin null mice are profoundly deaf [14].

3.3. Other ferlins

Caenorhabditis elegans' ferlin (fer-1) is the first ferlin to be identified; it is detected in the sperm acrosome membrane. Fer-1 mutant worms are infertile as they are defective in fusion of spermatid vesicular organelles with plasma membrane during spermatogenesis [3][15]. The fusion of these spermatid vesicular organelles adds extra membrane to the plasma membrane at the fusion site; which is required for the extension of the pseudopodia that cause crawling of the spermatids. Ten different mutations have been characterized within Fer-1, five of them fall within the C2 domains. These mutations disrupt Ca^{2+}-sensitive fusion potentials [5]. Very interestingly, fer-1 mutants exhibit vesicles accumulation on the cytoplasmic face of the plasma membrane.

Drosophila ferlin (Misfire) is found in the testes and ovaries of the adult flies where it plays a role in fertilization. Male *Drosophila* mutants show defects in sperm plasma membrane break-down post-fertilization, a process involving Ca^{2+}-dependent exocytosis and essential for release of the sperm DNA [3][16].

Fer1L4, Fer1L5 and Fer1L6 are poorly studied and their functions are still unknown. They all harbor five to six C2 domains and databases suggest that they can undergo alternative splicing. Except for Fer1L5, which shows high similarity to myoferlin; it binds to EHD family (EHD1 and EHD2) and is implicated in myoblast fusion [17].

4. Discovery and function of myoferlin

Fer1L3 was discovered in 2000 by Davis et al. [18] and Britton et al. [19]. They reported the expression of fer1L3 in both heart and skeletal muscles at both mRNA and protein levels. They named fer1L3 as "Myoferlin" due to its high similarity with dysferlin and its expression in muscles. This study has reported myoferlin to be expressed at the plasma membrane, cytosol, nucleus and nuclear envelope as well.

The study of its structure revealed that it is a 235 kDa protein predicted to have 2061 amino acids. It contains six C2 domains: C2A at the amino acids 1-82, C2B at 200-281, C2C at 359-458, C2D at 1126-1231, C2E at 1538-1638 and finally C2F at 1790-1903. Myoferlin possess also a SH3 domain at amino acids 676-689 lodging between the C2C and C2D domains; and FerI, FerA, FerB and two DysF domains.

It also has five potential tyrosine phosphorylation sites (629-953-1593-1611-1835) and 3 potential cAMP and cGMP-dependent protein kinase phosphorylation sites (35-183-575), which open the possibility of a potential role in cell signaling. It also bears a single transmembrane domain at 2027-2042 and finally an extracellular domain at 2043-2061 (figure 4).

Myoferlin has 8 isoforms with a molecular weight ranging from 17 to 235 kDa. But up to date, it is not known whether the different isoforms play different roles in the cell or not.

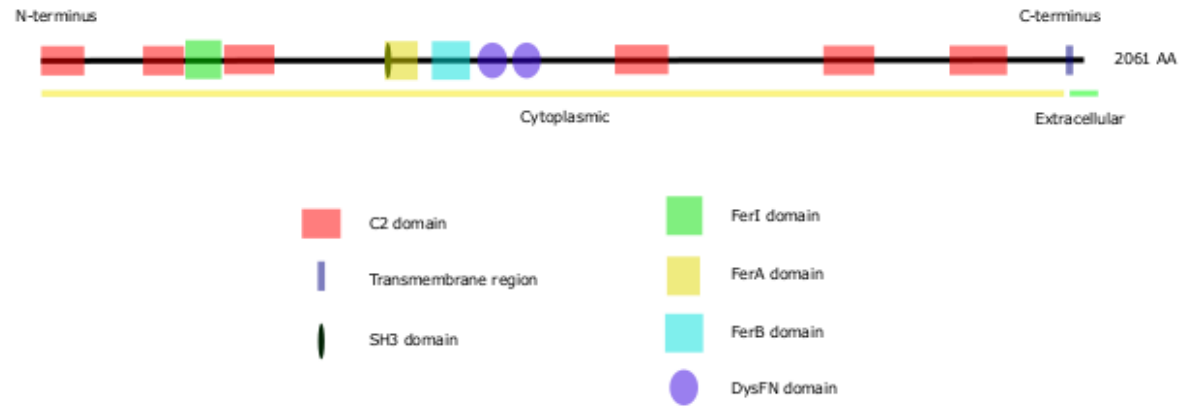

Figure 4: Schematic structure of myoferlin. Myoferlin structure showing multiple C2 domains, SH3 domain, FerI-A-B domains and two DysFN. Compiled from Davis et al, 2000; Britton et al, 2000; and GenPept at NCBI.

4.1. Myoferlin in non-cancer context

Myoferlin role has been described in different non-cancerous contexts. Myoferlin is highly expressed in myoblasts; especially those myoblasts that have begun to differentiate, and plays a role in myofusion. Myoferlin also plays a role in endocytosis and endocytic recycling in endothelial cells.

Myoferlin in muscles

Different studies have revealed myoferlin to be directly implicated in myoblast fusion where it is highly expressed in elongated myoblasts prior to fusion [20]. It has been shown that myoferlin is expressed, early in myofusion, at the plasma membrane area at the fusion site but then, its expression is declined and dysferlin expression increases instead; underlying a specialized fusion of each protein in myoblasts fusion [4]. Of interest, it has been observed that transient vesicular structures appear at the site of membrane fusion. Doherty et al. proposed that these structures may derive from the endocytic recycling compartment [21].

Finally, it has been observed that myoferlin is overexpressed in muscle cells after injury. This overexpression of myoferlin participates into the muscle repair as in myoferlin null mice, a delay in membrane resealing is observed. The mechanism of myoferlin response to injury has been suggested by Demonbreun et al. to be mediated by the increase in intracellular Ca^{2+} concentration that in turn, increases the activity of calcineurin and NFAT. NFAT is a transcription factor family of 4 proteins, of which, at least NFAT 1 and 3 bind the promotor of myoferlin increasing myoferlin expression. Myoferlin null mice phenotype was reproduced in NFAT null mice, implying an upper handed control of NFAT on myoferlin expression/role [22].

Myoferlin in endothelial cells

While myoferlin was initially thought to be a muscle specific protein, a study revealed its expression in endothelial cells. In this context, myoferlin plays an important role in VEGF-receptor signaling upon VEGF induction. VEGF-receptor is a primary tyrosine kinase receptor responsible for the majority of VEGF functions in endothelial cells. VEGF promotes proliferation, migration of endothelial cells via VEGF-receptor phosphorylation and downstream activation/phosphorylation of ERK, JUN and PLCγ. Myoferlin was proved to complex dynamin 2 and VEGF-receptor via its SH3 domain that is known to interact with proline rich proteins, like dynamin. This complex formation protects VEGF-receptor from casitas B-lineage lymphoma (CBL) dependent polyubiquitination and subsequent degradation. Myoferlin depletion provokes a decrease in VEGF-mediated proliferation, migration of endothelial cells and VEGF-receptor recycling to the surface. In myoferlin null mice, it was observed a decreased expression of VEGF-receptor and VEGF-mediated vascular hyper-permeability. Interestingly in endothelial cells, myoferlin also regulates membrane resealing after physical damage, like in myoblasts [6].

Another study by Yu et al. confirmed the expression of myoferlin in endothelial cells showing its implication in the regulation of Tie 2-receptor fate, another tyrosine kinase receptor. Yu demonstrated that in a non-Tie 2-challenged system, myoferlin depletion decreased Tie 2 expression without alteration of its mRNA level, neither in a CBL-polyubiquitination nor in a proteasome dependent manner [23].

Myoferlin in endocytic recycling and vesicle fusion

Previous data would make one think that myoferlin governs all surface proteins trafficking; but it has been shown that myoferlin silencing has no effect on trafficking of non-tyrosine kinase receptors, like G-coupled protein receptor and Bradykinin receptor (B2R). It seems that myoferlin plays a role in trafficking of only some specific receptors [23].

Myoferlin contains the amino acid sequence “Asparagine-Proline-Phenylalanine”, a known binding motif of EHD proteins, within the second C2 domain of myoferlin (C2B). This binding was shown to be Ca^{2+}-independent as the C2B domain lacks a Ca^{2+}-binding motif. EHD proteins have been linked to endosomal trafficking including cell surface recycling.

Of interest, Davis et al. showed that myoferlin null mice myoblasts displayed a delay in transferrin-receptor recycling to the surface after transferrin uptake with no effect on transferrin uptake itself. This means that myoferlin does not seem to play a role in the endocytosis of receptor as much as its subsequent fate determination, in myoblasts [21]. While Bernatchez et al. showed that myoferlin depletion in endothelial cells provoked a decrease in transferrin uptake itself with no effect on surface receptor expression. In this study, Bernatchez highlighted a role of myoferlin in receptor-mediated endocytosis for both Clathrin-Dependent Endocytosis and Non-Clathrin-Dependent Endocytosis.

In endothelial cells, myoferlin depletion provokes a 50% decrease in the internalization of both Transferrin and Cholera Toxin B, which are clathrin pits and caveolin/lipid raft

dependent endocytosis, respectively. Mechanistically, myoferlin and caveolin-1 act on receptor endocytosis in a similar rather than parallel manner to mediate endocytosis. In a cell free system, myoferlin was shown to directly interact with caveolin-1. This shows that myoferlin is involved in both caveolin-mediated endocytosis and clathrin dependent endocytosis [24].

4.2. Myoferlin in cancer context

Since the role of myoferlin has been starting to get revealed in endothelial and muscle cells, showing an important implication in fusion, fission and endocytosis; there was an increasing interest in understanding the probable role of myoferlin in cancer progression. Immunohistochemical evidences available from "Human Protein Atlas" database suggest that myoferlin is strongly expressed in several cancer types including pancreas, stomach, liver, colorectal, ovarian, cervical, endometrial, thyroid, breast and lung cancers.

Up to date, myoferlin has been studied mainly in four cancer types: pancreas, breast, lung and oropharyngeal cancers.

Myoferlin in pancreas cancer

Myoferlin has been first identified to be overexpressed on the transcriptomic level in Pancreatic Ductal Adenocarcinoma (PDAC) [25–27]. Afterwards, its overexpression has been confirmed on the proteomic level thanks to the proteomic profiling of PDAC samples [28][29]. Later on, another study not only confirmed the overexpression of myoferlin in PDAC, but also revealed that myoferlin expression is significantly correlated with the degree of histological differentiation and that it is an independent prognostic marker for survival. Patients having high myoferlin expression have higher tumor grade and shorter survival. This seems to fit, as it is well-known in PDAC, that tumor grade is an important variable of survival where patients with high grade (poorly differentiated tumors) tends to have the worst prognosis [30].

In their study, Wang et al. showed that PDAC cell lines BxPC-3 and CFPAC-1 express high level of myoferlin and that myoferlin silencing decreased cell proliferation, migration and colony forming ability. The decrease in cell proliferation seemed to be due to both an increase in apoptosis and a cell cycle arrest in S phase. Moreover, mouse model of subcutaneously injected myoferlin-knocked down cells; showed smaller tumors compared to control counterparts.

Myoferlin in breast cancer

Myoferlin has also been shown to be overexpressed in breast cancer. Eisenberg and Friedman proposed a potential role of myoferlin in the breast cancer cell motility [31]. Li et

al. showed that myoferlin expression in breast cancer cell lines correlates with their invasive potential. Myoferlin is highly expressed in highly invasive cells (MDA-MB-231 and BT 549); moderately expressed in less invasive cells (MCF7 and T47D); weakly expressed in non-invasive MCF10A cells. Li and coworkers also showed that myoferlin knockdown in MDA-MB-231 increased mesenchymal to epithelial transition (MET) and decreased invasive properties in matrigel and collagen-based assays. That was associated with a decrease in MMPs secretion and/or transcription [32].

The same group has further explained the effect of myoferlin knockdown on decreasing invasion potential to be related to the acquisition of an epithelial phenotype. This phenotype change, due to myoferlin depletion, promotes maintenance of cell-to-cell junctions and migration as cohesive communal cell sheet or cluster, not affecting Euclidian traveled distance but indeed affecting accumulated distance and velocity. In other words, myoferlin depletion promotes slow but directional migration. Blackstone et al. proposed that this effect is mediated via an increase in focal adhesion kinase phosphorylation/maturation [33].

While myoferlin knockdown did not have an effect on neither cell cycle nor cell growth *in vitro*; myoferlin knocked-down in MDA-MB-231 cells gave smaller tumors and demonstrated less tumor burden when injected subcutaneously in nude mice. The tumors displayed high circularity and no invasion of the surrounding [33]. Leung et al. also confirmed the expression of myoferlin in breast cancer cell lines showing no differential expression in terms of metastasis potential or transformation.

Interestingly, our laboratory has revealed the control of myoferlin on the fate determination of EGF-receptor and its consequent effects on breast cancer cell lines. Turtoi et al. showed that myoferlin silencing enhanced and sustained EGF-receptor and down-stream AKT phosphorylation but not affecting internalization kinetics of EGF-receptor during EGF challenge in MDA-MB-231 cell line compared to counterpart control. They demonstrated that myoferlin silencing provoked a decrease in proteasome degradation of activated/internalized receptor proposing that sustained signaling is linked to the inability of the cells to properly degrade the receptor in the absence of myoferlin. The myoferlin down regulation was also able to reduce EGF-induced cell migration in Boyden chamber. The authors also demonstrated a decrease in epithelial to mesenchymal transition (EMT) after EGF treatment in MDA-MB-468 cell line. Finally, Turtoi et al. showed that myoferlin silencing in MDA-MB-231 provoked a decrease in tumor volume in Chorioallantoic membrane (CAM) assay, this decrease in tumor volume could be at least partially explained by an increase in apoptosis observed after an EGF challenging *in vitro* [34].

Myoferlin in lung and oropharyngeal cancers

Myoferlin has been shown to play a key role in lung cancer progression. Myoferlin-depleted Lewis Lung Carcinoma (LLC) cells gave 50 % smaller tumors and demonstrated less tumor burden when injected into nude mice. This effect was further confirmed to be due to a direct effect on LLC cell growth as there were neither increase in apoptosis nor a decrease in

micro-vessels density nor a decrease in VEGF-A serum levels, compared to counterpart control [35].

Recently, myoferlin has been reported to be expressed in non-small cell lung cancers patients sections and correlates with VEGF-receptor 2 expression and poor prognosis [36]. Another study showed that myoferlin nuclear staining is associated with a poor overall survival, increased hazard death, tumor recurrence, perineural invasion, extracapsular spread, high T-stage and distant metastasis in oropharyngeal squamous cell carcinoma patients [37].

5. Exocytosis

Exocytosis is the trafficking of secretory vesicles to the plasma membrane. It is the base of cellular communication in multicellular organisms through the release of a wide range of extracellular acting molecules like hormones, growth factors, plasma proteins, antibodies and extracellular matrix components to exert various functions. Exocytosis could be whether constitutive, like the continuous secretion of extracellular matrix components and plasma proteins; or regulated, as a response to an external stimulus, like neurotransmitters release at the synapses (figure 5). Cells may show different kinds of secretory granules composed of different cargos that could be secreted by the same cell in a constitutive or regulated manner to ensure different physiological functions [38].

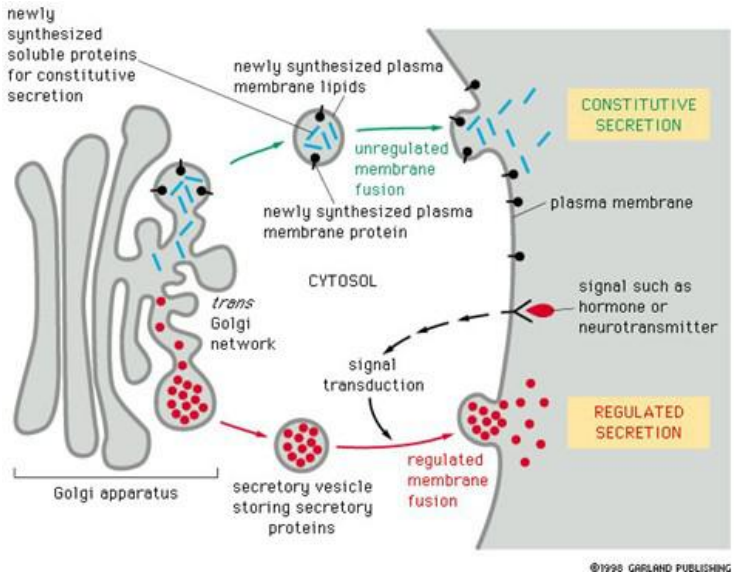

Figure 5: Illustration of exocytosis types. Constitutive exocytosis is when there is a non-stop secretion of extracellular components while regulated exocytosis is triggered upon extracellular stimuli. Alberts et al., 1998.

Exocytosis vesicles formation starts at the *trans*-Golgi network (TGN) where the cargo of soluble proteins assembles at the inner surface of the TGN. It is thought that this

aggregation of proteins is the main driver of the deformation of the TGN membrane and budding of vesicles. Nascent granules bud at the surface but they require a pinching to be released as immature secretory granules (ISG) into the cytoplasm. At that point, cholesterol seems to play an important role in mediating this fission process probably by directly facilitating negative membrane curvature at the bud-neck or indirectly by recruiting raft associated proteins that carry out the fission [38]. In ISG, the lipid bilayer of the secretory vesicle contains phospholipids, like phosphatidylserine, and proteins required for secretory vesicles acidification, transport, targeting and fusion. While the dense core of the secretory vesicles contains the soluble protein cargo to be secreted, which indeed differs in composition upon cell type and nature of the messenger [39] [40]. However, there are still unknowns in the exact composition of the vesicles membrane components that might be involved in vesicle transport, targeting and fusion.

The exact composition of the lipids and proteins of the unilamellar lipid bilayer membrane of vesicles are not yet totally revealed. However, studies have identified some negatively charged phospholipids like phosphatidylserine and phosphatidylinositol; also other phospholipids like phosphatidylcholine, phosphatidylethanolamine, sphingomyelin. There are evidences suggesting that negatively charged phospholipids on the outer surface of the vesicles membrane and cytoplasmic leaflet of the plasma membrane act as coupling factors enhancing interaction between negatively charged phospholipids and positively charged motifs of SNARE proteins. It is believed that the anionic characteristic of the serine head group of phosphatidylserine promotes interaction between phosphatidylserine and positively charged SNARE proteins. SNARE proteins like the vesicular Synaptobrevin (VAMP2), the membranous Syntaxin1a (Syt1a) and membranous synaptosome-associated protein of 25 kDa (SNAP25), mediate fusion events with the plasma membrane. All these proteins share a 60-70 long highly conserved juxtamembrane poly-basic amino acid sequence called SNARE motif that binds negatively charged phospholipids such as phosphatidylserine as mentioned [40]. It was proven that a mutation of the Ca^{2+}-calmodulin/phospholipids binding motif of VAMP2, decreasing its net positive charge, decreases its interaction with negatively charged phospholipids and interferes with the normal catecholamine secretion from chromaffin cells [41]. As well Synaptotagmin I (SYT1), a dual C2 domains bearing protein involved in membrane fusion events and exocytosis, binds to negatively charged phospholipids in a Ca^{2+}-dependent manner, thanks to its C2 domains. Increasing phosphatidylserine levels leads to an increase in Ca^{2+}-triggered vesicles fusion by stabilizing fusion pores in PC12 cells and increase Ca^{2+}-dependent SYT1 binding in liposomes [42].

5.1. VEGF and angiogenesis in pancreatic cancer

Pancreatic cancer is considered to be one of the most aggressive cancers. It is characterized by a very poor prognosis, with a five year survival rate ranging from 0 to 18% and most patients suffer from metastasis [43]. The median survival is reported to range between 2.5 to 8 months [44]. This is due to multiple factors including late diagnosis, resistance to

chemotherapy, high tendency of metastasis and a wide range of genetic and epigenetic alterations [45]. While surgical resection remains the only curative option; only 20% of the patients can benefit from this process [46]. However, the five year survival of that group of resectable patients grow only to 20% [47] and the median survival raises only from 13 to 21 months [44]. Overall, it is a common opinion that the decline in mortality observed in other cancer diseases has so far not taken place in pancreatic cancer [48].

Angiogenesis is required for invasive tumor growth and metastasis and it constitutes an important point in the control of cancer progression. For tumors to develop in size and metastasize, they must make an "angiogenic switch" through perturbing the local balance of pro-angiogenic versus anti-angiogenic factors [49]. Although pancreatic cancer is considered as a hypovascularized tumor due to its characteristics on imaging, yet angiogenesis is critical for the development and growth of pancreatic cancer [43]. The best-known pro-angiogenic protein is the vascular endothelial growth factor-A (VEGF-A). VEGF-A binds to VEGF-receptor 2 on endothelial cell and promotes the migration of endothelial cell [50].

In general, VEGF is an attractive therapeutic target. In pancreatic cancer the overexpression of VEGF and VEGF-receptors have been reported [51]. Furthermore, pancreatic cancer often exhibits enhanced foci of endothelial cell proliferation [49]. Overexpression of VEGF in pancreatic cancer is demonstrated to be significantly associated with microvesicles density (MVD) and that advanced stage of the disease is significantly more frequent with high MVD [52]. VEGF concentration correlates with poor prognosis in patients and animal models as well [51]. Actually, It has been described that VEGF transcription is increased in PDAC and that patients having a VEGF plasma concentration lower than 596 pg/ml have better survival chances [53]. Further studies showed that portal VEGF-A concentration is associated with tumor grade and size but not with survival [54]. Hypoxia is associated with the expression of pro-angiogenic factors, including VEGF that plays a pivotal role in pancreatic cancer liver metastasis [55]. Finally, it has been shown that patients having VEGF-receptor 2 negative tumors gain 10 months of survival compared to patients how are positive for the receptor [56].

6. Exosome biogenesis

Exosomes are small extracellular vesicles with a size ranging between 50 to 150 nm in diameter [57]. They correspond to the intraluminal vesicles (ILV) of the multivesicular bodies (MVB), which are secreted upon the fusion of the latter with the plasma membrane. ILV are formed by the reverse budding of the endosomal limiting membrane (figure 6); when hundreds of ILV are formed, this structure is referred as MVB. The latter structure could either fuse with lysosomes for the degradation of its content or fuse with the plasma membrane releasing ILV as exosomes to the extracellular milieu [57][58]. During the reverse budding of the endosomal limiting membrane, cargo proteins are segregated and packed within the lumen of the nascent vesicles. The mechanisms underlying the sorting of the proteins, genetic materials and lipids into the ILV, the docking and fusion of MVB with the plasma membrane are largely unknown [59].

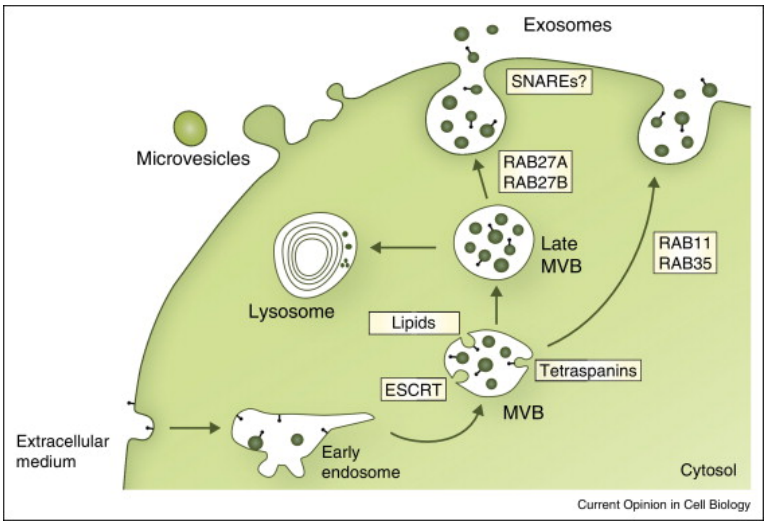

Figure 6: Biogenesis of exosomes from the endosomal compartment. Exosomes are produced by the reverse budding of the endosomal limiting membrane of the late endosomes. MVB can fuse with lysosomes for the degradation of their content or fuse with the plasma membrane for the release of exosomes. Kowal et al., 2014 [60].

Vesicles with hallmarks of exosomes have been isolated from diverse body fluids including blood [61], saliva [62], bile [63], breast milk [64], semen [65], urine [66], cerebrospinal fluid [67], ascites fluid [68] and amniotic fluid [69]. As well exosomes have been isolated from different cell culture supernatant of different cell types including erythrocytes [70], B-lymphocytes [71], dendritic cells [72], neurons [73], mast cells [74], intestinal cells [75] and more.

Since exosomes formation includes two inwards budding processes, exosomes maintain the same topological orientation as the cell, with membrane proteins on the outer side and some cytosolic content on the inner side [59]. The content of exosomes is very diversified as exosomes contain proteins, lipids and genetic materials.

Exosomes contain proteins that are usually present in the membrane regions prone to internalization, proteins of endosomal origin and cytosolic proteins. Generally, exosomal protein content is like heat shock protein (HSC70 and HSC90), cytoskeleton proteins (actin, tubulin, meosin), endosomal sorting complex required for transport “ESCRT” proteins (Alix and Tsg101), tetraspanins (CD9, CD63, CD81, CD82 and CD151) and proteins implicated in the transport (Rab2, Rab7 and Rab11) [59][76][57]. In consistency with their endosomal origin, no proteins from the nucleus, mitochondria or endoplasmic reticulum are found in exosomes. That is why markers of mitochondria (like complex IV) or endoplasmic reticulum (like GRP78) are often used as negative markers for exosomal isolation purity. Finally, some other protein content reflects the cellular origin of the exosomes, it means, harboring a specific marker of the cell of origin (figure 7).

Interestingly, it has also been reported that exosomes carry mRNA and miRNA [77][78]. This nucleic acid content of the exosomes was demonstrated to alter the transcriptome of the recipient cells and to provoke a phenotype change [79]. Moreover, it has been reported that

neuronal and myoblast-derived exosomes carry mitochondrial DNA, which is hypothesized to integrate into recipient cell's mitochondria, but yet this haven't been proven [57] [77].

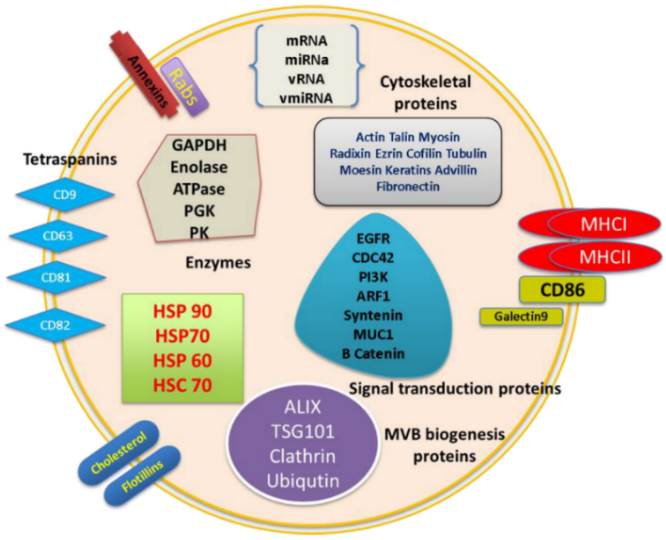

Figure 7: Exosomal content of proteins, lipids and nucleic acids. Charchar et al., 2015 [80].

Noteworthy, the total cellular content of mRNA was shown to be different from the exosomes, in other words, some mRNA were never reported to be present in exosomes, implying probably a selection mechanism. Some reports propose a Zip code at the 3'UTR that could interact with some sorting proteins [76]. On the other hand, the miRNA content is not reported to be different between cells and exosomes and it is believed that miRNA is just packed into exosomes during the reverse budding of the endosomal limiting membrane. However, some reports suggest that miRNA could be selectively packed via a physical interaction with the RISK complex proteins [76].

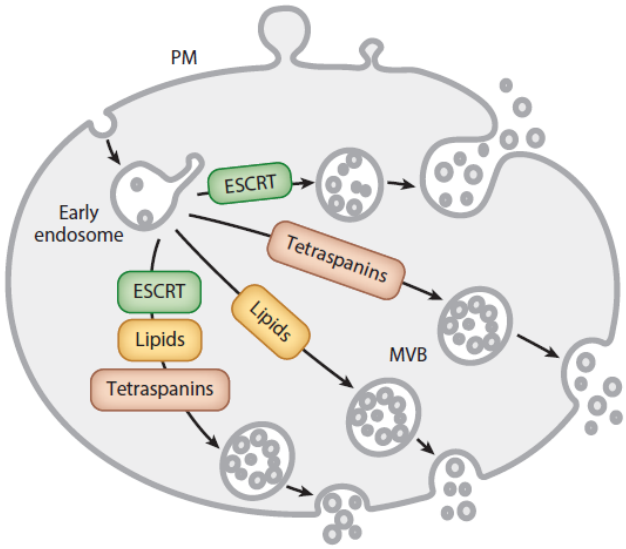

Figure 8: Illustration of the different possible exosomes biogenesis pathways. Colombo et al., 2014 [81].

In general, exosomes biogenesis is far from being totally understood. This could be due to the presence of different biogenesis pathways within the same cell and certainly within different cell types (figure 8). Overall, few biogenesis pathways were described, among then the ESCRT pathway. ESCRT proteins are a family of 20 proteins forming four soluble complexes, and they have been reported to play important roles in exosomes biogenesis. It has also been reported that lipids and tetraspanins are implicated in exosomes biogenesis via different mechanisms.

6.1. Exosomes in cellular communication

Pan and Johnston first described exosomes while studying the maturation of sheep reticulocytes during the eighties [82]. Exosomes were first thought to be a way for the cell to eliminate excess or unnecessary materials or materials that are unable to be degraded by the cell. However, a hypothesis was raised that exosomes could play a role in cellular communication. Since the discovery of genetic material in exosomes, this hypothesis was rendered stronger. Exosomes' cargos have been proven to alter the recipient cell's transcriptome and lead to a phenotype change.

In general, exosomes have been associated with the progression of some diseases like neurodegenerative, cardiovascular diseases and cancer; but also management of some of these conditions. It has been shown that exosomes derived from an *in vitro* culture of human embryonic stem cells are able to reduce infarction size when injected into murine and swine models of ischemia. Another example of exosome communication between endothelial cells and smooth muscles was shown when endothelial cell-derived exosomes, rich in mir143/145, were able to reduce atherosclerosis lesion formation when injected into a mouse model of atherosclerosis [79].

Many hypotheses have been proposed on how exosomes act on target cells. Exosomes can act directly on target cells via interaction with surface proteins/receptors of the recipient cells. Another possibility is that exosomes could be cleaved due to some protease activity releasing their content of soluble cargo that can interact with recipient cell surface receptors. Finally, exosomes can be totally internalized into recipient cells. The latter mechanisms have been proved by the transfer of labeled exosomes into recipient cells and the transfer of genetic material as well [77].

6.2. Exosomes in cancer communication

Since many cell types shed exosomes, one could expect cancer cells to adopt this very same communication pathway with their environment. Interestingly, it was reported that cancer patients' serum contain more exosomes than normal donors and that increased amount of exosomes correlates with advanced disease conditions. Further on, it has been shown that cancer cell-derived exosomes have higher miRNA content compared to normal cell-derived

exosomes. Moreover, miRNA content is also more abundant in exosomes derived from highly metastatic cancer cells compared to less metastatic ones. This enrichment of miRNA content in cancer cell-derived exosomes was not a reflection of an overall increase in cellular miRNA content when compared with normal cells but was a selective enrichment. Noteworthy, normal and cancer cell-derived exosomes also contain pre-miRNA but only cancer cell-derived exosomes harbors components essential for maturation of pre-miRNA into miRNA like DICER, RLC, AGO2 and TBRP. This group of factors were shown to be able to conduct the maturation of pre-miRNA in exosomes when exosomes were incubated in a cell free system [78]. This increase in exosome numbers and alteration of their content reflect the importance of exosomes in cancer cell communication.

Exosomes in tumor context have been proven to be associated to many events during cancer progression. Cancer-derived exosomes have been proven to be involved in angiogenesis where glioblastoma-derived exosomes' mRNA content was shown to be translated into pro-angiogenic proteins in the recipient cells [57]. Immune cell exosomes are reported to contribute in the battle against cancer. It has been reported that activated T cell exosomes expressing Fas ligand are able to promote apoptosis of Jurkat cells. While for B16 (murine melanoma) and 3LL (lung cancer) cells, FasL positive exosomes from activated T cells promote the invasiveness and migration potentials; in other words, it promoted a tumor immune escape [83].

It was very nicely shown by Melo et al. that the breast cancer cell MDA-MB-231 derived exosomes are able to provoke a global change of mRNA profile of the recipient non-tumorigenic epithelial MCF10A cells. This cancer cells derived exosomes were also able to increase viability, proliferation and colony forming ability of MCF10A cells *in vitro*. When MCF10A cells were co-injected with the cancer cell-derived exosomes into a mouse model, mice did develop tumors, while no tumors were detected in the non-exosomes condition. Moreover, cancer patients and normal donors' serum exosomes were isolated and co-injected with MCF10A cells into mice; almost fifty percent of the mice co-injected with cancer patients' serum derived exosomes developed tumors (5/11 mice) while no mice developed tumors when co-injected with normal donors' derived exosomes [78].

7. MYOFERLIN A THERAPEUTIC TARGET IN VESICLE-MEDIATED CANCER CELL COMMUNICATION

As previously mentioned, myoferlin has been identified to be overexpressed in different types of cancer including pancreas and breast cancer. Interestingly, our laboratory identified myoferlin as an accessible marker in PDAC in the aim of identifying new targets for diagnosis and therapeutic applications. This is based on the hypothesis that extracellular matrix and membrane proteins are the first line of physical interactions of cancer with the surrounding microenvironment. These proteins are of extreme interest in therapeutic applications if they are proved to be primordial for cancer cell viability.

Leung et al. proposed that in cancer cells, as they by-pass cell-cycle check points via genetic instabilities or DNA mutations, they are able to proliferate in a continuous manner increasing their requirement of nutrients uptake. This increase in nutrients uptake requires intense plasma membrane fusion and fission events. This uncontrolled proliferation may also imply an intense communication with the surrounding microenvironment [35]. The point is, since myoferlin is required for membrane integrity and remodeling to ensure homeostasis, it could be an interesting therapeutic target. Moreover, Bernatchez et al. raised the hypothesis that impaired cell growth and chemotaxis in endothelial cells lacking myoferlin may be due to a defect in the endocytic recycling [24].

A number of precious pieces of information would make one hypothesis that myoferlin is an important mediator for vesicle trafficking and cellular communication. Cellular communication is essentially based on sending and receiving information from one cell to the other. A great deal of cellular communications is handled by vesicles; like exocytotic vesicles or exosomes, which are both vesicles made of phospholipid.

Starting by otoferlin, as previously mentioned before, it has been proven that otoferlin is expressed in Inner Hair Cells where electron microscopy showed that it is located on the exocytosis vesicles. Otoferlin has been proven to interact with essential components for exocytosis like Syntaxin-1 and SNAP25 in a Ca^{2+}-dependent manner. Moreover, a mutation in otoferlin leads to deafness; implying a direct role in exocytosis of neurotransmitters.

Dysferlin, myoferlin and Fer-1 have all been shown to be involved in membrane fusion processes in different models, whether endothelial cell repair, myoblast fusion or *C. elegans* sperm motility. Interestingly, all three proteins down regulation were accompanied by the observation of an accumulation of different kind of vesicles, depending on the model and the protein, at the inner surface of the plasma membrane. This implies a key role in vesicles fusion with the plasma membrane. Moreover, during myoblast differentiation, myoferlin is observed to be present in vesicles [4]. Myoferlin, Dysferlin and Fer-1's structure-activity relationship implies a role in protein-protein, protein-lipid interaction in a Ca^{2+}-dependent manner, as they all possess multiple tandem C2 domains as shown previously. This has been confirmed by P. Johnson et al. where they showed that the ferlin proteins not only passively stick to membranes but concretely are able to sculpt lipid bilayers which result in high curved or distorted membrane regions that could facilitate membrane fusion and fission or recruitment of other membrane trafficking proteins [11]. Moreover, Posey and colleagues hypothesized that by analogy with synaptotagmin, a key protein in exocytosis, one could expect that exocytosis events such as those related to vesicles fusion, and membrane organelles fusion are regulated by ferlin proteins [5].

Of extreme interest, Redpath et al. have made a stunning discovery where they showed that dysferlin has a special splicing variant mRNA, called 40a, producing a protein that has a putative calpain cleavage site. This enzymatic cleavage was Ca^{2+}-dependent and gave rise to an about 70 KDa protein *in vitro*. As well *in vivo*, Dysferlin 70 kDa protein fragment, called mini-dysferlin, is also generated and has been proven to be functionally active in membrane repair after injury. Redpath's observation was extended to both myoferlin and otoferlin, where both protein generated a 70 kDa fragments similar to mini-dysferlin *in vitro*. But what

was extremely interesting is that calpain cleavage of myoferlin, unlike dysferlin and otoferlin, does not necessary require Ca^{2+}, however Ca^{2+} would just increase the cleavage yield [84].

These mini-proteins released harbor the very last two C2 domains at the carboxy terminal (C2E and C2F) with the transmembrane region and the extracellular domain as well. Previous phylogenetic studies revealed that the two most carboxy terminal C2 domains are the most evolutionally conserved within the ferlin family tree. This hypothesizes a preserved function among proteins harboring them. Also, these mini-proteins bearing these C2E-C2F domains have a structure similarity with synaptotagmin family (figure 9). Overall data strengthen the assumption of Doherty and coworkers, and proposed a role of myoferlin in constitutive processes like vesicles fusion events.

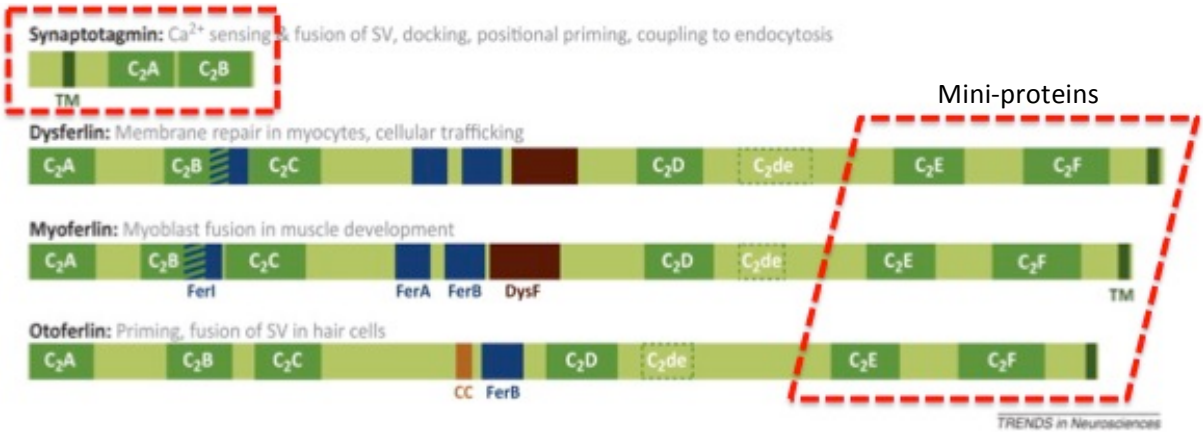

Figure 9: C2 domain bearing proteins. Calpain enzymatic cleavage produces mini-proteins resembling synaptotagmin. Modified from Pangrsic et al., 2012 [13]

Noteworthy to mention that a mathematical model was established to examine the role of myoferlin in cancer cell invasion; as a tool to generate hypotheses which then can be experimentally tested. This model proposed a diversified role of myoferlin in breast cancer invasion including a hypothesis that myoferlin impacts matrix metalloprotease production and/or secretion; hypothesizing a probable role of myoferlin in exocytosis [31].

Myoferlin has been reported to be located in different cellular compartments like plasma membrane, cytoplasm, nucleus envelope and nucleoplasm [85][5]. A recent study confirmed the previous observation but also emphasized on the colocalization of myoferlin with secretory pathway markers in general. Myoferlin strongly colocalizes with early endosomal Rab5-positive vesicles and most importantly with Rab7-positive large vesicles of late endosomes whether perinuclear or periplasmic. However, myoferlin showed occasional colocalization with Lamp-1-positive vesicles [86]. This may imply that myoferlin could be a player in endosomal recycling but, to a less extent, involved into the lysosomal degradation; raising a hypothesis that it could be involved in multivesicular bodies biogenesis and exosome biology.

Moreover, while investigating the role of myoferlin in myoblast fusion, Doherty et al. showed the presence of transient vesicle-like structures at the site of membrane fusion. Their data proposed that these vesicles may be derived from the endocytic recycling component [21]. The fact that myoferlin interacts with EHD protein family, known for its involvement in the endosomal trafficking [12], fortify the hypothesis that myoferlin could be present in endosomal recycling system and so implicated in exosome biogenesis or simply be a cargo of exosomes.

It was so worth verifying the presence of myoferlin in exosomes. Literature review showed the identification of myoferlin in exosomes isolated from trabecular meshwork cells [87] and urine [88]. Moreover, myoferlin has also been identified in cancer cell-derived exosomes from ovarian cancer [89], hepatocellular carcinoma [90], squamous carcinoma [91], prostate cancer [92], melanoma [93], colon cancer [94][95] and bladder cancer [96]. These studies did not directly investigate myoferlin expression or function in exosomes but myoferlin was present among a list of proteins, thanks to mass spectrometry proteomic profiling of these exosomes.

It is now concrete that myoferlin is present in exosomes but unfortunately no study has tried neither to validate the presence of myoferlin in exosomes by any other mean than mass spectrometry nor to investigate its role in exosome biogenesis and/or function.

Parts of this chapter are included in a review submitted for publication in Current Medicinal Chemistry.

Aims of the study

Breast cancer is the second leading cause of cancer death in females while pancreatic cancer is the fourth in the general population. Pancreatic cancer death rate is almost as its incidence rate, with a 5 years survival lower than 5%. This implies that more exhaustive research on breast and pancreatic cancers are required for a better understanding of the diseases. The comprehension of the molecular pathways and mechanisms that control the cancer progression is the key for developing new therapeutic tools aiding in the fight against this disease.

This study was based on the previous reports indicating an overexpression of myoferlin in different cancer types including breast and pancreatic cancers. These studies suggested that myoferlin might be implicated in tumor progression and metastasis dissemination, potentially and partially, by affecting different vesicular events. Myoferlin structure and reported activity suggest a potential role for this ferlin in cellular communication especially mediated by vesicles.

In this work, our first objective was to study the role of myoferlin in pancreatic cancer progression. We aimed to investigate whether myoferlin contributes to tumor growth and vascularization. Based on the reported role of myoferlin in different vesicle-mediated trafficking, we then investigated if myoferlin is involved in exocytosis of the main angiogenic factor VEGF-A; and if myoferlin is associated with angiogenesis and survival of PDAC patients.

The second objective of our work was to examine if myoferlin was present in pancreas and breast cancer cell-derived exosomes. We then aimed to determine myoferlin's putative role in both the formation and the secretion processes of exosomes by cancer cells and finally in the delivery of their cargo to the recipient cells, such as endothelial cells.

Results

PART I: STUDYING THE ROLE OF MYOFERLIN IN VEGF-A SECRETION AND VEGF-A MEDIATED ANGIOGENESIS IN PANCREAS CANCER MODEL

PUBLICATION I

Myoferlin plays a key role in VEGFA secretion and impacts tumor-associated angiogenesis in human pancreas cancer.

K. Fahmy, A. Gonzalez, M. Arafa, P. Peixoto, A. Bellahcène, A. Turtoi, Ph. Delvenne, M. Thiry, V. Castronovo, O. Peulen

International Journal of Cancer. 2016 Feb 1;138(3):652-63.

1. INTRODUCTION

Despite intensive research, pancreatic ductal adenocarcinoma (PDAC) remains one of the most deadly forms of cancers. Early-stage of the disease is clinically silent and the diagnosis of the disease is mostly made at an advanced stage. The late diagnosis contributes to one of the lowest 5-year survival rates (<5%). Today, PDAC are treated by surgery and/or adjuvant therapy, increasing only slightly the median survival of the patients. There is therefore an urgent need to develop new effective therapies for PDAC patients.

Myoferlin is a member of the ferlin family of proteins and is an ortholog of the ferlin-3 protein of C. elegans. Myoferlin is a calcium-sensitive phospholipid binding protein harboring several C2 domains and required for membrane processes, including cell fusion, endocytosis, membrane repair, and vesicle transport, in both normal [3,6,21,24,85] and cancer cells [35]. Our laboratory and others have reported the overexpression of myoferlin in several cancers including pancreatic adenocarcinoma at both gene [25,26,97] and protein levels [28–30]. However, very few studies have yet explored the potential role of this protein in cancer progression and development. Recently, myoferlin was found to be important for breast cancer invasion [31,32,98]. Our laboratory showed that myoferlin, is a key regulator of EGFR activity in human breast cancer cells [34]. Interestingly, it was reported, in a Kaplan-Meier survival curve, that patients with myoferlin-positive PDAC had a significant lower overall survival than those with a myoferlin-negative tumor [30]. As a consequence, myoferlin could represent a potential therapeutic target for PDAC, however the understanding of the mechanism by which myoferlin contributes to tumor growth and progression remains unclear.

Several genetically engineered-mouse models (GEMM) have been established and validate to study the PDAC and to address translational issues. Unfortunately, beside the fact that the GEMMs are not easily accessible and require dedicated facilities, their implementation in new therapy screening is time consuming. In consequence, there is an obvious need for simpler and faster PDAC models such as xenografts. We have recently optimized and validated a refined model based on the grafting of BxPC-3 cells on the chorioallantoic membrane (CAM) [99]. We used this model to evaluate the functional role of myoferlin in pancreatic tumor development. We observed a decrease in the tumor growth after myoferlin silencing. The tumor volume reduction was accompanied by a surprising pale color of the tumors, suggesting a decrease of the blood vessel density inside the tumor. We further investigated the VEGFA production/secretion by myoferlin-silenced BxPC-3 cells and showed that myoferlin is required for an optimal VEGFA secretion in cell-conditioned media.

2. Material and Methods

2.1 Cells and chemicals

Human pancreatic adenocarcinoma cells BxPC-3 (ATCC CRL-1687) were a generous gift from Prof. Bikfalvi (Inserm U1029, Bordeaux, France). PANC-1 cells (ATCC CRL-1469) were a generous gift from Prof. Muller and Burtea (NMR Laboratory, University of Mons, Belgium). All reagents were purchased from Sigma (Bornem, Belgium) unless mentioned otherwise.

2.2 Cell culture

BxPC-3 cells were culture in Roswell Park Memorial Institute - RPMI1640 medium supplemented with 2.5g/L glucose, 1mM sodium pyruvate and 10% heat inactivated Fetal Bovine Serum – (FBS). PANC-1 cells were maintained in DMEM supplemented with FBS (10%). Cells were cultured in a 37°C humid incubator supplied with 5% CO2. Cells were used between passage 1 and 10 and passed one to five by trypsin (200 mg/L) detachment when reaching near confluence.

2.3 Small interfering RNA transfection

BxPC-3 cells were transfected using Lipofectamine (Life Technologies, Carlsbad, NM) in DMEM medium supplied with 2mM Glutamine and 10% heat inactivated FBS and following the manufacturer's recommendation. Cells were transfected with 40nM siRNA against myoferlin (Eurogentec, Seraing, Belgium – siRNA#1 CCCUGUCUGGAAUGAGAUUTT; siRNA#2 GAUUGAGGGCCGACAGUUATT) or GL3 targeting luciferase (Eurogentec, Seraing, Belgium – CUUACGCUGAGUACUUCGATT) as irrelevant siRNA. Medium was changed 6h after transfection. Analyses were conducted 48h after medium change.

PANC-1 cells were transfected with 40nM siRNA against myoferlin using calcium phosphate as described previously [100]. Myoferlin siRNA sequences are the same as above. ON-TARGETplus non-targeting pool (Thermo Fisher-Dharmacon, Whaltham, MA) was used as irrelevant siRNA control.

2.4 Cell growth

Equal densities of cells were seeded in complete medium and were harvested at the indicated time-points. For rescue experiment, recombinant human VEGFA (R&D systems, Minneapolis, MN - 10 ng/ml) was added to 1% FBS culture media every 24h, cells were harvested 96h after the seeding. The cell numbers were indirectly determined using Hoechst incorporation. Results were expressed as DNA content.

2.5 Annexin V/propidium iodide staining

Proportion of apoptotic cells was determined by annexin V-FITC and non-vital dye propidium iodide (PI) staining with a FITC-Annexin V apoptosis detection kit I (BD Biosciences, Franklin Lakes, NJ) according to the manufacturer's instructions. Flow cytometry was performed on a FACSCalibur II™ and samples were analyzed using CellQuest™ software (BD Biosciences, Franklin Lakes, NJ).

2.6 Western blotting

Cells were lysed in RIPA buffer (NaCl 150mM, Na-deoxycholate 0.5%, Triton X-100 1%, SDS 0.2%, Tris-HCl pH 7.5 50mM) in the presence of protease and phosphatase inhibitors. Ten µg of heat denaturated proteins were separated by SDS-PAGE then electrotransfered onto nitrocellulose membrane.

Antibodies were purchased from Sigma (Bornem, Belgium): myoferlin antibody (rabbit - HPA014245), or Santa Cruz Biotechnology (Santa Cruz, CA): HSC70 antibody (mouse - sc7298). Antibodies were diluted in TBS-Tween (0.1%) containing milk (5%) and incubated for 2 h at room temperature. Corresponding secondary antibodies were diluted in TBS-Tween (0.1%) containing milk (5%) and incubated for 1 hour at room temperature.

2.7 Quantitative real-time PCR

Total RNA was extracted with trizol reagent (Life Technologies, Carlsbad, NM) using manufacturer's instructions. RNA concentration was measure using Nanodrop ND-1000 Spectrophotometer and RNA integrity was assessed using the Agilent 2100 Bioanalyser (Agilent Technology, Santa Clara, CA). Reverse transcription was realized using Transcriptor First Strand cDNA Synthesis kit (Roche, Basel, Switzerland). Human VEGFA was detected using FastStart Universal Probe Master (Rox) TaqMan reaction buffer (Roche, Basel, Switzerland), lab-designed probe (5'-CTTGCCTTGCTGCTCTACC-3') and primers (forward primer: 5'-AGTTCATGGATGTCTATCAGCGCAGCT-3', backward primer: 5'-CTTGCCTTGCTGCTCTACC-3'). Gene expression was normalized with the endogenous 18S rRNA using a commercially available probe based assay (Applied Biosystems, Carlsbad, NM; 4310893E).

2.8 VEGFA ELISA Assay

VEGFA protein level in the conditioned medium was quantified using Human VEGFA Quantikine ELISA Kit (R&D systems, Minneapolis, MN) following the manufacturer's protocol. VEGFA levels were normalized to the number of cells.

2.9 Immunofluorescence

After siRNA transfection procedure, 3.5 X 104 cells were plated on coverslip in 24-well plates. After 48 h of siRNA-mediated silencing, cells were washed with DPBS, fixed in PFA 4%, permeabilized with triton X-100 0.5% for 5 minutes, blocked with Bovine Albumin Serum 2% for 30 minutes. Coverslips were incubates overnight at 4°C with the primary antibody against VEGFA (Santa Cruz Biotechnology, mouse - sc73344) diluted in PBS-BSA solution. Afterwards, coverslips were washed 3 times in PBS-BSA solution and incubated with Alexa-fluor 488 conjugated (Invitrogen, A11029) or Alexa-fluor 633 conjugated (Invitrogen, A21070) secondary antibody for 1 h at room temperature. Followed by 3 additional washes and nuclei labeling with 10 ng/ml of Hoechst 33258 and sections were mounted with Mowiol. Colocalisation of myoferlin (Sigma, Bornem, Belgium, rabbit – HPA014245) and sec5/exoc2 (ProteinTech, Chicago, IL, mouse – 66011-1-Ig), a multiple protein complex essential for targeting exocytic vesicles, was evaluated after confocal image acquisition using Pearson's correlation coefficient, Mander's coefficient and Li's approach [101].

Images were acquired using Leica (Leica Microsystems, Wetzlar, Germany) TCS SP5 Confocal microscope and analyzed with ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2014).

2.10 Ultrastructural analysis

Cells were fixed for 1.5 h at 4 °C with 2.5% glutaraldehyde in a Sörensen 0.1 M phosphate buffer (pH7.4) and post-fixed for 30 min with 2% osmium tetroxide. After dehydration in graded ethanol, samples were embedded in Epon. Ultrathin sections obtained with a Reichert Ultracut S ultramicrotome were contrasted with uranyl acetate and lead citrate. Observations were made with a Jeol (Tokyo, Japan) JEM-1400 transmission electron microscope at 80 kV.

2.11 Tumor growth on chick chorioallantoic membrane (CAM)

As described previously, BxPC-3 cells were grown on CAM for 7 days then tumor volumes have been calculated as an ellipsoid. In order to visualize the tumor vasculature, tumors sections have been subjected to FITC-conjugated SNA (Sambucus nigra agglutinin) staining as previously described [99]. Densitometric profiling and quantification were performed using ImageJ (Rasband, W.S., ImageJ V1.48, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2014).

2.11 Immunochemistry and scoring

A series of 26 pancreas adenocarcinoma and 16 non-tumoral pancreatic tissues, including pancreatitis, were obtained from University Hospital, University of Pisa (Italy), as formalin fixed, paraffin embedded tissue blocks. These cases were previously described in a biomarker discovery study from our laboratory [28]. Sections were stained with antibodies against myoferlin (rabbit, Sigma) or PECAM-1/CD31 (mouse, Dako) followed by a second step using the according peroxidase-conjugate.

Sections were observed and scored by an independent pathologist (M.A.) who was blind to the purpose of the experiment. CD31 scoring was done according to the hotspot method. This technique is widely used in diagnosis and showed a significant correlation between microvessel density and PDAC grade [101]. The three most intense hotspots were identified and the average microvessel number per high power field (HPF – 400X, MCX100, Micros Austria, St Veit/Glan, Austria) was calculated. Myoferlin scoring was done by an evaluation of the intensity (ranging from 0 to 3) and of the extent of the staining (ranging from 0 to 4).

2.12 Myoferlin gene expression and survival analysis

Myoferlin gene expression in normal and PDAC tissue was analysed using the Pancreas Expression Database [102]. A population of PDAC patients (GSE2150122 [103]) were classify in high-risk and low-risk groups on the basis of survival. Data were censored on the survival months without stratification. Survival curves were compared by a log-rank Cox test. Myoferlin expression was analyzed in each group using the SurvExpress [104] web-based tool.

2.13 Ethics statement

All animal (CAM) experiments were approved by the Animal Welfare Committee of the University of Liège (approval #1590). All human sections were used with the agreement of the Human Ethic Committee of the University of Liège and from the University Hospital (approval #B707201420895).

2.14 Statistics

All results were reported as means with standard deviation (s.d.) or Standard Error Mean (s.e.m.) as described in legends. Statistical analysis was performed using one-way or two-way ANOVA depending on the number of grouping factors. Group means were compared by an unpaired Student’s t-test or Bonferroni’s post-test according to the group number.

P<0.05 was considered as statistically significant. All experiments were performed as several independent biological replicates.

3. RESULTS

3.1 Myoferlin is essential for BxPC-3 tumor growth on CAM

The functional role of myoferlin in pancreatic tumor growth on CAM was evaluated. Grafted BxPC-3 cells grew as a unique spheric nodule in the connective tissue of the CAM (Figure 1A). We observed a significant 50% decrease of the tumor final volume after myoferlin silencing by siRNA#1 or siRNA#2 (Figure 1B). 96h after silencing the myoferlin expression was reduced by 90%. The volume decrease was mainly due to a reduction of the tumor thickness. Intriguingly, the volume reduction was accompanied by a bleaching of the tumors compared to controls (Figure 1A), suggesting a decrease of the blood vessel density inside the tumor. The vessel density was evaluated after a FITC-conjugated Sambucus nigra (SNA) staining. It showed a drastic reduction of stained structures in the tumor core of the myoferlin-silenced condition compared to the no siRNA condition (Figure 1D-E). The lack of vessels in the tumor suggests an impairment of the tumor-associated neoangiogenesis.

3.2 Myoferlin-silencing reduces pancreatic cell growth

To investigate whether the tumor size reduction observed in the CAM assay was only due to the lack of vascularization or to a direct effect on the cell growth, we examined the BxPC-3 cell time-dependent growth after siRNA myoferlin silencing (Figure 2A). Our results showed an 80% and 50% cell growth reduction ($p<0.001$) respectively with siRNA#1 and siRNA#2. As VEGFA is known to stimulate the growth of pancreatic cells [105], we thaught that the cell growth reduction could have been the result of the alteration of a VEGFA autocrine loop by myoferlin-silencing. To test this hypothesis we tried to rescue the cell proliferation by adding VEGFA in the culture media of myoferlin-silenced cells. VEGFA was unable to lessen the inhibition of proliferation induced by the myoferlin-silencing (Figure 2D). We then asked the question whether this reduction was due to an induction of apoptosis and performed an annexin V/propidium iodide staining at 48, 72 and 96h (Figure 2B) following the silencing. None of the individual siRNA was able to significantly induce apoptosis. Comparable results were obtained using another human pancreatic cancer cell line, PANC-1. In these cells siRNA#1 and siRNA#2 reduced the cell growth respectively by 70% and 30% (Figure 2C, $p<0.001$) without inducing apoptosis (Figure 2D).

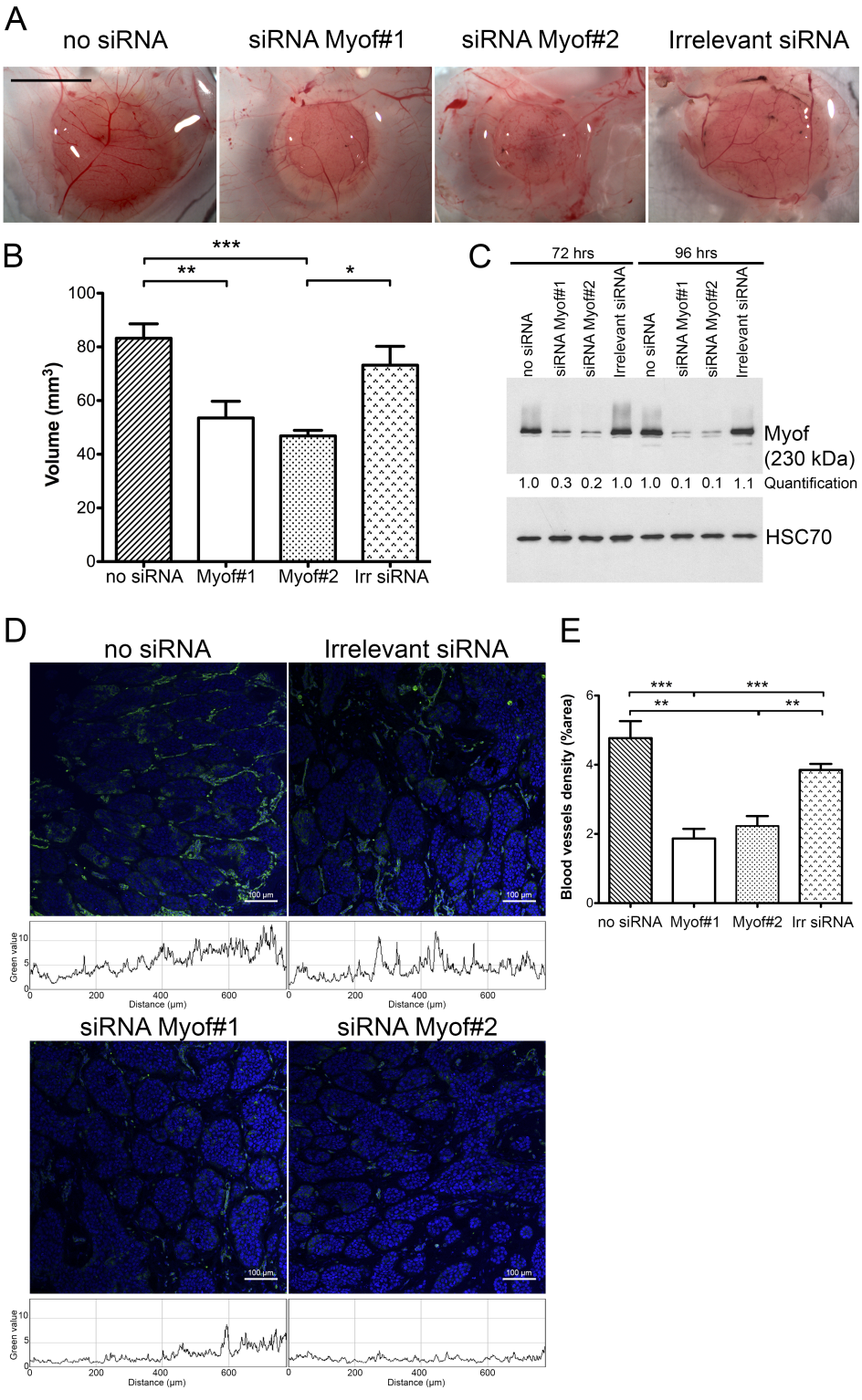

Figure 1. Effects of myoferlin silencing on BxPC-3 tumor growth on CAM and blood vessel density. (A) Macroscopic aspect of tumors obtained from control and myoferlin-silenced BxPC-3 cells implanted on CAM on day 11 of embryonic development and let to grow for 7 days. Pictures were taken from the bottom side view of the tumors. Scale bar represents 5 mm. (B) Tumoral volumes calculated using an ellipsoid formula at day 7 post-implantation. (C) Western blot detection of myoferlin in 10µg total proteins isolated from BxPC-3 cells implanted on CAM after siRNA transfection. HSC70 was used as a loading control. (D) Sections of tumors grown on CAM and collected at day 7 post-implantation. Sections were stained with FITC-conjugated SNA (green) visualizing blood vessels inside the tumors, and with DAPI (blue) visualizing nucleus. Scale bar represents 100 µm. Density profile of green channel (blood vessels) was represented under each section. (E) Blood vessel density (% of tumor area) in SNA stained sections. Results are expressed as mean ± s.e.m. ***P<0.001, **P<0.01, *P<0.05. n>8 in each condition.

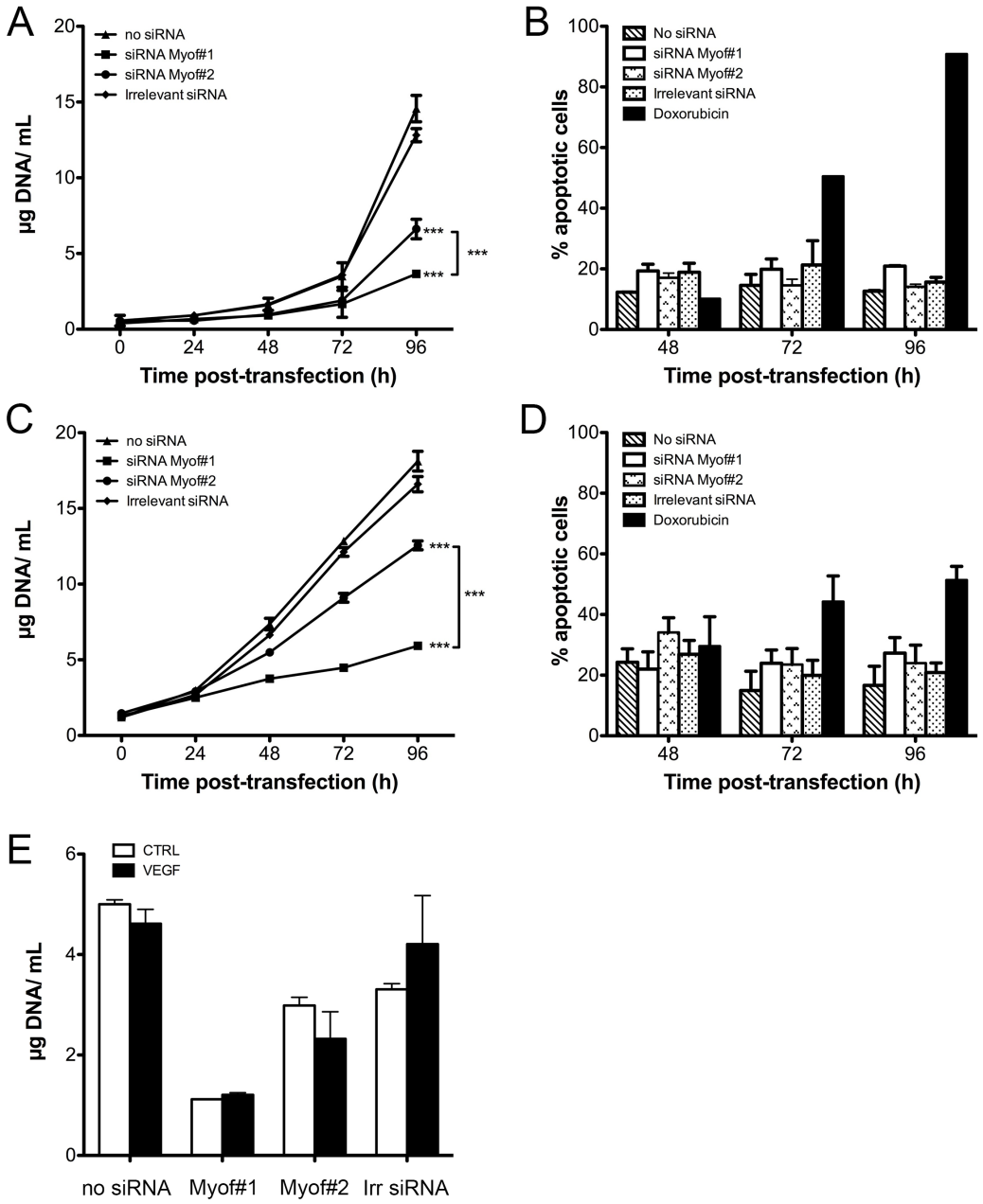

Figure 2. Effect of myoferlin silencing on BxPC-3 and PANC-1 cell growth. (A & C) Time-dependent effects of myoferlin siRNA transfection on (A) BxPC-3 and (C) PANC-1 cell proliferation. (B & D) Time-dependent effects of myoferlin siRNA transfection on (B) BxPC-3 or (D) PANC-1 apoptotic cell ratio by annexin V/PI flow cytometry. Doxorubicin was used as an apoptosis inducer positive control. (E) Rescue of a 96h BxPC-3 cell growth with addition of recombinant VEGFA (10 ng/ml). Results are expressed as mean ± s.d. ***P<0.001. n=3 in each condition.

3.3 Myoferlin is essential for VEGFA secretion by BxPC-3 cells

Since we showed that myoferlin-silencing reduced blood vessels density in BxPC-3 grown on CAM, we decided to focus on this aspect and checked whether myoferlin-silencing reduced VEGFA production. BxPC-3 cells were selected as a model, as they are known to produce more VEGFA than PANC-1 cells [106]. Thus, we measured the VEGFA concentration in conditioned medium and VEGFA gene expression in BxPC-3 cells after myoferlin-silencing.

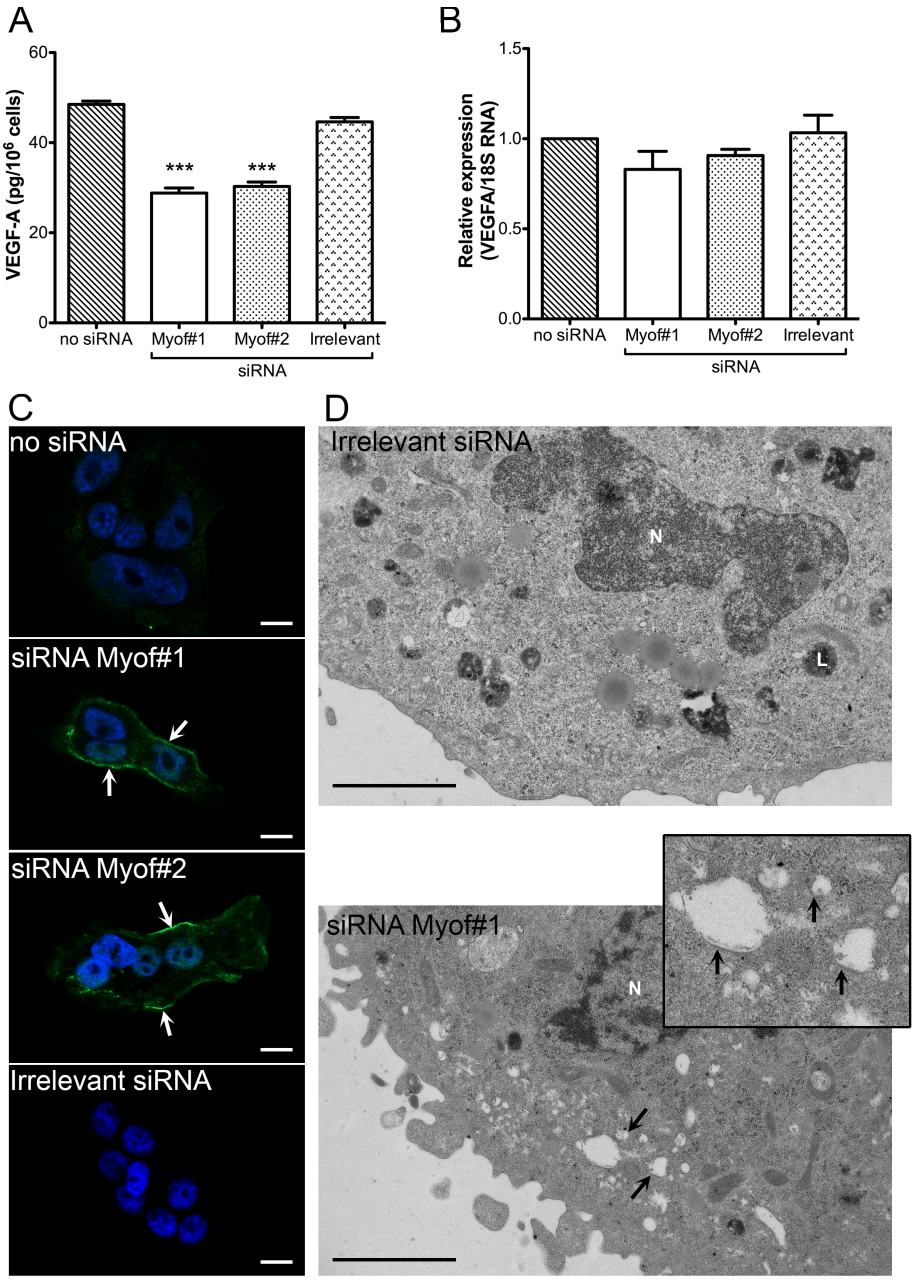

Figure 3. Effects of myoferlin silencing on VEGF secretion. (A) VEGFA concentration determined by ELISA in the conditioned medium of BxPC-3 cells after 48 h of siRNA-mediated myoferlin silencing. VEGFA concentration was standardized to the cell number. (B) Relative VEGFA gene expression of BxPC-3 after 48 h of siRNA-mediated myoferlin silencing. (C) Immunocytochemistry of BxPC-3 sparse cells 48 h after siRNA-mediated myoferlin silencing showing the effect of myoferlin silencing on VEGFA intracellular localization. VEGFA was stained with Alexa Fluor 488-conjugated antibody (green) and nucleuses were stained with Hoechst (blue). White arrows indicate VEGFA localization at plasma membrane. Scale bars represent 7,5µm. (D) Ultrastructural observation of BxPC-3 cells transfected during 48 h with siRNA against myoferlin. Scale bars represent 2µm. L = lysosomes, N = nucleus. Black arrows indicate accumulation of small vesicles close to the plasma membrane. Results are expressed as mean ± s.e.m. ***P<0.001. n=3 in each condition.

VEGFA concentration in the conditioned medium was determined by ELISA 48h after siRNA transfection and normalized to the cell number. Results showed a 40% decrease in VEGFA concentration in the conditioned medium of myoferlin-silenced condition ($p<0.001$) compared to both controls (Figure 3A). To determine whether VEGFA reduction occurs at transcriptional level, we analyzed VEGFA mRNA level by RT-qPCR 48h after siRNA transfection. We found that myoferlin silencing did not significantly affect VEGFA gene expression in BxPC-3 cells (Figure 3B).

Ferlins have been described as an active sculptor of lipid bilayers facilitating membrane fusion [11]. As such, we hypothesized that the observed VEGFA concentration decrease in conditioned medium after myoferlin silencing might be the result of exocytosis impairment. To test this hypothesis, we examined by immunofluorescence the VEGFA localization in BxPC-3 cells 48h after myoferlin siRNA transfection. Results showed no or low VEGFA staining in control conditions suggesting a dynamic exocytosis of the protein. In contrast to control cells, a clear accumulation of VEGFA at the vicinity of the plasma membrane was observed in myoferlin-silenced cells (Figure 3C). Furthermore, ultrastructural analysis revealed the accumulation of small vesicles, surrounded by a membrane, in the cytosol close to the plasma membrane (Figure 3D) in the myoferlin-silenced BxPC-3 but not in the control cells.

Immunofluorescence experiments indicate that in BxPC-3 cells, Myoferlin colocalized with sec5/exoc2, a component of a complex essential for targeting exocytic vesicles, mainly at the periphery of the cells (Figure 4A). Pearson's coefficient is the simplest way to measure the dependency of pixels in dual-channel images. Despite its sensitivity to background noise and its ambiguity with heterogeneous localization throughout the samples, it showed a 75 ± 6% (N=13) correlation (Figure 4D). In order to relieve the Pearson's limitations, we performed a Mander's correlation analysis.

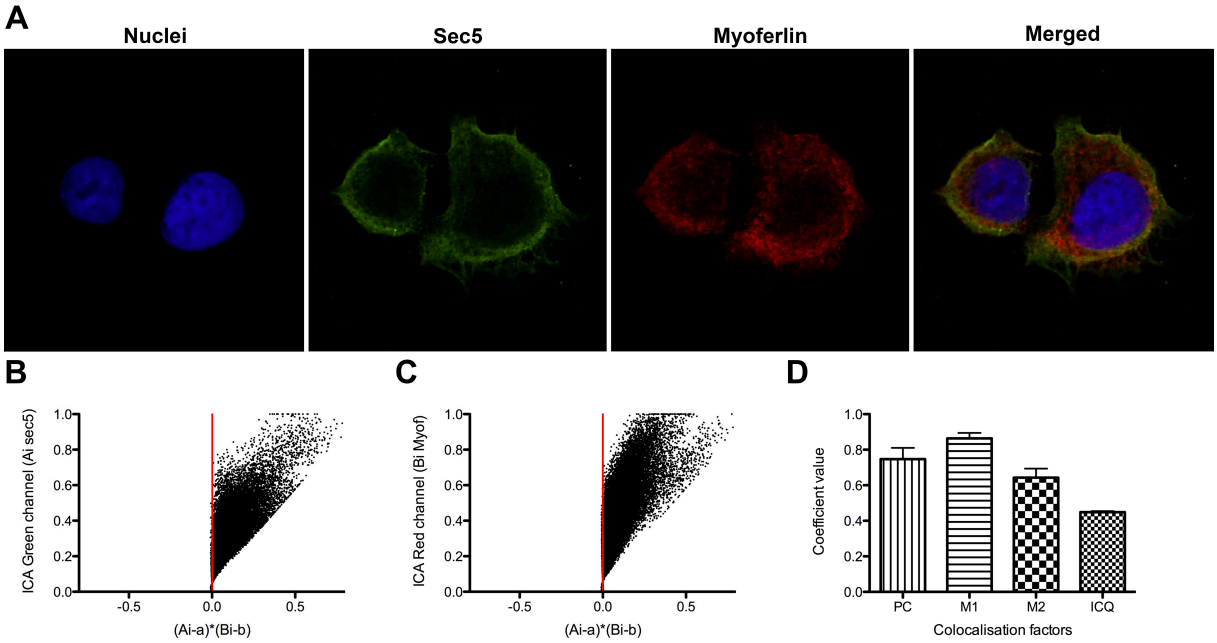

Figure 4. Colocalization of myoferlin and exocytic complex protein. (A) Representative image of BxPC-3 sparse cells stained for nuclei (blue channel), for sec5/exoc2 (green channel) and myoferlin (red channel). Merged image show partial colocalization of sec5/exoc2 and myoferlin. (B-C) Representative intensity correlation analysis (ICA) performed for complete colocalization in the (B) green channel (sec5/exoc2) and in the (C) red channel (myoferlin). (D) Mean value (± s.d.) of several several colocalization factors obtained from 13 different observation fields: Pearson's coefficient (PC), Manders' coefficient after background thresholding (M1 = green/red; M2 = red/green), Li's intensity correlation quotient (ICQ).

It showed that 86 ± 11% (M1) of the green channel (sec5/exoc2) coincide with a red (myoferlin) signal and that 64 ± 18% (M2) of the red channel (myoferlin) coincide with a green (sec5/exoc2) signal. The Mander's coefficient remains very sensitive to noise, thus we confirmed this result using the Li's approach able to discriminate coincidental events in a

heterogeneous situation. In the case of colocalization the product of (Ai-a)*(Bi-b) is positive and therefore the dot cloud of the intensity correlation analysis (ICA, Figure 4B-C) is mostly concentrated on the right side of the x=0 line. The Intensity Correlation Quotient (ICQ) varies from 0.5 (colocalization) to -0.5 (exclusion) when an artefactual colocalization give a value close to zero. In our experiment the mean ICQ is 0,45 ± 0,02, indicating a significant but partial colocalization between myoferlin and sec5/exoc2.

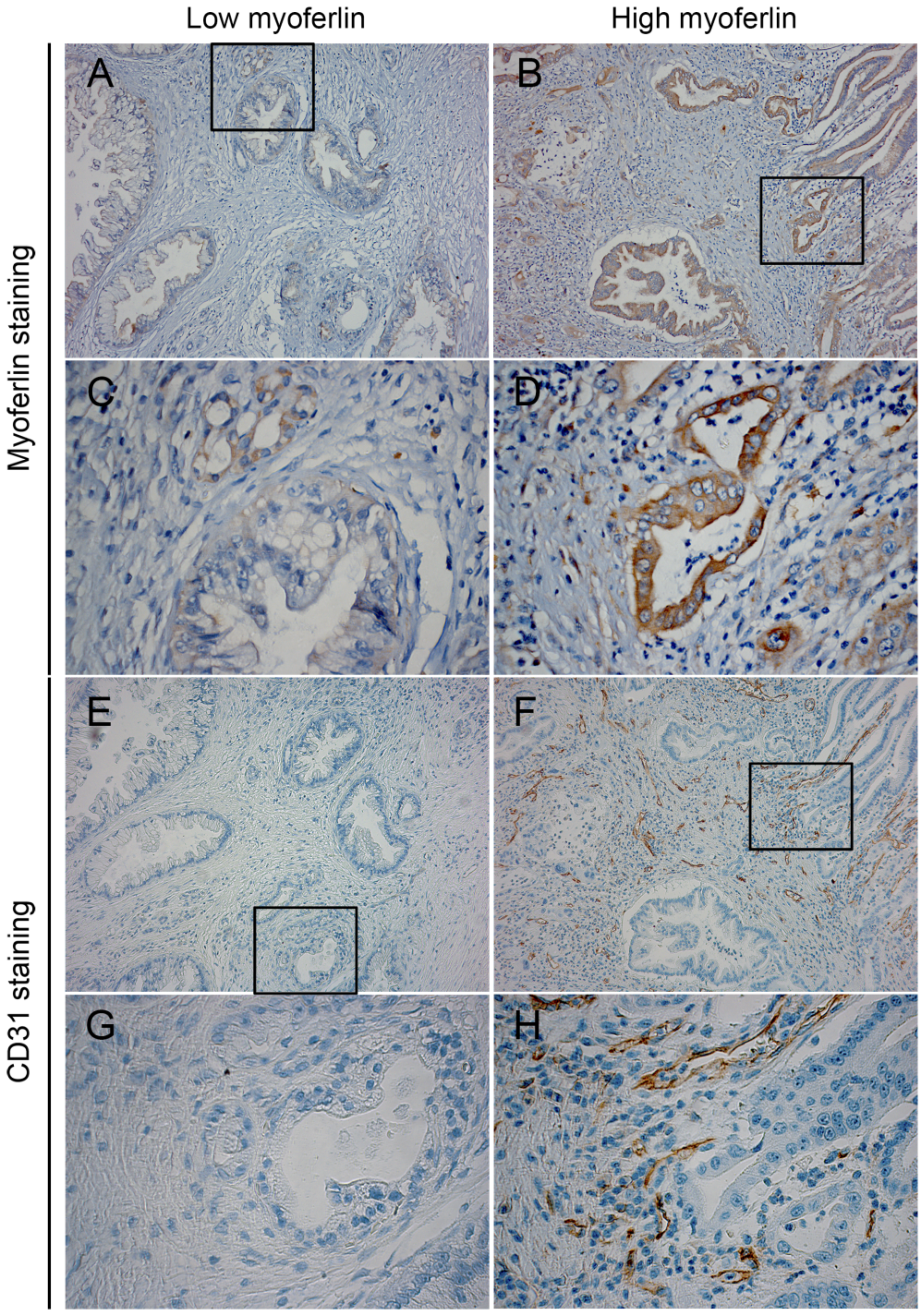

Figure 5. CD31 and myoferlin staining in PDAC sections. Immunoperoxydase staining of myoferlin (A-D) and CD31 (E-H) in PDAC sections sorted according to their myoferlin staining extent. Areas surrounded by a black rectangle in A, B, E, F are shown at higher magnification in C, D, G, H

3.4 Myoferlin staining extent is associated with blood vessel density and with survival in human PDAC

Since myoferlin silencing provoked a decrease of VEGFA secretion in cell medium and a decreased blood vessels density in BxPC-3 cells grown on CAM, we asked if a higher intratumoral vessel density characterizes patients bearing myoferlin-positive pancreatic tumor. Twenty-six PDAC and 19 non-tumoral pancreas sections, including pancreatitis, were immunostained for both myoferlin and CD31 (Figure 5). All endothelial cells showed consistent moderate CD31 staining. The vessel density in normal pancreatic tissue did not exceed 2/HPF except in 2 cases. Cancer cells were the main structures immunoreactive to myoferlin. Cases were grouped according to low (<50%) or high (≥50%) myoferlin extent.

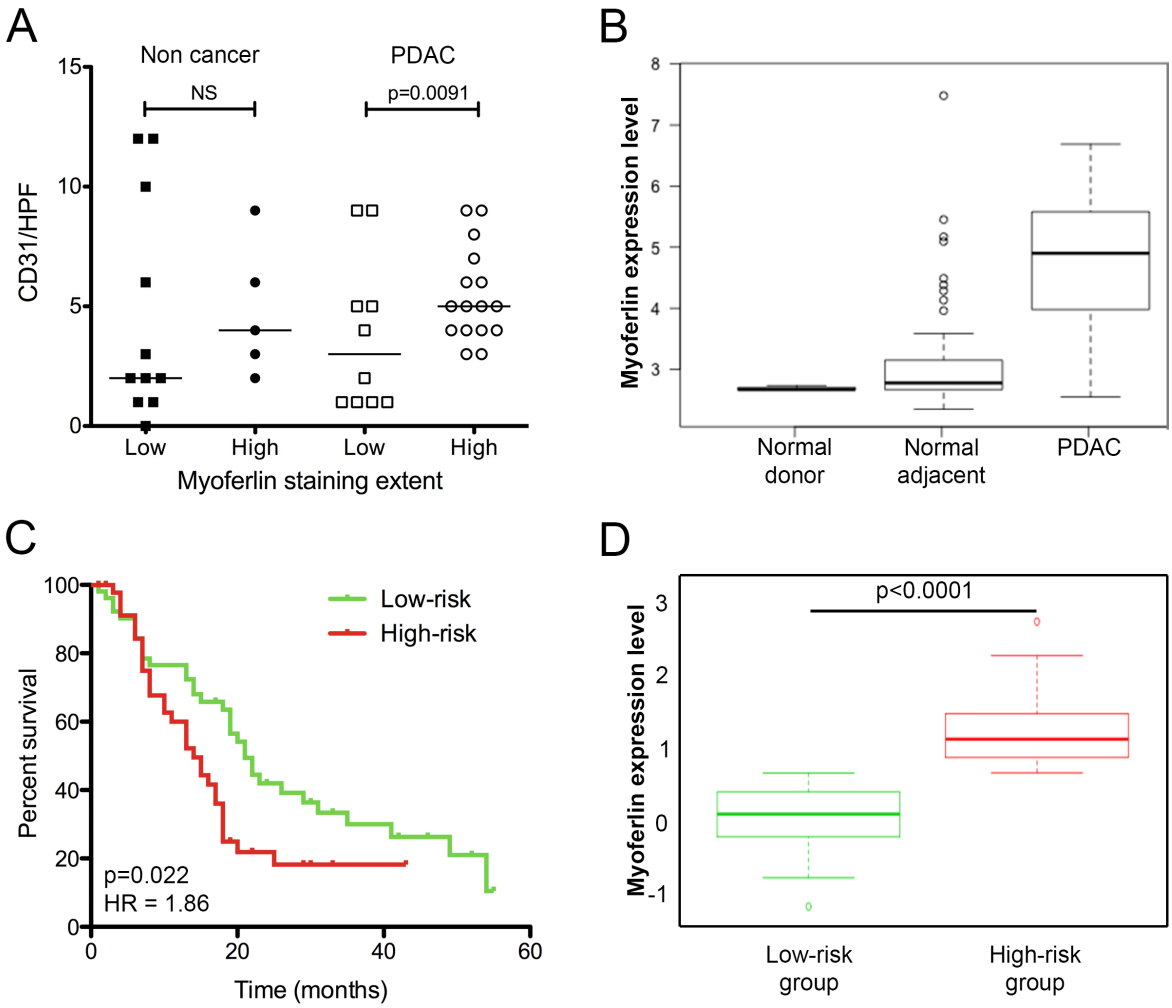

Figure 6. Correlation between myoferlin and microvessel density or survival in PDAC patients. (A) Twenty-six PDAC sections and 16 non-tumoral pancreas sections were stained for myoferlin and CD31. Each marker was scored individually before the sorting of the sections according to their myoferlin staining extent (low or high). Microvessel densities (CD31/HPF) were compared between low and high myoferlin staining within each category (non-tumoral or PDAC). Results are expressed as individual data with the median (line). NS = not significant. (B) Boxplot of myoferlin gene expression in normal pancreas tissue, normal adjacent to tumor and PDAC tissue (from Pancreas Expression Database). (C) Kaplan-Meier curve of PDAC patients divided in two groups significantly different regarding survival (low-risk and high-risk groups). (D) Myoferlin gene expression according to the survival group.

In non-cancer cases there was no significant differences in CD31 between low- and high-myoferlin groups. However, in cancer cases, a highly significant difference was observed: high-myoferlin cases having higher CD31 than low-myoferlin cases (Figure 6A). Publicly available dataset allowed us to show the overexpression of myoferlin in PDAC tumor in comparison to normal or normal adjacent tissue (Figure 6B) and to classify PDAC patients in two groups significantly different regarding survival (Figure 6C). The high-risk group was characterized by a hazard-ratio HR = 1.86. Myoferlin gene was significantly overexpressed (Figure 6D) in this group.

4. DISCUSSION

In this study, we demonstrate that myoferlin, a member of the ferlin family highly expressed in PDAC [28–30] and associated to a poor prognosis [30], plays a key role in PDAC progression, at least in part, by promoting tumor associated angiogenesis.

SiRNA-mediated myoferlin silencing in BxPC-3 cancer cells resulted in a significant reduction of their ability to grow in vitro and as a tumor in the stroma of chorioallantoic membrane, a PDAC model we recently optimized and validated [99]. We selected the widely used BxPC-3 cells for their ability to grow on CAM. This anti-tumor growth effect was associated with a massive inhibition of capillaries development within the tumoral stroma, suggesting a loss of pro-angiogenic activities of the cancer cells. Among the known proangiogenic factors, VEGFA is regarded as one of the most important in malignant diseases since it is the most predominant isoform secreted by human tumors [43] including pancreatic cancer [105,107]. Moreover, the involvement of VEGFA in our results is supported by the fact that VEGFA had demonstrated auto- and paracrine pro-proliferative effects respectively on cancer cells and endothelial cells [106,108]. In addition, the role of VEGFA in pancreatic tumor progression has also been studied in animal model. In these studies, inhibitors of the VEGFR tyrosine kinase or neutralizing antibodies against VEGFA or VEGFR2 reduced the growth and angiogenesis of pancreatic tumors [109,110].

As previously described, ferlin protein family is reported to be implicated in membrane fusion processes including myoblast fusion (dysferlin) [20] and synaptic vesicle exocytosis at the auditory ribbon synapse of inner hair cells (otoferlin) [14]. In consequence, we hypothesized that myoferlin may be also implicated in proangiogenic factors exocytosis by pancreatic cancer cells. In order to evaluate our hypothesis we analyzed VEGFA concentration in conditioned medium of myoferlin-silenced BxPC-3. A 40% reduction was observed and appeared as independent of a transcriptional effect. VEGFA immunodetection in myoferlin-silenced BxPC-3 cells revealed an accumulation of this protein inside the cell at the vicinity of the plasma membrane. This observation, together with the colocalization of myoferlin with sec5/exoc2, is in agreement with our ultrastructural observation of small vesicles accumulated near the plasma membrane suggesting that exocytosis vesicles, containing VEGFA, were stalled at the plasma membrane interface, unable to fuse with it. Several observations reported in the literature support our findings. Myoferlin is known to

possess several C2 domains, with a C2a domain able to bind phosphatidylserine in presence of Ca2+ [4], making it able to interact with the phospholipid bilayer after Ca2+ intracellular concentration increase, a common feature of exocytosis. Myoferlin is a substrate for calpain whose enzymatic cleavage gives rise to a synaptotagmin-like module [84]. Interestingly, synaptotagmin is a protein involved in Ca2+-triggered exocytosis [111], suggesting a potential role for the cleavage fragment of myoferlin in exocytosis. Finally, myoferlin has been considered as a regulator of protein secretion by breast cancer cells [31].

Although considered as an hypovascular tumor, angiogenesis is nonetheless critical for the development and growth of PDAC [43]. In patients with such neoplasia, VEGFA expression in the tumor and high serum concentration are significantly correlated to a decreased median survival time [53,112] and to metastasis progression, especially to the liver [107]. The correlation between VEGFA expression and the survival remains significant even after a surgery putatively considered as curative [113]. These results are consistent with the discovery that microvessel density and vascular proliferation index were significantly associated with larger tumor size and lymph node metastasis [114]. A recent study demonstrated that focal VEGFA locus amplification is regularly detected in PDAC [115]. A multi-institutional phase II study of bevacizumab, an antibody against VEGFA, gemcitabine and 5-fluorouracil in advanced pancreatic cancer demonstrated a continued interest in VEGFA as a valid therapeutic target [116].

Bevacizumab and axitinib, a VEGFR tyrosine kinase inhibitor, were tested in a phase III trial in advanced pancreatic cancer. Unfortunately, they failed to show any survival benefit for bevacizumab or axitinib in combination with gemcitabine [117,118]. However, these studies did not consider separately patients with a high basal production of VEGFA which correlates with better progression-free and overall survival in bevacizumab-treated patients versus those receiving placebo [119]. In another study, the pretreatment plasma VEGFA levels were described as ranging from 0 to 462 pg/ml [109], underlying the heterogeneity of this parameter in patients. According to the importance of a high VEGFA basal level in the response to targeted therapy, BxPC-3 cells, known for their high production of VEGFA [106], are a suitable model to investigate this aspect.

The clinical relevance of our study for PDAC patients is supported by the observation that a higher density of blood vessels characterized PDAC tumors with high myoferlin extent. This is of particular interest as vascular proliferation is associated in PDAC with a poor prognosis [114]. Interestingly, publicly available dataset indicates that myoferlin gene expression is also inversely correlated with the survival. Recently, the same association was described at the protein level [30].

Beside the role of myoferlin in exocytosis, this protein seems to control tightly the function of several receptor tyrosine kinase (RTK) in cancer and normal cells, including endothelial cells. Our laboratory recently described myoferlin as a key regulator of EGFR activity in breast cancer [34]. In endothelial cells, it was found to regulate VEGFR2 biological activity through preventing its polyubiquitination and its subsequent degradation by the proteasome [6]. In the same cell type the expression of angiopoietin-1 receptor (TIE-2) seemed to be myoferlin-dependent [23]. In normal myoblasts, myoferlin was demonstrated

as an essential component of the insulin-like growth factor receptor (IGF1R) trafficking and signaling [12]. In PDAC, as well as in PDAC cell lines, several RTK are overexpressed (VEGFR, EGFR, FGFR, PDGFR) and their targeting reduced the pancreatic cell growth or tumor growth [120–122]. However, in our conditions, exogenous VEGFA was unable to rescue the decrease of proliferation induced by myoferlin-silencing, indicating that the autocrine loop involving VEGFA is not the main event in the cell proliferation decrease.

In conclusion, myoferlin is functionally important in the context of PDAC. It is essential for cell and tumor growth but also for the establishment of a functional blood vessel network inside the tumor.

Acknowledgments

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Statement for author contributions

Conceived and designed the experiments: VC OP. Performed the experiments: KF AG. Performed electron microscopy: MT. Performed immunohistochemistry and scoring: PD, MA. Analyzed the data: KF AG AT PP AB VC OP. Wrote the paper: OP VC. All authors contributed to the redaction of their specific part and approved the final version.

PART II: STUDYING THE ROLE OF MYOFERLIN IN EXOSOMES BIOGENESIS, FUSION AND FUNCTION

PUBLICATION II

Myoferlin is a Novel Exosomal Protein that Determines the Protein Cargo and Function of Cancer-Derived Exosomes

Arnaud Blomme, Karim Fahmy, Marie Fontaine, Marc Thiry, Ingrid Struman, Olivier Peulen, Akeila Bellahcène, Dominique Baiwir, Edwin de Pauw, Vincent Castronovo, Andrei Turtoi

Submitted, in revision

1. Introduction

Exosomes are small extracellular vesicles, ranging in size from 50 to 150 nm. They are derived from endosomal vesicles and secreted into the extracellular medium upon fusion with the plasma membrane [123]. Many cell types produce exosomes, and these extracellular vesicles can bear multiple functions involved in many biological processes, including cancer progression [124]. Exosomes were shown to transform non-tumorigenic cells [125–127], modulate cancer cell metabolism [128], angiogenesis [129] and take part in conditioning the metastatic niche [130]. Exosome accessibility in liquid biopsies (blood, urine, lymph) and their correlation with patient clinical features (metastatic dissemination, tumor relapse or response to therapy) make them candidates for diagnostic, prognostic and therapy response indicators [131–133]. Recent advances in the field demonstrated that exosomes are not merely transport containers for intercellular trafficking. They are in fact fully functional entities that contain all the machinery required for de novo protein synthesis and processing [78]. These findings are the evidence for the existence of a specific proteome that is confined to the exosomes. The understanding of key factors that shape the proteome of exosomes is essential for identifying novel mechanisms that contribute to tumor progression [134,135].

Myoferlin is a 230kDa membrane protein that belongs to the ferlin family of proteins. It was first identified in muscle cells, where it contributes to cell/cell fusion and muscle regeneration [18,20]. Further studies performed in endothelial cells demonstrated that myoferlin was important for membrane repair and endocytosis [24] as well as receptor-mediated angiogenesis [6,12,23,34]. In cancer, overexpression of the protein has been reported in breast, lung, and pancreatic tumors [28,30,35,136], where it has been associated with increased tumorigenic potential and angiogenesis [9,32,34,98]. Mechanistically, myoferlin has been shown to control both endocytosis (EGFR, VEGFR2, IGFR and Tie-2) and exocytosis (VEGF) of several growth factors and their receptors [6,9,12,23,34,90]. Inspired by myoferlins' role in cell membrane biology, we hypothesized that myoferlin could be essential to exosomes in potentially regulating their maturation, secretion or internalization. Indeed, our literature research strengthens this hypothesis in showing that myoferlin was found in exosome proteomes from different cell lines. Using immunoblotting and electron microscopy, we confirm that myoferlin is indeed present in exosomes derived from breast and pancreas cancer cell lines. We show for the first time that myoferlin is determining factor for the proteomic diversity of the exosomes, having direct impact on their function. Collectively, our findings place myoferlin on the short list of key proteins important for cancer exosome biology.

2. Materials and Methods

2.1 Cell culture and myoferlin-depleted cell lines

The cell lines used in this study (MDA-MB-231 (HTB-26), MDA-MB-468 (HTB-132), BT-549 (HTB-122), Hs 578T (HTB-126), MCF7 (HTB-22), ZR-75-1 (CRL-1500), BT-474 (HTB-20), SK-BR-3 (HTB-30) and CFPAC-1 (CRL-1918)) were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). Human pancreatic adenocarcinoma cells BxPC-3 (ATCC CRL-

1687) were a generous gift from Prof. Bikfalvi (Inserm U1029, Bordeaux, France). PANC-1 cells (ATCC CRL-1469) were a generous gift from Prof. Muller and Burtea (NMR Laboratory, University of Mons, Belgium). All cells were cultured in their recommended medium at 37°C, 5% CO2, and 95% humidity. Isolation of human umbilical vein endothelial cells (HUVEC) (passages 6–11) was described previously [137]. HUVEC were seeded onto gelatin-coated culture flasks and cultured in EGM-2 medium (Cat. #: CC3162/6; Lonza, Basel, Switzerland) supplemented with 5% fetal bovine serum (Cat. #: DE-802F; Lonza).

Stable myoferlin depletion was achieved using lentiviral shRNA particles. Myoferlin shRNA plasmid (sequence CCCUGUCUGGAAUGAGA cloned into pLKO, Sigma, St. Louis, MO, USA) or control shRNA (Cat. #: SHC005; Sigma, St. Louis, MO, USA) were cotransfected with pSPAX2 (Addgene, Cambridge, MA, USA) and a VSV-G encoding plasmids [138] in lenti-X 293T cells (Clontech, Mountain view, CA, USA). Titration of the lentiviral vectors was performed using the qPCR Lentivirus Titration (Titer) Kit (Cat. #: LV900; ABM, Richmond, BC, Canada). After transduction, the stably transduced clones were selected following Puromycin treatment (10 µg/mL, ant-pr-1, Invivogen, Toulouse, FR).

2.2 Exosome purification and characterization

Cancer cells were seeded at 75% confluence and allowed to grow in their respective culture medium supplemented with exosome-depleted serum. After 48 hours, supernatant was first centrifuged for 20 minutes at 2,000g to remove floating cells. Cell-free supernatant was next centrifuged at 12,000g for 45 minutes and filtered (0.22 µm filter) to remove additional cell debris. The collected medium was then ultracentrifuged for 2 hours at 100,000g using SW-28 rotor and Optima LE80 ultracentrifuge (Beckman Coulter, Brea, CA, USA). The exosome containing pellet was washed once with PBS, centrifuged again for 2 hours at 100,000g and finally suspended either in PBS or SDS-based lysis buffer (50 mM Tris-HCl, 1% SDS, pH 7.5, supplemented with protease and phosphatase inhibitors, (Cat. #: 11873580001; Roche, Mannheim, Germany). The PBS fraction was used for functional assays and microscopy, whereas the SDS-lysate for Western blot and proteomic analysis.

2.3 Electron microscopy and immunogold labelling of isolated exosomes

Labelling of cell-derived exosomes was performed as previously described[137]. Briefly, exosomes attached on formvar-carbon coated grids were successively washed, fixed in 2% formaldehyde and incubated with primary (2 hours, RT, anti-MYOF (Cat. #: HPA014245; Sigma Aldrich, St. Louis, MO, USA) 1/20 dilution in PBS-BSA 0.2% supplemented with normal goat serum 1/50) and secondary antibodies (1 hour, RT, anti-rabbit coupled with gold particles (Aurion, Wageningen, The Netherlands) diluted 1/40 in PBS-BSA 0.2%, pH 8.2) for one hour. Samples were postfixed for 10 minutes in 2.5% glutaraldehyde and counterstained

using uranyl acetate and lead citrate. Pictures were made with a Jeol JEM-1400 transmission electron microscope at 80 kV (Jeol, Peabody, MA, USA).

2.4 Western blotting

Cells or exosome pellets were first lysed in SDS-based buffer. Protein quantification was performed using the BCA quantification kit (Pierce, Thermo Scientific, Rockford, IL, USA). Desired amount of proteins were mixed with Laemmli buffer (60 mM Tris-HCl pH 6.8, 25% glycerol, 2% SDS, 14.4 mM of 2-mercaptoethanol and trace of bromophenol blue) and denatured for 5 minutes at 99°C. Protein samples were separated by SDS-PAGE followed by electro-transfer on PVDF membrane. The membranes were incubated with the selected primary antibodies (outlined below) overnight at 4°C and then probed with corresponding secondary antibody conjugated to horseradish peroxidase (anti-rabbit antibody (Cat. #: G21234; Life-Technologies, Gent, BE) and anti-mouse antibody (Cat. #: P0260; Dako, Glostrup, Denmark) for one hour at room temperature. The immunoblots were revealed using the chemiluminescent substrate (ECL Western blotting substrate, Thermo Scientific, Rockford, IL, USA). Following antibodies were used: myoferlin (Cat. #: HPA014245; Sigma Aldrich, St. Louis, MO, USA), caveolin-1 (Cat. #: 3238; Cell Signaling, Danvers, MA, USA), flotilin-1 (Cat. #: 3253; Cell Signaling), CD-9 (Cat. #: 2125-0909; AbD Serotec, Düsseldorf, Germany), CD-63 (Cat. #: ab134045; Abcam, Cambridge, United Kingdom) and HSC70 (Cat. #: sc-7298; Santa Cruz, Dallas, TX, USA).

2.5 Proteomic analysis of cancer cell-derived exosomes

Exosome purifications were performed in biological duplicates. Exosome pellets were directly lysed in the SDS-based lysis buffer supplemented with protease and phosphatase inhibitors (for details see above). Protein extracts were further reduced with dithiothreitol (100 mM), alkylated with 150 mM of chloroacetamide and precipitated with 20% trichloroacetic acid. Protein pellets were then washed with acetone, suspended in ammonium bicarbonate buffer (50 mM NH4CO3, pH 8.0) and digested with trypsin (sequencing grade, 1/50: protease/ protein; Promega, Madison, WI, USA). Finally, 5 µg of peptides were desalted using C18 ZipTip (Millipore, Billerica, MA) and 2.5 µg were injected on an Acquity M-Class UPLC ((Waters, Milford, MA, USA) connected to a QExactive Plus (Thermo Scientific, Bremen, Germany), in nano electrospray positive ion mode. The samples were loaded on a Symmetry C18 5µm column (180 µm x 20 mm, 20 µL/min, pH 10, Waters), followed by a stepwise elution on the analytical column HSS T3 C18 1.8 µm (75 µm x 250 mm, pH 3, Waters). The samples were loaded on the trap column in 100% solvent A (water 0.1% formic acid) during 3 minutes and subsequently separated on the analytical column; flow rate 600 nL/min, solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile), linear gradient 0 min, 98% A; 5 min, 93% A; 135 min, 70% A; 150 min, 60% A.

The total run time was 180 min. The MS acquisition was conducted in data-dependent mode. The parameters for MS acquisition were: MS range from 400 to 1750 m/z, Resolution of 70,000, AGC target of 1e6 or maximum injection time of 50 ms. The parameters for MS2 spectrum acquisition are: isolation Window of 2.0 m/z, collision energy of 25, resolution of 17500, AGC target of 1e5 or maximum injection time of 50 ms. Protein identifications and quantifications were conducted using MaxQuant vXX using UniProt® human database. Peptide modification carbamidomethylation was set as fixed and oxydation (M) as variable.

2.6 Immunofluorescence

Exosomes isolated from cancer cells were labelled with PKH67 dye (Cat. #: P7333; Sigma Aldrich, Saint-Louis, USA) according to the manufacturer's instructions. Fluorescently-labelled exosomes were then incubated with endothelial cells (HUVEC, seeded at 30,000 cells in 24-well plate) for different time points. Following the treatment, the cells were fixed for 15 minutes in 4% PAF, washed two times with PBS and mounted on slides for visualization under a confocal microscope. Images were acquired using SP5 A0BS confocal microscope (Leica, Wetzlar, Germany) at a magnification of 63X.

2.7 miRNA overexpression and exosome-mediated transfer to endothelial cells

Murine specific miRNA miR298 (Cat. #: PM12525; Thermo Scientific, Rockford, IL, USA) was transfected at a concentration of 25 nM in cancer cells using Lipofectamine 2000 reagent (Cat. #: 11668-019; Invitrogen, Carlsbad, California, USA). MiR298-overexpressing exosomes were then isolated and incubated with HUVEC (seeded at 30,000 cells in 24-well plate) for 2 hours. Following exosome treatment, total RNA was extracted from the endothelial cells using the DNA, RNA, and protein purification kit - Nucleospin (Cat. #: 740955.250; Macherey-Nagel, Duren, Germany). Reverse transcription was performed using the RevertAid H Minus First Strand cDNA Synthesis Kit (Cat. #: K1631; Thermo Scientific). Detection of the miR298 miRNA was then performed using Quantitative PCR, with specific primers miR298 (Cat. #: 002598; Applied Biosystems, Foster City, California, USA) and miR-RNU44 (Cat. #: 001094; Applied Biosystems, Foster City, California, USA). RNU44 was used as an internal control.

2.8 Proliferation of exosome-treated endothelial cells

HUVEC were plated at 2 × 103 cells/well in 96-well plates and cultured overnight in EGM-2 (100 µl/well). Cells were incubated with 1.5 µg exosomes per well for 72h in EBM-2 (Cat. #: CC3156; Lonza) with 0.5% DBS. Bromodeoxyuridine (BrdU) was added and the culture was incubated for 8h. BrdU incorporation was measured with the Cell Proliferation ELISA BrdU (chemiluminescence) kit (Roche) according to the manufacturer's protocol.

2.9 Migration of exosome-treated endothelial cells

HUVEC were plated at 5 × 104 cells/well in 48-well plates and cultured overnight in EGM-2 (300 µl/well). Cells were incubated with 5 µg exosomes per well for 24h. To examine wound healing, the confluent monolayers were mechanically scratched using a pipette tip to create the wound. Cells were washed with PBS and EBM-2 (Cat. #:CC3156; Lonza) 0.5% DBS (Cat. #: 10371029; Gibco-Thermo Fisher,) was added to allow for wound healing. The distance between the two sides of the wound was measured with a graduated ocular lens and an epifluorescence microscope (CKX41; Olympus, Tokyo, Japan). The distance between the two sides of the wound after 6h of migration was subtracted from the distance at time 0 and represented on a graph.

3. RESULTS

3.1 Myoferlin is expressed in exosomes derived from breast and pancreatic cancer cell lines

In order to investigate the possibility that myoferlin is a constitutive part of exosomes, we sought to explore existing proteomic data from purified exosomes. To do so we have interrogated the "Exocarta" database (www.exocarta.org) for the presence of myoferlin in extracellular vesicles. Interestingly, we found that myoferlin had been reported in several proteomic analyses of purified exosomes from different cellular origins (Table 1, adapted from exocarta). To validate these in silico data, we purified exosomes from the supernatant of breast and pancreatic cancer cell lines, and have evaluated myoferlin expression using Western blot. Isolated exosomes were characterized by a size ranging from 40 to 400 nm with an average size of 155-160 nm. Most of the particles were surrounded by lipid bilayer as showen by electron microscopy (supplemental figure 1). On Figure 1A, the results confirmed the presence of myoferlin in exosomes purified from all the cancer cell lines tested. We also confirmed the myoferlin presence in exosomes by electron microscopy; using an anti-myoferlin immunogold labelling of purified exosomes from MDA-MB-231 breast cancer cells (Figure 1B).

3.2 Myoferlin silencing affects the size and the protein content of tumor-derived exosomes

In order to further elucidate the function of myoferlin in exosomes, we have assessed the exosome production and their size in myoferlin-depleted MDA-MB-231 and BxPC-3 cancer cells (Figure 2).

Gene Name	Analysis	Origin	Authors	Journal	Year
MYOF	Proteomic analysis of exosomes	Bladder Cancer Cells	Welton, et al.	Mol. Cell. Proteomics	2010
MYOF	Proteomic analysis of exosomes	Colon Cancer Cells	Mathivanan, et al.	Mol. Cell. Proteomics	2010
MYOF	Proteomic analysis of exosomes	Colon Cancer Cells	Demory Beckler, et al.	Mol. Cell. Proteomics	2013
MYOF	Proteomic analysis of exosomes	Hepatocellular Carcinoma Cells	He, et al.	Carcinogenesis	2015
MYOF	Proteomic analysis of exosomes	Melanoma Cells	Lazar, et al.	Pigment Cell. Melanoma Res.	2015
MYOF	Proteomic analysis of exosomes	Ovarian Cancer Cells	Liang, et al.	J. Proteomics	2013
MYOF	Proteomic analysis of exosomes	Prostate Cancer Cells	Kharazia, et al.	Oncotarget	2015
MYOF	Proteomic analysis of exosomes	Squamous Carcinoma Cells	Park, et al.	Mol. Cell. Proteomics	2010
MYOF	Proteomic analysis of exosomes	Urine	Gonzales, et al.	J. Am. Soc. Nephrol.	2009

Table 1: Exocarta recapitulated table. Myoferlin presence in different tumour-derived exosomes

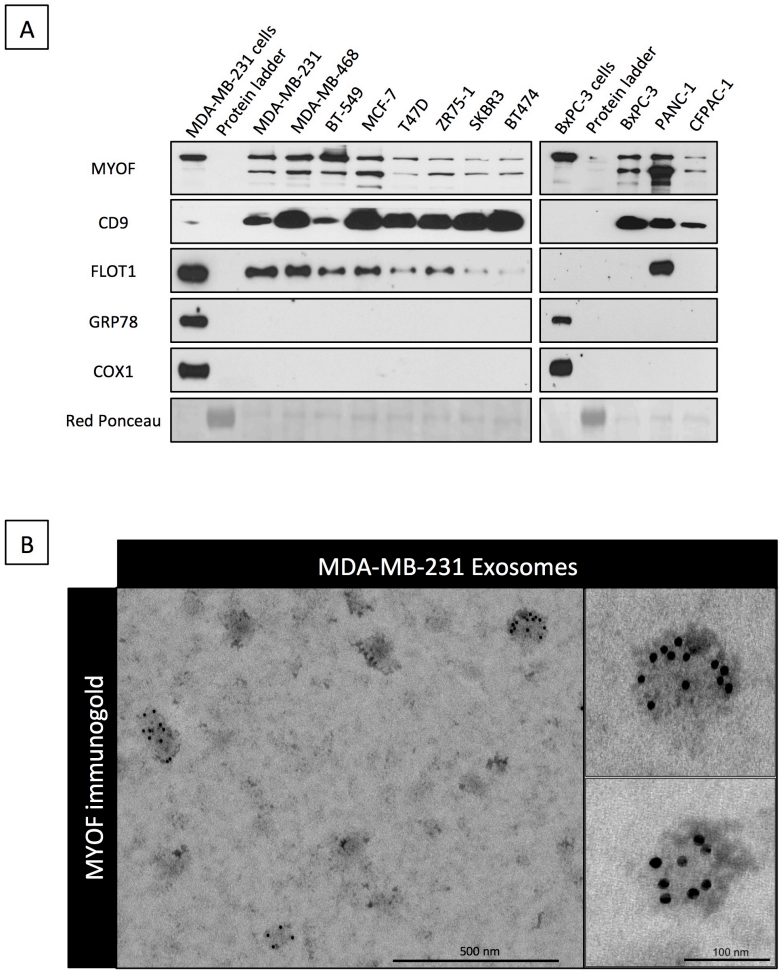

Figure 1: Myoferlin is expressed in exosomes from breast and pancreas cell lines. (A) Western blot analysis of myoferlin expression in exosome isolations from multiple breast and pancreas cancer cell lines. CD9 and FLOT1 were used as positive controls for exosomes whereas the GRP78 and COX1 were employed as negative controls. (B) Immunogold electron microscopy of exosome preparations from MDA-MB-231 cells stained for myoferlin.

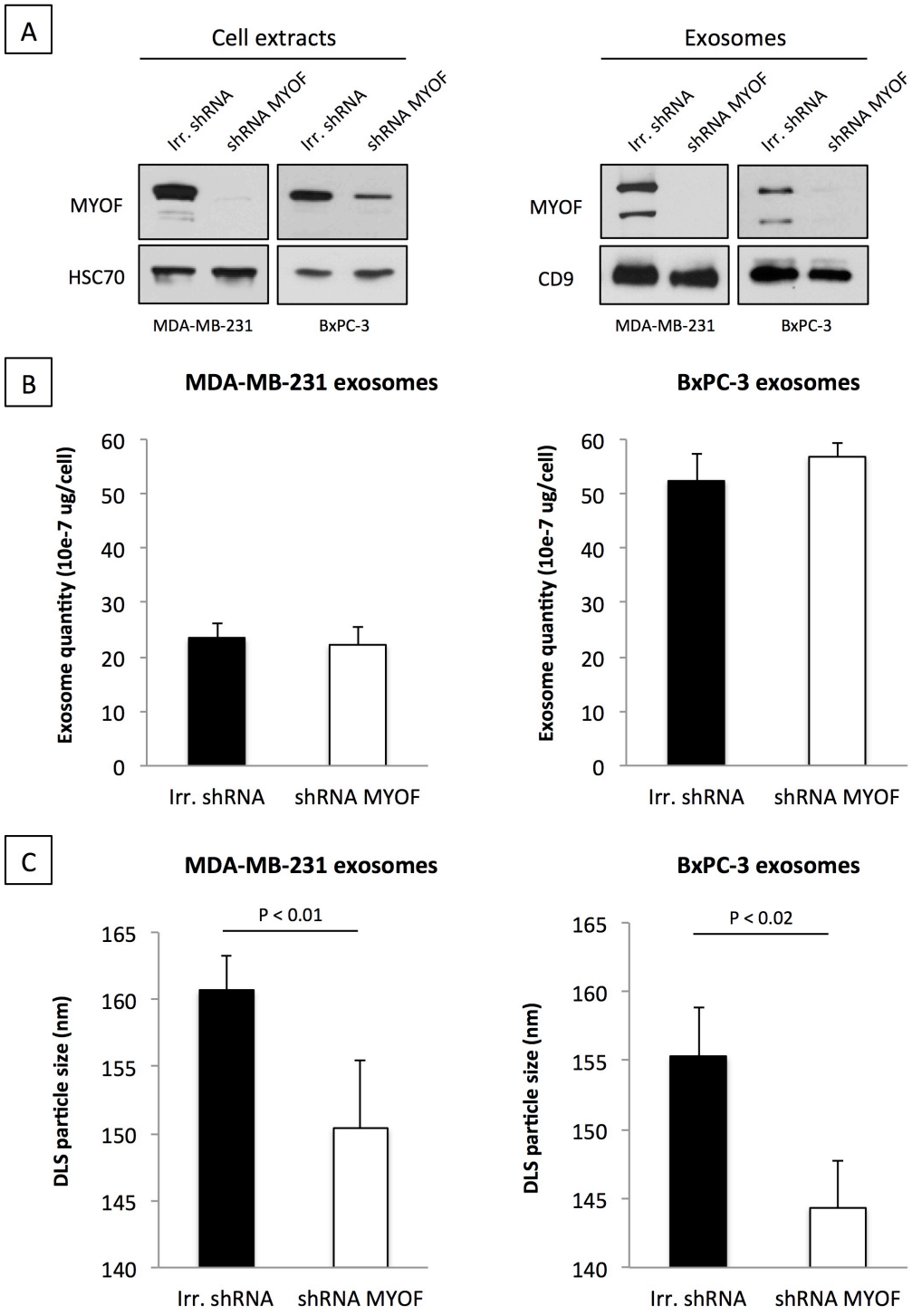

Figure 2: Loss of myoferlin decreases exosome size but not quantity. (A) Validation of myoferlin depletion in exosome preparation from breast (MDA-MB-231) and pancreas (BXPC3) cancer cells. (B) Measurement of exosome quantity. (C) Measurement of exosome size.

We found no major difference in the quantity of exosomes produced by myoferlin-depleted cells in comparison to the control condition (Figure 2B; measured as the relative protein quantity extracted from purified exosomes normalized to cell number). However, using dual light scattering, we observed that exosomes derived from myoferlin-depleted cells showed

consistently a significantly smaller particle size (Figure 2C). This suggested that exosomal content, rather than exosome production, could be affected by the absence of the protein.

To test this hypothesis, we sought to understand if the proteome content of myoferlin-depleted exosomes would be different from those who kept myoferlin. Therefore we performed a proteomic analysis of exosomes isolated from MDA-MB-231 and BxPC-3 that were silenced for myoferlin, and compared them to control exosomes. We identified 307 and 498 proteins, in MDA-MB-231 and BxPC-3 respectively, which were expressed in at least 50% of the replicates (Table S1). Expectedly, over 75% of the identified proteins were predicted to have an exosomal subcellular localization (Figure 3A). Although the identity of the modulated proteins was different between breast and pancreatic exosomes (only 34 % of proteins overlap, Table S2), we observed that the regulatory pathways down-regulated in absence of myoferlin were highly similar (Figure 3B). Indeed, in both cell types, these pathways were related to endocytosis and other vesicle-mediated processes. From these data, we generated a list of 32 proteins that were consistently decreased in myoferlin-depleted exosomes, independent of the cell type analyzed (Figure 3C-D and Table S3). Among these selected proteins we notably found proteins that are essential for proper intracellular vesicle transport (CAV1, FLOT1, FLOT2, SXN6) and key regulators of the endo-lysosomal trafficking (VAMP7, RAB7A), both processes requiring high number of vesicle/membrane fusion events. Interestingly, we also observed a consistent decrease in CD63 protein expression, which is an important marker of extracellular vesicles and of the major components regulating heme homeostasis (TFRC, FTH1). Decreased expression of several of these proteins was further validated using Western blot on purified exosome extracts (Figure 3E).

3.3 Myoferlin silencing alters exosome fusion and cargo delivery in target cells

In addition to endocytosis, tumor-derived exosomes can reach target cells (e.g. fibroblasts, endothelial cells…) by fusing with their plasma membrane, resulting in the delivery of molecular cargo [123]. We therefore aimed to test whether lack of myoferlin could impair exosome fusion with endothelial cells. For this purpose, we treated endothelial cells with PKH67-labelled exosomes, isolated from control and myoferlin-depleted cancer cells. We then used immunofluorescence to follow exosome uptake in HUVEC after their incubation with the labelled exosomes. Following incubation, endothelial cells treated with control exosomes displayed a strong fluorescent signal (Figure 4A). The absence of colocalization with CD31, a membrane protein specific of endothelial cells, confirmed that the vesicles have entered the target cells (Supplemental figure 2).

In contrast to this, we could not detect any fluorescence in HUVEC incubated with exosomes lacking myoferlin. We confirmed this effect of myoferlin silencing by following the transfer of a specific miRNA between the exosomes isolated from cancer cells and the endothelial cells. To do so, we have overexpressed a murine miRNA (miR-298) in the cancer cells (in both

control and myoferlin depleted condition), purified the exosomes from these cells and then incubated them with HUVEC. As shown in Figure 4A, the expression of the miR-298 was hardly detected in HUVEC treated with exosomes lacking myoferlin, in comparison to the control condition.

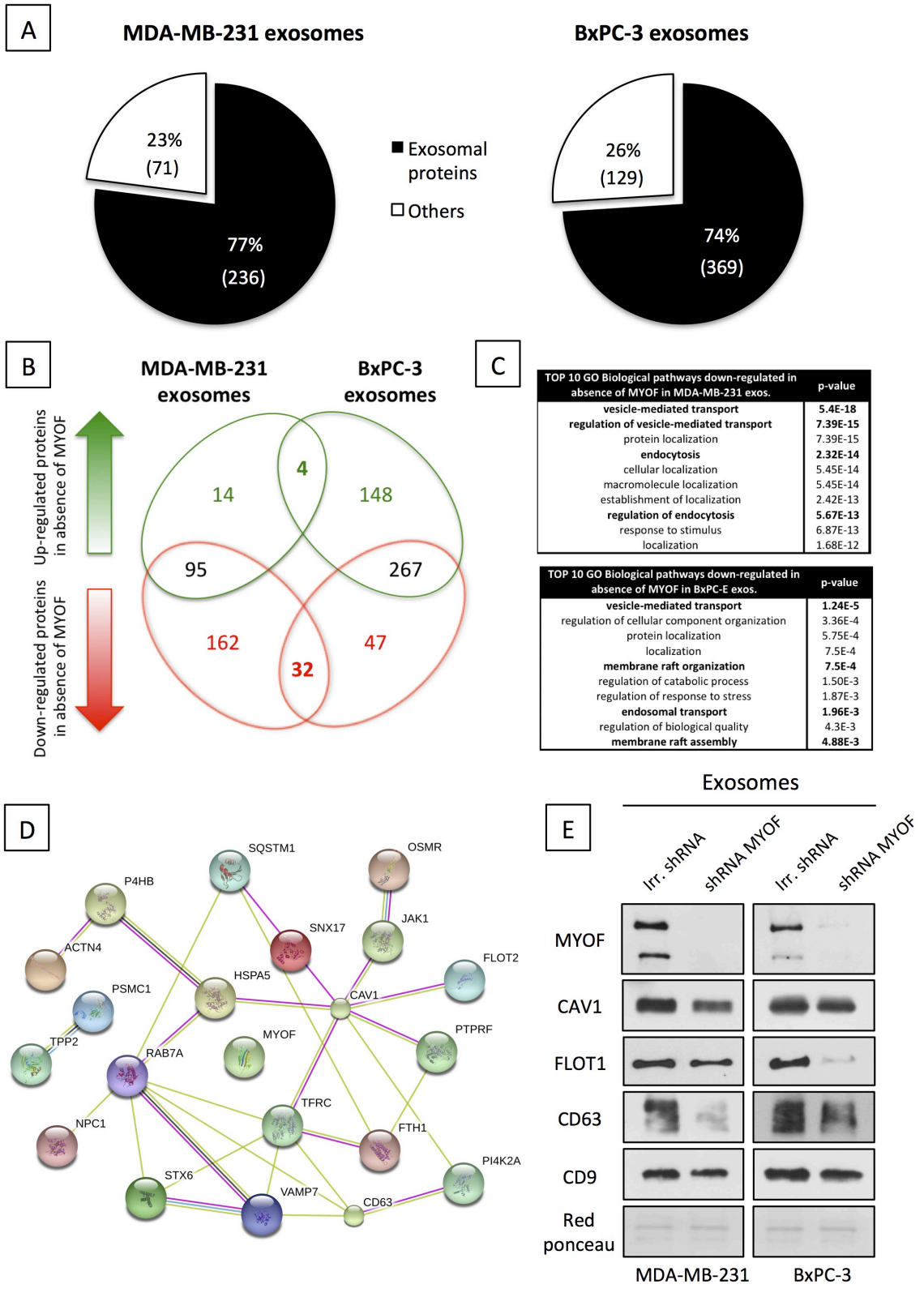

Figure 3: Myoferlin depletion from cancer exosomes induces major change in their proteome content.

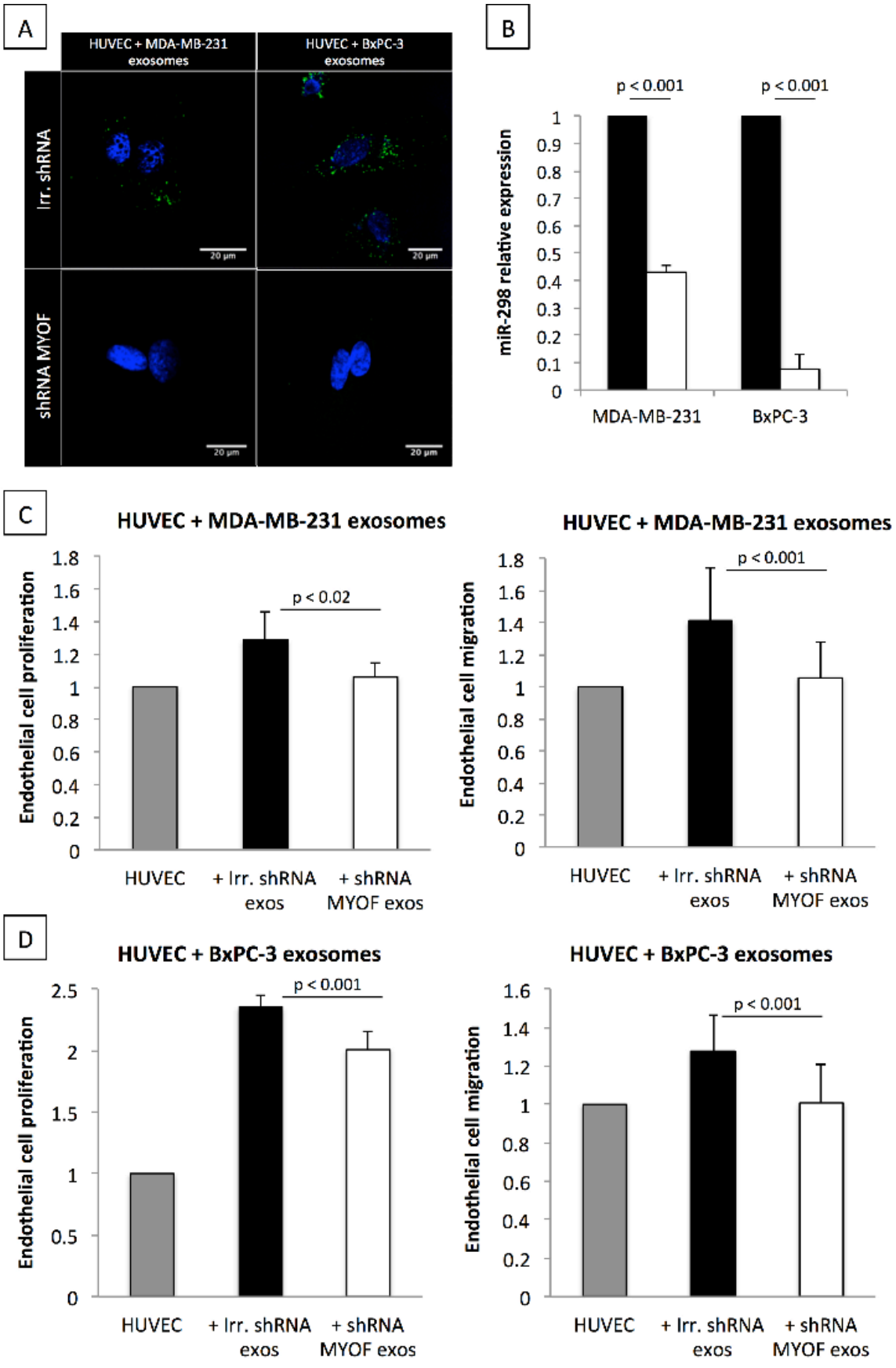

Figure 4: Myoferlin-deficient exosomes are functionally impaired to transfer biological message to neighboring cells.

3.4 Myoferlin silencing impairs exosome-mediated reprogramming of endothelial cells in vitro

Having established that myoferlin deficiency decreases exosome entry and cargo release, we next asked the question if this could have biological consequences for target cells. In order to evaluate this we have examined endothelial cell proliferation and migration following their incubation with control and myoferlin depleted exosomes from MDA-MB-231 and BxPC-3 cells. In comparison with naïve HUVEC, endothelial cells incubated with control exosomes displayed increased rates of proliferation and migration (Figure 4C-D). However, when the HUVEC were incubated with myoferlin-depleted exosomes, these effects were significantly attenuated, restoring the proliferation and migration rates similar to those of non-treated cells (Figure 4C-D).

4. DISCUSSION

Cancer cell/stroma communication represents a key mechanism required for both tumor growth and metastatic dissemination. A paucity of studies demonstrates that exosomes are mediators that facilitate inter-cellular exchange of biomolecules, assuming an important role in cell-to-cell communication [81]. In the current study we focused on myoferlin, a membrane associated protein, which has recently been demonstrated to be pro-oncogenic in several types of cancer [9,31,32,34,35,98]. In the breast cancer, in vivo tumor xenografts lacking myoferlin have been described as smaller, less invasive and less vascularized than their control counterparts [98]. Antitumor effects observed following myoferlin depletion have been attributed to impaired membrane repair/turnover [35], the inability to properly internalize growth factor receptors [34], impaired cancer cell motility [31,34,98] and the inability to sustain tumor-associated angiogenesis [9,23]. However, our study brings forward an additional important role for myoferlin in tumor progression, namely the ability to modulate tumor/stroma crosstalk. Indeed, although the presence of exosomal myoferlin had been reported in several proteomic analyses of tumor-derived exosomes (see Table 1), to our knowledge, the definitive proof of its presence in exosomes has not been provided to date. Starting from our original proteomic observation and strengthened by the previous studies, we demonstrated by Western blotting and electron microscopy that myoferlin is indeed a major component of cancer cells-derived exosomes. In the present study we confirm that myoferlin is present in exosomes derived from all major breast and pancreatic cancer cell lines from different molecular and genetic backgrounds. Following this, we next sought to examine if myoferlin depletion has any functional consequences for cancer-derived exosomes. We have thus characterized the size and the quantity of exosomes secreted by myoferlin depleted cells. The analysis showed that myoferlin depletion does not affect exosome quantity but it does affect their size. This suggests that myoferlin influences exosome cargo rather than the production by cancer cells. These findings have further motivated us to examine the protein content of myoferlin-depleted exosomes and compare this to the control counterparts. The proteomic analysis of isolated exosomes revealed that

myoferlin silencing leads to a significant alteration of the protein content. Notable was the decreased expression of several vesicular markers that are known to be expressed in exosomes (caveolin-1, flotilin-1 and 2, syntaxin 6). The analysis also demonstrated the modulation of several members of the RAB family of RAS-related GTP-binding proteins, which are important regulators of vesicular transport. Of these, RAB7A was commonly downregulated in myoferlin-depleted condition. RAB7A is a known marker of endo-lysosomal transition whose upregulation has been associated with tumorigenesis in multiple organs [139–141]. Recently, Redpath et al. showed that myoferlin strongly localized in RAB7-positive late endosomes [86]. The decrease of RAB7A expression observed in absence of myoferlin could therefore reflect another mechanism that could explain the observed anti-tumor effect. A further important observation resulting from the current proteomic study was the marked decrease of CD63 following myoferlin silencing. CD63 is a widely used exosomal marker and its expression correlates with increased aggressiveness and metastatic potential of breast tumors [142,143]. Our data have also pointed that ferritin and transferrin levels were reduced in myoferlin-depleted exosomes, suggesting that myoferlin could also be important factor regulating iron metabolism. Iron metabolism is frequently deregulated in cancer [144] and high levels of serum ferritin have been shown to correlate with adenoma formation in colon [145], clinical outcome of pancreatic cancer patients [146] and response to chemotherapy in breast cancer [147,148]. Exosomes are thought to support tumor growth by reprogramming cells from the tumor microenvironment, such as fibroblasts and endothelial cells [126,149]. Myoferlin was previously described as a key factor in the process of cell-cell fusion [20], suggesting that similar mechanism may here also be underway. Indeed, the entry of exosomes into target cells involves, among other mechanisms, a direct fusion with the plasma membrane [123]. The present study demonstrates for the first time that silencing myoferlin delays exosome uptake by endothelial cells. This, in addition to their altered protein content, led to diminished ability of myoferlin-depleted exosomes to stimulate endothelial cell migration and proliferation. Although the consequences for tumor progression are evident, additional studies need to be conducted in order to understand how myoferlin contributes to the exosome uptake in the target cells. Hints towards answering this question may be provided by the recent study of Melo et al. [78] who elegantly demonstrated that an exosomal maturation process outside the cell is required to achieve proper release of their proteic and nucleotidic content in the target cells and subsequently initiate tumorigenesis. The fact that myoferlin is able to determine protein cargo of cancer-derived exosome could explain why this protein is overexpressed in cancer, and further underlines its importance in the regulation of both intra- and extracellular vesicle trafficking of cancer cells.

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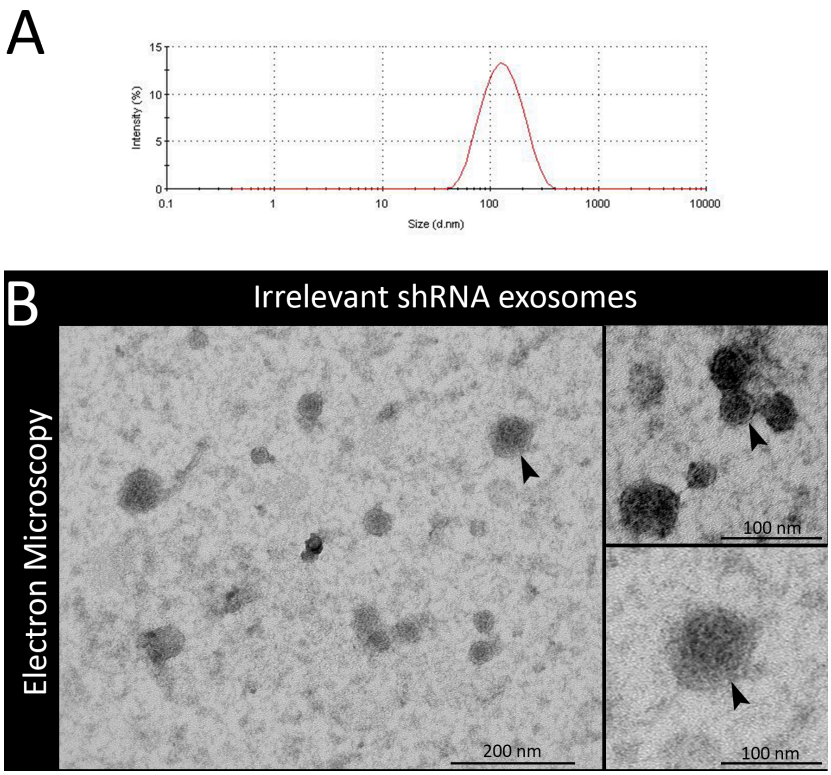
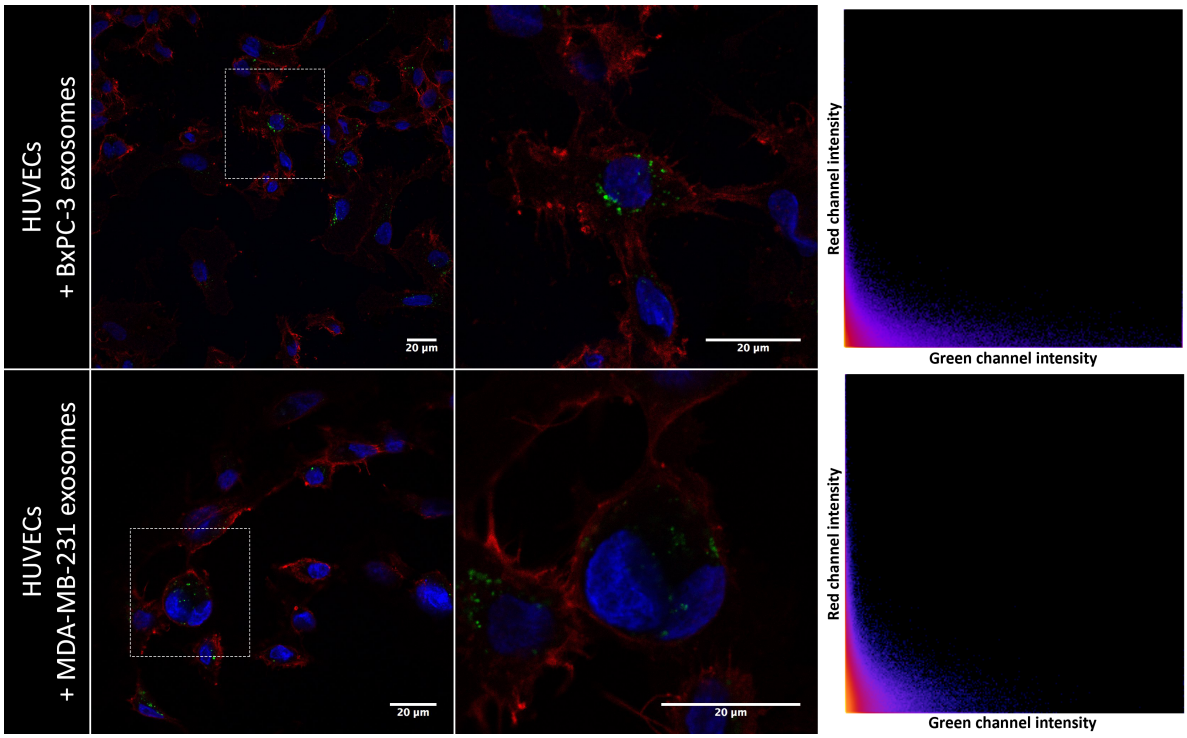
5. Supplemental Data



Supplemental Figure 1: Morphological characterization of exosomes isolated from BxPC-3 cells. A. Dynamic Light Scattering of isolated exosomes. B. Electron microscopy of isolated exosomes.



Supplemental Figure 2: Uptake of exosomes derived from MDA-MB-231 and BxPC-3 cell lines by the HUVEC cells. Immunofluorescence (red channel: CD31, green channel: PKH67-exosomes, bleu channel: nucleus) showing the uptake and localization of exosomes derived from MDA-MB-231 and BxPC-3 within HUVEC cells. Colocalization of CD31 and PKH67-exosomes was assessed by 2D intensity histogram.

General Discussion and Conclusion

Although the interest in cancer research rose exponentially during the last century but cancer is not a new disease. Autopsies of ancient Egyptian mummies have shown the presence of bone tumors [150] even also in some ancient prehistoric fossils [151]. Nowadays, according to the World Health Organization (WHO), cancers figure among the leading causes of morbidity and mortality worldwide, with approximately 14 millions new cases and 8.2 millions cancer related deaths in 2012.

Understanding the mechanisms that control cancer progression is the key for developing new therapeutic tools helping in the fight against this disease. Most of the research teams focus on the cancer cells themselves; while in fact; cancer acts like an organ-like structure. This means that cancer communicates with host systems including stromal cells, immune cells and blood vessels [152]. This cancer communication with its environment is now recognized as a key factor in cancer progression as it plays a role in local resistance, immune-escape and metastasis dissemination [153].

Myoferlin has been reported to be over expressed in pancreatic and breast cancers and to be associated with bad prognosis [30]. In this study, we confirm myoferlin expression at the cellular level in all tested breast and pancreatic cancer cell lines and we confirm its association with bad prognosis in PDAC cancer. We report here for the first time, myoferlin expression at the exosomal level in all tested breast and pancreatic cancer cell lines. We further show the implication of myoferlin in exocytosis and in exosome biology. We describe the role of myoferlin in the biogenesis and the fusion of exosomes and/or exocytosis vesicles.

There is actually an immense body of literature studying different factors implicated in different kinds of vesicle biogenesis, targeting and fusion. Most of these factors are being studied in normal physiological conditions, however, since myoferlin is being further and further proposed to be a potential biomarker of cancer, it is of extreme interest to investigate its role in cancer communication. Targeting specifically cancer communication would be an extremely useful tool to manage cancer, bypassing limitations and side effects of actual cancer management molecules.

We have first oriented our study to the pancreas cancer as our laboratory and others have reported that PDAC overexpresses myoferlin. Concretely, we have decided to use two pancreatic cancer cell lines, which are BxPC-3 and Panc-1 cell lines. Both cell lines express myoferlin but only BxPC-3 cells express high levels of VEGF-A, which makes it a suitable model to study angiogenesis. Moreover, our laboratory had previously shown that this cell line is able to grow on CAM model, actually growing inside the CAM connective tissue giving rise to functionally vascularized tumors; these tumors grow as unique spherical nodules mimicking to a great extent the human PDAC [99]. Overall, BxPC-3 tumors grown on CAM could be a very interesting, easy, economically and ethically suitable model to study pancreatic cancer growth and angiogenesis. Panc-1 cell line expresses almost the same level of myoferlin as BxPC-3 cell line, however, it does not neither grow on CAM nor produce high levels of VEGF-A but yet, it is widely used in the literature, so it is a valid cell line to perform functional studies.

The work of Turtoi et al, demonstrated that myoferlin is essential for breast cancer tumor growth on CAM. Myoferlin depletion in MDA-MB-231 cell line provoked a significant decrease in tumor volume [34]. Our results come in consistency with the findings of Turtoi, where we showed that myoferlin depletion in BxPC-3 tumors grown on CAM gave rise to significantly smaller tumors when compared to normal counterparts **(Publication 1)**. Moreover, our observations were extended to the tumor appearances, where we noticed that myoferlin-depleted tumors were whitish and pale compared to control ones, implying a probable alteration in the vascularization of the tumors.

Myoferlin depletion has been reported to provoke a decrease in cell proliferation in different cell types including endothelial cells [23]. We have demonstrated that myoferlin depletion in BxPC-3 cells *in vitro* provoked a significant decrease in the cell growth. We have also confirmed the same effect on Panc-1 cells; suggesting a VEGF-A-independent effect as Panc-1 cell line produces almost no VEGF-A. These results are in consistency with Wang et al, findings [30] as they have demonstrated a significant cell growth inhibition after myoferlin silencing on both BxPC-3 and CFPac-1 cell lines.

Wang demonstrated that myoferlin depletion provokes a slight (5%) but significant increase in apoptosis and an arrest of the cell cycle in the S phase in both BxPC-3 and CFPac-1 cell lines. Our results are not in consistency with Wang as we show that myoferlin depletion does not significantly increase apoptosis, but are in consistency with Leung et al. findings, where they showed that myoferlin depletion in Lewis Lung carcinoma cells (LLC) provoked a decrease in cell proliferation without an increase in apoptosis [35]. This difference between our results and the results of Wang et al. could be due to the different technique used, as they have used a methodology that may recognize necrotic cells as well.

Overall results explain, at least in part, the decrease in myoferlin-depleted tumor sizes on CAM but does not explain why myoferlin-depleted BxPC-3 tumors have whitish pale appearances. Our immunostaining analysis of the tumors showed a decrease in the vascularization of myoferlin-depleted tumors. While Wang et al. and Turtoi et al. did not investigate MVD in their respective work, Leung et al. showed that myoferlin-depleted LLC cells injected in mice lungs had no effect neither on MVD nor on serum VEGF-A levels [30,34,35].

We are reporting for the first time, according to our knowledge, that myoferlin depletion significantly decreased VEGF-A secretion into the conditioned medium. The implication of myoferlin in soluble factor secretion has previously been proposed by Eisenberg et at, in the context of breast cancer [31].

Our immunofluorescence data showed the accumulation of VEGF-A at the vicinity of the plasma membrane concomitantly with the accumulation of vesicle-like structure seen by the electron microscopy in the myoferlin-depleted condition. This hypothesizes that VEGF-A containing exocytosis vesicles are approaching the plasma membrane to fuse and secrete their cargo of VEGF-A to make the call for angiogenesis. However, this does not occur in the absence of myoferlin. Therefore, we hypothesize a role of myoferlin in vesicle-mediated fusion events. Actually this is in consistency with previous observations made for other

members of the ferlin family (dysferlin and Fer-1) and even for myoferlin itself; where their depletion in different cellular models provoked also an accumulation of vesicle-like structures in the plasma membrane area.

The major contribution of this work is to show that myoferlin plays a role in the trafficking of VEGF-A containing vesicles by pancreatic cancer cells. This is probably due to the possession of myoferlin of six C2 domains that sculpt, in an additive manner, the vesicular lipid bilayer resulting in high curved or distorted membrane regions that could facilitate membrane fusion and fission or recruitment of other membrane trafficking proteins, as shown by Marty [11].

Mechanistically, Xie et al, have shown that Exoc2/Sec5 is expressed at the surface of insulin secretory granules of the beta-cell of the pancreas and that it plays a key role in mediating docking of these vesicles to the plasma membrane [154]. Our data show a partial colocalization of myoferlin with Exoc2/Sec5, very interestingly, at the vicinity of the plasma membrane fortifying our hypothesis of myoferlin's implication in exocytosis machinery.

However, we do not know where myoferlin is located in the exocytosis process. Myoferlin is a type II transmembrane protein reported to be present at the inner leaflet of the plasma membrane. In the current work, we report myoferlin to be expressed on the external surface of exosomes (**Publication 2**). So myoferlin could also be present on the external surface of exocytotic vesicles to mediate their fusion with the plasma membrane, like SNARE proteins [155,156].

Plasma membrane-anchored myoferlin could interact with negatively charged phospholipids present on the outer surface of exocytotic vesicles, which may explain why insulin secretory granules increase their composition of negatively charged phospholipids, as mentioned in the introduction. Another figure is that myoferlin could be recruited on the outer surface of the exocytotic vesicles, like v-SNARE proteins, to bring extra-fluidity to the exocytotic vesicle membranes and facilitate their docking and fusion. Rein et al. proposed that v-SNARE proteins could be directly recruited at the external surface of exocytotic vesicles [157]. Which in all cases, is in consistency with the reported role of myoferlin in mediating fusion events at the membrane site [5,20,22].

Redpath et al, demonstrated the constitutive production of mini-myoferlin that has a great structure similarity with synaptotagmins [84]. While synaptotagmin is expressed on the outer surface of exocytotic vesicles [158,159] mediating their fusion in a Ca^{2+}-dependent manner; it is very speculative that myoferlin could act exactly like synaptotagmin (Figure 10).

To further fortify our hypothesis, we showed that myoferlin expression in PDAC sections is positively associated with the MVD, while it is known that VEGF-A is associated with MVD [160]. This means that myoferlin could play a role in VEGF-A exocytotic machinery and so, VEGF-A mediated angiogenesis in PDAC patients as it does for BxPC-3 tumors grown on CAM model.

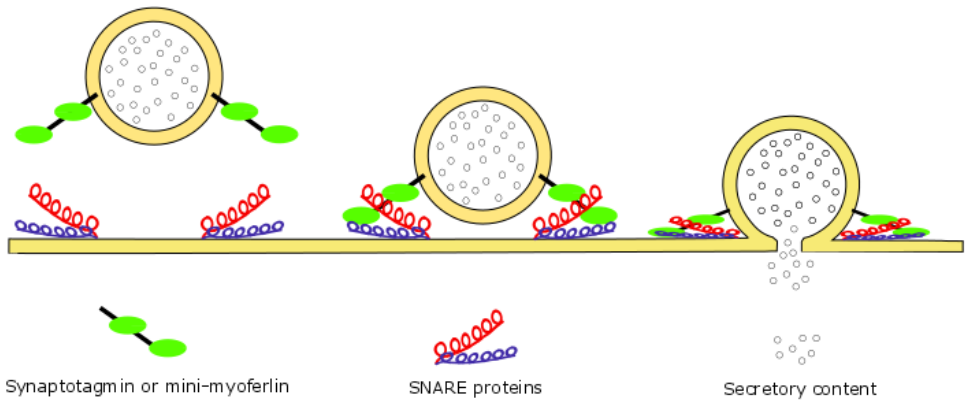


Figure 10. Hypothetic model of myoferlin involvement in exocytosis. Exocytosis vesicles fusion at the plasma membrane and the interaction between C2 domains bearing proteins (Synaptotagmin or myoferlin) with SNARE proteins. Fahmy et al., 2015 [9].

It is of particular interest to further investigate the localization of myoferlin during exocytosis. In other words, knowing if myoferlin is present on the surface of exocytotic vesicles or on the inner leaflet of the plasma membrane interacting with the other element's phospholipids or, may be, proteins. As well, it would also bring a great importance to investigate if BxPC-3 cells, and PDAC in general, do express mini-myoferlin or not. In general terms, this will help to understand if myoferlin is implicated in other soluble factors secretion or if it plays solely a specific role in VEGF-A secretion. If myoferlin is a general player in exocytosis, this implies that myoferlin plays a pivotal role in cancer communication and cross talk with its microenvironment. In that case, a deeper exhaustive research would provide more information about myoferlin in cancer communication.

However, we show in the present study that myoferlin does not solely play a role in exocytosis vesicle trafficking; our work demonstrated also the role of myoferlin in exosomes mediated communication.

As mentioned earlier, we have demonstrated by immunoblotting the presence of myoferlin in exosomes derived from breast and pancreatic cancer cell lines of different molecular and genetic backgrounds (**Publication 2**). Actually, myoferlin presence in exosomes has been previously mentioned, indirectly, in the literature. A query on exocarta (www.exocarta.org), which is a database of proteomic studies of exosomes, shows that myoferlin has been identified in a multitude of cancer cell-derived exosomes. These studies did not target studying myoferlin in exosomes, however myoferlin was one protein among a list of proteins identified thanks to proteomic profiling of exosomes. None of these studies have further investigated myoferlin role in exosomes or event confirmed its identity by other technique than proteomic profiling.

We further confirmed the presence of myoferlin in exosomes derived from MDA-MB-231 cell line by immunogold on entire adsorbed exosomes. Our data show, for the first time, the abundant presence of myoferlin on the external surface of exosomes. The antibody used for immunogold is oriented against an epitope within the C2 domains region meaning that

myoferlin has its C2 domains region oriented outside of the exosomes. This myoferlin orientation is unexpected as exosomes undergo two reverse budding events, one from the cell surface to form primary endosome and the second at the late endosomal limiting membrane to form the ILV [124]; so exosomes normally maintain the same topology of the cell. For example, tetraspanins are transmembrane surface receptors [161]; they preserve their external-surface orientation in exosomes [162], but in exosomes myoferlin orientation was reversed. This reversed orientation of myoferlin could be interpreted in different ways. Myoferlin could have been recruited on the external surface of exosomes, or could have undergone a flipping event to revert its orientation or finally, myoferlin could have simply passively stuck on the exosome surface.

In all cases, this external-surface localization of myoferlin hypothesizes a role in exosome fusion with the target cells or even selection of the target cells. Some external-surface proteins, like tetraspanins and ICAM-I, are proven to mediate selection of the target cells [163], however, we have no data regarding myoferlin implication in that process. Regarding fusion of exosomes, again some external-surface proteins, like tetraspanins and integrins, are implicated in exosomal fusion via the establishment of a network with target cell surface molecules and phospholipids [164][165]. Noteworthy, CD9 has been further reported to mediate other fusion processes, e.g., it is implicated in skeletal muscle fusion events [166].

Since myoferlin mediates different fusion processes, it is probable that it could mediate exosomal fusion with the target cells. In the present work, we showed that exosomes derived from myoferlin-depleted cancer cells are less able to fuse or to be up taken by the target HUVEC cells. We also showed a decrease in the exosomal shuttling of a murine miRNA from donor cells to recipient HUVEC cells after myoferlin silencing in cancer cells.

On the other hand, myoferlin has been reported to colocalize with $Rab7^{+}$ large vesicles of late endosomes near nucleus and plasma membrane. It occasionally colocalizes with Lamp-1 positive vesicles [86] meaning that it is most likely present in the endosomal trafficking compartment and least likely implicated in the lysosomal degradation pathway. We hypothesized that myoferlin could then play a role in MVB biogenesis. Our results showed that myoferlin depletion did not alter exosomes quantity but it did, indeed, alter their size. It was then worthy investigating exosomal content.

We demonstrated in this study that myoferlin depletion from BxPC-3 and MDA-MB-231 cells provoked a significant modulation of their corresponding exosomal cargo, implying a role of myoferlin in cargo selection. Among several modulated factors, we reported the down regulation of several vesicular markers like caveolin-1, flotillin-1, flotillin-2 and syntaxin-6; also a down regulation of tetraspanins markers like CD63. This observation could be of extreme interest, as for instance, flotillin-1, flotillin-2 and CD63 have all been reported to play a role in exosome biogenesis and cargo selection [167][168].

Moreover, we showed also the down regulation of Rab7a in the exosomes. Although its role in cancer progression is controversial, Rab7a has been reported to have some pro-tumor effects [139,169],[140]. On the same path, CD63 has been reported to act as pro-metastatic [143]. Overall, our results propose that myoferlin is a key player in the exosome biogenesis

whether directly or even indirectly by down regulating other proteins implicated in MVB biogenesis. Strikingly, it seems that myoferlin mediates the packaging of a selected pro-cancerous cargo, which adds to the already proved explanation of the anti-tumor effect observed in the myoferlin-depleted tumors in various studies.

It has been previously reported that cancer-derived exosomes are able to induce proliferation and migration of HUVEC cells [170–172]. So to investigate the role of myoferlin in exosome-mediated tumorigenicity, we have decided to investigate a phenotypic change in the HUVEC cells after incubation with exosomes that are originated from myoferlin-depleted cells or not. In consistency with the literature, we showed that BxPC-3 and MDA-MB-231 derived exosomes increase the proliferation and the migration of HUVEC cells. But interestingly, we showed that myoferlin depletion from these exosomes provoked a decrease or abolishment of these effects. It means, myoferlin-depleted exosomes provoked less or no effect on endothelial cell proliferation and migration.

There are two possible explanations for why myoferlin-depleted exosomes mediated less or no phenotypic changes in endothelial cells. First explanation is that the absence of myoferlin from the surface of exosomes provoked a decrease in their uptake by the HUVEC cells and so less transfer of their cargo, as we reported in here. Another explanation is that, as we had also shown, myoferlin plays a role in exosome biogenesis especially in cargo selection. It is very possible that exosomal content in the absence of myoferlin lacks pro-angiogenic factors. Further investigations will be needed to confirm this hypothesis.

Exosomes are of extreme importance in cancer communication. Communication with the tumor microenvironment is vital for tumor progression and metastasis. Cancer cells secrete exosomes to reprogram their niche and establish favorable conditions for tumor growth and invasion of healthy tissues. Cancer-derived exosomes are reported to be implicated in metastasis dissemination of primary tumors [129,], immune-escape of the cancer cells [83], and also angiogenesis to guaranty tumor oxygenation, nutrients arrival and the transition from hyperplasia to neoplasia [174,175].

Generally speaking, PDAC is considered as a hypovascularized tumor, however high VEGF-A correlates with decreased median survival time [112] and with decreased survival even after surgical removal of the tumor [113]. VEGF-A also correlates with liver metastasis [107] in PDAC patients. As well for breast cancer, VEGF-A is associated with MVD [176], poor survival and relapse [177]. This implies that targeting angiogenesis is an important tool for the fight against cancer. However, treatment failure of anti-angiogenic therapies is a fact; and this is so far explained by the multiplicity of the pro-angiogenic factors and the redundancy of angiogenic signaling pathways within the tumor [45]. Interestingly, we reported myoferlin, in here, as a novel player in vesicle-mediated communication. We have demonstrated the implication of myoferlin in exosome-mediated communication in different cancer cell types. We have also showed that myoferlin plays an important role in VEGF-A exocytosis in pancreatic cancer. This means that myoferlin could be a very interesting target in the anti-angiogenic therapy especially if proven that myoferlin is a general player in cellular communication, i.e., not solely playing a role in specific soluble factors secretion but also in different exocytosis events, and in different types of tumors.

Generally, myoferlin-depleted breast cancer in mice models have been described to be smaller and less invasive [98]; while for pancreas cancer, myoferlin-depleted tumors in mice model are also smaller [30]. This is thought to be due to an impairment in membrane repair [24,85], internalization of growth factor receptors [5,21,178] and motility [32,33,98]. Our current work brings a new aspect of the story, is the ability of myoferlin to mediate extracellular communication with the tumor microenvironment via exosomes and exocytosis vesicles.

A more thorough research is moreover required to investigate myoferlin implication in selective pro-cancerous cargo selection and target cell selection. Myoferlin has many domains with yet unknown functions. An investigation targeting myoferlin implication in mRNA and miRNA cargo selection could provide new insight for further myoferlin implication in cancer communication. As previously mentioned, tetraspanins for instance are reported to mediate target cell selection. So due to function resemblance with myoferlin in terms of cargo selection and fusion, it would also be interesting to check if myoferlin could play a role in that process too.

As a conclusion of this study, we confirm myoferlin to be an important factor in PDAC progression. Myoferlin plays a role in tumor cell growth as well as VEGF-A mediated angiogenesis. Mechanistically, myoferlin plays a role in VEGF-A secretion without altering its transcription. Myoferlin seems to mediate VEGF-A secretory granules docking and fusion with the plasma membrane in order to secrete their cargo and to make the call for angiogenesis. In vivo chicken chorioallantoic membrane model, myoferlin depletion reduced both tumor volumes and tumor vascularization. On the patients level, myoferlin staining in PDAC sections correlates with both high MVD and poor survival.

We also report in here for the first time, that myoferlin is expressed in different breast and pancreatic cancer cell-derived exosomes. We prove that myoferlin is expressed on the outer surface of exosomes and is involved in the fusion and uptake of exosomes by HUVEC cells. Myoferlin depletion of exosomes reduces uptake of exosomes by the target cells and so decreases the cargo transfer into them. Myoferlin also plays a role in exosome biogenesis as myoferlin-depleted exosomes are smaller and harbor altered protein content compared to normal counterparts. Overall, myoferlin plays a role in exosome functions, as myoferlin-depleted exosomes are able to mediate less phenotypic changes in HUVEC cells.

Annexes

REVIEW: STUDYING THE ROLE OF MYOFERLIN IN CELL COMMUNICATION

PUBLICATION III

Myoferlin, a C2 Domain-Rich Protein, Is a First League Player in Vesicle-Mediated Cellular Communication.

K. Fahmy, V. Castronovo, O. Peulen

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1. INTRODUCTION

Cell communication broadly includes all types of inter-cellular communications. Since cells do not live isolated, Cell survival is dependent on sending and receiving information from their environment and neighbors. McCrea described cell communication as “The music that the nucleus hears” as cell’s response to the surrounding environment affects gene transcription programs [2]. It means that cell communication intervenes in morphogenesis, cell differentiation, homeostasis, cell growth and cell interactions [1].

In this review, we focus on the vesicle-mediated cellular communication, especially exocytosis vesicles and receptor-mediated endocytosis/endocytic recycling, and on the importance of Myoferlin in the vesicle-mediated communication.

1.2. Vesicle mediated cellular communication

It is noteworthy to mention at first that endocytosis and exocytosis are related fundamental biological processes. The endo-exocytosis relation could be described as an endless circle where endocytosis is maintained by endocytic-vesicle recycling and exocytosis, to prevent plasmic membrane exhaustion.

Exocytosis is the trafficking of secretory vesicles to the plasma membrane. It is the basis of cellular communication in multicellular organisms through the release of a wide range of extracellular acting molecules like growth factors, plasma proteins, antibodies and extracellular matrix components to exert various functions. Exocytosis vesicles formation starts at the trans-Golgi network (TGN) where the cargo of soluble proteins assembles at the inner surface of the TGN. It is sought that this aggregation of proteins is the main driver of the deformation of the TGN membrane and budding of vesicles. Nascent granules bud at the surface but they require a pinching to be release as immature secretory granules (ISG) into the cytoplasm. At that point, cholesterol seems to play an important role in mediating this fission process probably by directly facilitating negative membrane curvature at the bud-neck or indirectly by recruiting raft associated proteins that carry out the fission [179]. In ISG, the lipid bilayer of the secretory vesicle contains phospholipids and proteins required

for transport, targeting and fusion. While the dense core of the secretory vesicles contains the soluble protein cargo to be secreted [39,40]. However, there are still unknowns in the exact composition of the vesicle membrane components that might be involved in vesicle transport, targeting and fusion.

Endocytosis-endosomal recycling is a more complex process than exocytosis. Endocytosis is the internalization of extracellular materials, membrane proteins and lipids while the endosomal recycling returns much of these components back to the plasma membrane. The balance between endocytosis, exocytosis and endosomal recycling controls the composition of the plasma membrane.

Endocytosis has two modes of action, Clathrin Dependent Endocytosis (CDE) that is a well-studied mode and Non-Clathrin Dependent Endocytosis (NCE). The latter mode is subdivided into Dynamine-dependent and Dynamine-independent NCE but yet, it remains not so much studied as CDE. Primary endocytic vesicles are membrane bound intracellular vesicles formed by invagination of the plasma membrane around an extracellular substrate, they could either be coated or stripped vesicles so they are by definition composed of plasma membrane and may be a coat [180]. The cargo of the endocytosis arrives in primary vesicles to the early endosomes where cargos are being sorted to go back to the surface, to the endosomal recycling compartment, to late endosomes for degradation or to Multivesicular Bodies (MVB) for exosomes biogenesis.

1.3. Phospholipid binding proteins mediating fusion and fission processes of vesicles

As previously mentioned for exocytosis vesicles, the exact composition of lipids and proteins of the unilamellar lipid bilayer membrane of vesicles are not yet totally revealed. However, studies have identified some negatively charged phospholipids like phosphatidylserine (PS) and phosphatidylinositol (PI), and some positively charged phospholipids like phosphatidylcholine (PC), phosphatidylethanolamine (PE), sphingomyelin (SM). An interesting observation was made for “Insulin Secretory Granules” of the pancreatic beta cells; they increase their membrane composition of negatively charged phospholipids in

response to glucose stimulation, increasing exocytosis of insulin. Moreover, they also increase their composition of unsaturated short fatty acids which brings more fluidity and favors membrane curvature of vesicles that would enhance docking and fusion of vesicles with the plasma membrane [40].

There are evidences suggesting that negatively charged phospholipids on the outer (cytoplasmic) leaflet of the vesicles membrane and inner (cytoplasmic) leaflet of the plasma membrane act as coupling factors enhancing interaction between negatively charged phospholipids and positively charged motifs of SNAP (synaptosomal-associated protein) receptors (SNARE) which are a large protein superfamily with a primary role in vesicle fusion. It is believed that the anionic characteristic of the serine head group of PS promotes interaction between PS and positively charged SNARE proteins. SNARE proteins like the vesicular Synaptobrevin (VAMP2), the membranous Syntaxin1a (Syxt1a) and membranous synaptosome-associated protein of 25 kDa (SNAP25), mediate fusion events with the plasma membrane. All these proteins share a highly conserved juxtamembrane poly-basic amino acid sequence called SNARE motif that binds negatively charged phospholipids such as PS as mentioned [40]. It was proven that a mutation of the Calcium-Calmodulin/phospholipids binding motif of VAMP2, decreasing its net positive charge, decreases its interaction with negatively charged phospholipids and interferes with the normal catecholamine secretion from chromaffin cells [41]. As well Synaptotagmin I (SYT1), a dual C2 domains bearing protein implicated in membrane fusion events and exocytosis, binds to negatively charged phospholipids in a Ca2+-dependent manner, thanks to its C2 domains. Increasing PS levels leads to an increase in Ca2+ triggered vesicles fusion by stabilizing fusion pores in PC12 cells and increase Ca2+ dependent SYT1 binding in liposomes [42].

While for endocytosis, phospholipid binding C2 domains bearing proteins also seem to be implicated, it has been proven that SYT1 binds with high affinity to the AP-2 adaptor protein of clathrin-coated vesicles via a dual interaction with the second C2 domain of SYT1, C2B. Mutants SYT1 or SYT7 lacking the C2B domain reduce receptor mediated endocytosis [181]. Moreover SNARE protein, previously mentioned above; also seem to be implicated in endocytosis. Tetanus toxin and Botulinum neurotoxin E treatment results in the cleavage of VAMP and SNAP25 – Syxt1 respectively, and that these cleavages resulted in a decrease of both rapid and slow endocytosis at the calyx of Held, a particularly large synapse in the

mammalian auditory central nervous system. These results were a surprising insight for the dual role of SNARE proteins in both exocytosis and endocytosis [182].

All these information emphasize on the importance of an interaction between negatively charged phospholipids of the vesicles membranes with positively charged motifs/domains, like SNARE motif and C2 domains, to mediate fusion and may be fission events. What brings the attention to ferlin protein family which play important roles in fusion events as they harbors multiple tandem phospholipid binding C2 domains; and especially to their recently identified new member, myoferlin.

2. THE FERLIN FAMILLY

There are six mammalian ferlin proteins (Fer1L1-6). Mutation in Fer1L1 (Dysferlin) and Fer1L2 (Otoferlin) are known to be responsible for inherited autosomal recessive diseases in human [5,13,14].

2.1. Myoferlin's structure reveals multiple domains

Fer1L3, also known as myoferlin, a member of mammalian ferlin protein family was first identified and characterized in 2000 to harbor multiple C2 domains, a unique characteristic of the ferlin family [18,19]. Myoferlin shares some common characteristics with all the members of the mammalian ferlin family; it is a type II transmembrane proteins with a single pass transmembrane region located at the carboxy terminus. The rest of the protein resides in the cytosol bearing multiple tandem C2 domains. Myoferlin harbors several other specific domains as SH3, Ferlin (Fer) and Dysferlin (DysF) domains.

An unique characteristic of ferlin family is to possess multiple C2 domains, while there are about one hundred proteins harboring only a couple of C2 domains. These proteins are all membrane-associated proteins implicated in protein-protein, protein-Ca2+ or protein-lipid interactions [5]. Myoferlin harbors six C2 domains: C2A at amino acids 1-85, C2B at 200-281, C2C at 359-458, C2D at 1126-1231, C2E at 1538-1638 and C2F at 1790-1903; SH3 domain at

676-689 between the C2C and C2D domains; a transmembrane region at 2027-2042 and finally an extracellular domain at 2043-2061 [6,18] (Fig. 1A).

C2 domain is a Ca2+-dependent phospholipid-binding domain found in a variety of membrane-associated proteins. C2 domain is an independently folding domain composed of eight beta-strands forming a sandwich-like structure and coordinating 2 or 3 Ca2+ ions by conserved aspartic acid residues (Fig. 1B). In vitro, the C2A domain, the first C2 domain from the animo-terminal extremity of myoferlin, bounds to negatively charged phospholipids mixture similar to the phospholipid composition of the inner leaflet of the plasma membrane. It has been shown that the C2A domain is able to bind a mixture of 50% PS and 50% PC in the presence of 1mM of Ca2+. However, it failed to bind a mixture of 25% PS in PC meaning that this domain requires a certain amount of negatively charged phospholipids to bind. The requirement of negatively charged phospholipids may be influenced by the number of positively charged residues in the Ca2+ binding loop of the C2A domain. This could hypothesize that myoferlin interacts with specific regions of the plasma membrane or vesicles that are enriched with high concentration of negatively charged phospholipids [85].

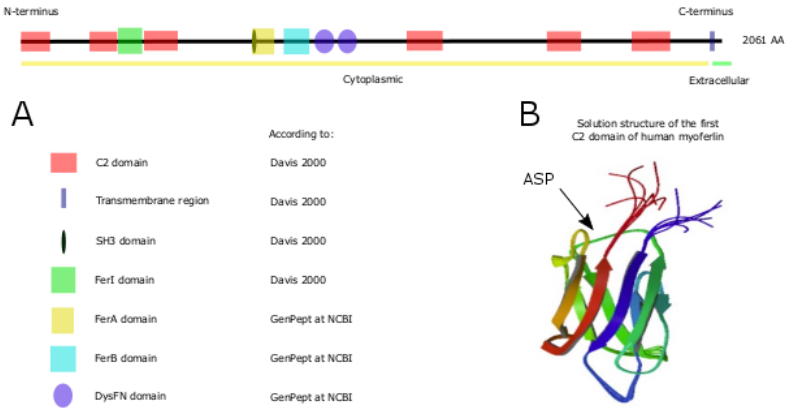

Figure 1. Schematic representation of myoferlin. A. Schematic representation of myoferlin structure according to Davis and colleagues[14] and GenPept at NCBI (Accession number: AAF27176). Myoferlin possesses 6 C2 domains (C2A-F), SH3, FerI, FerA, FerB and 2 DysFN domains within the cytoplasmic region. B. Solution structure of the first C2 domains (C2A) of human myoferlin (2DMH, 10.2210/pdb2dmh/pdb).

However, it has been shown that myoferlin C2A domain cannot bind phospholipids in basal physiological Ca2+ concentration, which is about 0,1µM. Indeed, it requires 1µM of Ca2+ concentration [4]. Suggesting that it is implicated in specific processes inside the cell requiring Ca2+ release from intracellular stock like in Ca2+-regulated exocytosis. When cells

are stimulated by various mean, including depolarization and ligand binding, the cytosolic Ca2+ concentration increases to levels of 1 µM or more [183].

More interestingly, a point mutation in myoferlin (I67D) provokes an abolishment of the Ca2+- dependent phospholipids binding. The same study has also showed that unlike the C2A domain, the remaining C2 domains (C2B to C2F) were unable to bind the mixture of 50% PS and 50% PC. But this doesn't mean that they do not bind to other mixtures. In fact, their role is still poorly known but like all the other C2 bearing proteins, they are probably implicated in protein-protein interactions [4]. Further on, a pilot study has shown that recombinant fragments of the first three C2 domains (C2A, C2B and C2B) of myoferlin are able to bind small unilamellar vesicles composed of 25% PS in PC [11].

Myoferlin bears also Fer and DysF domains which functions are yet unknown, but it has been observed in myoferlin and other members of mammalian ferlin family, that Fer domain appears concomitantly with DysF domain, implying probably a functional relation. Myoferlin also bears a SH3 domain that is known to interact with proline rich domains of proteins [6].

2.2. Structure activity relationship of myoferlin

Unlike most of the C2 domain bearing proteins, myoferlin possesses six C2 domains. Where not only the number of the C2 domains but also special characteristics make the difference between myoferlin and the other C2 domain bearing proteins, implying probably a special function of this protein that is dictated by its unique structure.

Like most of the membrane-associated proteins, myoferlin has a typical plasma membrane-targeting C2 domain as its C2 domains target negatively charged lipids; unlike the C2 domains of "Golgi localized proteins" which bind to zwitterionic lipids. Also and unlike C2 domains of Synaptotagmin I, myoferlin's C2 domains have been proven to bind negatively charged phospholipid vesicles in a salt insensitive manner, suggesting an hydrophobic and non-electrostatic interaction contributing to the interaction of C2 domains and lipid bilayer [11]. These information provide insights that myoferlin could be a key player in vesicles trafficking thanks to its ability to stably bind negatively charged phospholipids at the vesicles or plasma membrane sites.

Further on, it has been shown that myoferlin C2 domains do not solely passively stick to the membranes thanks to electrostatic attraction but they sculpt lipid bilayers which results in high curvature or disorder of membrane regions that could facilitate membrane fusion and fission or recruitment of other membrane trafficking proteins by an increase in fluidity and alteration of the lipid order of the lipid bilayer. Of interest, a study has shown that the more the C2 domains number increases the more they perturb the structural state of lipid bilayer in an additive manner. It means that myoferlin possession of six C2 domains probably implies a more special role than most of the proteins bearing maximum two C2 domains. Moreover, this structural disruption property of lipid vesicles was shown to be Ca2+-enhanced specially for domains bearing calcium binding motifs [11]. Overall, this means that, the "Insulin Secretory Granules" fluidity brought by increasing their composition of short, unsaturated and negatively charged phospholipids could also be brought instead by recruiting myoferlin at the vesicles surface.

The C2 domain structure folds independently and reveals eight antiparallel beta-strands connected by highly variable surface loop forming a beta-sandwich with a highly conserved Ca2+-binding loop at one end of the sandwich. The C2 domains are classically observed to function as Ca2+-dependent lipid binding modules. However, some C2 domains, like the C2B domain of myoferlin, lost their Ca2+-sensing ability and instead specialized in protein-protein interaction [8].

While most of the research focus on the first C2 domain, C2A, phylogenetic analysis reveals that it is the least conserved domain among mammalian ferlin family. Homology modeling among mammalian ferlin proteins suggests a higher conservation of the two most carboxy terminus C2 domains (C2E and C2F) relative to C2A and C2C that showed great variability in predicted structure and electrostatic properties [3]. It has been also demonstrated that the three most carboxy-terminus C2 domains (C2DEF) of otoferlin and dysferlin perturb more the structural state of small unilamellar lipid bilayer vesicles than the three most amino-terminus C2 domains (C2ABC) [11].

Of interest to these two particular C2 domains (C2E and C2F), it has been shown that myoferlin possesses an alternative splicing variant that gives rise to an isoform bearing a putative motif for calpain cleavage. This cleavage produce a fragment (mini-protein) composed of the last two C2 domains at the carboxy terminus (C2E and C2F) and the

transmembrane region, and being structurally similar to synaptotagmin. Unlike dysferlin and otoferlin, myoferlin cleavage by the calpain was Ca2+-independent but the Ca2+ would solely increase the yield of the reaction; in fact, this fragment was observed constitutively in ectopic expression model [84]. Moreover, sequence analysis of myoferlin revealed that C2D, C2E and C2F domains have putative Ca2+-binding motives at positions similar to the first C2 domain of synaptotagmin-1 [19]. Allover, these information and the constitutive expression of myoferlin mini-protein may imply its implication in a constitutive cellular processes, liked vesicle trafficking.

2.3. Myoferlin's implication in exocytosis

Apart from the structure-based activity of ferlins, the observation of ferlin proteins implication in exocytosis events was first revealed for otoferlin. Otoferlin is expressed in the auditory Inner Hair Cells (IHC) ribbon synapse; precisely at the surface of synaptic exocytosis vesicles, mediating their exocytosis via its binding to exocytosis component syntaxin-1 and SNAP25 in a Ca2+-dependent manner. Otoferlin mutation in human causes an autosomal recessive form of non-syndromic deafness (DFNB9); as well, otoferlin null mice are profoundly deaf [13,14].

A recent study published by our group highlighted a new function of myoferlin in exocytosis. We showed that myoferlin silencing provoked a decrease in VEGF-A secretion by pancreas cancer cell line BxPC-3. This decrease in secretion was not due to a transcriptional effect of myoferlin but due to an accumulation of VEGF-A at the vicinity of the plasma membrane coincidently with the ultrastructure observation of vesicles accumulated near the plasma membrane. We hypothesized that myoferlin depletion disabled VEGF-A exocytic vesicles to fuse with the plasma membrane and release their cargo. In order to confirm myoferlin's implication in the exocytosis machinery, we further demonstrated a colocalization of myoferlin with Sec5/Exoc2, a component of a complex essential for targeting exocytosis vesicles, mainly at the periphery of the cells where exocytosis vesicles fusion with the plasma membrane occurs. This myoferlin control over the fusion of exocytic vesicles containing VEGF-A was confirmed by a decrease in micro-vessels density (MVD) in animal

model and a statistical significance was observed between myoferlin expression and MVD in pancreas cancer patient sections [9].

Noteworthy to mention that a mathematical model was established to examine the role of myoferlin in cancer cell invasion; as a tool to generate hypotheses which then can be experimentally tested. This model proposed a diversified role of myoferlin in breast cancer invasion including a mathematical hypothesis that myoferlin impacts matrix metalloprotease production and/or secretion; hypothesizing a probable role of myoferlin in exocytosis [31].

As previously mentioned above, myoferlin was proven to be processed by the calpain enzyme releasing a fragment; while this observation was initially conducted for dysferlin it has been then extended for myoferlin and otoferlin. Where all three proteins gave rise to a 70 kDa fragment called mini-protein. Mini-dysferlin was further shown to be generated in myoblast cells after injury and to be present and active in cell membrane repair at the injury site confirming that these mini-proteins could be functionally active. Of interest, these fragments released are structurally similar to synaptotagmin. Mini-myoferlin, unlike dysferlin and otoferlin, was constitutively generated in ectopic expression model in a Ca2+-independent but favored circumstances implying that it could be implicated in a constitutive process like exocytosis.

Based on structural data, on the constitutive expression of mini-myoferlin and on the regulation of VEGF-A secretion by myoferlin, we hypothesize that this protein could be present either or both on the outer leaflet of exocytosis vesicle or the inner leaflet of the plasma membrane mediating the fusion process of secretory vesicles (Fig. 2).

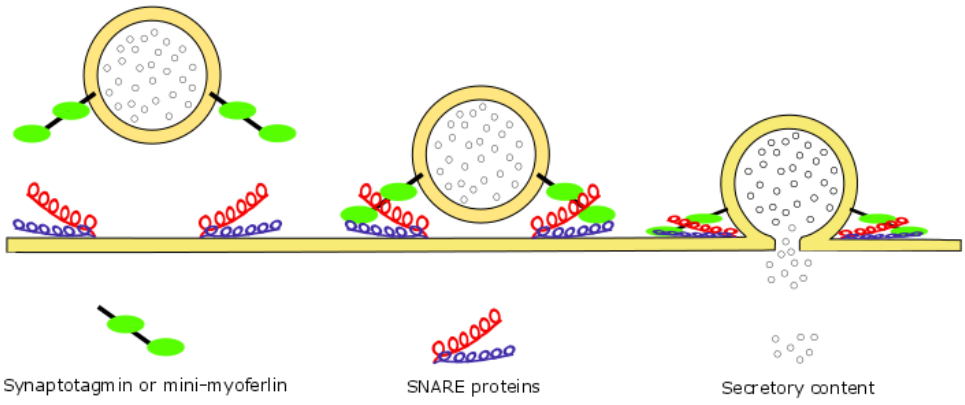

Figure 2. Schematic representation of possible myoferlin role in exocytosis vesicles fusion. Synaptotagmin or mini-myoferlin could be present on the external (cytoplasmic) leaflet of exocytosis vesicles and interact with SNARE protein present on the inner (cytoplasmic) leaflet of cytoplasmic membrane mediating exocytosis vesicles fusion and exocytosis of the content.

2.4. Myoferlin's implication in endocytosis/endocytic recycling

While myoferlin harbors multiple tandem C2 domains, it also harbors several domains and motifs that promote other functions. Where it has been shown that myoferlin intervenes in different surface receptor fate determination via different mechanisms.

Myoferlin plays an important role in Insulin Growth Factor Receptor (IGFR) stability after activation and internalization. In normal conditions, once IGFR is internalized it should be processed again to the surface of the cell, where normally 80% of the receptor is recycled and 20% is degraded. However, in myoferlin depletion this ratio is not respected and IGFR accumulates in aggregates in the cytoplasm and colocalizes with LAMP-2 positive vesicles, implying that it is shuttled in to the lysosomal degradation pathway (Fig. 3A). It was also observed an increase in LAMP-2 positive vesicles number and size in myoferlin null mice myoblast. In absence of myoferlin, this degradation also leads to a decrease in IGFR activation of downstream signaling pathways like AKT and MAPK. Mechanistically, myoferlin physically interacts with IGFR and EHD-2 preventing IGFR degradation but mediating surface export [12]. The provoked defect on internalized vesicles recycling after a myoferlin loss resembles the phenotype due to mutations in the EHD protein family. EHD family contains 4 proteins (EHD1-4) that are linked to endosomal trafficking including recycling of surface receptor to the plasma membrane. It has been shown in myoblasts that at least EHD-2 physically interacts with the amino acid sequence "Asparagine-Proline-Phenylalanine"-(NPF) present in the C2B domain of myoferlin. This binding of both protein was calcium insensitive as the C2B domain of myoferlin lacks a Ca2+ sensing motif and solely implicated in protein-protein interactions, as mentioned before [21]. It was also observed a decrease in transferring receptor recycling after myoferlin silencing in myoblast cells.

Another role of myoferlin is the control of the signaling and the stability of Vascular Endothelial Growth Factor Receptor-2 (VEGFR2) after VEGF stimulation. Myoferlin depletion in endothelial cells leads to the polyubiquitination of VEGFR2 and its subsequent degradation by the proteasome, leading to less surface receptor recycling (Fig. 3A). This leads to the decrease of VEGFR phosphorylation itself and downstream phosphorylation of ERK1/2, JNK and PLC-γ and subsequently endothelial cells migration and proliferation. While myoferlin has a SH3 domain that is known to interact with proline rich proteins, it has been

shown that it directly interacts with the proline rich protein: dynamine-2 via this domain, and complexes VEGFR-2 preventing casitas B-lineage lymphoma (CBL) dependent polyubiquitination and degradation [6].

In breast cancer cells, myoferlin depletion provoked a sustained Epidermal Growth Factor Receptor (EGFR) phosphorylation, after stimulation with EGF, with downstream phosphorylation of Akt. Mechanistically, caveolin was shown to colocalize with phosphorylated-EGFR and myoferlin, and that the interaction between myoferlin and caveolin was essential for the proper assembly of caveolae. In the absence of myoferlin, caveolin showed less colocalization with phosphorylated-EGFR and aberrant homo-oligomerization and no alteration of the EGFR internalization kinetics after EGF treatment. This hypothesizes that myoferlin depletion does not alter non-clathrin-mediated endocytosis but hinders the degradation of the activated receptor in that model [34].

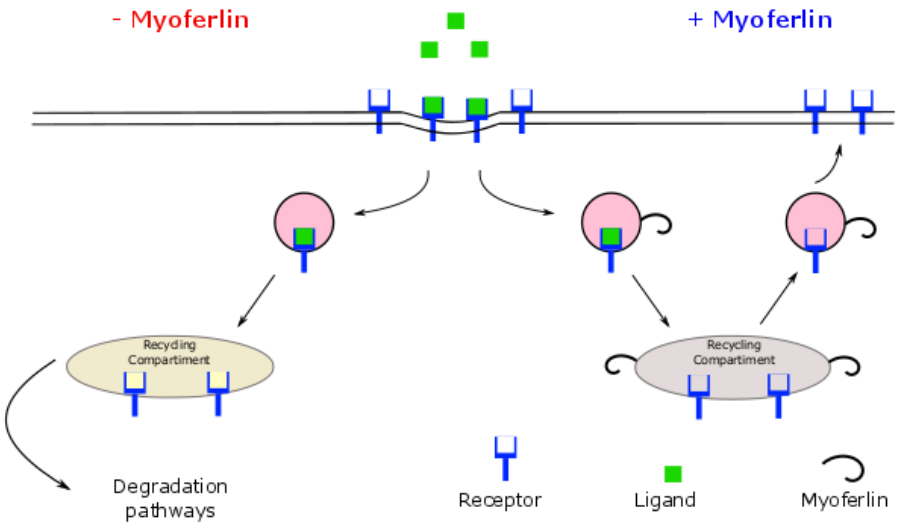

Figure 3. Schematic representation of possible myoferlin implication in endocytic recycling. Myoferlin interferes via different mechanism in surface receptor internalization and could be present on early endosomes until its trafficking to the recycling compartment. In the presence of myoferlin, surface receptor are protected from degradation and recycled to the surface. However, in absence of myoferlin, surface receptors can undergo degradation via different pathways.

However, another study highlighted a new role of myoferlin in receptor-mediated endocytosis for both clathrin-mediated endocytosis and non-clathrin mediated endocytosis. It has been shown that myoferlin depletion in endothelial cells provoked a 50 % decrease in the internalization of both transferrin and cholera toxin-b chain. The first one is a CD71 ligand requiring clathrin pits for endocytosis. The second is a GM-1 ligand requiring

caveolin/lipid raft for endocytosis. The myoferlin ectopic expression increased both the uptake of transferrin and cholera toxin. Mechanistically, it has been proven that both myoferlin and caveolin-1 act on receptor endocytosis in a similar rather than a parallel manner to mediate caveolin-mediated endocytosis and that they physically interact together in a cell free system. Furthermore, that myoferlin colocalizes with Dynamine-2 in endothelial cells. These data show that myoferlin regulates caveolin-mediated endocytosis and also clathrin mediated endocytosis [24].

Again in endothelial cells, myoferlin depletion provokes a decrease of Tie-2 receptor in a non-angiopoietin challenged system without neither altering its transcription level nor in a CBL-dependent polyubiquitination and degradation [23]. This implies yet unknown mechanisms of myoferlin implication in endocytic recycling other than the previously mentioned ones. Noteworthy, is was observed that myoferlin depletion leads to a down regulation of the phosphorylation of several Tyrosine Kinase receptors including EphB4, FGFR-2, HcK, Jak-2, and TXK but yet the mechanism has not been investigated [31,32].

2.5. What is the importance of myoferlin in translational oncology?

Myoferlin has been reported to be overexpressed at protein level in different cancer types including pancreas [28–30], breast [34], lung [35]and oropharyngeal squamous cell carcinoma [37]. In these cases, myoferlin expression is correlated with a bad outcome for the patients.

Self-sufficiency in growth factor signaling is a hallmark of cancer cells. Cancer cells overproduce growth factor to stimulate unregulated proliferation of themselves in an autocrine, juxtacrine or paracrine fashions. In this context, myoferlin can be considered as a growth support for the cancer cells as it helps the exocytosis of growth factors.

Myoferlin was described in breast cancer [34] and in endothelial cell [6] as involved in receptor tyrosine kinase (EGFR, VEGFR) recycling or expression, allowing as such the cell response to growth factors. Knowing that some cancer cells exhibit mutations in tyrosine kinase receptors, which lead to a constitutive receptor activation triggering the downstream pathways, we can speculate that myoferlin depletion could impede cell proliferation in these cases.

Exosomes are small extracellular vesicles released on exocytosis of multivesicular bodies filled with intraluminal vesicles. They represent an important role in intercellular communication, serving as carrier for transfer of miRNA, proteins, … between cells. The exosomes are more and more described as cancer biomarker [184] and involved in the preparation of the tumor microenvironment [185]. The exosome biogenesis, particularly the docking of the multivesicular bodies to the plasmic membrane, required SNARE complexes [186]. Interestingly, myoferlin was demonstrated to be present in exosomes isolated from several cancer cell types, including bladder, colon and ovary [89,94–96,187,188]. In this context, we suggest that myoferlin can be used as therapeutic target in order to prevent exosome release from cancer cells.

Myoferlin, being overexpressed in several cancer types and a potential modulator of exocytosis and surface receptor fate determination, offers a very promising therapeutic advantage in cancer management. Myoferlin's promising properties make one think that its targeting may confer to the therapy a multi-functional aspect, starting from tumor-detection, to tumor-targeting, to the growth factor signaling. Targeting myoferlin at the expression or functional levels remains however the next challenge.

CONFLICT OF INTEREST

Authors have no conflict of interest to declare.

Supplemental data

Accession	Protein Description	Gene Name	CTRL/MYOF Ratio
P62258	14-3-3 protein epsilon	YWHAE	0.72
P61981	14-3-3 protein gamma	YWHAG	CTRL
P31947	14-3-3 protein sigma	SFN	0.62
P63104	14-3-3 protein zeta/delta	YWHAZ	0.85
P09543	2,3-cyclic-nucleotide 3-phosphodiesterase	CNP	1.10
P62333	26S protease regulatory subunit 10B	PSMC6	3.01
P62191	26S protease regulatory subunit 4	PSMC1	CTRL
P17980	26S protease regulatory subunit 6A	PSMC3	CTRL
P43686	26S protease regulatory subunit 6B	PSMC4	4.51
P35998	26S protease regulatory subunit 7	PSMC2	2.49
P62195	26S protease regulatory subunit 8	PSMC5	CTRL
Q99460	26S proteasome non-ATPase regulatory subunit 1	PSMD1	CTRL
O00231	26S proteasome non-ATPase regulatory subunit 11	PSMD11	1.30
Q13200	26S proteasome non-ATPase regulatory subunit 2	PSMD2	1.78
P48556	26S proteasome non-ATPase regulatory subunit 8	PSMD8	3.68
P62277	40S ribosomal protein S13	RPS13	CTRL
P62249	40S ribosomal protein S16	RPS16	CTRL
P15880	40S ribosomal protein S2	RPS2	CTRL
P61247	40S ribosomal protein S3a	RPS3A	CTRL
P62701	40S ribosomal protein S4, X isoform	RPS4X	CTRL
P62241	40S ribosomal protein S8	RPS8	CTRL
P46781	40S ribosomal protein S9	RPS9	3.31
P08865	40S ribosomal protein SA	RPSA	CTRL
P21589	5-nucleotidase	NT5E	1.08
P50914	60S ribosomal protein L14	RPL14	CTRL
P61313	60S ribosomal protein L15	RPL15	2.65
P18621	60S ribosomal protein L17	RPL17	CTRL
P83731	60S ribosomal protein L24	RPL24	CTRL
P36578	60S ribosomal protein L4	RPL4	2.62
P46777	60S ribosomal protein L5	RPL5	CTRL
Q02878	60S ribosomal protein L6	RPL6	5.92
P18124	60S ribosomal protein L7	RPL7	CTRL
P62424	60S ribosomal protein L7a	RPL7A	5.21
P11021	78 kDa glucose-regulated protein	HSPA5	2.56
P61158	Actin-related protein 3	ACTR3	CTRL
P07741	Adenine phosphoribosyltransferase	APRT	0.79
P23526	Adenosylhomocysteinase	AHCY	0.87
P84077	ADP-ribosylation factor 1	ARF1	1.78
P18085	ADP-ribosylation factor 4	ARF4	1.71
P62330	ADP-ribosylation factor 6	ARF6	1.91
Q9NVJ2	ADP-ribosylation factor-like protein 8B	ARL8B	8.22
O00468	Agrin	AGRN	2.83
P14550	Alcohol dehydrogenase [NADP(+)]	AKR1A1	0.65
P00330	Alcohol dehydrogenase 1	ADH1	0.98
O43707	Alpha-actinin-4	ACTN4	1.51
P06733	Alpha-enolase	ENO1	1.07
P50995	Annexin A11	ANXA11	1.29
P07355	Annexin A2	ANXA2	0.80
P12429	Annexin A3	ANXA3	CTRL
P08758	Annexin A5	ANXA5	1.24
P08133	Annexin A6	ANXA6	1.17
P20073	Annexin A7	ANXA7	CTRL
P63010	AP-2 complex subunit beta	AP2B1	2.64
P04114	Apolipoprotein B-100	APOB	0.59
P00966	Argininosuccinate synthase	ASS1	0.41
Q8N5I2	Arrestin domain-containing protein 1	ARRDC1	CTRL
P14868	Aspartate--tRNA ligase, cytoplasmic	DARS	1.33
P53396	ATP-citrate synthase	ACLY	1.98
Q01813	ATP-dependent 6-phosphofructokinase, platelet type	PFKP	2.60
O00148	ATP-dependent RNA helicase DDX39A	DDX39A	CTRL
O00571	ATP-dependent RNA helicase DDX3X	DDX3X	CTRL
P98160	Basement membrane-specific heparan sulfate proteoglycan core protein	HSPG2	1.50
P35613	Basigin	BSG	0.72
P61769	Beta-2-microglobulin	B2M	0.55
P07814	Bifunctional glutamate/proline--tRNA ligase	EPRS	4.63
Q9UQB8	Brain-specific angiogenesis inhibitor 1-associated protein 2	BAIAP2	CTRL

Table S1/ publication 2: List of proteins identified by MS in MDA-MB-231 and BxPC-3 derived exosomes. Protein commonly expressed in at least 50% of the replicated.

Q5VW32	BRO1 domain-containing protein BROX	BROX	CTRL
P11586	C-1-tetrahydrofolate synthase, cytoplasmic	MTHFD1	1.29
Q9Y376	Calcium-binding protein 39	CAB39	0.68
P17655	Calpain-2 catalytic subunit	CAPN2	0.70
O15484	Calpain-5	CAPN5	CTRL
Q8NEV1	Casein kinase II subunit alpha 3	CSNK2A3	CTRL
P35222	Catenin beta-1	CTNNB1	3.62
P07339	Cathepsin D	CTSD	0.83
Q03135	Caveolin-1	CAV1	2.17
P48509	CD151 antigen	CD151	0.81
Q9Y5K6	CD2-associated protein	CD2AP	CTRL
P08962	CD63 antigen	CD63	CTRL
P21926	CD9 antigen	CD9	1.22
P60953	Cell division control protein 42 homolog	CDC42	0.82
Q16739	Ceramide glucosyltransferase	UGCG	CTRL
O43633	Charged multivesicular body protein 2a	CHMP2A	CTRL
Q8IWA5	Choline transporter-like protein 2	SLC44A2	3.68
Q00610	Clathrin heavy chain 1	CLTC	2.59
P09497	Clathrin light chain B	CLTB	CTRL
P00742	Coagulation factor X	F10	1.02
P20908	Collagen alpha-1(V) chain	COL5A1	1.79
P12109	Collagen alpha-1(VI) chain	COL6A1	0.75
P39060	Collagen alpha-1(XVIII) chain	COL18A1	1.72
Q99829	Copine-1	CPNE1	CTRL
O75131	Copine-3	CPNE3	2.15
Q86YQ8	Copine-8	CPNE8	3.29
P31146	Coronin-1A	CORO1A	0.78
Q9H5V8	CUB domain-containing protein 1	CDCP1	1.27
P06493	Cyclin-dependent kinase 1	CDK1	3.00
Q14204	Cytoplasmic dynein 1 heavy chain 1	DYNC1H1	3.08
O00154	Cytosolic acyl coenzyme A thioester hydrolase	ACOT7	CTRL
Q14126	Desmoglein-2	DSG2	3.16
P15924	Desmoplakin	DSP	0.54
Q16555	Dihydropyrimidinase-related protein 2	DPYSL2	0.91
Q9P265	Disco-interacting protein 2 homolog B	DIP2B	2.26
O14672	Disintegrin and metalloproteinase domain-containing protein 10	ADAM10	1.27
P11387	DNA topoisomerase 1	TOP1	CTRL
P78527	DNA-dependent protein kinase catalytic subunit	PRKDC	CTRL
O60884	DnaJ homolog subfamily A member 2	DNAJA2	2.82
P25685	DnaJ homolog subfamily B member 1	DNAJB1	2.19
P50570	Dynamin-2	DNM2	3.98
Q14258	E3 ubiquitin/ISG15 ligase TRIM25	TRIM25	CTRL
P22413	Ectonucleotide pyrophosphatase/phosphodiesterase family member 1	ENPP1	0.63
O43854	EGF-like repeat and discoidin I-like domain-containing protein 3	EDIL3	1.54
Q9NZN4	EH domain-containing protein 2	EHD2	2.20
P26641	Elongation factor 1-gamma	EEF1G	2.55
P13639	Elongation factor 2	EEF2	1.10
P14625	Endoplasmin	HSP90B1	CTRL
P29317	Ephrin type-A receptor 2	EPHA2	1.28
Q99808	Equilibrative nucleoside transporter 1	SLC29A1	1.20
P60842	Eukaryotic initiation factor 4A-I	EIF4A1	1.28
P38919	Eukaryotic initiation factor 4A-III	EIF4A3	CTRL
P63241	Eukaryotic translation initiation factor 5A-1	EIF5A	2.93
P56537	Eukaryotic translation initiation factor 6	EIF6	CTRL
P49327	Fatty acid synthase	FASN	1.78
P02794	Ferritin heavy chain	FTH1	2.63
P02792	Ferritin light chain	FTL	1.97
P02751	Fibronectin	FN1	1.41
P21333	Filamin-A	FLNA	1.31
O75369	Filamin-B	FLNB	8.50
O75955	Flotillin-1	FLOT1	4.22
Q14254	Flotillin-2	FLOT2	4.25
P04075	Fructose-bisphosphate aldolase A	ALDOA	0.76
P09382	Galectin-1	LGALS1	0.74
P11413	Glucose-6-phosphate 1-dehydrogenase	G6PD	5.14
P06744	Glucose-6-phosphate isomerase	GPI	1.31
Q06210	Glutamine--fructose-6-phosphate aminotransferase [isomerizing] 1	GFPT1	2.94

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Q7RTV2	Glutathione S-transferase A5	GSTA5	0.54
P04406	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	1.32
P62993	Growth factor receptor-bound protein 2	GRB2	CTRL
P01111	GTPase NRas	NRAS	1.29
P62826	GTP-binding nuclear protein Ran	RAN	0.76
Q15382	GTP-binding protein Rheb	RHEB	2.32
P04899	Guanine nucleotide-binding protein G(i) subunit alpha-2	GNAI2	0.88
P62879	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2	GNB2	1.32
P63092	Guanine nucleotide-binding protein G(s) subunit alpha isoforms short	GNAS	1.38
Q14344	Guanine nucleotide-binding protein subunit alpha-13	GNA13	1.16
P63244	Guanine nucleotide-binding protein subunit beta-2-like 1	GNB2L1	CTRL
P48723	Heat shock 70 kDa protein 13	HSPA13	0.74
P08107	Heat shock 70 kDa protein 1A/1B	HSPA1A	1.59
P11142	Heat shock cognate 71 kDa protein	HSPA8	1.50
Q92598	Heat shock protein 105 kDa	HSPH1	2.69
P04792	Heat shock protein beta-1	HSPB1	1.18
P08238	Heat shock protein HSP 90-beta	HSP90AB1	1.18
P26927	Hepatocyte growth factor-like protein	MST1	MYOF
P61978	Heterogeneous nuclear ribonucleoprotein K	HNRNPK	CTRL
P52272	Heterogeneous nuclear ribonucleoprotein M	HNRNPM	CTRL
P30825	High affinity cationic amino acid transporter 1	SLC7A1	2.37
P16403	Histone H1.2	HIST1H1C	1.33
Q16778	Histone H2B type 2-E	HIST2H2BE	1.43
Q71DI3	Histone H3.2	HIST2H3A	1.36
P62805	Histone H4	HIST1H4A	1.88
P01892	HLA class I histocompatibility antigen, A-2 alpha chain	HLA-A	1.74
Q04826	HLA class I histocompatibility antigen, B-40 alpha chain	HLA-B	0.71
Q969P0	Immunoglobulin superfamily member 8	IGSF8	CTRL
P12268	Inosine-5-monophosphate dehydrogenase 2	IMPDH2	3.36
P17301	Integrin alpha-2	ITGA2	0.89
P08648	Integrin alpha-5	ITGA5	0.98
P23229	Integrin alpha-6	ITGA6	0.84
P06756	Integrin alpha-V	ITGAV	1.52
P05106	Integrin beta-3	ITGB3	1.68
P16144	Integrin beta-4	ITGB4	0.86
Q13418	Integrin-linked protein kinase	ILK	1.09
O75874	Isocitrate dehydrogenase [NADP] cytoplasmic	IDH1	CTRL
P41252	Isoleucine--tRNA ligase, cytoplasmic	IARS	CTRL
P53990	IST1 homolog	IST1	1.52
P14923	Junction plakoglobin	JUP	0.67
Q08431	Lactadherin	MFGE8	2.12
Q9P2J5	Leucine--tRNA ligase, cytoplasmic	LARS	CTRL
Q86X29	Lipolysis-stimulated lipoprotein receptor	LSR	3.77
P00338	L-lactate dehydrogenase A chain	LDHA	0.85
P07195	L-lactate dehydrogenase B chain	LDHB	0.81
O60488	Long-chain-fatty-acid--CoA ligase 4	ACSL4	2.14
Q7Z4F1	Low-density lipoprotein receptor-related protein 10	LRP10	CTRL
Q9H3U5	Major facilitator superfamily domain-containing protein 1	MFSD1	CTRL
Q14764	Major vault protein	MVP	2.18
P20774	Mimecan	OGN	0.58
O15427	Monocarboxylate transporter 4	SLC16A3	1.14
O95297	Myelin protein zero-like protein 1	MPZL1	0.27
Q96S97	Myeloid-associated differentiation marker	MYADM	CTRL
Q9NZM1	Myoferlin	MYOF	CTRL
Q9Y2A7	Nck-associated protein 1	NCKAP1	CTRL
Q09666	Neuroblast differentiation-associated protein AHNAK	AHNAK	1.20
O14786	Neuropilin-1	NRP1	1.37
Q15758	Neutral amino acid transporter B(0)	SLC1A5	1.84
Q96TA1	Niban-like protein 1	FAM129B	MYOF
P43490	Nicotinamide phosphoribosyltransferase	NAMPT	2.53
O15118	Niemann-Pick C1 protein	NPC1	3.85
P22392	Nucleoside diphosphate kinase B	NME2	0.91
Q99650	Oncostatin-M-specific receptor subunit beta	OSMR	CTRL
Q9C0B5	Palmitoyltransferase ZDHHC5	ZDHHC5	CTRL
P62937	Peptidyl-prolyl cis-trans isomerase A	PPIA	1.17
Q06830	Peroxiredoxin-1	PRDX1	1.16
Q9BTU6	Phosphatidylinositol 4-kinase type 2-alpha	PI4K2A	CTRL
P20020	Plasma membrane calcium-transporting ATPase 1	ATP2B1	1.36

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P05121	Plasminogen activator inhibitor 1	SERPINE1	CTRL
Q15149	Plectin	PLEC	CTRL
O15031	Plexin-B2	PLXNB2	1.48
Q9UKK3	Poly [ADP-ribose] polymerase 4	PARP4	4.87
P11940	Polyadenylate-binding protein 1	PABPC1	CTRL
P0CG47	Polyubiquitin-B	UBB	1.62
Q8WUM4	Programmed cell death 6-interacting protein	PDCD6IP	1.33
O75340	Programmed cell death protein 6	PDCD6	2.18
O00622	Protein CYR61	CYR61	3.88
P07237	Protein disulfide-isomerase	P4HB	CTRL
Q92734	Protein TFG	TFG	CTRL
Q9C0H2	Protein tweety homolog 3	TTYH3	2.15
P12931	Proto-oncogene tyrosine-protein kinase Src	SRC	2.35
Q9Y315	Putative deoxyribose-phosphate aldolase	DERA	CTRL
Q5VTE0	Putative elongation factor 1-alpha-like 3	EEF1A1P5	1.30
O43143	Putative pre-mRNA-splicing factor ATP-dependent RNA helicase DHX15	DHX15	CTRL
P14618	Pyruvate kinase PKM	PKM	0.87
P50395	Rab GDP dissociation inhibitor beta	GDI2	1.83
Q14699	Raftlin	RFTN1	2.35
P46940	Ras GTPase-activating-like protein IQGAP1	IQGAP1	3.04
Q15404	Ras suppressor protein 1	RSU1	0.50
P63000	Ras-related C3 botulinum toxin substrate 1	RAC1	0.78
P15153	Ras-related C3 botulinum toxin substrate 2	RAC2	3.12
P61026	Ras-related protein Rab-10	RAB10	1.23
Q15907	Ras-related protein Rab-11B	RAB11B	1.13
P61106	Ras-related protein Rab-14	RAB14	1.29
Q9H0U4	Ras-related protein Rab-1B	RAB1B	0.87
Q9UL26	Ras-related protein Rab-22A	RAB22A	4.12
P61019	Ras-related protein Rab-2A	RAB2A	CTRL
P61020	Ras-related protein Rab-5B	RAB5B	2.33
P51148	Ras-related protein Rab-5C	RAB5C	1.74
P51149	Ras-related protein Rab-7a	RAB7A	1.55
P61006	Ras-related protein Rab-8A	RAB8A	0.78
P61224	Ras-related protein Rap-1b	RAP1B	0.83
P10301	Ras-related protein R-Ras	RRAS	1.92
P62070	Ras-related protein R-Ras2	RRAS2	2.29
P18433	Receptor-type tyrosine-protein phosphatase alpha	PTPRA	CTRL
P10586	Receptor-type tyrosine-protein phosphatase F	PTPRF	3.97
P23470	Receptor-type tyrosine-protein phosphatase gamma	PTPRG	CTRL
Q15262	Receptor-type tyrosine-protein phosphatase kappa	PTPRK	4.22
Q8NFJ5	Retinoic acid-induced protein 3	GPRC5A	4.24
Q9Y265	RuvB-like 1	RUVBL1	4.07
Q9Y230	RuvB-like 2	RUVBL2	CTRL
O14828	Secretory carrier-associated membrane protein 3	SCAMP3	3.23
Q13501	Sequestosome-1	SQSTM1	CTRL
O95747	Serine/threonine-protein kinase OSR1	OXSR1	4.35
P30153	Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform	PPP2R1A	0.74
Q92783	Signal transducing adapter molecule 1	STAM	3.98
P62318	Small nuclear ribonucleoprotein Sm D3	SNRPD3	CTRL
Q8NCG7	Sn1-specific diacylglycerol lipase beta	DAGLB	CTRL
P05023	Sodium/potassium-transporting ATPase subunit alpha-1	ATP1A1	1.27
P05026	Sodium/potassium-transporting ATPase subunit beta-1	ATP1B1	1.30
P54709	Sodium/potassium-transporting ATPase subunit beta-3	ATP1B3	CTRL
Q9H2H9	Sodium-coupled neutral amino acid transporter 1	SLC38A1	1.50
P55011	Solute carrier family 12 member 2	SLC12A2	2.76
P11166	Solute carrier family 2, facilitated glucose transporter member 1	SLC2A1	1.71
Q8NBI5	Solute carrier family 43 member 3	SLC43A3	1.24
Q15036	Sorting nexin-17	SNX17	CTRL
O60493	Sorting nexin-3	SNX3	3.65
P31948	Stress-induced-phosphoprotein 1	STIP1	1.09
Q6UWP8	Suprabasin	SBSN	0.65
O43752	Syntaxin-6	STX6	CTRL
O00560	Syntenin-1	SDCBP	1.80
Q9Y490	Talin-1	TLN1	1.12
Q86VP1	Tax1-binding protein 1	TAX1BP1	CTRL
P78371	T-complex protein 1 subunit beta	CCT2	1.26
P50991	T-complex protein 1 subunit delta	CCT4	1.46
P48643	T-complex protein 1 subunit epsilon	CCT5	2.08

Table S1/ publication 2: List of proteins identified by MS in MDA-MB-231 and BxPC-3 derived exosomes. Protein commonly expressed in at least 50% of the replicated.

Q99832	T-complex protein 1 subunit eta	CCT7	CTRL
P49368	T-complex protein 1 subunit gamma	CCT3	1.17
Q9P273	Teneurin-3	TENM3	CTRL
Q8NG11	Tetraspanin-14	TSPAN14	4.47
P37173	TGF-beta receptor type-2	TGFBR2	CTRL
P10599	Thioredoxin	TXN	CTRL
Q9H3M7	Thioredoxin-interacting protein	TXNIP	CTRL
P13726	Tissue factor	F3	CTRL
P02786	Transferrin receptor protein 1	TFRC	3.02
Q15582	Transforming growth factor-beta-induced protein ig-h3	TGFBI	0.36
P37802	Transgelin-2	TAGLN2	0.92
P55072	Transitional endoplasmic reticulum ATPase	VCP	1.43
Q92616	Translational activator GCN1	GCN1L1	CTRL
P13693	Translationally-controlled tumor protein	TPT1	0.87
P60174	Triosephosphate isomerase	TPI1	1.03
P29144	Tripeptidyl-peptidase 2	TPP2	2.77
Q9BQE3	Tubulin alpha-1C chain	TUBA1C	1.99
P68366	Tubulin alpha-4A chain	TUBA4A	1.31
P07437	Tubulin beta chain	TUBB	1.82
P68371	Tubulin beta-4B chain	TUBB4B	1.59
Q99816	Tumor susceptibility gene 101 protein	TSG101	5.07
P23458	Tyrosine-protein kinase JAK1	JAK1	CTRL
P30530	Tyrosine-protein kinase receptor UFO	AXL	2.61
P07947	Tyrosine-protein kinase Yes	YES1	CTRL
P09012	U1 small nuclear ribonucleoprotein A	SNRPA	MYOF
A0AVT1	Ubiquitin-like modifier-activating enzyme 6	UBA6	CTRL
O60701	UDP-glucose 6-dehydrogenase	UGDH	CTRL
O00159	Unconventional myosin-Ic	MYO1C	2.69
P00749	Urokinase-type plasminogen activator	PLAU	0.66
Q16851	UTP--glucose-1-phosphate uridylyltransferase	UGP2	0.68
Q9UK41	Vacuolar protein sorting-associated protein 28 homolog	VPS28	6.45
Q9H9H4	Vacuolar protein sorting-associated protein 37B	VPS37B	CTRL
O75351	Vacuolar protein sorting-associated protein 4B	VPS4B	6.04
P51809	Vesicle-associated membrane protein 7	VAMP7	CTRL
P08670	Vimentin	VIM	0.91
P18206	Vinculin	VCL	0.74
P13010	X-ray repair cross-complementing protein 5	XRCC5	CTRL
P12956	X-ray repair cross-complementing protein 6	XRCC6	2.50

Table S1/ publication 2: List of proteins identified by MS in MDA-MB-231 and BxPC-3 derived exosomes. Protein commonly expressed in at least 50% of the replicated.

Accession	Protein Description	CTRL/MYOF Ratio
P62191	26S protease regulatory subunit 4	CTRL
P43686	26S protease regulatory subunit 6B	0.43
O00232	26S proteasome non-ATPase regulatory subunit 12	0.37
Q9UNM6	26S proteasome non-ATPase regulatory subunit 13	0.49
P48556	26S proteasome non-ATPase regulatory subunit 8	0.54
P25398	40S ribosomal protein S12	MYOF
P62277	40S ribosomal protein S13	MYOF
P62249	40S ribosomal protein S16	0.51
P60866	40S ribosomal protein S20	0.23
P23396	40S ribosomal protein S3	0.23
P61247	40S ribosomal protein S3a	0.38
P62701	40S ribosomal protein S4, X isoform	0.40
P08865	40S ribosomal protein SA	0.32
P52209	6-phosphogluconate dehydrogenase, decarboxylating	0.57
P05388	60S acidic ribosomal protein P0	MYOF
P27635	60S ribosomal protein L10	0.41
P62906	60S ribosomal protein L10a	MYOF
P18621	60S ribosomal protein L17	0.52
Q02878	60S ribosomal protein L6	0.35
P11021	78 kDa glucose-regulated protein	3.66
P61160	Actin-related protein 2	0.52
O15144	Actin-related protein 2/3 complex subunit 2	0.42
O15145	Actin-related protein 2/3 complex subunit 3	0.49
P61158	Actin-related protein 3	0.37
P55263	Adenosine kinase	0.42
Q9NVJ2	ADP-ribosylation factor-like protein 8B	1.85
P11766	Alcohol dehydrogenase class-3	MYOF
P47895	Aldehyde dehydrogenase family 1 member A3	0.61
Q04828	Aldo-keto reductase family 1 member C1	0.38
O43707	Alpha-actinin-4	1.98
P50995	Annexin A11	0.63
P20073	Annexin A7	0.64
P04424	Argininosuccinate lyase	MYOF
P08243	Asparagine synthetase [glutamine-hydrolyzing]	CTRL
P14868	Aspartate--tRNA ligase, cytoplasmic	0.36
P06576	ATP synthase subunit beta, mitochondrial	MYOF
P61221	ATP-binding cassette sub-family E member 1	MYOF
Q01813	ATP-dependent 6-phosphofructokinase, platelet type	0.30
Q08211	ATP-dependent RNA helicase A	0.22
O00148	ATP-dependent RNA helicase DDX39A	0.33
O00571	ATP-dependent RNA helicase DDX3X	0.40
Q9H4G0	Band 4.1-like protein 1	1.54
P61769	Beta-2-microglobulin	0.20
P07686	Beta-hexosaminidase subunit beta	1.80
P07814	Bifunctional glutamate/proline--tRNA ligase	MYOF
P31939	Bifunctional purine biosynthesis protein PURH	0.47
P11586	C-1-tetrahydrofolate synthase, cytoplasmic	0.45
P10644	cAMP-dependent protein kinase type I-alpha regulatory subunit	CTRL
Q9HCP0	Casein kinase I isoform gamma-1	0.43
Q9Y6M4	Casein kinase I isoform gamma-3	MYOF
Q03135	**Caveolin-1**	1.77
P08962	**CD63 antigen**	2.29
Q9HD42	Charged multivesicular body protein 1a	0.51
O43633	Charged multivesicular body protein 2a	0.41
Q9BY43	Charged multivesicular body protein 4a	0.52
Q9H444	Charged multivesicular body protein 4b	0.48

Table S2/ publication 2: List of proteins identified by MS in MDA-MB-231 and BxPC-3 derived exosomes. Protein commonly expressed in exosomes derived from both cell lines MDA-MB-231 and BxPC-3.

Q9Y696	Chloride intracellular channel protein 4	2.11
O75390	Citrate synthase, mitochondrial	MYOF
O95832	Claudin-1	0.50
P53618	Coatomer subunit beta	0.36
P23528	Cofilin-1	0.65
Q99715	Collagen alpha-1(XII) chain	3.49
Q9UMD9	Collagen alpha-1(XVII) chain	0.53
P08174	Complement decay-accelerating factor	MYOF
Q12860	Contactin-1	2.16
Q86YQ8	Copine-8	0.58
P06493	Cyclin-dependent kinase 1	0.64
Q9H1C7	Cysteine-rich and transmembrane domain-containing protein 1	CTRL
Q14204	Cytoplasmic dynein 1 heavy chain 1	0.45
O00154	Cytosolic acyl coenzyme A thioester hydrolase	MYOF
Q13443	Disintegrin and metalloproteinase domain-containing protein 9	0.45
P31689	DnaJ homolog subfamily A member 1	0.33
Q02750	Dual specificity mitogen-activated protein kinase kinase 1	0.29
O95834	Echinoderm microtubule-associated protein-like 2	CTRL
Q9H223	EH domain-containing protein 4	0.60
P13639	Elongation factor 2	0.48
P27105	Erythrocyte band 7 integral membrane protein	0.31
P60842	Eukaryotic initiation factor 4A-I	0.57
P38919	Eukaryotic initiation factor 4A-III	MYOF
Q14152	Eukaryotic translation initiation factor 3 subunit A	MYOF
P56537	Eukaryotic translation initiation factor 6	MYOF
P52907	F-actin-capping protein subunit alpha-1	MYOF
Q16658	Fascin	0.55
P49327	Fatty acid synthase	0.55
Q96NE9	FERM domain-containing protein 6	0.43
Q86UX7	Fermitin family homolog 3	0.49
P02794	Ferritin heavy chain	1.75
Q14512	Fibroblast growth factor-binding protein 1	1.53
P02751	Fibronectin	2.25
O75369	Filamin-B	0.19
O75955	**Flotillin-1**	1.89
Q14254	**Flotillin-2**	2.09
Q9NQ84	G-protein coupled receptor family C group 5 member C	0.40
P09382	Galectin-1	0.17
P06744	Glucose-6-phosphate isomerase	2.12
Q7RTV2	Glutathione S-transferase A5	0.44
P41250	Glycine--tRNA ligase	MYOF
P11216	Glycogen phosphorylase, brain form	MYOF
P35052	Glypican-1	1.64
P49915	GMP synthase [glutamine-hydrolyzing]	MYOF
Q7Z5G4	Golgin subfamily A member 7	0.49
P62826	GTP-binding nuclear protein Ran	0.64
P63244	Guanine nucleotide-binding protein subunit beta-2-like 1	0.30
P09651	Heterogeneous nuclear ribonucleoprotein A1	0.47
Q14103	Heterogeneous nuclear ribonucleoprotein D0	2.20
P61978	Heterogeneous nuclear ribonucleoprotein K	0.32
Q16778	Histone H2B type 2-E	2.48
P62805	Histone H4	2.02
P52292	Importin subunit alpha-1	MYOF
Q14974	Importin subunit beta-1	0.42
Q13308	Inactive tyrosine-protein kinase 7	2.17
P08648	Integrin alpha-5	0.34
Q9NPH3	Interleukin-1 receptor accessory protein	MYOF

Table S2/ publication 2: List of proteins identified by MS in MDA-MB-231 and BxPC-3 derived exosomes. Protein commonly expressed in exosomes derived from both cell lines MDA-MB-231 and BxPC-3.

O15554	Intermediate conductance calcium-activated potassium channel protein 4	CTRL
O75874	Isocitrate dehydrogenase [NADP] cytoplasmic	2.76
P41252	Isoleucine--tRNA ligase, cytoplasmic	0.29
Q96J84	Kin of IRRE-like protein 1	3.15
O43278	Kunitz-type protease inhibitor 1	0.34
Q13751	Laminin subunit beta-3	0.21
Q13753	Laminin subunit gamma-2	0.53
O43813	LanC-like protein 1	MYOF
Q08722	Leukocyte surface antigen CD47	0.43
Q7Z4F1	Low-density lipoprotein receptor-related protein 10	1.73
P11279	Lysosome-associated membrane glycoprotein 1	4.56
P40926	Malate dehydrogenase, mitochondrial	MYOF
Q99102	Mucin-4	3.01
P33527	Multidrug resistance-associated protein 1	MYOF
Q9NZM1	**Myoferlin**	11.68
Q15223	Nectin-1	1.54
Q9BT67	NEDD4 family-interacting protein 1	3.46
Q9NV92	NEDD4 family-interacting protein 2	CTRL
Q09666	Neuroblast differentiation-associated protein AHNAK	0.61
Q14697	Neutral alpha-glucosidase AB	0.66
Q96TA1	Niban-like protein 1	MYOF
P43490	Nicotinamide phosphoribosyltransferase	MYOF
O15118	Niemann-Pick C1 protein	5.71
P06748	Nucleophosmin	0.30
Q9NTK5	Obg-like ATPase 1	CTRL
Q99650	Oncostatin-M-specific receptor subunit beta	1.68
Q9BTU6	Phosphatidylinositol 4-kinase type 2-alpha	CTRL
P36969	Phospholipid hydroperoxide glutathione peroxidase, mitochondrial	MYOF
Q99569	Plakophilin-4	0.41
P05120	Plasminogen activator inhibitor 2	0.16
P13797	Plastin-3	0.32
Q15149	Plectin	0.59
Q9UKK3	Poly [ADP-ribose] polymerase 4	2.05
Q15365	Poly(rC)-binding protein 1	0.63
Q15366	Poly(rC)-binding protein 2	MYOF
P11940	Polyadenylate-binding protein 1	0.63
P09668	Pro-cathepsin H	0.44
Q9H3G5	Probable serine carboxypeptidase CPVL	CTRL
Q02809	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1	MYOF
P12004	Proliferating cell nuclear antigen	MYOF
Q9UQ80	Proliferation-associated protein 2G4	0.14
P28072	Proteasome subunit beta type-6	2.55
Q99873	Protein arginine N-methyltransferase 1	0.43
P07237	Protein disulfide-isomerase	1.60
Q92520	Protein FAM3C	1.60
Q92597	Protein NDRG1	0.39
Q9UGV2	Protein NDRG3	MYOF
P60903	Protein S100-A10	4.45
Q9C0H2	Protein tweety homolog 3	1.59
Q9GZT5	Protein Wnt-10a	CTRL
O75695	Protein XRP2	1.58
Q14517	Protocadherin Fat 1	0.49
Q9UN70	Protocadherin gamma-C3	1.52
O43865	Putative adenosylhomocysteinase 2	MYOF
O43143	Putative pre-mRNA-splicing factor ATP-dependent RNA helicase DHX15	MYOF
P14618	Pyruvate kinase PKM	0.50
P35241	Radixin	2.10

Table S2/ publication 2: List of proteins identified by MS in MDA-MB-231 and BxPC-3 derived exosomes. Protein commonly expressed in exosomes derived from both cell lines MDA-MB-231 and BxPC-3.

P46940	Ras GTPase-activating-like protein IQGAP1	0.55
P51149	Ras-related protein Rab-7a	1.59
Q9Y3L5	Ras-related protein Rap-2c	0.46
P10586	Receptor-type tyrosine-protein phosphatase F	1.54
O14828	Secretory carrier-associated membrane protein 3	2.25
Q12884	Seprase	CTRL
Q13501	Sequestosome-1	2.51
O15269	Serine palmitoyltransferase 1	1.52
Q92743	Serine protease HTRA1	3.01
Q7KZI7	Serine/threonine-protein kinase MARK2	MYOF
P30153	Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform	0.54
P62318	Small nuclear ribonucleoprotein Sm D3	MYOF
Q8NCG7	Sn1-specific diacylglycerol lipase beta	CTRL
Q96QD8	Sodium-coupled neutral amino acid transporter 2	0.52
Q08357	Sodium-dependent phosphate transporter 2	2.11
P19634	Sodium/hydrogen exchanger 1	MYOF
P05026	Sodium/potassium-transporting ATPase subunit beta-1	0.45
P54709	Sodium/potassium-transporting ATPase subunit beta-3	2.25
Q9UMY4	Sorting nexin-12	2.37
Q15036	Sorting nexin-17	CTRL
P38646	Stress-70 protein, mitochondrial	MYOF
Q8TED4	Sugar phosphate exchanger 2	CTRL
O43760	Synaptogyrin-2	1.65
O43752	Syntaxin-6	1.79
P17987	T-complex protein 1 subunit alpha	0.60
P78371	T-complex protein 1 subunit beta	0.60
P50991	T-complex protein 1 subunit delta	0.55
P48643	T-complex protein 1 subunit epsilon	0.44
Q99832	T-complex protein 1 subunit eta	0.60
P50990	T-complex protein 1 subunit theta	0.67
P40227	T-complex protein 1 subunit zeta	0.65
Q86VP1	Tax1-binding protein 1	2.09
O95858	Tetraspanin-15	CTRL
P26639	Threonine--tRNA ligase, cytoplasmic	0.46
P07204	Thrombomodulin	0.36
P00750	Tissue-type plasminogen activator	3.70
P02786	Transferrin receptor protein 1	1.69
Q15582	Transforming growth factor-beta-induced protein ig-h3	0.10
P29401	Transketolase	1.87
Q7Z403	Transmembrane channel-like protein 6	0.42
Q9BXS4	Transmembrane protein 59	1.51
P29144	Tripeptidyl-peptidase 2	2.95
P07437	Tubulin beta chain	0.66
Q14166	Tubulin--tyrosine ligase-like protein 12	MYOF
P21580	Tumor necrosis factor alpha-induced protein 3	0.59
O75509	Tumor necrosis factor receptor superfamily member 21	2.13
P23458	Tyrosine-protein kinase JAK1	2.10
O75643	U5 small nuclear ribonucleoprotein 200 kDa helicase	MYOF
P61088	Ubiquitin-conjugating enzyme E2 N	0.37
Q8IX04	Ubiquitin-conjugating enzyme E2 variant 3	0.64
P22314	Ubiquitin-like modifier-activating enzyme 1	0.53
Q9P206	Uncharacterized protein KIAA1522	MYOF
Q16851	UTP--glucose-1-phosphate uridylyltransferase	0.53
O75436	Vacuolar protein sorting-associated protein 26A	MYOF
A5D8V6	Vacuolar protein sorting-associated protein 37C	2.25
P50552	Vasodilator-stimulated phosphoprotein	2.03
Q9UEU0	Vesicle transport through interaction with t-SNAREs homolog 1B	2.59

Table S2/ publication 2: List of proteins identified by MS in MDA-MB-231 and BxPC-3 derived exosomes. Protein commonly expressed in exosomes derived from both cell lines MDA-MB-231 and BxPC-3.

P51809	Vesicle-associated membrane protein 7	1.88
O75083	WD repeat-containing protein 1	0.52
Q9Y6W5	Wiskott-Aldrich syndrome protein family member 2	MYOF
P13010	X-ray repair cross-complementing protein 5	MYOF

Table S2/ publication 2: List of proteins identified by MS in MDA-MB-231 and BxPC-3 derived exosomes. Protein commonly expressed in exosomes derived from both cell lines MDA-MB-231 and BxPC-3.

Accession	Protein Description	CTRL/MYOF Ratio MDA-MB-231	CTRL/MYOF Ratio BXPC-3
P62191	26S protease regulatory subunit 4	CTRL	CTRL
P11021	78 kDa glucose-regulated protein	2.56	3.66
Q9NVJ2	ADP-ribosylation factor-like protein 8B	8.22	1.85
O43707	Alpha-actinin-4	1.51	1.98
Q03135	Caveolin-1	2.17	1.77
P08962	CD63 antigen	CTRL	2.29
P02794	Ferritin heavy chain	2.63	1.75
O75955	Flotillin-1	4.22	1.89
Q14254	Flotillin-2	4.25	2.09
P62805	Histone H4	1.88	2.02
O75874	Isocitrate dehydrogenase [NADP] cytoplasmic	CTRL	2.76
Q7Z4F1	Low-density lipoprotein receptor-related protein 10	CTRL	1.73
Q9NZM1	Myoferlin	CTRL	11.68
O15118	Niemann-Pick C1 protein	3.85	5.71
Q99650	Oncostatin-M-specific receptor subunit beta	CTRL	1.68
Q9BTU6	Phosphatidylinositol 4-kinase type 2-alpha	CTRL	CTRL
Q9UKK3	Poly [ADP-ribose] polymerase 4	4.87	2.05
P07237	Protein disulfide-isomerase	CTRL	1.60
Q9C0H2	Protein tweety homolog 3	2.15	1.59
P51149	Ras-related protein Rab-7a	1.55	1.59
P10586	Receptor-type tyrosine-protein phosphatase F	3.97	1.54
O14828	Secretory carrier-associated membrane protein 3	3.23	2.25
Q13501	Sequestosome-1	CTRL	2.51
Q8NCG7	Sn1-specific diacylglycerol lipase beta	CTRL	CTRL
P54709	Sodium/potassium-transporting ATPase subunit beta-3	CTRL	2.25
Q15036	Sorting nexin-17	CTRL	CTRL
O43752	Syntaxin-6	CTRL	1.79
Q86VP1	Tax1-binding protein 1	CTRL	2.09
P02786	Transferrin receptor protein 1	3.02	1.69
P29144	Tripeptidyl-peptidase 2	2.77	2.95
P23458	Tyrosine-protein kinase JAK1	CTRL	2.10
P51809	Vesicle-associated membrane protein 7	CTRL	1.88

Table S3/ publication 2: List of proteins identified by MS in MDA-MB-231 and BxPC-3 derived exosomes. Protein commonly down regulated in myoferlin-deplete exosomes of both cell lines MDA-MB-231 and BxPC-3.

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