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Moteurs et contraintes de la recombinaison des norovirus murins

– une étude *in vitro* des processus évolutifs des norovirus

Drivers and constraints of murine norovirus recombination

– an *in vitro* study of norovirus evolutionary processes

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"We must find time to stop and thank the people who make a difference in our lives."
John F. Kennedy

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I end with a rather less illustrious, but no less meaningful, citation from one the great works of literature. It rather aptly expresses my feelings:

"Piglet noticed that even though he had a Very Small Heart, it could hold a rather large amount of Gratitude."

A.A. Milne

Abbreviations

aa	Amino acid
APC	Antigen presenting cell
bp	Base pair
cDNA	Complementary deoxyribonucleic acid
Ct	Cycle threshold
DMEMc	Complemented Dulbecco's modified Eagle's medium
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
dpi	Days post infection
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
FCS	Foetal calf serum
G I – X	Genogroup I – X
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
HBGA	Histo-blood group antigens
HIE	Human intestinal enteroid
hpi	Hours post infection
HuNoV	Human norovirus
ICTV	International Committee on Taxonomy of Viruses
IEC	Intestinal epithelial cell
IFN	Interferon
kb	Kilobases
MDPI	Multidisciplinary Digital Publishing Institute
ml	Millilitre
m.o.i.	Multiplicity of infection
mRNA	Messenger RNA
MuNoV	Murine Norovirus
NCWG	Norovirus Classification Working Group
nm	Nanometre
NoV	Norovirus
NS	Non-structural protein
ORF	Open reading frame
P	Polymerase
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
P-domain	Protruding domain
PFU	Plaque forming unit
qPCR	Quantitative polymerase chain reaction
RdRp	RNA-dependent RNA polymerase
RNA	Ribonucleic acid
RT	Reverse transcription
RT qPCR	Quantitative reverse transcription polymerase chain reaction
RT PCR	Reverse transcription polymerase chain reaction
S-domain	Shell domain
SF	Small fragment

SNP	Single nucleotide polymorphism
s/n/c	Substitutions per nucleotide site per cell infection
s/n/y	Substitutions per nucleotide site per year
µl	Microlitre
UTR	untranslated region
VF1	Virulence Factor 1
VLP	Virus-like particle
VP1	Viral protein 1 (major capsid protein)
VP2	Viral protein 2 (minor capsid protein)
VPg	Genome-linked protein
WB	Western blot
WT	Wild type

Résumé - Abstract

Résumé

Les norovirus (genre *Norovirus*, famille *Caliciviridae*) représentent la cause principale de gastro-entérite sporadique et épidémique non bactérienne chez l'homme au niveau mondial. La recombinaison et l'accumulation de mutations ponctuelles sont des mécanismes clés de l'évolution et de la diversité des norovirus; de plus en plus de preuves indiquent que la recombinaison façonne la pathogenèse et l'aptitude réplicative des norovirus et entraîne l'évolution de souche émergentes de norovirus humains.

La compréhension générale de la biologie des norovirus humains et en particulier de leur recombinaison est peu connue par rapport à celle d'autres virus, en raison, entre autres, de la difficulté que représente l'étude *in vitro* des norovirus humains. Malgré des avancées spectaculaires au niveau de l'étude *in vivo* et *in vitro* des norovirus humains, des questions importantes restent sans réponse en raison des limites techniques de ces systèmes expérimentaux. L'étude du norovirus murin, qui est génétiquement et biologiquement apparenté aux norovirus humains, combine plusieurs avantages, à savoir : une infection expérimentale *in vivo* relativement aisée sur un type d'hôte propice, d'une culture *in vitro* efficace et reproductible, et d'une large disponibilité d'outils de manipulation génétique. Il reste ainsi le modèle de choix pour l'étude de norovirus.

Cette thèse étudie les différents points de contrôle de la recombinaison: la co-infection de l'hôte, la co-infection de la cellule cible, la recombinaison en tant que processus et la sélection fonctionnelle des souches résultantes sont examinées. La thèse discute aussi qui les facteurs qui les favorisent ou le défavorisent.

L'article de revue «Norovirus recombinants: recurrent in the field, recalcitrant in the lab – a scoping review of recombination and recombinant types of noroviruses» (Ludwig-Begall et al., 2018) donne un aperçu complet de la recombinaison chez les norovirus et de son rôle dans leur évolution moléculaire. De plus, elle identifie les inconnues concernant les processus se déroulant avant et après la recombinaison *stricto sensu*; dans l'étude de la co-infection cellulaire à la sélection fonctionnelle, les études expérimentales 1 et 2 fournissent de nouvelles informations sur ces étapes cruciales.

In vivo, la co-infection unicellulaire synchrone par plusieurs virus est susceptible d'être un événement rare et les infections secondaires retardées sont plus probables. L'étude 1 détermine l'effet d'une séparation temporelle des infections *in vitro* avec les deux souches parentales de norovirus murins homologues MNV-1 WU20 et CW1 et leur impact sur la composition des populations de norovirus murins. En résumé, WU20 et CW1 ont été inoculés, soit de manière simultanée sur des monocouches de cellules macrophages murines (co-infection), soit en différé (surinfection avec des titres variables de CW1, d'une demi-heure à 24 heures de délai). Vingt-quatre heures après la co- ou surinfection initiale,

la quantification du nombre de copies génomiques et du criblage des virus de descendance infectieuses prélevés sur plaque ont démontré une prédominance dépendante du temps pour une primo-infection avec WU20 dans la majorité des nouvelles générations. Nos résultats indiquent qu'un intervalle de temps d'une à deux heures entre deux infections consécutives à norovirus permet l'établissement d'une barrière qui réduit ou empêche la surinfection; ceci représente la première démonstration d'interférence virale temporelle pour les norovirus et a des conséquences claires sur la compréhension de l'épidémiologie des norovirus, l'évaluation des risques et potentiellement sur le traitement.

L'étude 2 examine les processus ayant lieu directement après la recombinaison et vise à caractériser la capacité d'adaptation du norovirus murin recombinant WU20-CW1 précédemment généré *in vitro*, RecMNV, et examine ainsi comment l'accumulation de mutations ponctuelles à travers des passages viraux successifs peut compenser les pertes de capacité réplicative subies lors de la recombinaison. En comparant l'aptitude réplicative (replicative fitness) et les caractéristiques génétiques des descendants de RecMNV aux stades précoces et tardifs d'une expérience d'adaptation, le rétablissement de l'aptitude réplicative (replicative fitness) du recombinant a été démontré avant et après le passage *in vitro* en série. Les profils phénotypiques ont été associés à des modifications génétiques au niveau de la population. Pour étudier l'effet des changements génomiques séparés ou non au sein d'un norovirus murin infectieux chimérique, obtenu artificiellement, des mutations ont été introduites dans un ADNc recombinant WU20-CW1 en vue d'obtenir une récupération génétique inverse basée sur l'ADN. Cette expérience a prouvé que la perte de l'aptitude réplicative (replicative fitness) de RecMNV était ainsi liée à une mutation C7245T et à une troncature de la protéine de capside mineure (cadre de lecture ouvert 3) fonctionnelle; les effets compensatoires individuels et cumulatifs d'une mutation synonyme au niveau de la protéine majeure de capside (cadre de lecture ouvert 2) et de deux mutations non synonymes de la protéine non structurale 1/2 (cadre de lecture ouvert 1) acquises au cours de cycles successifs de réplication *in vitro* ont été démontrés, suggérant que les interactions entre les protéines virales et / ou les structures secondaires de l'ARN des cadres de lecture ouverts différents peuvent jouer un rôle dans la régulation de l'aptitude réplicative (replicative fitness) après recombinaison.

Cette thèse sert à fournir un aperçu des points critiques affectant le processus de recombinaison et, via l'étude de l'exclusion de la surinfection et de la sélection fonctionnelle, fournit de nouvelles informations sur les processus ayant lieu avant et après la génération d'un norovirus recombinant.

Abstract

Noroviruses (genus *Norovirus*, family *Caliciviridae*) are recognised as the major global cause of sporadic and epidemic non-bacterial gastroenteritis in humans. Recombination and the accumulation of point mutations are key mechanisms in the evolution and diversity of noroviruses; increasing evidence indicates that recombination shapes norovirus pathogenesis and fitness and drives the evolution of emerging human norovirus strains.

The understanding of human norovirus biology in general and norovirus recombination in particular has lagged behind that of other viruses due to the difficulties historically associated with robust *in vitro* human norovirus propagation. While recently developed *in vivo* and *in vitro* human norovirus assays have provided invaluable tools to dissect the norovirus life cycle, significant questions remain unanswered due to the technical limitations of many of these experimental systems. The genetically and biologically closely related murine norovirus combines the advantages of easy *in vivo* infection of a genetically tractable native host, efficient and robust *in vitro* culture, and availability of tools for genetic manipulation and thus remains the model of choice for many norovirus studies.

In the context of this thesis, the various norovirus recombination checkpoints, namely host coinfection, single cell coinfection, recombination, and functional selection, are examined and their drivers and constraints are discussed.

The review "Norovirus recombinants: recurrent in the field, recalcitrant in the lab – a scoping review of recombination and recombinant types of noroviruses" (Ludwig-Begall et al., 2018) provides a comprehensive overview of norovirus recombination and its role in norovirus molecular evolution and identifies knowledge gaps pertaining to prerequisite processes both directly prior to and post actual recombination *in sensu stricto*; in investigating conditions governing cell coinfection and functional selection, respectively, experimental studies 1 and 2 provide novel insights into these crucial steps.

In vivo, synchronous single-cell coinfection by multiple viruses, the ultimate prerequisite to viral recombination, is likely to be a rare event and delayed secondary infections are a more probable occurrence. Study 1 determines the effect of a temporal separation of *in vitro* infections with the two homologous parental murine norovirus strains MNV-1 WU20 and CW1 on the composition of murine norovirus populations. WU20 and CW1 were either synchronously inoculated onto murine macrophage cell monolayers (coinfection) or asynchronously applied (superinfection with varying titres of CW1 at half-hour to 24-hour delays). Twenty-four hours after initial co- or superinfection, quantification of genomic copy numbers and discriminative screening of plaque picked infectious progeny viruses demonstrated a time-dependent predominance of primary infecting WU20 in the majority of viral progenies. Our results indicate that a time interval from one to two hours onwards between two

consecutive norovirus infections allows establishment of a barrier that reduces or prevents super-infection; this first demonstration of time-dependent viral interference for noroviruses has clear implications for norovirus epidemiology, risk assessment, and potentially treatment.

Study 2 examines the processes directly following recombination and aims to characterise the adaptive capacity of previously *in vitro*-generated WU20-CW1 recombinant murine norovirus RecMNV, thus investigating how the accumulation of point mutations through successive viral passaging may compensate for initial replicative fitness losses incurred during deleterious recombination processes. By comparing the replicative fitness and genetic characteristics of RecMNV progenies at early and late stages of an adaptation experiment, replicative fitness regain of the recombinant was demonstrated between viral progenies prior to and post serial *in vitro* passaging and observable phenotypic profiles of viral fitness were associated to population-level genetic modifications. To investigate the effect of genomic changes separately and in combination in the context of an infectious lab-generated inter-murine norovirus chimera, mutations were introduced into a recombinant WU20-CW1 cDNA for subsequent DNA-based reverse genetics recovery. Fitness loss of RecMNV was thus linked to a C7245T mutation and functional minor capsid protein (open reading frame 3) truncation; individual and cumulative compensatory effects of one synonymous major capsid protein (open reading frame 2) and two non-synonymous non-structural protein 1/2 (open reading frame 1) consensus-level mutations acquired during successive rounds of *in vitro* replication were demonstrated, suggesting that interactions of viral proteins and/or RNA secondary structures of different open reading frames may play a role in the regulation of replicative fitness after a recombination event. This *in vitro* proof-of-concept study thus simulates successful adaptation (genetic drift) of a nascent norovirus after recombination (genetic shift) and serves to conceptualise how the emergence of recombinant human norovirus field strains, held to represent an adapted and functionally selected subset of all generated recombinants, may be regulated by an interplay between the two evolutionary processes of recombination and point mutation accumulation.

This thesis serves to provide a comprehensive overview of the recombination checkpoints to be bypassed and, in investigating both superinfection exclusion and functional selection, provides novel insights into prerequisite processes both before and after generation of a recombinant norovirus genome.

Preamble

Preamble

Noroviruses are recognised as the major global cause of sporadic and epidemic non-bacterial gastroenteritis in humans. Recombination and the accumulation of point mutations are key mechanisms in the evolution and diversity of noroviruses; increasing evidence indicates that recombination influences norovirus pathogenesis and fitness and contributes to the evolution of emerging human norovirus strains. Despite its importance, many aspects of norovirus recombination have hitherto remained unresolved.

In the context of this thesis, the various norovirus recombination checkpoints, namely host coinfection, single cell coinfection, recombination, and functional selection, are examined and their drivers and constraints are discussed.

The manuscript comprises four sections. The first chapter of the introduction encompasses an overview of the various aspects of norovirus biology. This is followed by a detailed description of RNA virus evolutionary processes and the molecular evolution of noroviruses in chapter 2, which closes with a scoping review of recombination and recombinant types of noroviruses (published in *Journal of General Virology*). The thesis objectives are succeeded by the experimental section which is subdivided into two parts. Experimental Study 1 describes how the analysis of synchronous and asynchronous *in vitro* infections with homologous murine norovirus strains reveals time-dependent viral interference effects (published in *Viruses*). Experimental Study 2 focuses on the replicative fitness recuperation of a recombinant murine norovirus and describes the *in vitro* reciprocity of genetic shift and drift (published in *Journal of General Virology*). In the last section of this manuscript, the main results of this thesis are discussed and perspectives of the work are presented.

Introduction

1. Noroviruses

1.1 Phylogeny

1.1.1 The *Caliciviridae* family

The *Caliciviridae* family of small, non-enveloped, positive sense, single-stranded RNA viruses derives its name from the Latin *calix* for chalice with reference to the cup-shaped depressions that commonly contour the virion surface of caliciviruses. The family is currently comprised of eleven approved genera, *Norovirus*, *Sapovirus*, *Nebovirus*, *Recovirus*, *Lagovirus*, *Vesivirus*, *Valovirus*, *Bavovirus*, *Nacovirus*, *Minovirus* and *Salovirus* (Figure 1), which are distinguished based on over 60% amino acid sequence difference in the complete major capsid protein (VP1) sequence (Vinjé et al., 2019).

Caliciviridae infect a wide range of host species and cause a variety of mainly species-specific diseases (Desselberger, 2019). Within the seven genera of which members infect mammals, noroviruses and sapoviruses typically cause gastroenteritis of varying severity in their animal and human hosts (Oka et al., 2015; Robilotti et al., 2015; Scipioni et al., 2008a), while neboviruses and recoviruses are enteric pathogens of cattle (Bridger et al., 1984) and rhesus macaques (Farkas, 2015; Farkas et al., 2008), respectively. Some lagoviruses and vesiviruses cause severe systemic infections in their mammalian hosts; pathogenic lagovirus infections provoke necrotic hepatitis and systemic haemorrhagic disease in lagomorphs (Abrantes et al., 2012; Le Pendu et al., 2017; Ohlinger et al., 1990; Wirblich et al., 1994), and vesivirus infections cause respiratory infections in cats (Radford et al., 2007), vesicular disease and foetal damage in swine, and vesicular exanthema and diseases of the reproductive system in marine mammals (Neill et al., 1995). The disease association of swine valoviruses remains unknown (L'Homme et al., 2009). Members of the two genera *Bavovirus* and *Nacovirus* have been associated with enteritis in poultry (Wolf et al., 2012); members of the two genera *Minovirus* and *Salovirus* infect various fish species (Mikalsen et al., 2014; Mor et al., 2017).

1.1.2 The *Norovirus* genus

The genetically diverse noroviruses (NoVs), which infect a broad range of mammalian hosts, derive their name from the city of Norwalk, Ohio, where an acute gastroenteritis outbreak in a school was caused by the prototypic Norwalk virus (Kapikian et al., 1972). In the early 2000s, classification into NoV genogroups and genotypes was initially based on amino acid sequence analysis of the complete VP1 capsid protein, with an amino acid divergence of 14.1% within a genotype and an adjusted cut-off threshold of a minimum of 15% pairwise difference proposed for classification of new genotypes (Vinjé et al., 2000; Zheng et al., 2006).

In 2013, the international *Norovirus Classification Working Group* (NCWG) put forth a proposal for a unified NoV nomenclature and genotyping, whereby NoVs were genetically classified into six established genogroups (GI–GVI), with a seventh proposed (GVII), and genogroups were further divided into at least 38 genotypes based on phylogenetic clustering of complete VP1 amino acid sequences (Kroneman et al., 2013; Vinjé, 2015); GII.4 strains were additionally subtyped into variants based both on phylogenetic clustering and on the condition of their having become epidemic in at least two separate geographical locations and were named according to year and location of the first full-length capsid sequence in the public domain. To account for the common occurrence of recombination in the overlapping region between the first two of three open reading frames (ORF1/2) encoded by NoVs, a dual-nomenclature system based on complete capsid sequences in ORF2 and partial sequences of the RNA-dependent RNA polymerase (RdRp) region in ORF1 was established (Kroneman et al., 2013). According to this nomenclature, e.g. "norovirus GII/Hu/FR/2004/GII.P12_GII.3/Paris23" designated a GII recombinant strain with known partial ORF1 RdRp type (GII, P type = 12) and complete ORF2 (genotype = 3) sequences; "norovirus GII/Hu/FR/2004/GII.12/Paris25" denoted a strain with known capsid sequence (GII, genotype 12), but unidentified RdRp. Naming of "orphan" ORF1 polymerase types, also known as "obligatory NoV recombinants" and designating known RdRp sequences lacking attributed capsid sequences but promiscuously associated with capsids of different genotypes, followed a preliminary alphabetical naming system (e.g. GI.Pa).

Adhering to the established criteria for genotype attribution and numbering of complete capsid sequences, the prior classification was recently updated to encompass ten accepted genogroups (GI to GX) and 49 confirmed genotypes (Figure 1), as well as two tentative genogroups (GNA1 and GNA2) and three proposed genotypes (Chhabra et al., 2019). To more easily accommodate ORF1/2 recombination of NoVs and to eliminate the necessity of the letter-based orphan ORF1 naming system, partial RdRp sequence clusters were grouped into eight confirmed and two tentative polymerase (P)-groups as well as 60 accepted and 14 tentative P-types independently of the classification of their capsid genogroups or genotypes. Accordingly, nine VP1 genotypes in GI, 27 in GII, three in GIII, two each in GIV, GV and GVI, and one each in GVII to GX are currently recognised; of the P-types, 14 cluster in GI, 37 in GII, two in GIII, one in GIV, two each in GV and GVI, and one each in GVII and GX (Figure 2). Separate phylogenetic clusters for both VP1 and partial RdRp sequences are confirmed according to the 2 × standard deviation criteria, which state that the average distance between all sequences within a newly identified cluster and its nearest established cluster, should not overlap within two standard deviations of each other (Chhabra et al., 2019). The previous dual typing nomenclature of norovirus strains was abandoned in favour of an updated version first listing the capsid genotype followed by the P-type between brackets (e.g. previous designation: GII.P12-GII.3; current designation: GII.3[P12]). For strains where ORFII and ORF1 amino acid sequences cluster in different genogroups, the designations are Genogroup.genotype[Pgroup.P-type] (e.g. previous designation: GVI.P1-GIV.2; current designation: GIV.2[GVI.P1] (Chhabra et al., 2019).

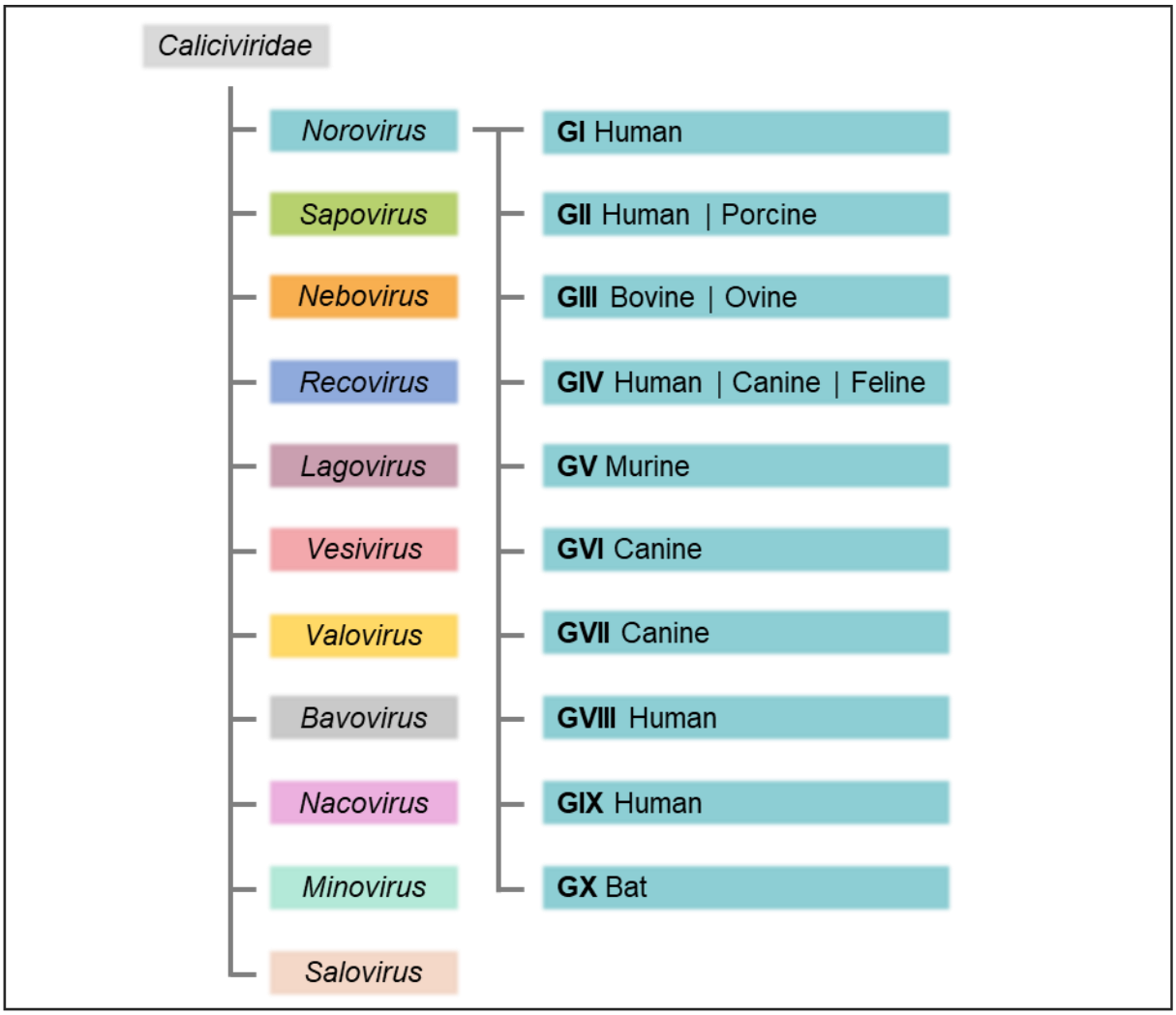

Figure 1. Schematic diagram of the *Norovirus* genus within the *Caliciviridae* family. The ten established genera (GI – GX), as defined by Chhabra et al. 2019, are shown.

Genogroups GI, GII, GIV, GVIII and GIX (previously GII.15) infect humans and cause acute gastroenteritis (Chhabra et al., 2019; van Beek et al., 2018). Of these, viruses from the genotype GII.4 are responsible for the majority of NoV outbreaks worldwide with novel pandemic GII.4 variants emerging every 2 to 3 years (Bruggink et al., 2017; de Graaf et al., 2016; Mathijs et al., 2011). Other species from which NoVs have been isolated include pigs (GII) (L'Homme et al., 2009), cattle and sheep (GIII) (Di Felice et al., 2016; Oliver et al., 2003; Scipioni et al., 2008a), rats and mice (GV) (Karst et al., 2003), dogs (GVI and GVII) (Mesquita et al., 2010) and bats (GX) (Wu et al., 2016). Tentative new genogroups GNA1 and GNA2 are detected in harbour porpoises (de Graaf et al., 2017a) and sea lions (Teng et al., 2018), respectively. The remarkable level of variability within the NoV genus reflects the high level of continuous viral evolution therein.

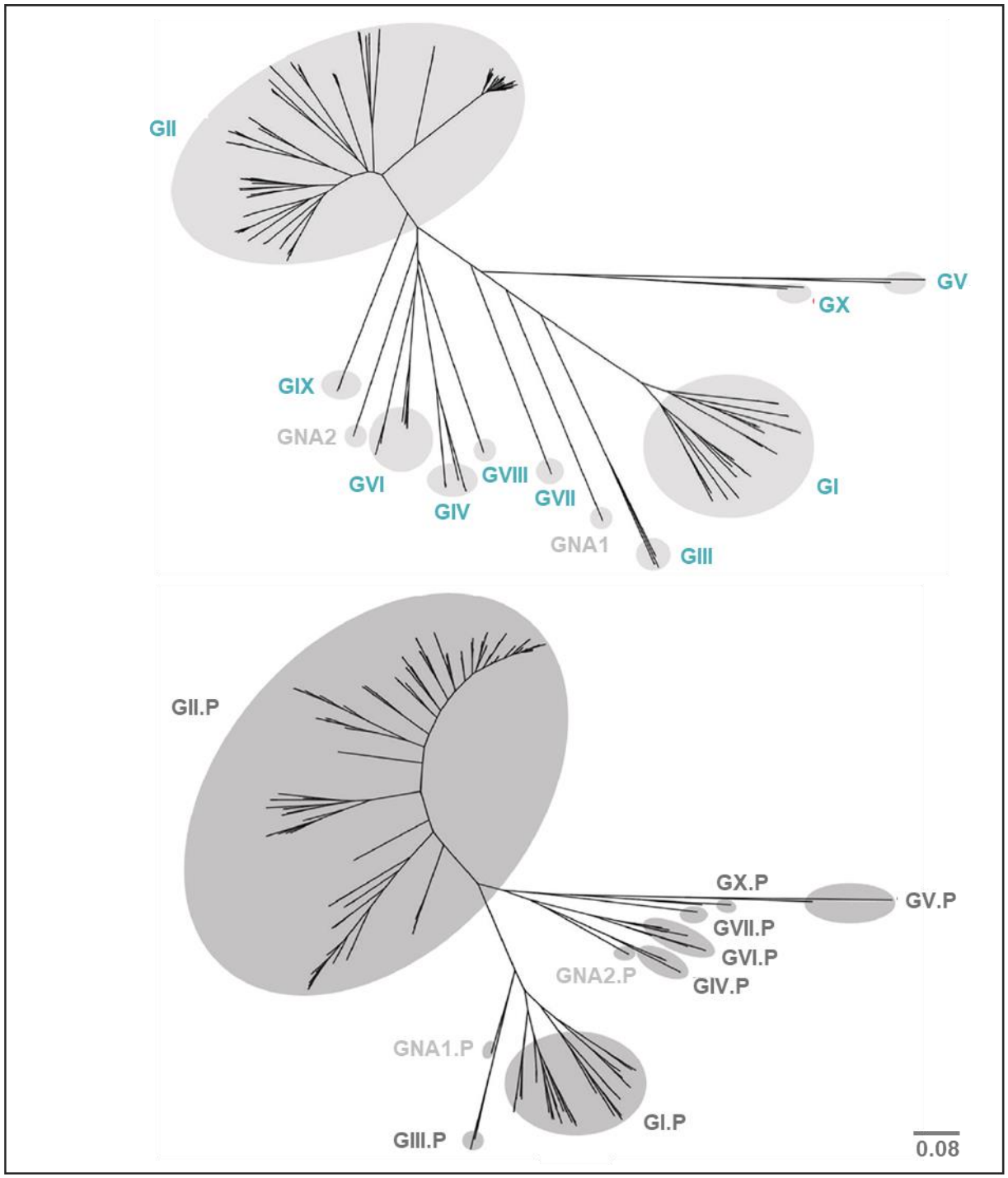

Figure 2. Phylogenetic classification of noroviruses. Above: Phylogenetic tree of ten established (turquoise) and two non-assigned (NA; light grey) norovirus genogroups based on major structural protein amino acid sequences. Below: Phylogenetic tree of eight established (dark grey) and two tentative (NA; light grey) norovirus P-groups based on partial RNA-dependent RNA polymerase sequences (762 nucleotides). Phylogenetic analyses were performed using maximum likelihood. Adapted from Chhabra et al. 2019.

In the following chapters, the focus of this thesis will be on both HuNoVs, the major aetiologic agents of global sporadic and epidemic viral gastroenteritis (Robilotti et al., 2015), and on the genetically and biologically closely related MuNoVs, which combine the advantages of efficient *in vitro* culture systems (Wobus et al., 2006, 2004) and availability of tools for genetic manipulation (Arias et al., 2012a; Yunus et al., 2010), and were used as a model for the NoV studies included in the context of this work.

1.2 Genome organisation

The linear, positive sense, single-stranded RNA genomes of NoVs are between 7.3 – 7.5 kilobases (kb) in length (Thorne and Goodfellow, 2014); a subgenomic RNA identical to approximately the last 2.3 kb of the genome is found in viral particles and is expressed, at higher levels than the viral genomic RNA, in infected cells (Asanaka et al., 2005). The 5' ends of NoV genomic and subgenomic RNA are linked to viral protein VPg (Goodfellow, 2011; Lee et al., 2018; Olspert et al., 2016), the 3' ends are polyadenylated (Lambden et al., 1993). At their extremities, NoV genomes contain short untranslated regions (UTRs) (Bertolotti-Ciarlet et al., 2003) which contain evolutionarily conserved RNA secondary structures that extend into the coding regions and are repeated throughout the genome, playing functional roles for viral replication, translation and pathogenesis by binding viral and host factors (Simmonds et al., 2008); a highly conserved non-coding RNA stem-loop structure upstream of the start site for subgenomic RNA initiation at the overlap of ORFs 1 and 2 has been identified as the core promoter for NoV subgenomic RNA synthesis by binding with the viral RNA-dependent RNA polymerase (RdRp) (Bull et al., 2005; Lin et al., 2015; Thorne and Goodfellow, 2014; Yunus et al., 2015).

The NoV genome is organised into three or, for MuNoV, four ORFs (Figure 3) (McFadden et al., 2011). The 5' proximal ORF1 encodes a large polyprotein that is co-and post- translationally cleaved by a virus-encoded protease into six non-structural proteins (NS) involved in replication complex formation (NS1/2, NS3, NS4), genome linkage (NS5, VPg), polyprotein processing (NS6), and genome replication (NS7, RdRp) (Thorne et al., 2012; Thorne and Goodfellow, 2014). Alternative names exist for HuNoV- and MuNoV NS proteins (Sosnovtsev et al., 2006); throughout this thesis the names related to MuNoV will be utilised. ORF2 and ORF3, both translated from subgenomic RNA, encode the structural components of the virion, the major viral protein (VP1) and minor viral protein (VP2), respectively. Open reading frame 4, unique to MuNoVs, overlaps ORF2 and is also translated primarily from subgenomic RNA; it encodes the virulence factor 1 (VF1) which is involved in regulation of innate immunity and apoptosis. The functions of various NoV proteins are discussed further in the context of the NoV replicative cycle (chapter 1.4.).

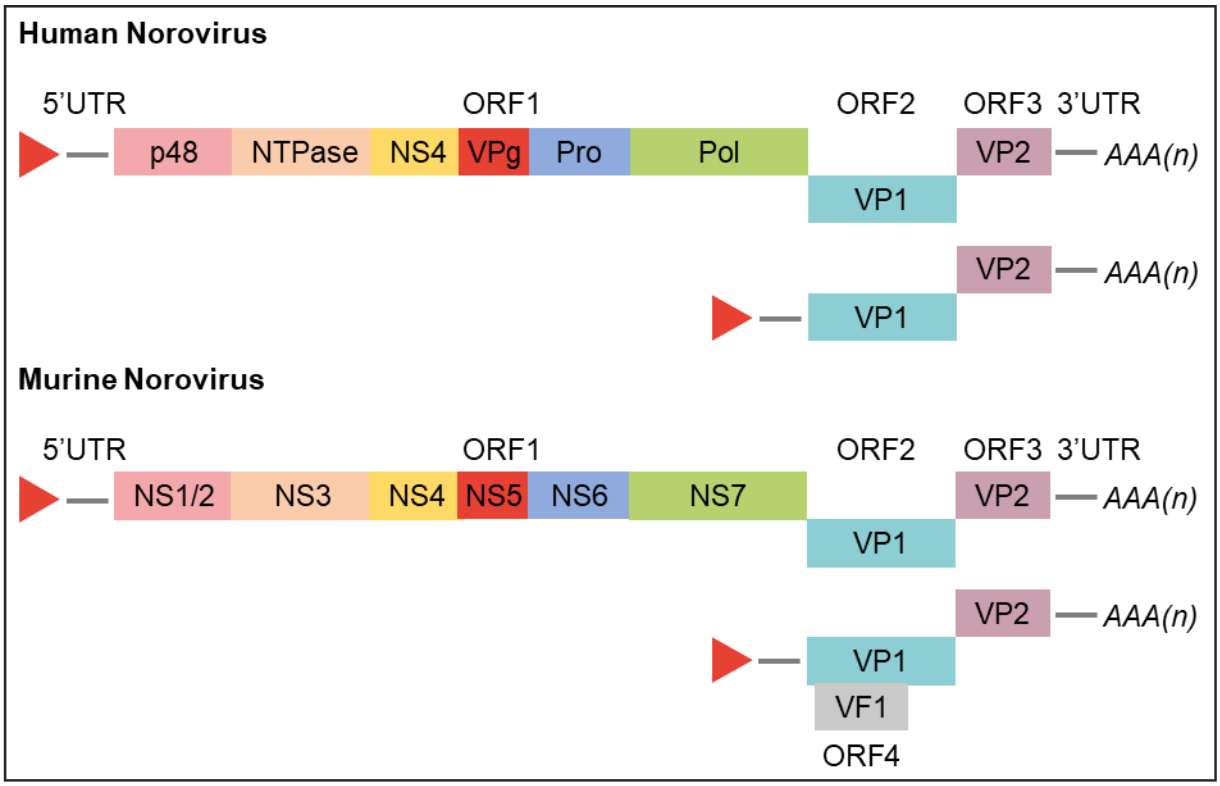

Figure 3. Schematic diagram showing the organisation of norovirus genomes.
Above: The human norovirus genome is covalently attached to genome-linked viral protein VPg at the 5'end and is polyadenlyated at the 3'end. The genome is divided into three open reading frames (ORFs), which are common to all noroviruses. ORF1 is translated as a polyprotein, which is cleaved by the viral protease (Pro) to produce the non-structural proteins (p48, NTPase, p22, VPg, Pro and Pol). ORF2 and ORF3 are translated from a subgenomic RNA. They encode the major structural protein, VP1 and the minor structural protein, VP2, respectively. The 5' and 3' genome extremities contain short untranslated regions (UTRs).
Below: The murine norovirus shares a similar genome organisation but has an additional fourth ORF which overlaps with ORF2 and is also translated primarily from subgenomic RNA into the virulence factor 1 (VF1) protein; adapted from Thorne & Goodfellow 2014

1.3 Virion morphology

The NoV capsid is typically 27-30 nm in diameter and displays a T=3 icosahedral symmetry; cup-like depressions, characteristic for caliciviruses, are localised at the three- and fivefold symmetry axes (Figure 4). Each capsid is composed of 180 copies of monomeric major structural protein VP1 which form 90 dimeric capsomers (Prasad et al., 1994, 1999). Each VP1 comprises a short N-terminal arm of unknown function, a shell domain (S), and a protruding domain (P) forming dimeric VP1 arches (Figure 4) (Prasad et al., 1999). The well-conserved N-terminal S domain faces the interior of the capsid and forms a continuous surface surrounding the viral RNA. The P domain, linked to the S domain through a flexible hinge, corresponds to the C-terminal part of VP1. It is postulated to confer increased stability to the icosahedral capsid and to provide a control for the size of viral particles (Bertolotti-Ciarlet et al., 2002). The P domain is further divided into a proximal P1 stalk subdomain at the base of the arches and the highly variable distal P2 subdomain. Localised at the tips of the arches, the exposed P2

subdomain interacts with neutralizing antibodies and contains the defined host receptor binding site for MuNoVs (Graziano et al., 2020) and putative receptor binding site for HuNoVs (Chakravarty et al., 2005; Graziano et al., 2019; Hutson et al., 2004; Orchard et al., 2016).

Minor structural protein VP2 (Glass et al., 2000), encoded by all caliciviruses, is located at the interior of the viral capsid and bound to a conserved motif in the VP1 S domain. It is postulated to be involved in MuNoV encapsidation via an interaction with viral genomic RNA (Thorne and Goodfellow, 2014; Vongpunsawad et al., 2013) and acidic regions of VP1 (Thorne et al., 2012) and is held to regulate expression and stability of VP1 in HuNoVs (Bertolotti-Ciarlet et al., 2003; Liu et al., 2019). VP2 integrity has been shown to be essential for productive replication of infectious feline calicivirus (Sosnovtsev et al., 2005). Feline calicivirus VP2 forms a portal-like assembly following host cell receptor engagement and is hypothesised to function as a channel for viral genome release from the endosome into the cytoplasm of a host cell (Conley et al., 2019).

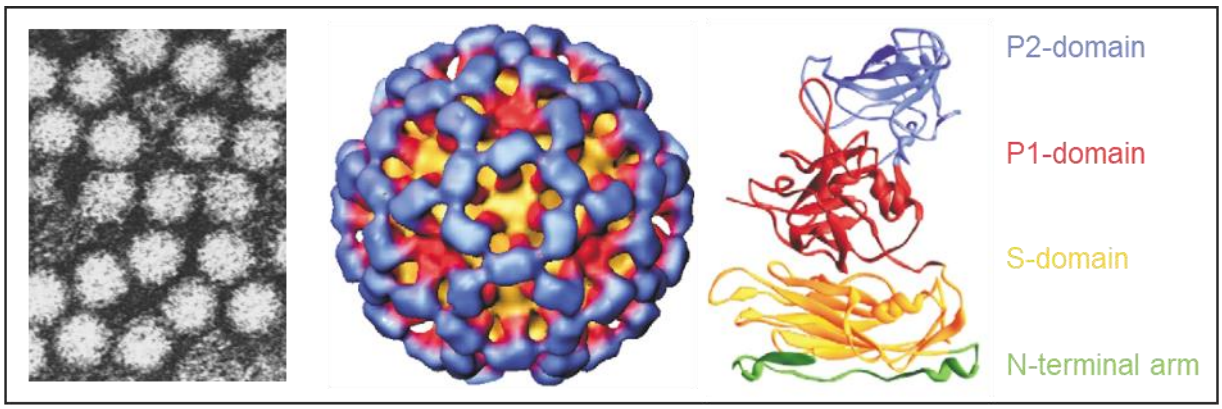

Figure 4. Norovirus virion morphology.
Left: An aggregate of Norwalk virus particles in stool filtrate as visualized by immune electron microscopy; adapted from Kapikian et al., 1972. Middle: Surface reconstruction of a T=3 icosahedral Norwalk virus-like particle (the structure has been resolved both by cryo-electron microscopy and x-ray crystallography; Protein Data Bank ID: 1IHM; DOI: 10.2210/pdb1IHM/pdb); adapted from Prasad et al., 1999 and Hutson et al., 2004. Right: The ribbon diagram represents the monomeric VP1 capsid protein divided into an N-terminal arm (green) facing the capsid interior, a shell domain (S-domain, yellow) that forms the continuous surface, and a protruding domain (P-domain) that extends from the S-domain surface. The P-domain is further divided into subdomains, proximal P1 (red) and distal P2 (blue); adapted from Hutson et al., 2004.

1.4 Replicative cycle

1.4.1 Attachment, receptor engagement, endocytosis, and uncoating

As the initial step of the NoV replicative cycle and decisive early determinant of cell tropism, host range, and pathogenesis, the multi-phasic process of viral entry commences via virion attachment to the cell surface (Marsh and Helenius, 2006). Attachment of NoVs is mediated by binding of the virus to both cell-associated and soluble host factors (Graziano et al., 2019).

HuNoVs are bound by histo-blood group antigens (HBGAs), as evidenced by *in vitro* assays (Marionneau et al., 2002) as well as multiple volunteer studies documenting the correlation between long-term resistance to infection with certain HuNoV strains and FUT2 gene-mediated genetic polymorphisms that determine host secretor status (Johnson et al., 1990; Thorven et al., 2005). The ability to secrete a diverse set of fucosylated HBGAs into body fluids and on mucosal cells (secretor) is associated with HuNoV susceptibility; expressing only a limited array of HBGAs (non-secretor) is linked to resistance to certain HuNoV strains (including genogroups GI.1 and prevalent GII.4) (Lindesmith et al., 2003; Nordgren and Svensson, 2019). While non-secretors thus experience infections with a lesser variety of NoV strains, the resistance to HuNoV is not absolute and they can become infected by secretor-independent strains (GII.3, GII.7, and GII.6), implicating non-HGBA ligands (fucosylated and sialylated carbohydrates (Wegener et al., 2017)) and co-factors in HuNoV binding (Almand et al., 2017; Graziano et al., 2019; Lindesmith et al., 2020).

In addition to binding to host HBGAs, NoVs may also bind directly to HBGAs expressed by commensal bacteria in the gut (Miura et al., 2013b), which may thus act as proviral co-factors for HuNoV infection; in this context, HuNoV B cell infection is enhanced by HBGA-producing bacteria or free synthetic HBGAs (Jones et al., 2014). While HGBA binding apparently plays no role in MuNoV infection, a dependency on faecal microbiota has been demonstrated for MuNoVs *in vitro* (Jones et al., 2014) and *in vivo* (Baldridge et al., 2015); thus, bacterial depletion via antibiotic treatment of mice prevents infection with both acute and persistent MuNoV strains (Baldridge et al., 2015) and susceptibility to persistent strains has been linked to changes in target cell numbers (tuft cells are targets of persistent MuNoV strains; see below) which may be regulated by the presence and composition of gut microbiota and their metabolites (Wilen et al., 2018).

For MuNoVs, non-essential carbohydrate attachment factors including heparan sulfate proteoglycans and terminal sialic acid have been shown to enhance viral VP1 binding in a strain-dependent manner (Orchard et al., 2016; Taube et al., 2012, 2009). Notably, sialic acids have also been implicated in facilitating the attachment of bovine NoVs and feline calicivirus (FCV) to susceptible cells (Mauroy et al., 2011; Stuart and Brown, 2007).

Suggested host and microbial cofactors that enhance NoV attachment to cells (also in a strain-dependent manner) include bile acids (MuNoV and HuNoV) (Ettayebi et al., 2016; Kilic et al., 2018; Nelson et al., 2018), phospholipids (MuNoV) (Orchard et al., 2018), and divalent cations (MuNoV) (Nelson et al., 2018).

The second step of viral entry is the engagement of host receptors to actively promote viral access to cells. CD300lf, an immunoglobulin domain-containing integral membrane protein expressed in myeloid cells, lymphoid cells and intestinal epithelial tuft cells (Borrego, 2013), has been identified as the primary physiologic cellular MuNoV receptor (Orchard et al., 2016). It functions by binding the apical side of the P2 subdomain and is essential for infection of diverse MuNoV strains both *in vitro* and *in vivo* independent of infection route (Graziano et al., 2020); its paralogue CD300ld has also been demonstrated to be sufficient for MuNoV infection *in vitro*. Ectopic expression of murine CD300lf on human and other mammalian cells has been shown to be sufficient to confer cross-species permissivity, effectually breaking the species barrier and allowing MuNoV replication in non-murine cells (Orchard et al., 2016). Human CD300lf is not a receptor for HuNoVs and the HuNoV receptor remains unknown (Graziano et al., 2020).

The details of which mechanisms are involved in the endocytic internalisation of HuNoV particles following receptor engagement are unknown. For MuNoVs, entry into permissive macrophages and dendritic cells is known to be rapid, requiring host cholesterol and dynamin (Gerondopoulos et al., 2010; Perry and Wobus, 2010). This viral endocytosis is independent of pH (Perry et al., 2009), clathrin and caveolae, and is neither mediated by phagocytosis nor micropinocytosis (Perry and Wobus, 2010). For bovine NoVs, VLP internalisation into permissive cells involves both the cholesterol-dependent pathway and macropinocytosis (Mauroy et al., 2011).

After endocytosis, endosomal escape and viral uncoating are required to release the viral genome into the host cytoplasm. While the process remains unsolved for NoVs, a recent near-atomic resolution analysis of FCV yielded important basic information regarding these important last steps of calicivirus entry. In the process of clathrin- and pH-dependent endocytosis, binding of FCV to its receptor feline junctional adhesion molecule A (fJAM-A) was shown to induce formation of a portal-like assembly made up of twelve copies of VP2 arranged with their hydrophobic N termini pointing away from the virion surface around a pore in the capsid shell. The funnel-like structure is hypothesised to function as a channel for the delivery of the viral genome through the endosomal membrane into the cytoplasm of a host cell, thereby initiating infection (Conley et al., 2019).

1.4.2 Translation and polyprotein processing

Following its release into the cytoplasm of a permissive cell, the VPg-linked NoV RNA acts as a messenger RNA (mRNA) template for an initial round of viral RNA translation. Attached covalently to the 5' end of the genome, NoV VPg (NS5) functions as a cap substitute to recruit eukaryotic initiation factors and mediate translation of viral RNA into protein via multiple direct interactions with the cellular translational apparatus of the host cell and core stress granule components (Brocard et al., 2020; Chaudhry et al., 2006; Daughenbaugh et al., 2006; Emmott et al., 2017; Hosmillo et al., 2019). Interactions between various host cell RNA-binding proteins and conserved RNA secondary structures of complementary sequences at the 3' and 5' genome extremities are further postulated to enhance and regulate viral protein translation, putatively by stabilising sequence-mediated, long-range physical RNA interactions that promote genome circularisation (López-Manríquez et al., 2013; Simmonds et al., 2008). Translation of the viral proteins VP1 and VP2 occurs primarily from the ORFs of the polycistronic subgenomic RNA which, following its transcription from genomic RNA by the NoV nonstructural proteins, is expressed at higher levels than the viral genomic RNA in infected cells (Asanaka et al., 2005) in a probable strategy to augment levels of VP1 production for virus assembly (Thorne and Goodfellow, 2014). Translation of ORF4 in MuNoV from subgenomic RNA yields VF1 which has been implicated in interfering with innate immune signalling at the cellular level and was recently found to delay the upregulation of IFN-β and other interferon stimulated genes (ISGs) *in vitro* (McFadden et al., 2011).

1.4.3 Viral genome replication

Once translated, the ORF1 polyprotein is co- and post-translationally cleaved by the viral protease (NS6) to release NS precursors and mature viral proteins (NS1/2 to NS7) (Emmott et al., 2019; Sosnovtsev et al., 2006) that then serve to assemble the replication complex by recruitment of cellular membranes to the perinuclear region of the cell (Hyde et al., 2009).

MuNoV NS1/2, the least conserved NoV NS (Thorne et al., 2012), is hypothesised to be one of the main drivers of replication complex formation by associating with components of the endocytic and secretory pathway together with co-localizing NS4 (Hyde and Mackenzie, 2010; Kaiser, 2006). NS1/2 contains an N-terminal disordered region and a C-terminal predicted trans-membrane domain (Baker et al., 2012). MuNoV NS1/2 has been shown to induce rearrangement of the endoplasmic reticulum. It is implicated in viral persistence *in vivo* (Nice et al., 2013) and, once unconventionally secreted via caspase-3 cleavage, is essential for intestinal pathogenesis of MuNoV infection and resistance to endogenous IFN-γ (Lee et al., 2019). Its HuNoV equivalent p48 promotes Golgi disassembly dependent upon the C-terminal hydrophobic region and disrupts expression and trafficking of cell surface proteins

by interfering with cellular vesicle transport (Doerflinger et al., 2017; Fernandez-Vega et al., 2004); a secreted form of HuNoV NS1 is also observed (Lee et al., 2019).

As a constituent of the MuNoV replication complex, NS4 localises to endosomes (Hyde et al., 2009; Hyde and Mackenzie, 2010). HuNoV NS4 (P22) has been shown to induce Golgi disassembly (Sharp et al., 2010) and has been identified as a key determinant in the formation of membrane alterations by HuNoVs (Doerflinger et al., 2017); both MuNoV and HuNoV NS4 inhibit cellular protein secretion (mildly in MuNoV and potently in HuNoV) (Hyde and Mackenzie, 2010; Sharp et al., 2010).

While NS1/2 and NS4 are acknowledged to be key main mediators of replication complex formation, NS3, to which RNA-chaperoning and helicase activities have been attributed (Han et al., 2018; Li et al., 2018), has also been shown to localise to cellular membranes (Hyde et al., 2009). Both HuNoV and MuNoV NS3 induce formation of motile membrane-derived vesicular structures that colocalise with the Golgi apparatus and the endoplasmic reticulum (Cotton et al., 2017; Doerflinger et al., 2017).

Norovirus genome replication occurs via a negative-strand intermediate (Thorne and Goodfellow, 2014); subsequent to the initial round of translation of the incoming positive-stranded parental RNA, this mRNA serves as a template for the synthesis of negative-strand RNA from its 3' end and the formation of a double-stranded replicative form. The negative-sense genomic and subgenomic RNAs are then used as templates for the synthesis of positive sense genomic and subgenomic RNAs (Thorne and Goodfellow, 2014). These transcription reactions are catalysed by the RNA-dependent RNA-polymerase (RdRp, NS7), using *de novo* mechanisms for synthesis of negative-stranded RNA (Subba-Reddy et al., 2017, 2012), and VPg-dependent mechanisms of positive sense genomic and subgenomic RNA synthesis in which the NS7 uses multifunctional VPg as a proteinaceous primer (Lee et al., 2018; McSweeney et al., 2019; Olspert et al., 2016). Two, not-mutually exclusive, models have been proposed for the generation of NoV subgenomic RNA; based on the detection of negative-sense subgenomic RNA copies in Norwalk virus replicon-bearing and MuNoV infected cells (Chang et al., 2006; Yunus et al., 2015), the pre-mature termination model proposes synthesis of negative-sense subgenomic RNA linked to an unidentified termination signal, and subsequent generation of positive sense subgenomic RNA from this template. The internal initiation model postulates that the highly conserved stem-loop structure upstream of the subgenomic start site in the negative-sense genomic RNA acts as the core of an internal subgenomic promoter and binds to the RdRp to direct initiation at the overlap of ORFs 1 and 2. In this case, newly synthesised subgenomic RNA may function as a template for further rounds of replication via a negative-sense subgenomic RNA intermediate (Bull et al., 2005; Lin et al., 2015; Simmonds et al., 2008; Yunus et al., 2015).

1.4.4 Assembly and exit

Self-assembly of VP1 into virus-like particles (VLPs) that are morphologically and antigenically comparable to native virions (Bertolotti-Ciarlet et al., 2002), suggests that VP1 alone may be able to drive assembly of infectious NoV particles. While not essential for assembly, the 3' UTR of the Norwalk mRNA can stimulate VP1 expression via putative RNA-capsid interactions and the presence of VP2 is held to enhance the stability of nascent particles (Bertolotti-Ciarlet et al., 2003; Lin et al., 2014; Pogan et al., 2018). Associated with a conserved acidic motif in the VP1 S domain at the capsid interior (Thorne et al., 2012; Vongpunsawad et al., 2013), the highly basic VP2 may provide the link between capsid subunits and acidic viral RNA (Thorne and Goodfellow, 2014; Vongpunsawad et al., 2013).

Upon completed assembly, virion exit is the last step of the replicative cycle. Active viral replication of MuNoVs in permissive cells, and indeed the expression of the MuNoV polyprotein alone, have been shown to regulate and induce apoptosis and programmed cell death in conjunction with downregulation of pro-survival factor surviving in infected cells (Bok et al., 2009; Herod et al., 2014). While its role in viral exit remains undetermined, inhibition of apoptosis has been shown to accelerate cell death, change the death pathway to rapid necrosis, and to ultimately result in an over 10-fold reduction in infectious NoV yield (Furman et al., 2009).

While MuNoVs lytically infect innate immune cells including macrophages and dendritic cells *in vitro* (Karst et al., 2003), the nature of *in vitro* B cell infection by HuNoVs and MuNoVs is distinct in that mature B cells are infected noncytopathically (Jones et al., 2015; Karst, 2015a), suggesting that different mechanisms of cellular regulation and cell exit can be employed by NoVs. The paradigm that nonenveloped viruses must lyse their target cells in order for progeny virions to be released extracellularly has further been challenged by the discovery that, amongst other enteric viruses, NoVs can be secreted from cultured cells inside extracellular membrane-bound vesicles and that they are shed in faeces within vesicles of exosomal or plasma membrane origin presenting highly virulent units of faecal-oral transmission (Santiana et al., 2018).

An outline of the entire NoV replication cycle is provided in Figure 5.

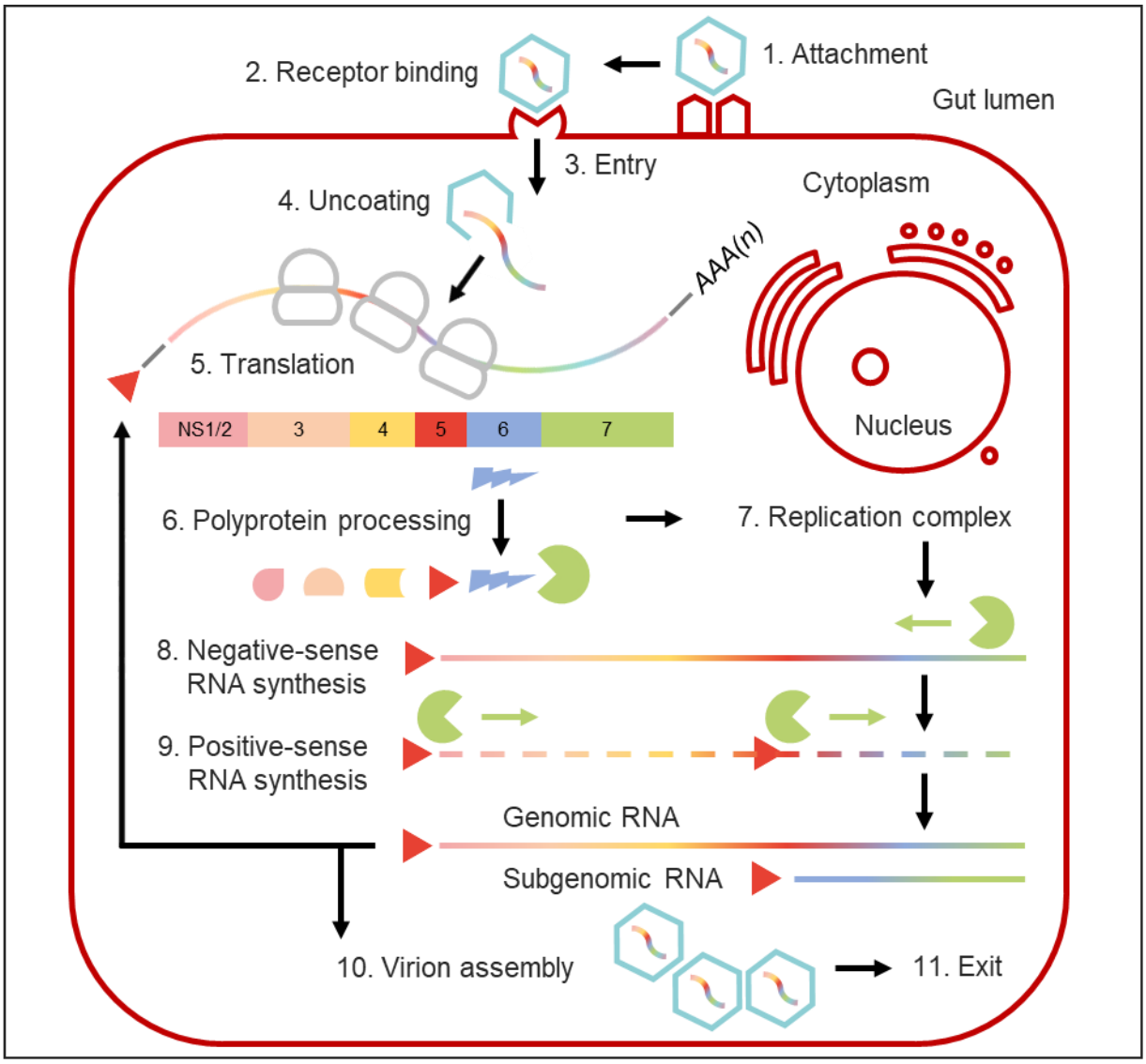

Figure 5. Outline of the norovirus replication cycle.
Human and murine noroviruses (turquoise hexagons) attach to the cell surface using carbohydrate attachment factors and cofactors (1). To mediate entry, binding to a protein receptor is required (2). After entry (3) and uncoating (4), the incoming viral genome is translated through interactions with the genome-linked protein VPg (non-structural protein NS5; red triangle) at the 5' end of the genome and the cellular translation machinery (5). The open-reading frame 1 polyprotein is co- and post-translationally cleaved by the viral protease (NS6; blue flash) (6). The replication complex is formed by recruitment of cellular membranes to the perinuclear region of the cell, through interactions in part with NS1/2 (rose shape) and NS4 (yellow shape) (7). Genome replication occurs via a negative-strand intermediate (dashed line) (8), and genomic and subgenomic RNA (unbroken lines) are generated by the viral RNA-dependent RNA polymerase (NS7; green jagged circle), using de novo and VPg- or internal promoter-dependent mechanisms of RNA synthesis (9). Replicated genomes are translated or packaged into the capsid, composed mainly of viral protein 1 (VP1), for virion assembly (10) and exit (11).

1.5 Clinical aspects of norovirus infection

1.5.1 Human noroviruses

HuNoVs are recognised as the major global cause of sporadic and epidemic viral gastroenteritis (Patel et al., 2008; Robilotti et al., 2015). After a short incubation period of 24–48 hours (Lee et al., 2013), clinical symptoms typically last for two to three days (Robilotti et al., 2015), followed by a median of four weeks of post-clinical shedding (Atmar et al., 2008) with peak viral titres varying between 10^5–10^9 genome copies/g of faeces (Teunis et al., 2015).

Characteristic symptoms of HuNoV infection are acute onset of watery, non-bloody diarrhoea and projectile vomiting (Kaplan et al., 1982). Other symptoms include abdominal cramps, nausea, bloating, mild fever, chills, headaches and myalgia (Atmar and Estes, 2006; Gallimore et al., 2004a; Tseng et al., 2011). While self-limiting gastrointestinal infections are the norm, more severe intestinal pathologies such as necrotising enterocolitis in neonates (Stuart et al., 2010; Turcios-Ruiz et al., 2008), post-infectious irritable bowel syndrome (Marshall et al., 2007), and exacerbation of inflammatory bowel disease (Khan et al., 2009) have been described. Atypical extraintestinal pathologies such as seizures in young children (Chen et al., 2009; Hu et al., 2017; Ueda et al., 2015), encephalopathy (Ito et al., 2006), and acute liver dysfunction (Lok Tung Ho et al., 2020; Nakajima et al., 2012) have also been reported in association with NoV infections; NoV RNA has been detected in sera (Takanashi et al., 2009) and cerebrospinal fluids (Ito et al., 2006) of infected individuals, suggesting possible spread to peripheral tissues.

Despite typically eliciting severe gastroenteritis, HuNoVs cause only modest intestinal pathologies. Histopathological changes in the small intestine include broadening and shortening of the microvilli, crypt hypertrophy, as well as increased epithelial mitoses and apoptosis (Schreiber et al., 1973). Decreased brush border enzyme activity, transient malabsorption of D-xylose, fat, and lactose, disruption of epithelial barrier functions, reduction of tight junctional sealing proteins, and stimulation of active anion secretion, suggest that both a leak flux and alterations of secretory and/or absorptive processes cause HuNoV-induced diarrhoea (Blacklow et al., 1972; Karst et al., 2015; Troeger et al., 2009). Vomiting episodes may be linked to abnormal gastric motor functions and delays in gastric emptying, however the underlying pathophysiology remains unclear (Meeroff et al., 1980).

Asymptomatic infections and viral shedding similar to that of symptomatic infections (Teunis et al., 2015) have been both experimentally observed in volunteer studies (Graham et al., 1994) and detected in various epidemiological analyses of clinically healthy individuals and those with various underlying illnesses resulting in impaired immunity (Ayukekbong et al., 2011; Lopman et al., 2014; Siebenga et al., 2008; Utsumi et al., 2017).

Customarily an acute and self-limiting illness, HuNoV infection can become persistent in the elderly (Harris et al., 2008), malnourished and/or immunocompromised (individuals with genetic or acquired immune-deficiencies, cancer patients undergoing treatment, transplant patients) (Brown et al., 2017, 2016; Gallimore et al., 2004b; Vega et al., 2014; Woodward et al., 2017). These individuals often experience severe, even lethal, persistent or recurring NoV infections during which prolonged diarrhoea and vomiting can lead to weight loss and malabsorption; in these patient cohorts viral RNA remains detectable in stool samples for months to years (Brown et al., 2019; Gallimore et al., 2004b; Green, 2014; Sukhrie et al., 2010).

1.5.2 Animal noroviruses

Animal NoVs have been linked to gastroenteritis outbreaks and acute diarrhoeic episodes of varying severity in cattle (Di Felice et al., 2016), pigs (Mauroy et al., 2008; Shen et al., 2012b, 2012a), cats (including a captive lion cub that succumbed to severe haemorrhagic enteritis) (Martella et al., 2007; Pinto et al., 2012), and dogs (Mesquita and Nascimento, 2012). While a clinical association typically exists in these domesticated mammalian hosts, asymptomatic infections have been observed (at lower prevalences) in epidemiological screening studies (Cho et al., 2013; Scipioni et al., 2008a; Villabruna et al., 2019); the only documented GIII sheep NoVs were reportedly isolated from animals that showed no obvious clinical signs (Wolf et al., 2009). In wild animals such as bats (Wu et al., 2016), harbour porpoises (de Graaf et al., 2017a), and Californian sea lions (Teng et al., 2018), where NoVs are typically detected in the context of metagenomics analyses and/or retrospective analyses of stored samples, a potential disease association often remains undetermined.

1.5.3 Murine noroviruses

Murine noroviruses have been isolated from asymptomatic wild populations of both field and wood mice (*Apodemus agrarius* and *Apodemus sylvaticus*) (Farkas et al., 2012; D. B. Smith et al., 2012) and have been detected in various cohorts of domesticated mice (*Mus musculus),* including mice sold as pets or snake food, show animals, and those bred for academic research (D. B. Smith et al., 2012).

Indeed, MuNoVs are recognised as one of the most prevalent, albeit often undetected, pathogens of contemporary laboratory mice, as evidenced by serologic testing and reverse transcription polymerase chain reaction (RT PCR) screening (Hsu et al., 2006, 2005; Müller et al., 2007). Thirty fully sequenced MuNoV strains have been isolated from specific-pathogen-free mice in academic research colonies across the globe; while these strains comprise a single genetic cluster, they broadly segregate into two categories regarding their pathogenesis and disease profile (Kahan et al., 2011). The prototype acute strain MNV-1, which infects immune cells in the gut-associated lymphoid tissue, reaches peak intestinal titres 1-2 days post-infection (dpi) and is cleared by 7-14 dpi; persistent strains MNV-3 and MNV-CR6

can establish life-long infections, linked to replication in the caecum, colon, mesenteric lymph nodes, and rare intestinal epithelial tuft cells (Arias et al., 2012a; Hsu et al., 2006; Wilen et al., 2018). Persistent asymptomatic infection with typically nonpersistent strain MNV-1 CW3 has been associated with adaptive changes to viral proteins NS1/2, NS7 and VP2 (Borin et al., 2014; Nice et al., 2013).

Notwithstanding differences in clearance kinetics and cell tropism, all MuNoV strains elicit subclinical infections of the intestine, lacking any association with diarrhoea or other overt disease in juvenile and adult mice of wild-type- and certain knock-out strains. A MNV-1-induced decrease in faecal consistency as measured by visual scoring of faecal samples of immunocompetent mice remains the only modest disease-association (MNV-3 failed to induce this pathology) (Kahan et al., 2011). Despite a subclinical presentation, quantifiable intestinal pathology and detection of viral RNA in the liver, spleen, mesenteric lymph nodes, and proximal intestine (but not in the lung, brain, blood, or faeces) have been described in association with experimental MuNoV infection of wild-type hosts (Hsu et al., 2005; Karst et al., 2003; Shortland et al., 2014; Wobus et al., 2006).

In severely immunodeficient adult mice lacking functional components of the innate immune system and interferon (IFN) pathways, MuNoV infection has been shown to be associated with lethal disease (Karst et al., 2003; Wobus et al., 2006). Following oral MNV-1 inoculation, mice deficient in signal transducer and activator of transcription 1 (STAT1) and recombination-activating gene 2 (RAG2) rapidly succumb to systemic disease associated with severe weight loss, diarrhoea, bloating, pathologies in intestinal and peripheral tissues, and the presence of viral RNA in all organs. Persistent strains MNV-3 and MNV-CR6 cause less overt symptoms than MNV-1 in IFN-deficient mice (Strong et al., 2012).

Recently, self-resolving diarrhoea in the absence of systemic disease was reported in MuNoV-infected wild-type neonatal mice, mirroring key clinical features of HuNoV disease; diarrhoeic episodes were neither associated with disruption of the intestinal epithelium nor notable inflammation. Oral MNV-1 inoculation, and to a lesser extent that of MNV-3 and MNV-CR6, caused acute diarrhoea in three-day-old BALB/c mice (Roth et al., 2020).

1.6 Human norovirus epidemiology and transmission

1.6.1 The societal burden of norovirus infections and the role of genotype GII.4

HuNoVs are recognised as major aetiologic agents of global sporadic and epidemic non-bacterial gastroenteritis (Patel et al., 2008; Robilotti et al., 2015), causing significant morbidity and mortality in developing countries and engendering enormous economic losses in developed countries (Bartsch et al., 2016). Causing a median number of 669 million illnesses and an estimated 219.000 deaths across all ages per year globally, HuNoVs have been calculated to result in a yearly total of USD

4.2 billion in direct health care costs (outpatient visits and hospitalisation) and USD 60.3 billion in societal costs (productivity losses due to absenteeism or mortality) (Bartsch et al., 2016).

GII.4 infections, which are responsible for the majority of past HuNoV outbreaks (55-85%) and also sporadic cases, have been associated with a higher probability of severe outcomes and lead to higher hospitalisation and mortality rates (Desai et al., 2012).

GII.4 NoVs have been the predominant genotype circulating in humans for over two decades, with novel circulating GII.4 strains emerging every two to three years and replacing their predecessors in an immune-driven selection process known as epochal evolution (Ji et al., 2013; Siebenga et al., 2007; Wang et al., 2012). Postulated mediators for the GII.4 dominance include selective advantages and improved adaptation to host receptors via physicochemical P2 changes in the virion of new GII.4 subtypes and the evasion of herd immunity against predominant genotypes (Giammanco et al., 2012; Hoffmann et al., 2013; Lam et al., 2012; Motomura et al., 2010). The elevated number of novel nonsynonymous mutations in GII.4 capsid sequences and changing HBGA binding patterns (Boon et al., 2011), as well as intragenotypic recombination have long been postulated to be a driving force of GII.4 NoVs. Strain-dependent differences in NoV molecular evolution via the accumulation point mutations are briefly discussed in chapter 2.2; complex patterns of intragenotypic recombination within the GII.4 lineage are discussed in chapter 2.3 (Ludwig-Begall et al., 2018).

The position of the rapidly evolving dominant GII.4 variants has only recently been challenged by emergence and re-emergence of different intra- and intergenotype recombinants modifying long-term global NoV genetic diversity trends (Bruggink et al., 2016, 2014; De Graaf et al., 2015; Fu et al., 2017; Hoffmann et al., 2013; Mahar et al., 2013; van Beek et al., 2018).

1.6.2 Norovirus shedding and human infectious dose

Norovirus particles are shed for weeks to months via the faeces or vomit of both infected symptomatic and asymptomatic patients (Davis et al., 2020; Leon et al., 2008; Siebenga et al., 2008). While the main NoV transmission route is faecal-oral transmission, with faecal loads reaching up to 10^9 genomic copies/g faeces (Atmar et al., 2008; Teunis et al., 2015), transmission via vomiting has also been identified as a risk (de Graaf et al., 2017b). Unlike shedding through stool, vomiting is more likely to result in significant environmental contamination, leading to transmission through fomites and airborne vomitus droplets ($1.7x10^8$ genome equivalent copies are typically shed in emesis (circa $4x10^4$ genomic equivalent copies/ml vomitus) (Atmar et al., 2014; Kirby et al., 2016; Tung-Thompson et al., 2015). The high doses of virus shedding stand in clear contrast to the low 50% human infectious dose which has been calculated to lie between 1320 and 2800 genome equivalents (Atmar et al., 2014).

1.6.3 Transmission routes

Highly tenacious and resistant in the face of various decontamination methods (Ludwig-Begall et al., 2021; Zonta et al., 2015), HuNoVs are transmitted either via direct person-to-person contact or by consumption of contaminated water or food (Verhoef et al., 2015) (Figure 6). Infectious viruses can enter environmental waters either via direct discharge or release of improperly-treated sewage from industrial-scale or small private waste-water treatment plants, discharges from vessels, as well as urban runoff, the latter especially in times of flooding or heavy rainfall which have been linked to a high prevalence of HuNoVs in coastal waters (Campos et al., 2013; Campos and Lees, 2014; Hassard et al., 2017; Wyn-Jones et al., 2011). Suspended or precipitated NoVs have been shown to retain infectivity for weeks to months (Bosch et al., 2006; Campos and Lees, 2014; McIntyre et al., 2012; Seitz et al., 2011) and have been detected up to 10 km distant from their discharge point (Campos et al., 2017; Wyn-Jones et al., 2011).

The foodborne proportion of HuNoV outbreaks is estimated at 14% (Verhoef et al., 2015). Foods implicated in outbreaks are contaminated either directly with faecal matter at the source or by infectious food-handlers (Hardstaff et al., 2018). The most common food vehicles remain fresh or frozen soft fruits and vegetables, ready-to-eat foods (such as sandwiches and salads) which require handling but no or little subsequent cooking, and undercooked or raw seafood (bivalve molluscs) (Razafimahefa et al., 2019). Bivalve molluscs, including cockles, mussels, clams, scallops, and oysters, accumulate NoVs via filter feeding; large volumes of water are pumped through the ctenidia, the molluscs' respiratory and feeding organs, in a process which filters not only nutrients but also contaminating bacteria and viruses. Depuration practices, which aim at eliminating such bioaccumulated pathogen charges are unsuccessful in the face of NoV contamination. Increasingly, this effect is attributed to the fact that NoVs are not only filtered and concentrated through nonspecific interactions, but are also bound in a genogroup- and strain-dependent manner to molluscan gastrointestinal carbohydrate structures (HBGA-like moieties and sialic acid-residues) (Almand et al., 2017). As known "hotspots" for the accumulation of multiple NoV strains (de Graaf et al., 2016; Lysén et al., 2009), bivalve molluscs have been postulated to present opportunities for infectious HuNoV inter-and intragenotype co-infection (thus facilitating subsequent viral recombination within the host), and have been pinpointed as high-risk vectors for the introduction of novel recombinant strains into the human population (Ludwig-Begall et al., 2018; Rajko-Nenow et al., 2013). In a similar context, bivalve molluscs, as potential interfaces of shared species exposure through filtration of human and animal waste, have also tentatively been implicated as a putative way of introducing both human and different animal NoVs into a single host (Ludwig-Begall et al., 2018; Takano et al., 2015).

Norovirus outbreaks are often reported in the context of communal dining at restaurants, festivals, picnics, schools, cruise ships and military bases (Pringle et al., 2015; Rha et al., 2016; A. J.

Smith et al., 2012; Verhoef et al., 2008) or in institutional settings such as hospitals and care homes, where spread of infection from a common-source exposure is facilitated by enclosed living quarters and reduced personal hygiene (Mathijs et al., 2012; Patel et al., 2009; Sukhrie et al., 2012, 2010).

Recently, wild birds and rodents were named as new potential HuNoV transmission routes; GI and GII HuNoV genome copies were detected in faecal samples of gulls and crows (31%) and rats (2%), implicating them as mechanical carriers, capable of spreading HuNoVs in the environment and possibly transmitting the virus to humans directly or indirectly by contaminating foods (Summa et al., 2018). Determination of the replication capability of HuNoVs in these new potential carriers (e.g. by detection of viral antibodies in blood or whole virus particles in faeces) is still pending.

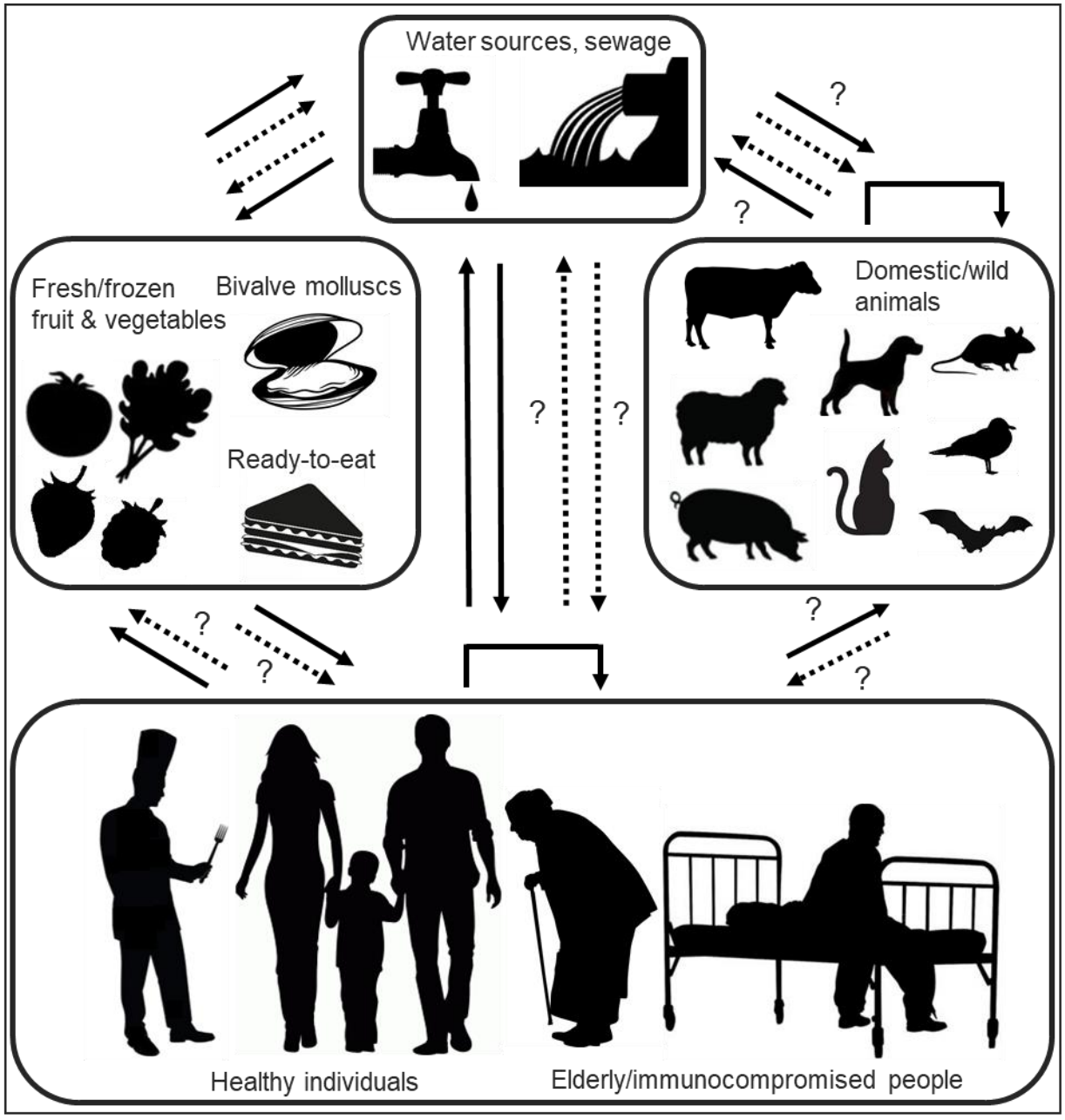

Figure 6. Norovirus transmission.
Transmission routes of human noroviruses (solid arrows) and animal noroviruses (dashed arrows) are shown. Unconfirmed transmission is indicated by a question mark.

1.6.4 Immunity to noroviruses

Many gaps remain in the understanding of natural immunity to HuNoVs. In addition to genetic resistance to infection based on secretor status (see chapter 1.4.1), with non-secretors representing as much as 20% of the European population (Le Pendu et al., 2006), NoV infection has been shown to result in development of clinical immunity.

Upon RNA virus invasion, two main innate immune cell pathways are rapidly launched against intestinal RNA viruses such as NoVs. Recognition of conserved viral pathogen–associated molecular patterns by germline-encoded pathogen recognition receptors upregulates transcription of genes involved in antiviral responses and activates both type I and type III IFN systems to control viral replication, clear pathogen-infected cells, and coordinate adaptive immune responses (Campillay-Véliz et al., 2020; Jensen and Thomsen, 2012; Lee and Baldridge, 2017). Both toll-like and retinoic acid-inducible gene I-like receptor family members sense cytosolic viral RNA and signal via mitochondrial antiviral-signalling protein to stimulate transcription of type I and III IFNs by members of the IFN regulatory factor family. Both type I IFNs (13 subtypes of IFN-α, IFN-β, κ, ω, ε, δ, and τ), which signal through the ubiquitous IFNα/β receptor to regulate IFN-stimulated gene expression through phosphorylation of STAT proteins (Cho and Kelsall, 2013), and type III IFNs (IFN λ or interleukin-28/9), which are produced by leukocytes and epithelial cells and signal through the IFNλ receptor expressed on epithelial cells, but also type II IFNs (IFN γ), have been shown to be critical for control of HuNoV and MuNoV replication. Thus, findings show that natural HuNoV infection results in the production of proinflammatory and anti-inflammatory cytokines (Cutler et al., 2017), that HuNoV replication in zebrafish larvae results in a measurable innate response (Van Dycke et al., 2019), and that the innate immune response partially restricts HuNoV replication in human intestinal epithelial cells (IECs) through IFN-induced transcriptional responses and production of pro- and anti-inflammatory cytokines (Hosmillo et al., 2020). Mice lacking functional type I and II IFN pathways succumb to lethal MuNoV infections (Karst et al., 2003). Type I and II IFNs play a role in the control of acute MuNoV infections both *in vivo* and *in vitro* (Changotra et al., 2009) but are dispensable for intestinal regulation of persistent strains for which IFN-λ instead plays a critical regulatory role (Lee and Baldridge, 2017; Nice et al., 2015).

Adaptive immunity against HuNoVs is postulated to include both cellular and humoral responses (Campillay-Véliz et al., 2020; van Loben Sels and Green, 2019). While information on cellular responses to HuNoV infection is scarce, increases of various pro- and anti-inflammatory cytokines in volunteer serum samples indicate involvement of both Th1 and Th2 immune responses (Lindesmith et al., 2005). Humoral immunity to HuNoVs is considered to be stronger and more long-lasting than cellular immunity; based on human challenge studies, first estimates of immunity duration suggested short term, adaptive immunity to homotypic Norwalk re-challenge with high viral doses to

last from two months to two years (Parrino et al., 1977) or for longer than six months (Johnson et al., 1990). Epidemiological data and mathematical modelling have since suggested that naturally induced immunity in the absence of major strain changes may actually last for much longer and potentially span up to a decade (Simmons et al., 2013). While seroprevalence studies have shown an estimated 90% of adult populations to be seropositive to NoV (O'Ryan et al., 1998), probably only a small fraction of the total HuNoV specific antibodies represent partial or even absolute neutralising antibodies, i.e. correlates of protection that mediate reduced infection or disease severity (van Loben Sels and Green, 2019). Strain dependent differences in the induction of protective immune responses (Zhu et al., 2013), antigenic diversity and known lack of heterotypic cross-protection between certain NoV genogroups, genotypes and strains (Rockx et al., 2005a) further confound the determination of immunity duration (Cates et al., 2020).

A recent model of adaptive immune responses to MuNoV infection suggests that presentation of MuNoV peptides on major histocompatibility complex class I molecules leads to the stimulation of primary Th1 proinflammatory responses, whereupon CD4+ Th1 cells release various cytokines that upregulate the activity of CD8+ cytotoxic T lymphocytes (van Loben Sels and Green, 2019). Humoral immunity supplements the Th1 response and has been shown to play a critical role in MuNoV clearance (Chachu et al., 2008) and protection from subsequent challenge (in this context it is noteworthy that MuNoVs are less diverse genetically and constitute a single genotype) (Zhu et al., 2013); proposed responses involve migration of antigen presenting cells to mesenteric lymph nodes where they present MuNoV antigens on major histocompatibility complex class II molecules and elicit upregulation of Th2 responses to help mature B cells (van Loben Sels and Green, 2019).

1.6.5 Seasonality of human norovirus infections

True to the name "winter vomiting disease", HuNoV infections follow a typical seasonality with incidents peaking during the winter months from October to March (Lopman et al., 2009). While not fully elucidated, this pattern is attributed to a complex combination of host, climactic environmental and viral factors. On the host side, winter peaks in NoV infections are linked to changes in societal behaviour, an upsurge in hospitalisations due to other infectious diseases, and fading herd immunity; inverse linear associations of NoV laboratory reports and daily temperatures have been reported, linking cold, dry conditions to higher NoV activity. NoV levels typically peak in winter in sewage (Nordgren et al., 2009; Victoria et al., 2010), freshwater (Westrell et al., 2006; Pérez-Sautu et al., 2012) and seawater as conditions for NoV persistence in waters are improved by colder water temperatures and reduced solar irradiation (Katayama et al., 2004; Lopman et al., 2009; Nordgren et al., 2009).

1.6.6 Reservoirs

The excretion of infectious NoV from persistently infected individuals (Davis et al., 2020; Teunis et al., 2015) is purported to be one of the sources of NoV outbreaks. Not only has the involvement of chronic shedders in hospital outbreaks indicated them to be a reservoir for nosocomial transmission of NoVs (Sukhrie et al., 2010), but persistently infected patients have also been suggested to contribute to HuNoV transmission as reservoirs for emerging strains.

Intra-host evolution via point mutation accumulation (Hoffmann et al., 2012; Yu et al., 2020) and the acquisition of superinfections over the protracted period of persistent infections (Brown et al., 2017) implicate persistently infected patient cohorts as potential reservoirs for novel HuNoV variants (de Graaf et al., 2016). Multiple phylogenetic analyses have identified viral populations in persistently infected patients to be highly diverse and genetically distinct from viruses circulating in the general population (Bull et al., 2012; Green, 2014).

The within-host viral variation via the acquisition of point mutations in chronic shedders is typically not random but has been shown to be a result of positive selection, as evidenced both by nonsynonymous versus synonymous substitution ratios (>1) and the clustering of amino acid changes at VP1 blocking epitopes (hypervariable P2 domain) and HBGA binding sites on the capsid surface (Hoffmann et al., 2012; Nilsson et al., 2003; Siebenga et al., 2008; Van Beek et al., 2017; Yu et al., 2020). Indeed, the intra-host emergence of antigenically distinct strains comparable to the variation between chronologically predominant GII.4 strains has been observed, suggesting that in certain individuals the evolution during a persistent NoV infection translates into relevant phenotypic variability, thus potentially selecting for viruses able to escape herd immunity to earlier isolates (Debbink et al., 2014).

At an average of five to nine mutations per 100 days (Hoffmann et al., 2012) or 1.85 to 2.66 x 10^{-2} substitutions per nucleotide site per year (s/n/y) in the viral capsid gene (Nilsson et al., 2003), NoV evolution rates in immunocompromised hosts are generally significantly elevated compared to those in healthy hosts. The process, whereby NoV strains can acquire enough mutations to constitute novel epidemic subtypes within weeks to months (on a global scale this would normally take years), has been attributed to the particularities of a reduced but constant intra-individual selection pressure in immunocompromised hosts (Hoffmann et al., 2012; Karst and Baric, 2015; Siebenga et al., 2008). Siebenga et al. reported that the number of VP1 amino acid changes selected per time in intra-individual quasispecies was higher in patients with intermediate immunocompromise than in severely immunocompromised patients (Siebenga et al., 2008). This is concurrent with the phylodynamic framework for RNA virus evolution proposed by Grenfell et al., which argues that highest rates of pathogen adaptation occur at intermediate levels of immunity when medium immune pressure coincides

with appreciable virus replication and the co-evolutionary competition between host and pathogen is most intense (Grenfell et al., 2004).

Over the prolonged period of persistent NoV infections, superinfections with a second genotype have been shown to occur in a sixth of patients; in such cases, temporary mixed infections can be detected in a single sample (Brown et al., 2017). Mixtures of NoV strains further heighten the complexity of intra-individual quasispecies in immunocompromised hosts and provide opportunities for viral recombination, which constitutes another possible factor towards driving the emergence of new epidemic strains (Ludwig-Begall et al., 2018; Parra, 2019).

While multiple analyses have highlighted the diversity of NoV variants in immunocompromised patients and have shown that chronic variants have the propensity to rapidly generate novel variants, the contribution of this diversity to NoV evolution at the inter-host population level is still unclear. Recent mathematical modelling based on the standard epidemiological categorisation of susceptible, infected and recovered individuals, suggested that despite the capacity of immunocompromised hosts to generate significant diversity, the relative isolation and rarity of such hosts limits their impact on broader pathogen evolution and epidemiology. Specifically, only a minor role for immunocompromised individuals in shaping large scale evolutionary patterns and processes and the global emergence of new HuNoVs was inferred (Eden et al., 2017). However, the model presented several inherent caveats, notably the implicit assumption within the modelling framework for there to be no selective advantage of novel genetic variants (all nucleotide substitutions were considered to be effectively neutral), the fact that varying immune pressures were not accounted for, the disregard of superinfections and the potential for mixed viral recombination in immunocompromised patients, and the failure to account for complex host population structures in institutional settings.

While the reservoir of novel NoV strains is yet to be definitively identified, NoV diversity could also be originated at inter- and intra-host levels in otherwise healthy populations of different age groups (from infants in day care centres (Hebbelstrup Jensen et al., 2019) to adults in the context of communal living and dining as described above). Thus, mutations could arise during transmission events which present an evolutionary bottleneck in outbreak settings, and/or during shedding in healthy individuals (Bull et al., 2012; Parra, 2019).

The lack of certitude regarding the source of newly emerging HuNoVs and the close genetic relatedness between certain animal and human NoVs have generated interest in the possible role of animals as a potential zoonotic reservoir for emerging strains (Villabruna et al., 2019). More than two thirds of human emerging infectious diseases are thought to originate from animal reservoirs (Jones et al., 2008); for other members of the *Caliciviridae* family, interspecies transmission has been reported (Smith et al., 1998, 1973). The as yet unproven existence of a zoonotic potential for NoVs has long been discussed, potential interfaces of shared species exposure being food, water or animal contact. Despite

known NoVs exhibiting marked host specificity, the discussion about interspecies and/or zoonotic transmission is fuelled by the close relationship of certain animal and human NoV strains, detection of HuNoVs in animal faeces, detection of antibodies against HuNoVs in swine, and the demonstration of experimental HuNoV GII infection in gnotobiotic pigs (Bank-Wolf et al., 2010; Mathijs et al., 2012; Scipioni et al., 2008a; Wilhelm et al., 2015). Questions concerning species barrier determinants preventing HuNoV infection of murine cells were recently resolved with the identification of a CD300If proteinaceous receptor as the primary determinant of MuNoV species tropism. All other components of cellular machinery required for NoV replication are conserved between humans and mice (Orchard et al., 2016); expression of MuNoV CD300 family receptor molecules rendered non-murine mammalian cells susceptible to MuNoV infection (Haga et al., 2016). If the key to cross-species transmission lies only at a structural virus-host receptor level, this presents ORF1/2 NoV recombination (discussed further in chapter 2.3.1), by which a nascent recombinant virus gains a complete novel capsid protein set, in an interesting light, in that a "lucky" intragenogroup recombination event between two co-infecting viruses might tender a zoonotic/ interspecies recombinant. Indeed, putative GIV.2_GVI.I interspecies recombinant FNoVM49, isolated from a cat captured near a Japanese oyster farm in 2015 (Takano et al., 2015), may have originated via a similar mechanism. However, since conclusive data supporting inter-species transmission is yet lacking, the continuous emergence of new HuNoV through zoonotic events is unlikely.

1.7 Detection and typing of noroviruses

1.7.1 Diagnostic methods

Since NoV infections present a major public health issue, rapid diagnosis is vital for the initiation of appropriate control measures to curtail viral spread and curb the extent of outbreaks.

Based on the typical clinical presentation of NoV infections, the Kaplan criteria can assist in diagnosis when laboratory resources are unavailable to determine an outbreak aetiology. Developed from pooled data of gastroenteritis outbreaks between 1967 and 1980, the Kaplan criteria consist of four patterns that characterise NoV outbreaks; accordingly, stool cultures negative for bacterial pathogens, mean (or median) duration of illness of 12–60 hours, vomiting in greater than or equal to 50% of cases, and a mean (or median) incubation period of 24–48 hours satisfy the criteria for a Norwalk-like infection (Kaplan et al., 1982). While a useful diagnostic aid in discriminating confirmed foodborne gastroenteritis outbreaks due to NoVs from those due to bacteria with a reportedly high specificity (99%), these criteria are only moderately sensitive (68%) (Turcios et al., 2006), necessitating further laboratory confirmation of the viral aetiology.

Electron microscopy, utilised for the first ever identification of NoV particles in stool (Kapikian et al., 1972), permits rapid and direct visualisation of NoVs and other gastroenteritis viruses such as rotaviruses, adenoviruses, astroviruses, and sapoviruses. However, the method lacks sensitivity and facile implementation (highly trained personnel is a prerequisite to its use), rendering it ineligible for routine diagnostics (Vinjé, 2015). In lieu of this costly method, and in the absence of a stable and inexpensive HuNoV cell culture system, routine laboratory diagnostics for NoVs are typically either performed via immunological assays or amplification of viral nucleic acids.

While the development of a broadly reactive NoV antigen enzyme immunoassay (EIA) has proven challenging owing to the number of antigenically distinct HuNoV genotypes and the continuous antigenic drift of certain strains (Chan et al., 2016), several EIAs are commercially available for the detection of NoV GI and GII antigens in stool specimens. Most commercial kits consist of solid-phase, sandwich-type immunoassays and include combinations of multiple cross-reactive monoclonal and polyclonal antibodies. Sensitivity and specificity of these kits, typically around 70% and 90%, respectively, are subject to significant variation depending on the viral load and NoV genotypes present in the sample. The clinical context of sample collection (sporadic case versus outbreak) and the number of samples tested are recognised to influence the sensitivity of EIAs to such an extent that their use, while undoubted for rapid screening of multiple faecal samples during an outbreak of acute gastroenteritis, is not recommended in interpreting test results from sporadic cases and that negative results should be further confirmed by molecular methods (RT-PCR) (Costantini et al., 2010; Gray et al., 2007). Similarly, immunochromatographic lateral flow assays, designed for rapid and uncomplicated testing of individual faecal samples, have been shown to have a varying, genogroup-dependent sensitivity and, while useful for preliminary screening in outbreaks, their negative results should be verified by RT-PCR (Ambert-Balay and Pothier, 2013).

Amplification-based techniques for the detection of NoVs in clinical samples, environmental samples, and food and water include conventional RT PCR (Vinjé et al., 2003) and one- or two-step quantitative real-time RT PCRs (RT qPCR) (Kageyama et al., 2003). Most contemporary assays use genogroup-specific oligonucleotide primers and fluorescent probes typically targeting a small conserved genome region at the ORF1/ORF2 junction (Katayama et al., 2002). Increasingly, such assays are multiplexed, allowing simultaneous detection of multiple NoV genotypes within different genogroups, e.g. the simultaneous detection of GI and GII strains (Rolfe et al., 2007; Shigemoto et al., 2011) or GI, GII, and GIV strains (Miura et al., 2013a); several different multiplex molecular gastrointestinal diagnostic pathogen platforms are commercially available (Claas et al., 2013).

Quantitative RT qPCR assays, which implement either intercalating dyes (Scipioni et al., 2008b) or fluorescent probe-based chemistries (Miura et al., 2013a), can be used to determine the amount of nucleic acid (genomic copies) in a sample. However, a distinction between infectious and non-infectious

virus particles is not possible and virus detection by RT qPCR does not necessarily correlate with a true infectious NoV burden. Methods to evaluate the correlation between genomic copies and infective NoV particles are under investigation. Amongst these, the binding long-range PCR (Li et al., 2014) has been proposed to assess genome integrity and the use of a ligand binding step prior to RT qPCR (Afolayan et al., 2016; Dancho et al., 2012) or viability PCR assays (Karim et al., 2015; Razafimahefa et al., 2021) are utilised to investigate capsid integrity. Comparison of RT qPCR results with newly developed HuNoV infectivity assays (further discussed in chapter 1.9) may help determine cycle threshold cut offs for clinical diagnostic RT qPCRs, allowing estimation of infectious virus burdens to help guide infection control (Chan et al., 2019; Straub et al., 2013).

Increasingly, the spectrum of analytical techniques is being widened; promising developments in the field include biosensors (such as monoclonal antibodies, aptamers, porcine gastric mucin, and HBGAs), investigated for their potential of concentrating NoVs, microarray-based assays (Yu et al., 2016) and omics-based analyses (Liu and Moore, 2020; Strubbia et al., 2019).

1.7.2 Genotyping

With the increasing implementation of molecular methods in NoV diagnostics, virus typing through (partial) sequence analysis has become increasingly common. The web-based, open access Norovirus Automated Genotyping Tool (Version 2.0; NoroNet) for sequence-based typing, available online from the NoroNet website of the Dutch National Institute for Public Health and the Environment (http://www.rivm.nl/mpf/norovirus/typingtool), provides direct and internationally standardised genotyping of NoVs. Based on genetic homology and phylogenetic inferences, the tool assigns sequences to a NoV genogroup, maps query sequences to a specific location on the reference genome(s), and offers information on RdRp- and capsid affiliation on either side of the ORF1/2 overlap. Briefly, the tool, updated periodically with new names and reference strains, employs a typing algorithm on ORF1 and ORF2 sequences of GI and II NoVs, starting with BLAST analysis of the query sequence against a reference set of *Caliciviridae* sequences. This is followed by phylogenetic analysis of the query sequence and a sub-set of the reference sequences to assign NoV genotype and/or variant (GII.4), with profile alignment, construction of phylogenetic trees and bootstrap validation (Kroneman et al., 2011).

1.8 Treatment and prophylaxis

Despite the clinical significance and societal burden of NoV infections, neither approved antivirals nor licensed vaccines are yet available to combat this pathogen.

1.8.1 Antivirals

While medical intervention is rarely needed in typical NoV infections of immunocompetent individuals, safe and effective antivirals are essential for treatment of high-risk, persistently infected immunocompromised individuals and other vulnerable populations (juvenile/elderly). In the absence of specific therapeutic measures, treatment is focused on providing supportive care such as rehydration.

Research efforts towards antiviral development have been furthered by a deeper understanding of the NoV replicative cycle and recent breakthroughs in culturing HuNoVs; direct acting antiviral therapies target various stages of the NoV replication cycle (Arias et al., 2013; Netzler et al., 2019).

Strategies to prevent NoV attachment and entry include HBGA binding inhibition via various glycomimetic compounds (Koromyslova et al., 2017, 2015; Zhang et al., 2013) and passive immunotherapy with monoclonal antibodies (Chen et al., 2013) or nanobodies (Koromyslova and Hansman, 2017).

The activity of NS6 protease inhibitors depends on preventing polyprotein processing by the viral NS6. Candidate drugs targeting this step include broad-spectrum antivirals that covalently bind to the catalytic site of 3C or 3C-like proteases (Kim et al., 2012), enzymatic transition state inhibitors or -analogues (Galasiti Kankanamalage et al., 2016).

Compounds targeting viral polymerase NS7 to interfere with NoV replication comprise chain-terminating and mutagenic nucleoside analogues as well as non-nucleoside inhibitors. Nucleoside analogues under investigation include the cytidine analogue 2′-C-methylcytidine (Rocha-Pereira et al., 2015b) and its derivatives, and purine analogues favipiravir (Arias et al., 2014) and ribavirin, the latter of which is licensed to treat chronic hepatitis C infections (Chang and George, 2007; Perales et al., 2013; Woodward et al., 2017). Their inhibitory effects are attributed to multiple modes of action including chain termination, provocation of an error catastrophe scenario for the viral quasispecies via ambiguous base pairing (lethal mutagenesis), direct RdRp inhibition, and unbalancing of intracellular NTP pools (Crotty et al., 2002; Graci and Cameron, 2006). Non-nucleoside inhibitors target binding pockets of the RdRp thus preventing conformational changes required for formation of an active replication complex (Mastrangelo et al., 2012).

Host factor drugs with the potential to treat NoV infections include immunomodulators (type I, II and III IFNs) (Changotra et al., 2009; Nice et al., 2015; Rocha-Pereira et al., 2015a) and small molecule inhibitors that downregulate viral RNA secondary structure-binding host factors (Arias et al., 2013).

1.8.2 Vaccines

The development of HuNoV vaccines is desired to protect vulnerable populations (immunocompromised/juvenile/elderly) and high-risk groups, including health care workers, military personnel, and (cruise ship) travellers experiencing crowding conditions. Prophylactic applications may also include the vaccination of food handlers to reduce the occurrence of food-borne outbreaks.

Key challenges for NoV vaccine development pertain to vaccine effectiveness in the face of NoV strain diversity and continuing evolution, which call for multivalent vaccines and periodic updates to protect against a range of current and emerging epidemiologically important genotypes. Further, the lack of a universally accepted correlate of protection against NoV, documented varying seroresponse and uncertainty regarding the duration of long-term immunity conferred by NoV infection (see chapter 1.6.4) or vaccination are barriers faced in NoV vaccine development (Hallowell et al., 2019).

Nevertheless, a bivalent GI.1/GII.4 VLP vaccine (Treanor et al., 2020) and a recombinant adenovirus vector vaccine expressing GI.1 or GII.4 VP1 with monovalent or bivalent dosing (Kim et al., 2018), are currently in clinical trials. Further vaccines have been approved for clinical trial testing or are in the pre-clinical phase of development (Cates et al., 2020; Lucero et al., 2018).

1.9 Model systems to study norovirus biology

1.9.1 *In vivo* model systems for human noroviruses

Early volunteer challenge studies and epidemiological observations of HuNoVs in their natural hosts have yielded important *in vivo* data to further the understanding of HuNoV infections (Johnson et al., 1990; Le Pendu et al., 2006; Meeroff et al., 1980). However, since the interpretation of results from such studies may not only be complicated by small sample sizes, variations in susceptibility to infection, previous history of exposure and cross-reactivity of antibodies, but may also pose potential health risks to participants, a robust HuNoV animal model has long been sought.

Various non-human primates have been tested as HuNoV infection models (Todd and Tripp, 2019). While neither baboons, common marmosets, cotton top tamarins nor cynomolgus seem susceptible to HuNoV infection (Rockx et al., 2005b), rhesus macaques and chimpanzees produce serum antibodies and shed virus upon oral HuNoV infection but do not develop clinical symptoms (Bok et al.,

2011; Rockx et al., 2005b; Wyatt et al., 1978); only infection of pigtail macaques has been shown to result in typical clinical illness including vomiting, thus potentially presenting a model to study the emetic response to HuNoVs (Subekti et al., 2002).

Large animal models for symptomatic HuNoV infection include gnotobiotic pigs and calves. Infection of gnotobiotic piglets with a GII.4 HuNoV results in mild diarrhoea, faecal shedding of viral RNA, expression of viral RNA in intestinal enterocytes and extra-intestinal lymphoid tissues, and seroconversion (Cheetham et al., 2006; Park et al., 2018). Prolonged HuNoV infections and viral dissemination beyond the intestine have been observed in gnotobiotic pigs with a severe combined immunodeficiency phenotype (Lei et al., 2016). Gnotobiotic piglets provide a useful experimental model as the pig intestine anatomy resembles that of humans and protection from disease provides a valuable read-out in vaccine trials and testing of therapeutics (Bui et al., 2013; Kocher et al., 2014). Gnotobiotic calves infected orally with HuNoV develop diarrhoea associated with intestinal damage and faecal viral shedding for up to six days, as well as local and systemic immune responses (Souza et al., 2008) .

Double knockout recombination activation gene ($Rag^{-/-}$) and common gamma chain ($\gamma c^{-/-}$) deficient BALB/c mice support subclinical HuNoV GII.4 replication upon infection via the intraperitoneal route (Taube et al., 2013). The model has been used to assess the anti-HuNoV activities of antiviral compounds (Kolawole et al., 2016). However, since these mice cannot be infected orally and lack both gut-associated lymphoid tissues and the ability to produce numerous cytokines and mature B and T cells, the model cannot recapitulate typical HuNoV infection.

Recently, multiple HuNoV GI and GII strains were shown to replicate to high titres in cells of both the hematopoietic lineage and the intestine of zebrafish larvae (*Danio rerio*) following yolk inoculation (larval food reserve) (Van Dycke et al., 2019). Yielding over three orders of magnitude ($3\log_{10}$) increases in GII.4 viral RNA copies, zebrafish larvae were shown to constitute a simple and robust *in vivo* HuNoV replication model and were also demonstrated to be suited to antiviral studies.

1.9.2 Human norovirus tropism and i*n vitro* culture of human noroviruses

In lieu of a stable HuNoV culture system, HuNoV *in vitro* assays were, until very recently, conducted using the Norwalk virus replicon (Chang et al., 2006) and/or virus-like particles (VLPs).

The Norwalk virus RNA replicon consists of an intact ORF1 and ORF3, and an ORF2 disrupted by a neomycin gene engineered into the VP1-encoding region (thus blocking expression of intact VP1). Self-replicating and stably expressed following transfection into cell lines of human (Huh-7) or hamster (BHK21) origin, the replicon has proven useful for the study of HuNoV genome replication and screening of antiviral compounds (Chang and George, 2007; Rocha-Pereira et al., 2014).

The RNA replicon is complemented by VLP systems, in which expression of capsid protein VP1 results in the self-assembly of recombinant VLPs that are morphologically and antigenically indistinguishable from native HuNoV virions and consequently represent useful tools to study physical virion properties, antibody responses, and attachment factor interactions (Bertolotti-Ciarlet et al., 2002).

Notwithstanding the utility of these two systems, the fact that the understanding of HuNoV biology has lagged behind that of other positive strand RNA viruses has been, in great part, due to the difficulties historically associated with robust *in vitro* HuNoV propagation (Duizer et al., 2004; Lay et al., 2010); in turn, issues with HuNoV cell culture stem from the uncertainties still surrounding HuNoV tropism and the lack of a known (proteinaceous) entry receptor (see chapter 1.4.1).

Recent data support a dual cell tropism of epithelial cells and nonepithelial cells of hematopoietic origin both *in vivo* (Karandikar et al., 2016) and *in vitro* (Wobus, 2018) and illustrate a complex interplay with the host microbiome (Jones et al., 2014; Walker and Baldridge, 2019). Currently, two different HuNoV cell culture systems successfully capitalise on this dual tropism.

The development of the *in vitro* BJAB human B cell line demonstrated that HuNoV (and MuNoV) can either infect B cells directly or in a coculture system in which the virus must cross a confluent epithelial monolayer to access underlying B cells; productive GII.4 HuNoV infection of B cells required the presence of the HGBA-expressing commensal bacteria (or free synthetic HBGA), identifying them as a stimulatory cofactor for bridging NoV attachment to and infection of B cells (Jones et al., 2015, 2014). This and other available data directed the development of a working model for NoV intestinal infection whereby NoVs bind to specific glycans expressed on the surface of members of the gut microbiota and/or enterocytes and are then transcytosed across the polarized intestinal epithelial barrier to gain access to their target immune cells (Karst, 2015b; Karst and Wobus, 2015). Notably, this model provides an explanation for how NoVs may achieve co-infection of host cells in conditions when the number of cells far outweighs that of virions; multiple genetically distinct virions can be effectively concentrated by binding to the surface of a single bacterium, thereby increasing the opportunity for co-infection (Erickson et al., 2018; Jones and Karst, 2018). While the technical simplicity and use of a commonly used cell line are strengths of the BJAB assay, current drawbacks are the modest level of viral replication and varying reproducibility.

In a technically more complicated approach, but with more robust infection levels overall, cultivation of multiple HuNoV strains has recently been demonstrated in stem cell–derived, human intestinal enteroid (HIE) cultures (epithelial mini guts) which recapitulate the multicellular, physiologically active human intestinal epithelium (Estes et al., 2019; Ettayebi et al., 2016). Grown from single multipotent stem cells of the human intestinal crypts (isolated from endoscopic biopsies), HIEs can be maintained continuously as three-dimensional cultures. Differentiation into distinct mature cell types present in the epithelium, such as absorptive enterocytes, multiple secretory cells (Paneth cells,

goblet cells, enteroendocrine cells, and tuft cells), and the M cells of Peyer's patches can be achieved by modifying culture conditions (Sato et al., 2011, 2013). Propagation (and limited passaging) of HuNoVs in enterocytes of differentiated HIEs (either three-dimensional or trypsinised and seeded into monolayers) has been shown to be dependent on HBGA expression in a strain-dependent manner (secretor-negative HIEs are permissive to GII.3, but not GII.4 replication); in addition, bile acids have been shown to be required for productive infection of certain strains (GI.1, GII.3, and GII.17), but bile is not necessary for cultivation of HuNoV GII.4/Sydney. The expense and complexity of the HIE system, relatively low sensitivity of the cultures to infection, issues with sustained passaging, and the unresolved basis for strain specific replication requirements remain challenges faced in the ongoing enhancement of HuNoV HIE cultures (Estes et al., 2019).

Different *in vivo* and *in vitro* HuNoV assays have all provided invaluable tools to dissect the NoV life cycle. However, there is still a lack of detailed understanding of NoV replication and significant questions remain unanswered due to the technical limitations of many of these experimental systems.

1.9.3 The murine norovirus - an *in vivo* and *in vitro* human norovirus surrogate

The genetically and biologically closely related murine norovirus (MuNoV) combines the advantages of easy *in vivo* infection of a cost-effective, genetically tractable, bona-fide native host (Karst et al., 2003), efficient and robust *in vitro* culture systems (Wobus et al., 2006, 2004), and availability of tools for genetic manipulation (Arias et al., 2012a, 2012b; Yunus et al., 2010), and thus remains the model of choice to study both the host response to NoV infection and basic aspects of NoV biology.

Caveats to the model include differences between HuNoV and MuNoV carbohydrate attachment factors and proteinaceous receptors (see chapter 1.4.1), the fact that HuNoVs replicate in intestinal enterocytes, a cellular tropism that MuNoV does not seem to share, and the typically asymptomatic nature of MuNoV infections in wild-type mice. *In vivo* MuNoV infections of adult immunocompetent and immunocompromised mice as well as those of neonatal mice are described in chapter 1.5.3. The adult *in vivo* models have long yielded valuable information concerning the biology of a NoV in its natural host (Wobus et al., 2006). The newly described model of NoV diarrhoea in which key clinical features of HuNoV disease are mirrored in MuNoV-infected neonatal mice will open up new avenues of research and the finding that disease severity is regulated by viral genetics (MNV-3 and MNV-CR6 cause a reduced incidence of diarrhoea relative to MNV-1) will facilitate identification of viral virulence determinants (Roth et al., 2020).

Until very recently, MuNoVs were the only cultivable NoVs, replicating efficiently and to high titres in cultured bone marrow-derived murine macrophages (RAW264.7 cells) (Wobus et al., 2006, 2004) and murine-derived microglial cells (BV-2 cells) (Cox et al., 2009) as well as B cells (M12 and

WEHI-231), where peak titres are reached one day later than in RAW264.7 macrophages (Jones et al., 2014).

The panel of techniques described to study MuNoV biology (Hwang et al., 2014) includes both DNA-based and RNA-based reverse genetics systems (Arias et al., 2012b). The DNA-based system is implemented in Study 2 of the Experimental Section of this thesis. Briefly, complementation in baby hamster kidney cells constitutively expressing the bacteriophage T7 RNA polymerase by a helper fowlpox virus encoding for T7 RNA polymerase allows transcription of an infectious plasmid containing MuNoV cDNA under control of a truncated T7 polymerase promoter (pT7: MNV 3' Rz), expression of the viral RNA, and subsequent recovery of infectious virus (Arias et al., 2012a) (Figure 7). A more sensitive RNA-based approach allows efficient recovery of infectious MuNoV from cDNA via *in vitro* transcription, *in vitro* capping and subsequent transfection into permissive RAW264.7 or BV2 cells (Yunus et al., 2010).

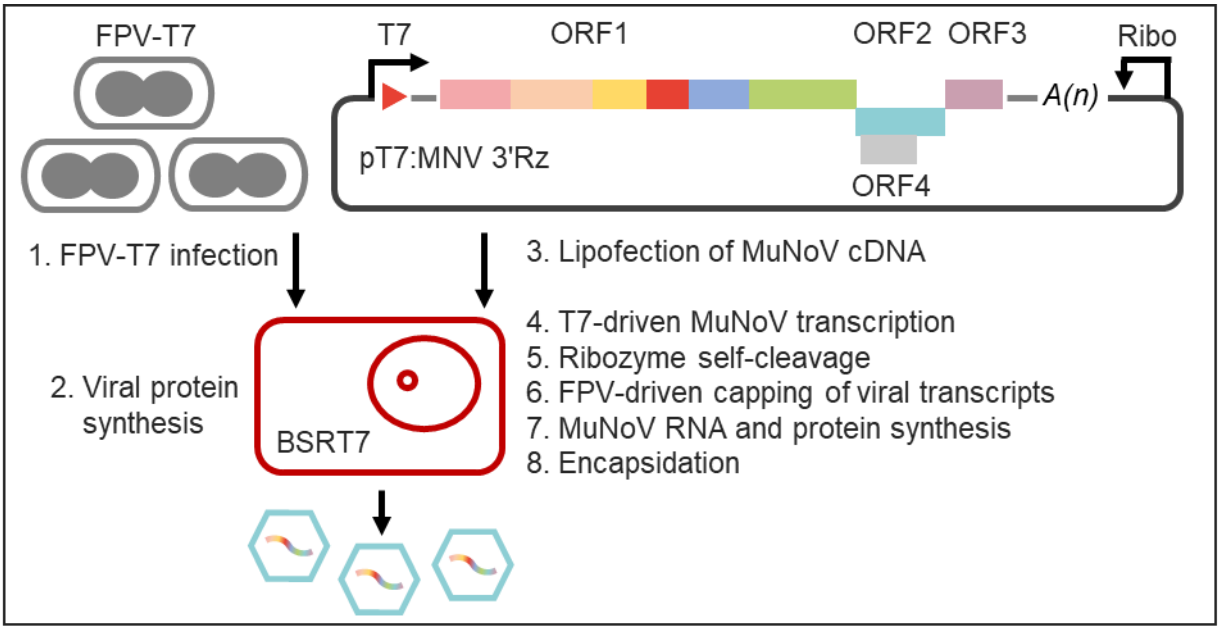

Figure 7. DNA-based reverse genetics recovery of infectious MuNoV, as adapted from Arias et al., 2012.
BSRT7 cells are infected with a recombinant fowlpox virus expressing T7 RNA polymerase (FPV-T7)) (1). The infected cells are incubated for two hours to allow for expression of FPV proteins (including the T7 RNA polymerase) (2). The plasmid pT7:MNV 3'Rz is transfected into the cells (3). Once inside the cell, pT7:MNV 3'Rz is recognised by the T7 RNA polymerase which synthesises murine norovirus (MuNoV) RNA transcripts (4). The presence of a self-cleaving δ-Ribozyme sequence (Ribo) at the 3' end of the genome guarantees that the transcript 3' terminus is located just after the polyA tail (5). Some viral transcripts are intracellularly capped by an FPV capping enzyme (6). The resulting capped MuNoV transcripts are translated to generate MuNoV proteins which catalyse replication of MuNoV transcripts. Newly synthesised non-structural protein 5 (VPg) -linked MuNoV RNA molecules then undergo successive cycles of replication accompanied by viral translation (7). Replicated genomes are packaged into the capsid for virion assembly and release of infectious virus (8). ORF= open reading frame.

2. Molecular evolution of noroviruses

2.1 General concepts of RNA virus evolution

RNA viruses, particularly those of the positive-strand Baltimore class IV, account for the majority of the virome diversity in eukaryotes (Koonin et al., 2015); RNA viruses pose major threats to human and animal health and number prominent agents of emerging and re-emerging infectious diseases (Holmes, 2010a; Woolhouse, 2002). A catalogue of all known human-infective RNA virus species comprises over 200 listings, is thought to be by no means complete, and remains subject to continuous revisions (Woolhouse and Brierley, 2018).

The variability within the RNA virosphere originates with the reliance of its constituents on the error prone RdRp, the viral hallmark protein that is universally conserved in RNA viruses (Koonin et al., 1993). The low-fidelity RdRp both introduces mutations (genetic drift) and mediates recombination between nascent RNA genomes (genetic shift). The viral diversity thus intrinsically produced is then modulated by extrinsic evolutionary forces, including random genetic drift (driven by frequent bottlenecking events) and natural selection (episodes of strong purifying pressure) (Grenfell et al., 2004).

2.1.1 Point mutation accumulation of RNA viruses

Mutations in viral genomes may originate from a range of sources such as spontaneous nucleic acid damage (all viruses), diversity-generating retro-elements (encoded by prokaryotic DNA viruses), and editing of the genetic material by host-encoded proteins (enzyme-driven hypermutation acts on a number of RNA viruses) (Sanjuán and Domingo-Calap, 2016). Intrinsic polymerase fidelity, the ability to incorporate the correct base and exclude incorrect bases from the active site during synthesis, is the primary determinant of genetic diversity (Sanjuán and Domingo-Calap, 2016). While error frequencies between viral polymerases are comparable prior to exonuclease correction, viral mutation rates are modulated by the ability of a virus to correct mismatches via polymerase-associated proofreading and/or post-replicative repair, a characteristic typically encoded by DNA viruses. Low-fidelity RNA virus RdRps lack exonuclease activity and consequently intrinsically misincorporate at higher frequencies (Smith, 2017) (exception among RNA viruses: viruses from the order *Nidovirales* encode a proofreading 3′- to -5′ exoribonuclease (Ogando et al., 2019)). At 0.1 to 1.0 mutations per genome per RdRp-mediated replication (Duffy et al., 2008), or 10^{-6} to 10^{-4} substitutions per nucleotide site per cell infection (s/n/c), average RNA virus mutation rates are several orders of magnitude higher than those of most DNA-based organisms (Peck and Lauring, 2018; Sanjuán, 2012).

High mutation rates confer genetic plasticity to a viral population; “mutational fitness effects” may be neutral, beneficial or deleterious to the overall fitness of a given virus within a viral population

(Wargo and Kurath, 2012). Within the typically compact RNA virus genomes (notable exception: *Nidovirales*) that encode only a few proteins, even single nonsynonymous mutations can be sufficient to alter the structure or function of virus-encoded proteins (Borin et al., 2014; Elde, 2012). Synonymous mutations (which do not change encoded amino acids) may impact viral fitness via non-neutral epistatic effects influencing RNA stability and splicing (Draghi et al., 2010; Lauring and Andino, 2010) and silent tuning for increased adaptability (Chamary et al., 2006; Elde, 2012; Lauring et al., 2012; Wilke and Drummond, 2010). In RNA viruses, most mutations are deleterious or lethal and result in the generation of less-fit or non-replicative variants, and beneficial mutations are comparatively rare (Sanjuán et al., 2004). Consequently, RNA virus populations commonly contain large numbers of defective RNAs or defective interfering particles, virus-like by-products of replication that carry deleterious mutations (typically large deletions). These degenerate non-viable particles may "interfere" with standard virus particles by competing for resources (Stauffer Thompson and Yin, 2010) but may also modulate the course of infection by acting as immune stimulants or immune decoys (Rezelj et al., 2018). The incorporation of deleterious mutations in an irreversible, ratchet-like manner is termed Muller's ratchet and can lead to a rapid debilitation of viral fitness unless relieved by compensatory mechanisms (Muller, 1964).

RNA virus replication may be described as a balancing act between the generation of sufficient diversity on which natural selection can act and the maintenance of genetic integrity and infectivity (Smith, 2017). This is illustrated by the fact that alterations to intrinsic RdRp fidelity have been demonstrated to have a negative impact on viral fitness in complex environments (Bordería et al., 2016), suggesting that RNA virus mutation rates have been evolutionarily optimised.

2.1.2 Selection and genetic drift of RNA viruses

Within the epidemiological triad of host, agent, and environment, viruses are locked in a perennial arms race with their hosts as they attempt to comply with the biological imperative of genetic survival (of the fittest) (Hurst and Lindquist, 2000).

The deterministic force of natural selection acts on the phenotypic diversity of mutant genomes in a viral population and drives viral populations as a whole towards increased overall viral fitness; positive selection drives fixation of beneficial mutations in a population, purifying selection removes deleterious reduced fitness mutants (Dolan et al., 2018).

The stochastic influence of random genetic drift, the change of variant frequencies in a viral population which occurs as a result of sampling error from generation to generation, can lead to the fixation of neutral and deleterious mutations in finite populations (Gillespie, 2001). RNA viruses, which can experience significant fluctuation in their population sizes, are subjected to the strong influence of genetic drift when within-host and transmission bottlenecks mediate transient reduction of the number

of viral genomes and the ensuing population is derived from a small sample of the ancestral population (Gutiérrez et al., 2012; Li and Roossinck, 2004).

Viral substitution rates, which describe the rate at which mutations become fixed within a population subsequent to selection and genetic drift, largely correlate with mutation rates. Thus, the high mutation rates of RNA viruses are mirrored in their mean nucleotide substitution rates of 10^{-4} to 10^{-3} s/n/y (Duffy et al., 2008; Holmes, 2010a).

The high evolutionary rates of RNA viruses are held to be inextricably linked to their typically short genomes, large population sizes, and their existence as viral quasispecies (Andino and Domingo, 2015; Holmes, 2010a, 2009; Sanjuán et al., 2010).

2.1.3 RNA viruses as viral quasispecies

Building on classical population genetics, quasispecies theory seeks to explore the consequences of error-prone replication of simple RNA and RNA-like replicons and near-infinite population sizes for genome evolution (Eigen, 1993). More recently, quasispecies theory has been used to describe the mutant distributions that are generated upon replication of rapidly mutating RNA viruses at large population sizes (Andino and Domingo, 2015; Domingo, 2016, 1998; Domingo et al., 2012; Domingo and Perales, 2019; Lauring and Andino, 2010; Más et al., 2010).

According to viral quasispecies theory, virus populations (mutant "spectra", "clouds", "swarms") are depicted as collections of closely related viral genomes connected by a network of single mutations which surround a modal master or consensus sequence; variants are linked within the viral population through antagonistic and cooperative functional interactions and collectively contribute to the characteristics of the population (Andino and Domingo, 2015; Domingo et al., 2012; Holmes, 2010b). The target of selection is the population as a whole, wherein variant distributions can swiftly shift and adapt to altered selective conditions by virtue of the expansive repertoire (or reservoir) of potentially beneficial mutations. The effect of deleterious mutations, which result in low individual fitness variants or defective interfering particles, can be relieved through complementation (Segredo-Otero and Sanjuán, 2019; Vignuzzi et al., 2006), cooperation (Shirogane et al., 2016), and, notably, recombination between different viruses (Muller, 1964).

Viral quasispecies theory has been extended to include not only the effects of point mutation accumulation but also recombination, which can buffer viral populations against deleterious and lethal mutations, prevent extinction of advantageous mutants during selective sweeps, combine co-circulating adaptive mutations to generate new variation that enhances virus fitness, but may also push a

quasispecies over a critical error threshold (Andino and Domingo, 2015; Boerlijst et al., 1996; Domingo et al., 2012).

2.1.4 RNA virus recombination – definitions and mechanisms of viral genetic shift

The concept of recombination prevalent in evolutionary genetics describes the complex molecular process by which a fragment of DNA is reciprocally exchanged between homologous chromosomes in the context of sexual reproduction in eukaryotes (exchange of genetic material between chromatids in the first meiosis division) (Posada et al., 2002). In prokaryotes and viruses, recombination, more aptly described as lateral gene transfer or gene conversion, involves nonreciprocal replacement or addition of genome sequences rather than exchange (Pérez-Losada et al., 2015; Posada et al., 2002).

Viral recombination occurs when at least two viruses infect the same host cell and exchange genetic sequences; less frequently, recombination may occur between viral and cellular sequences (Becher and Tautz, 2011). Between two (or more) RNA viruses, recombination can occur either via a replicative copy-choice mechanism or via non-replicative breakage and re-joining of genome fragments; both processes can theoretically result in homologous recombination involving the same site in both parental strands or non-homologous (illegitimate) recombination at different sites of the donor molecules (Galli and Bukh, 2014). Consequently, irrespective of the underlying recombination mechanism, homologous recombinants have the same genome architecture as their parental viruses, whereas nascent non-homologous recombinants bear atypical structures including deletions, insertions, or duplications (Galli and Bukh, 2014; Worobey and Holmes, 1999).

Focusing on the underlying mechanisms at play, three classes of replicative RNA recombination have been described (Nagy and Simon, 1997); accordingly, recombination events may be classed as similarity-essential (base-pairing dependent; class I), similarity-nonessential (base-pairing independent; class II), and similarity-assisted (base-pairing assisted; class III). For class I recombination, sequence similarity between parental RNAs is held to facilitate annealing between nascent and acceptor RNAs within the complementary region and is described as the major determinant of a recombination event. For class II recombination, sequence similarity between parental RNAs is not a requirement and recombination depends on RNA features other than base-pairing (e.g. RdRp binding sequences, RNA secondary structures, and heteroduplex formation between parental RNAs) which may bring parental RNAs into proximity and mediate template-switching by stalling the viral RdRp. Class III recombination combines features of both class I and II recombination in that both base-pairing and additional RNA features influence the occurrence of recombination events (Figure 8).

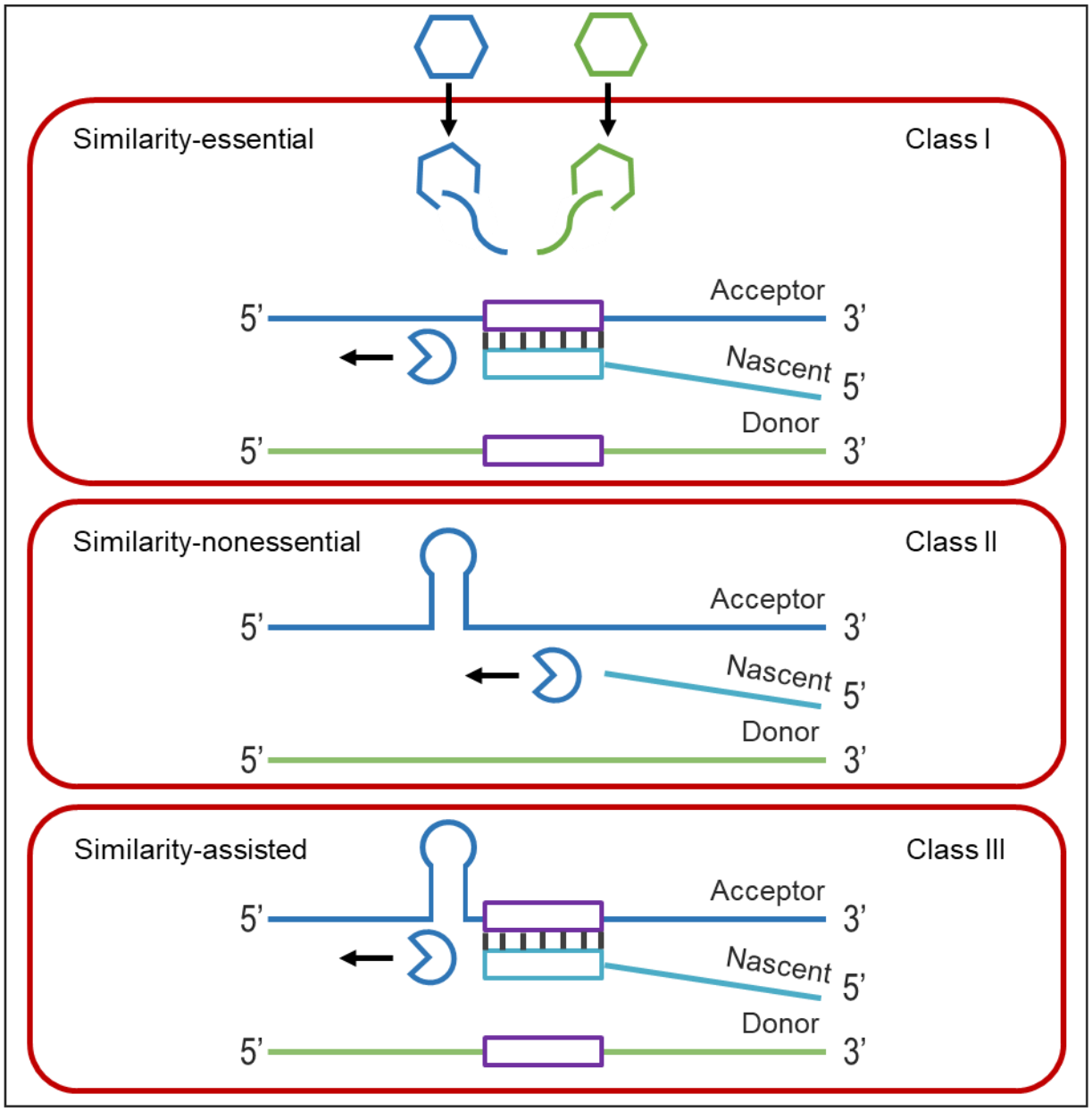

Figure 8. The three classes of RNA virus recombination. Similarity-essential (base-pairing dependent) recombination (top), similarity non-essential (base-pairing independent) recombination (middle), and similarity-assisted (base-pairing assisted) recombination (bottom) are shown. RNA polymerase-mediated RNA synthesis after the template switch is indicated by the jagged circle and arrow. The hairpin structure represents various RNA features that are required for Class II and Class III recombination (Figure adapted from Nagy and Simon, 1997).

The replicative copy-choice model of recombination typically (but not exclusively) depends on sequence similarity and mostly results in homologous recombination (if the viral polymerase continues to copy the new strand precisely where it left the old one); it is generally accepted to be the prevalent recombination mechanism in RNA viruses (Simon-Loriere and Holmes, 2011; Worobey and Holmes, 1999). Following this model, a mid-replication switch of the viral polymerase and the replication

complex from "donor" to "acceptor" template during synthesis of the nascent strand results in a chimeric RNA that contains fragments of both parental templates (Galli and Bukh, 2014).

The non-replicative mechanism of recombination describes self-ligation or host-factor-mediated joining of genetic fragments randomly cleaved through external influences such as physical shearing, electromagnetic radiation damage, and the activity of cellular endonucleases or cryptic ribozymes. Breakage and re-joining may occur between fragments of the same virus, amongst different viruses, and also between viral and cellular molecules, and more frequently results in non-homologous than homologous recombination (Galli and Bukh, 2014). Non-replicative recombination has been demonstrated for a number of positive sense single-stranded RNA viruses (Büning et al., 2017; Gallei et al., 2004; Galli and Bukh, 2014; Lowry et al., 2014). Proposed models for replicative and non-replicative NoV recombination, but which may also serve to illustrate RNA virus recombination in a wider context, are shown in Fig. 1 of the review on NoV recombination that comprises chapter 2.3 of this thesis (Ludwig-Begall et al., 2018).

Reassortment (or shuffling), a particular type of recombination unique to segmented or multipartite viruses (Sicard et al., 2016), can interchange discrete genome segments of co-infecting parental viruses; without involving intramolecular crossovers, entire genome segments of different origins are packaged into progeny viruses during viral replication, thereby giving rise to novel segment combinations (Pérez-Losada et al., 2015). Reassortment is frequently observed in segmented DNA and RNA viruses (Nelson et al., 2008; Thiry et al., 2005), however since it does not apply to monopartite NoVs which experience recombination in *sensu stricto*, the mechanism is not further discussed in the context of this thesis.

2.1.5 **RNA virus recombination frequencies**

Recombination frequencies are known to vary extensively amongst different RNA viruses; while large-scale comparative studies of RNA virus recombination rates are as yet lacking, significant variation has been reported both for the intrinsic rates of replicative and non-replicative RNA virus recombination prior to selection, as well as recombination rates that can be inferred at the population level. The former are typically measured *in vitro* via single-cycle assays in co-infected cells, population level estimations are typically based on sequence analysis and necessarily exclude deleterious recombinant forms that have been removed by purifying selection (Simon-Loriere and Holmes, 2011).

Recombination is frequent in retroviruses, notably in the human immunodeficiency virus where recombination rates, at approximately two to three recombination events per genome per virus replication cycle or 1.38×10^{-4} to 1.4×10^{-5} recombination events/adjacent sites/generation, may exceed those of mutation (Jetzt et al., 2000; Shriner et al., 2004). Such high recombination rates probably reflect

mechanistic aspects of retrovirus biology and genome architecture; specifically, their pseudodiploidy may facilitate recombination when two RNA molecules are packaged into the same virion, the physical proximity thus increasing the likelihood of template switching which is, in itself, an intrinsic component of the retrovirus replication strategy (Simon-Loriere and Holmes, 2011). Meanwhile, negative sense RNA viruses only infrequently experience recombination (Chare et al., 2003). Reasons for low negative sense RNA virus recombination rates remain to be fully elucidated but may include both ecologic and mechanistic constraints; their low recombination rates have been tentatively linked to the presence of the RNA-bound ribonucleoprotein complex which may affect the ability of the RNA polymerase to switch templates during replication (Chare et al., 2003). In positive sense RNA viruses, recombination occurs at highly variable frequencies between different virus families; recombination is frequently observed in the *Caliciviridae*, *Picornaviridae* and *Coronaviridae* (vertebrate viruses; in the latter, recombination is likely facilitated by discontinuous transcription involving jumps of the replication-transcription complex during minus strand RNA synthesis) (Desselberger, 2019; Lin et al., 2019; Simmonds, 2006), *Bromoviridae* and *Potyviridae (*plant viruses*)*, but appears to be non-existent in the *Leviviridae* (bacteriophages), *Barnaviridae,* and *Narnaviridae* (mycophages) (Bentley and Evans, 2018; Simon-Loriere and Holmes, 2011). In addition, rates may also vary significantly between different genera of the same family. Thus, the incidence of recombination varies amongst the four genera of the *Flaviviridae*, where recombination events are easily detected in pestiviruses (Becher and Tautz, 2011; Büning et al., 2017), pegiviruses (Zhang et al., 2019), the hepacivirus, hepatitis C virus (Galli and Bukh, 2014), and certain mosquito-borne flaviviruses (Durães-Carvalho et al., 2019), but are rarely reported in any of the tick-borne flaviviruses (Bentley and Evans, 2018; Norberg et al., 2013).

The range of recombination rates that characterises RNA viruses may be held to either reflect purely mechanistic features of particular viral ecologies or genome architectures, or may be attributed to certain advantages of recombination over asexual evolution and the fact that natural selection may favour specific genetic variants produced by recombination (Holmes, 2009; Simon-Loriere and Holmes, 2011). A dissection of the various checkpoints or steps that give rise to a recombinant a viral RNA, and ultimately a viable recombinant RNA virus, illustrates how drivers and constraints at each stage can determine whether a recombination event may be achieved for a given virus (Worobey and Holmes, 1999).

2.1.6 **RNA virus recombination checkpoints**

For the generation of a recombinant viral RNA, and ultimately a viable recombinant RNA virus, several requirements must be met (Galli and Bukh, 2014; Worobey and Holmes, 1999). Five steps or checkpoints must be successfully completed *in vivo* to generate a viable, replicating recombinant RNA virus following the classical copy-choice model of replicative recombination; four steps are necessary to obtain the same result via non-replicative recombination (Figure 9).

The first step necessarily preceding any recombination event is the simultaneous infection of a host with at least two parental strains (or clonal within-host replication allowing for subsequent recombination between nascent progeny viruses). Co-circulation of different viral strains in the same geographic area and within the same risk population are prerequisites to synchronous host co-infection. An overlap in space, if not in time, may nevertheless enable simultaneous infection of a host, provided superinfection by a secondary virus is not prevented by the host immune system and the primary virus has not been cleared before the event (Worobey and Holmes, 1999).

Once a host has been successfully co-infected, the second step is co-infection of a single target cell. The uptake of multiple viruses into a single cell is dependent both on the quantity of co-circulating viruses, the mode of their uptake (Erickson et al., 2018), and on factors that may limit consecutive entry of more than one virus particle per cell in a process known as superinfection exclusion. Superinfection exclusion is defined as the ability of an established virus to prevent a secondary infection by the same or a closely related virus (Folimonova, 2012); the primary infecting virus may render cells refractory to subsequent infection through interference at various stages of the replicative cycle of the secondary invader in a time-dependent manner. Viral pre-and post-entry blocks have been described for a number of RNA viruses (Adams and Brown, 1985; Bergua et al., 2014; Bratt and Rubin, 1968; Claus et al., 2007; Huang et al., 2008; Lee et al., 2005; Tscherne et al., 2007; Zhou et al., 2019), but may be overcome by certain strains after a period of adaptation (Lee et al., 2005; Webster et al., 2013; Zou et al., 2009).

The third step to obtaining a recombinant virus can either consist of a step of non-replicative recombination or a combination of replication and template switch (step four) between two co-infecting viruses within a cell. Co-localisation to the same subcellular region within said cell is necessary for interaction between viral genomes via either mechanism; co-occupancy of replication complexes is a prerequisite to recombination via the replicative pathway. Specific features of the viral genome and replicative proteins may further advance or hinder copy-choice recombination. Thus, the distribution of recombination junctions is frequently biased towards regions of sequence identity between RNA templates, the presence of tertiary genome structures is held to expedite replicative recombination, and the fidelity of the polymerase itself plays a role in determining how often particular genomes recombine (Bentley and Evans, 2018; Worobey and Holmes, 1999).

By whichever way a recombinant viral genome is generated, it is by no means a foregone conclusion that the process will result in a replicating recombinant RNA virus. Any given recombination event, switching out large genome segments in a nascent virus, presents a significant modification. It follows that initial imprecise recombination events (e.g. introduction of mutations or faulty epistatic interrelationships between the parts of an incipient recombinant) present an evolutionary bottleneck that

can result in the generation of non-functional genome chimeras (defective or defective interfering RNAs) or recombinant viruses of reduced replicative fitness. If recombinants are not able to function at the same level as their parental strains or do not possess selective advantages over their progenitors, it is unlikely that they will survive in a viral population (Lowry et al., 2014; Sackman et al., 2015). Studies in various RNA viruses have shown that circulating recombinants probably only represent a subset of those that are actually generated, and are the ones that are maintained in the viral population after a rigorous functional selection, having bypassed this fifth and final step of successful RNA virus recombination (Bagaya et al., 2017; Banner and Mc Lai, 1991; Lowry et al., 2014).

A conceptual model illustrating RNA virus recombination checkpoints is shown in Figure 9 (and is, with corresponding adaptations, reprised in Figure 3 of the review on NoV recombination that comprises chapter 2.3 of this thesis (Ludwig-Begall et al., 2018)).

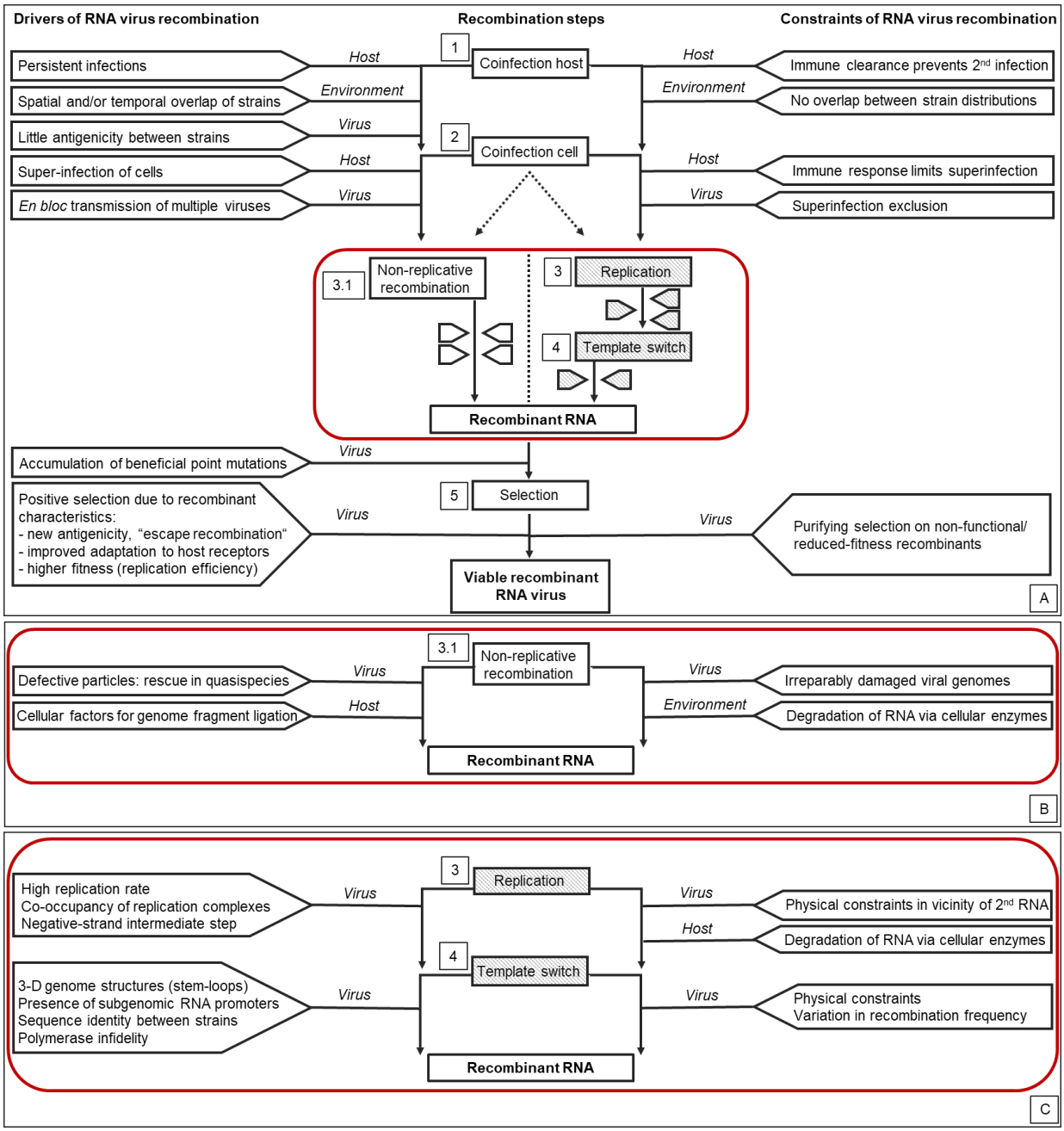

Figure 9. Conceptual model of steps, drivers and constraints of RNA virus recombination. (A) gives an overview of the different recombination steps and accompanying host, virus, or environmental drivers and constraints (predictive risk factors), as adapted from Worobey and Holmes (1999). (B) and (C) focus on putative drivers and constraints of both non-replicative recombination and template-switch-mediated recombination, respectively.

2.1.7 **Consequences of RNA virus recombination**

Successful recombination, whether it represents an accidental by-product of virus biology (reflecting breakage and joining (non-replicative) and/or association and dissociation of RNA template and replication complex (replicative)), or a key adaptive and evolutionarily selected process, may profoundly influence an individual virus and a virus population as a whole.

A single recombination event can switch out entire genome sections and simultaneously transfer multiple mutations previously incorporated into a genomic region; deleterious mutations can be purged via this process (an escape from Muller's ratchet) and advantageous genetic combinations may unlink from deleterious backgrounds and be spread at a rate unattainable by purely clonally reproducing organisms (Bentley and Evans, 2018; Simon-Loriere and Holmes, 2011).

The extensive genetic changes achievable through successful recombination can result in rapid and extreme changes in virus phenotype, allowing for antigenic shifts (Hahn et al., 1988; Malim and Emerman, 2001), pathogenesis and fitness modifications (including the facilitated spread of drug-resistant mutants) (Moutouh et al., 1996), and changes in receptor or even host tropism (Jackwood et al., 2010; Li et al., 2020).

Various prominent human pathogenic disease outbreaks have been linked to recombination events. Thus, bouts of vaccine-derived paralysis have been linked to recombination between live attenuated poliovirus vaccine strains and circulating enterovirus C species (Bentley and Evans, 2018; Kew et al., 2002); the three most pathogenic human coronaviruses (SARS, MERS, and most recently SARS-2) are the result of recombination among coronaviruses (Graham and Baric, 2010; Li et al., 2020). Consequently, the potentially dire fallout of viral recombination calls for an improved insight into and closer monitoring of these processes.

2.2 Norovirus point mutation accumulation

2.2.1 Human norovirus mutation rates and sources of point mutation

The NoV RdRp, a key enzyme for transcription and replication of the NoV genome, shares functional and structural features with other RNA virus polymerases (Deval et al., 2017). *In vitro* RdRp fidelity assays have been implemented to experimentally determine mutation rates of various HuNoV strains (Bull et al., 2010). These assays demonstrated overall mutation rates to lie within the range of those typically described for RNA viruses, but pinpointed strain-dependent differences. Globally predominant GII.4 strains had five- to 36-fold higher mutation rates (average of 7.95×10^{-4} substitutions per nucleotide site or 5.97 ±1.96 substitutions per genome replication event) compared to less frequently detected strains, GII.b (1.53×10^{-4} or 1.15 substitutions per genome replication event) and GII.7 (2.21×10^{-5} or 0.17 substitutions per genome replication event).

Recently, single-cycle viral replication of a Norwalk virus infectious cDNA clone transfected into human embryonic kidney cells yielded a mutation rate estimate of 1.5×10^{-4} s/n/c (Cuevas et al.,

2016). Interestingly, a large fraction of NoV spontaneous mutations constituted U-to-C and A-to-G substitutions occurring as bouts of mutations in the same RNA molecule; such sequence changes are characteristic of adenosine to inosine editing (inosines subsequently base-pair with cytosines) by double-strand RNA-dependent adenosine deaminases (ADARs) (Samuel, 2012), suggesting that host-driven extrinsic NoV hyper-mutation acting on double-stranded replication intermediates may be a source of NoV diversity comparable to intrinsic viral RdRp fidelity. In depth analysis of NoV spontaneous mutations in clinical GII.4 samples supported the hypothesis that hyper-mutation may reflect a relevant mutational process in NoVs (Cuevas et al., 2016).

2.2.2 **Evolutionary rates of human noroviruses**

Early bioinformatics analysis of published ORF2 sequence data revealed strain dependent differences in NoV evolutionary rates, estimating 1.7-fold higher average rates of evolution within GII.4 capsid sequences (3.9×10^{-3} n/s/y) than other NoVs (GII.3, GII.3[Pb], GII.7 with 1.9×10^{-3}, 2.4×10^{-3}, and 2.3×10^{-3} n/s/y, respectively) (Bull et al., 2010). Higher ratios of nonsynonymous to synonymous amino acid changes in GII.4 NoV capsids were held to indicate that GII.4 strains experience faster rates of antigenic drift than other NoV strains as a probable consequence of their higher RdRp mutation rates (Bull et al., 2010). Nonsynonymous mutations for NoV GII.4 and all other analysed genotypes (albeit at lower numbers) were shown to cluster to common structural surface-exposed residues of the hypervariable P2 capsid domain, corresponding to known HBGA-binding targets and hypervariable GII.4 "evolution hotspots" (Lindesmith et al., 2008), suggesting that these sites are likely to be subject to immune-driven selection (Bull et al., 2010).

Other long-term evolutionary analyses of archival NoV sequences have calculated similar population-level evolutionary rates for GII.4 VP1 capsid sequences (4.3×10^{-3} n/s/y) and have identified preferential sites for evolution under positive selection to be located in the VP1 shell domain as well as P2 (Karin et al., 2009; Mori et al., 2017). However, in contrast to previous observations, evolutionary rates of various non-GII.4 genotypes, e.g. GII.3 VP1 (4.16×10^{-3} n/s/y) (Boon et al., 2011), GII.2[P2] (1.75×10^{-3} n/s/y) or GII.2[P16] (2.37×10^{-3} n/s/y) (Tohma et al., 2017), have been estimated to be close to those of GII.4 strains. Differences in mutation rates may provide higher diversity at a given time (e.g. after a recombination event) and so confer an advantage to GII.4 strains; however, they seem to have a limited impact on overall NoV evolutionary rates. Strain-dependent differences of NoV evolutionary patterns are thus not entirely attributable to differences in viral RdRp fidelity and remain to be fully elucidated.

Full-genome deep sequencing analyses have revealed that evolutionary rates are not uniform across the NoV genome, with surface- and immune-exposed regions experiencing more variation than

less malleable sections; correspondingly, ORF2 (VP1) and ORF3 (VP2)-specific rates are typically higher than those reported for ORF1 (NS). Within ORF1, regions encoding NS1/2 and NS4 have been shown to exhibit the highest levels of change (Cotten et al., 2014; Hasing et al., 2016).

Overall NoV evolutionary dynamics at inter-host population levels may differ from intra-host dynamics where, subsequent to transmission typically characterised by a strong genetic bottleneck, evolutionary rates fluctuate by several orders of magnitude dependent on the host immune status (Bull et al., 2012; Hoffmann et al., 2012; Karst and Baric, 2015) (as described in chapter 1.6.6).

2.2.3 **Impact of human norovirus diversification via point mutation accumulation**

The epochal emergence of GII.4 variants is commonly ascribed to the accumulation of novel VP1 GII.4 amino acid mutations (linear evolution with intermediate periods of stasis), while non-GII.4 genotypes experience limited changes and can persist for decades with minimal VP1 modification as so-called static genotypes (Boon et al., 2011; Mori et al., 2017; Parra, 2019; Parra et al., 2017).

The emergence of both GII.4 and non-GII.4 viruses has been linked to changes in the viral RdRp, highlighting it, and potentially other non-structural proteins, as drivers of NoV evolution. Thus, the emergence of certain GII.4 variants (since their establishment as prevalent genotype in the mid-1990s) has been associated with mutations in the GII.4 RdRp gene (Lopman et al., 2004) or acquisition of a new viral polymerase via recombination (the genetic diversity of GII.4 variants due to recombination is discussed in chapter 2.3) (Cannon et al., 2017; Parra, 2019). Both the predominance of re-emerging (2016-2017) (Ao et al., 2018; Tohma et al., 2017) recombinant GII.2[P16] viruses (Parra et al., 2017; Tohma et al., 2017) and GII.17[P17] viruses between 2013 and 2015 (Parra and Green, 2015) have been putatively associated to substitutions in the viral RdRp. Notably, single HuNoV RdRp point mutations have been experimentally demonstrated to affect replication kinetics (Bull et al., 2010).

Norovirus diversification and emergence is thus associated (in varying measure) with changes to two regions of the NoV genome, non-structural protein-encoding ORF1 and VP1-encoding ORF2. Recombination events can create chimeric viruses to generate new recombinants and further contribute to NoV strain diversification by combining and modifying existing mutational profiles (discussed in chapter 2.3).

2.2.4 **Murine norovirus evolution via point mutation accumulation**

In vitro mutation rates have been inferred for representative genome regions of MuNoV isolate MNV1-CW1 (Mauroy et al., 2017). Mutation rates were shown to not significantly differ between

regions encompassing partial coding sequences for NS1/2, NS5, NS6, and NS7 within ORF1, where they were within the same range as those reported for various HuNoV strains, but were estimated to be at least one order of magnitude higher for partial ORF2, 3 and 4 sequences (Mauroy et al., 2017). Interestingly, the existence of defective RNAs or defective interfering particles, commonly associated with the population dynamics of error-prone virus replication (Stauffer Thompson and Yin, 2010), was indicated by diverging infectious NoV virus titres and genomic copy values determined during MuNoV serial passaging (Mauroy et al., 2017).

Highlighting the importance of point mutation as an evolutionary mechanism for NoVs, a single point mutation in NS1/2 (changing aspartic acid to glutamic acid) has been shown to dramatically alter the biological behaviour of a MuNoV, rendering non-persistent MNV1-CW3 persistent and causing an increased growth of CW3 in the proximal colon, a tissue reservoir of MuNoV persistence (Borin et al., 2014; Nice et al., 2013).

Furthermore, *in vivo* assays have shown that single point mutations modulating MuNoV RdRp fidelity may affect MuNoV pathogenesis; Arias et al. demonstrated a high-fidelity MNV-3 NS7 active-site mutant to exhibit delayed replication *in vivo* (but not *in vitro*) and reduced transmission between hosts, suggesting that the generation of sufficient genetic diversity (via a low-fidelity RdRp) may be linked to efficient intra-host virus transmission (Arias et al., 2016). Conversely, artificially increased mutagenesis above the inherently high mutation rates of NoVs has been shown to lead to extinction of MuNoV populations (Arias et al., 2014), highlighting the NoV RdRp as an important target for the development of anti-noroviral therapies (see also chapter 1.8.1) (Rocha-Pereira et al., 2016).

2.3 Norovirus recombinants: recurrent in the field, recalcitrant in the lab – A scoping review of recombination and recombinant types of noroviruses

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Norovirus recombinants: recurrent in the field, recalcitrant in the lab – a scoping review of recombination and recombinant types of noroviruses

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Abstract

Noroviruses are recognized as the major global cause of sporadic and epidemic non-bacterial gastroenteritis in humans. Molecular mechanisms driving norovirus evolution are the accumulation of point mutations and recombination. Intragenotypic recombination has long been postulated to be a driving force of GII.4 noroviruses, the predominant genotype circulating in humans for over two decades. Increasingly, emergence and re-emergence of different intragenotype recombinants have been reported. The number and types of norovirus recombinants remained undefined until the 2007 *Journal of General Virology* research article 'Norovirus recombination' reported an assembly of 20 hitherto unclassified intergenotypic norovirus recombinant types. In the intervening decade, a host of novel recombinants has been analysed. New recombination breakpoints have been described, *in vitro* and *in vivo* studies supplement *in silico* analyses, and advances have been made in analysing factors driving norovirus recombination. This work presents a timely overview of these data and focuses on important aspects of norovirus recombination and its role in norovirus molecular evolution. An overview of intergenogroup, intergenotype, intragenotype and 'obligatory' norovirus recombinants as detected via *in silico* methods in the field is provided, enlarging the scope of intergenotypic recombinant types to 80 in total, and notably including three intergenogroup recombinants. A recap of advances made studying norovirus recombination in the laboratory is given. Putative drivers and constraints of norovirus recombination are discussed and the potential link between recombination and norovirus zoonosis risk is examined.

INTRODUCTION

Noroviruses (NoVs) belong to the *Caliciviridae* family of small, non-enveloped, positive sense, single-stranded RNA viruses, currently divided into the five approved genera *Vesivirus, Lagovirus, Nebovirus, Sapovirus* and *Norovirus,* while additional genera have been proposed [1]. Detected in a wide range of mammalian species, NoVs cause gastroenteritis of varying severity in their animal hosts [2]. Human noroviruses (HuNoVs) are recognized as the major global cause of sporadic and epidemic non-bacterial gastroenteritis [3, 4], with significant morbidity and mortality in impoverished developing countries [5, 6] and a high economic impact in developed countries [7]. Despite their significance, no viable cell culture system existed for the study of HuNoVs until the recent report of low-level infection of cultured human B cells [8] and the advent of the human enteroid system [9]. While practicability of these new cell culture systems still presents hurdles, the murine norovirus (MuNoV), replicating efficiently in murine dendritic or macrophagic cells [10, 11], currently remains the model of choice for *in vitro* study of NoVs and *in vivo* infection of a genetically tractable host.

HuNoVs and MuNoVs share many similarities in terms of their genome structure. The HuNoV linear, single-stranded, polyadenlyated positive-sense, ca. 7.5 kb long RNA genome is classically divided into three ORFs, with a fourth described for MuNoVs [12]. The 5′ proximal ORF1 encodes a large polyprotein that is co- and post-translationally cleaved by protease-catalysed mechanisms into the six non-structural viral proteins (NS1/2 to NS7) [6]. ORF2 and ORF3 encode the structural components of the virion, major and minor capsid protein, VP1 and VP2, respectively. VP1 itself consists of a conserved shell (S) and two protruding (P) domains, of which the conserved P1 enhances particle stability, while the exposed, variable P2 forms binding clefts for virus receptors and harbours antigenic epitopes at

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Abbreviations: HuNoV, human norovirus; MuNoV, murine norovirus; pORF, partial ORF.

the capsid exterior. ORF4, entirely overlapping the 5′end of ORF2, encodes an antagonist of innate immunity, virulence factor VF1 [6].

NoVs are genetically classified into six established genogroups (GI–GVI), while a seventh (GVII) was recently proposed [13]. Genogroups are further divided into at least 38 genotypes [14, 15] and GII.4 strains are additionally subtyped into variants. Since the mid-1990s, classification into genogroups and genotypes has been based on amino acid sequence analysis of the complete VP1 capsid protein, with an updated cut-off threshold of a minimum of 15 % pairwise difference proposed for classification of a new genotype. To account for the common occurrence of recombination in the ORF1-ORF2 overlapping region, a dual-nomenclature system based on complete capsid sequences and the RNA polymerase region in ORF1 [14] was recently established by consensus of the *International Norovirus Working Group*. The remarkable level of variability within the NoV genus reflects the high level of continuous viral evolution therein.

Molecular mechanisms driving NoV evolution are the accumulation of point mutations (or genetic drift) and recombination. In any given virus, the accumulation of point mutations generally leads gradually to genetic variation and the generation of viral quasispecies [16]. Recombination, the complex molecular process by which a fragment of DNA is reciprocally exchanged between homologous chromosomes, serves as the basis of evolution/sexual reproduction in eukaryotes [17] and, in fact, deeply impacts the evolution of all biological entities. In prokaryotes and viruses, recombination (or lateral gene transfer or gene conversion) involves non-reciprocal replacement or addition of genome sequences rather than exchange [17, 18] and can create considerable changes in a given viral genome, allowing for antigenic shifts [19], changes in receptor or host tropism [20], and pathogenesis and fitness modifications to shape viral epidemiology [21].

Intragenotype recombination has long been postulated to be a driving force of GII.4 NoVs, which have been the predominant genotype circulating in humans for over two decades [22–24]. This position has only recently been challenged by emergence and re-emergence of different intergenotype recombinants modifying long-term global NoV genetic diversity trends [25–31]. Recombination is clearly a widespread evolutionary mechanism used by NoVs.

Here, we review the different aspects of NoV recombination and its role in NoV molecular evolution. We give a comprehensive overview of intergenogroup, intergenotype and intragenotype (and their importance in the GII.4 lineage) and obligatory NoV recombinants, as detected via *in silico* methods in the field. We then provide a recap of advances made studying NoV recombination in the lab. Putative drivers and constraints of NoV recombination are discussed and the potential link between recombination and norovirus zoonosis risk is briefly examined.

MECHANISMS OF NOV RECOMBINATION – THE ORF1/2 OVERLAPPING HOTSPOT AND LESS COMMON BREAKPOINTS

Typical as well as atypical recombination breakpoints have been described along the length of the NoV genome. While predictive recombination tools and similarity plots between putative recombinant genomes and suspected parental genomes have suggested recombination at breakpoints within ORF2 in several genomes of MuNoV field strains [32], sequence analysis of field HuNoV strains has overwhelmingly shown the predominant recombination breakpoint to lie in the highly conserved ORF1/ORF2 overlap corresponding to the junction of RdRp and capsid sequences [33]. The region is considered as a negative-strand subgenomic RNA promoter site, leading to the development of a model for NoV recombination, which combines the copy-choice model of recombination in which recombinant RNA molecules are generated via template switch of the RdRp, with an internal initiation mechanism for subgenomic synthesis [33, 34]. Both the standardized NoV nomenclature (as described above) and current genotyping assays are designed to accommodate the ORF1/2 recombination hotspot [14]. Thus, e.g. the Norovirus Automated Genotyping Tool (NoroNet) [35] assigns NoV sequences to a NoV genogroup, and offers unequivocal information on RdRp and capsid-affiliation on either side of the ORF1/2 overlap based on genetic homology and phylogenetic inferences.

However, atypical recombination breakpoints have been observed, amending the paradigm of ORF1/2 recombination. As such, an atypical recombination event located at the 3′ end of the NoV polymerase gene (at nt position 4889) was first described in the GII.4 recombinant Hu/771/2005/IRL (GenBank accession number EF219487) [36]. Recently, similar atypical breakpoints were observed in epidemic GII.4 variants at nt position 4.834 of the GII.4 US95_96/GII.4 Kaiso_2003 recombinant strain Hu/GII.P4/VIG246/2003/BRA (GenBank accession numbers KU756290–KU756293) and at nt position 5.002 of the strain Hu/GII.P4/2A1049/2009/BRA GII.4 Den_Haag-2006b/GII.4 Yerseke_2006a (GenBank accession numbers: KU756294 and KU756295) [37]. Interestingly, there seems a marked tendency for GII.P7/GII.6 viruses to also harbour breakpoints located near the 3′ end (C terminus) of the RdRp and at least 40 nt upstream of the overlapping region of ORF1 and 2. The breakpoint in 105 of 112 analysed GII.P7/GII.6 sequences was located at nt position 5009 in reference to strain GII/Hu/China/2009/GII.P7-GII.6/Beijing (GenBank accession number KX752057) in the absence of a known RNA promoter at this site [38]. An additional ORF2/3 junction breakpoint was reported for GII/4 variants [39–41] and sequences of GII.4 2008 variant viruses have been reported to consistently exhibit a 300–500 bp long mosaic fragment in the ORF2 P2 domain in addition to the typical ORF1/2 breakpoint [42]. In a recent study, only in one of 21 GII.2 recombinant strains was the recombination breakpoint located within the ORF1/2 overlapping region, while in 15

strains it was located within ORF1 and in five strains within ORF2 [43]. Low frequencies of recombination in the VPg, protease and 3′ end of the RdRp coding region as well as the VP1 S domain of MuNoVs were reported by Zhang *et al.* [44]. Such a recombination in the absence of an obvious RNA promoter or triggering secondary structure has been tentatively purported to suggest that at atypical recombination sites, recombination may have arisen by other mechanisms to those that induce a breakpoint in or around the ORF1/2 overlap [34]. The possibility of non-replicative recombination by which randomly cleaved RNA strands are self-ligated or joined by cellular enzymes has been demonstrated for other positive-sense single-stranded RNA viruses [45–48], and may be considered in this context. Proposed models for replicative and putative non-replicative NoV recombination, as adapted from Bull *et al.* [34] and a general model for RNA virus recombination [48], respectively, are shown in Fig. 1. Studies in other RNA viruses have shown that observed recombination breakpoints probably only represent a subset of those that are actually generated, and are the ones that are maintained in the viral population after a rigorous functional selection [45, 49]. The same probably holds true for the typical ORF1/2 NoV breakpoint as well as less frequent other breakpoints distributed across the virus genome (discussed further below).

NOROVIRUS RECOMBINATION IN THE FIELD

Intergenogroup and intergenotype recombinants

Since the first description of a naturally occurring HuNoV recombinant in 1997 [50], recombinant NoVs have been reported worldwide. The actual number and types of recombinants remained undefined until 2007, when Bull *et al.* published an assembly of 20 hitherto unclassified intergenotypic NoV recombinant types. The authors confirmed seven NoV GI recombinants collectively belonging to the recombinant genotype GI.2/GI.6, 17 prototype GII recombinants, all of which were a combination of one of eight different polymerase genotypes and one of nine different capsid genotypes, and three GIII recombinants,

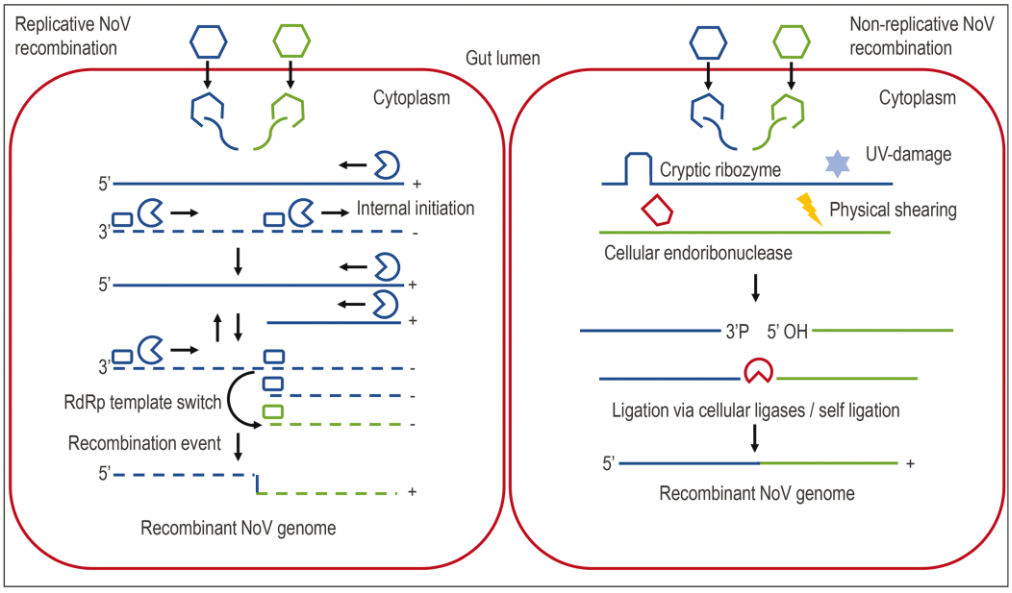

Fig. 1. Proposed models for replicative and non-replicative recombination in noroviruses, as adapted from Bull *et al.* [34] and Galli and Bukh [48], respectively. Left: this model of replicative norovirus recombination combines the copy-choice model of recombination, in which recombinant RNA molecules are generated via template switch of the RdRp, with an internal initiation mechanism for subgenomic synthesis. After infection of the cell by two different NoV strains (blue and green hexagons), RNA-dependent RNA polymerase (RdRp) (blue jagged circle) transcription of incoming genomes generates a negative-stranded intermediate. Binding of the RdRp to both a 5′ and an internal promoter (indicated by blue and green rectangles), initiates positive-stranded genome and subgenomic RNA synthesis. RNA synthesis from the 3′ end then generates negative-stranded genomes and subgenomic RNA. Recombination occurs during replication when the RdRp, having initiated positive-stranded synthesis at the 3′ end of the negative strand, stalls at the subgenomic promoter (near the ORF1/2 overlap), disassociates from the donor template and switches to subgenomic RNA of a co-infecting virus. Right: a putative model of non-replicative norovirus recombination as based on Galli and Bukh's model of non-replicative recombination in positive-sense RNA viruses [48]. After infection of the cell by two different norovirus strains, RNA strands are randomly cleaved via physical shearing, UV-damage, cryptic ribozyme activity or cellular endoribonucleases. Fragments, carrying 3′-phosphate and 5′-hydroxyl ends, are subsequently self-ligated or re-joined by cellular ligases to form a recombinant norovirus genome.

categorized into two recombinant types NoV GIII.1/GIII.2 and NoV GIII.2/GIII.1 [34].

In continuation of this list, Table 1 of the present review comprehensively compiles first reports (to the knowledge of the authors) of novel intergenogroup and intergenotype recombinants reported over the intervening ten years. The recombinant type list (Table 1), was assembled following an exhaustive Medline (accessed via PubMed) (www.ncbi.nlm.nih.gov/pubmed) and GenBank (www.ncbi.nlm.nih.gov/genbank/) search, including literature and database hits published from July 2007 to February 2018. PubMed search terms were as follows: ('norovirus'[MeSH Terms] OR 'norovirus'[All Fields]) AND ('recombination, genetic'[MeSH Terms] OR ('recombination'[All Fields] AND 'genetic'[All Fields]) OR 'genetic recombination'[All Fields] OR 'recombination'[All Fields]) AND recombinant[All Fields]. In total, 96 full-text English-language research articles, either direct PubMed search results hits or identified via perusal of selected articles' bibliographies, were included in this compilation. Corresponding sequence searches in the NCBI nucleotide database yielded a total of 83 NoV recombinant sequences. Table 1 thus enlarges the scope of intergenotypic recombinant types to 80 in total and notably includes three intergenogroup recombinants.

While Bull *et al.* rigorously applied three methods, namely phylogenetic analysis, SimPlot analysis and the χ^2 method, to identify recombinants and excluded (certain intergenotypic and all intragenotypic) reported recombinant strains that failed to meet all three criteria, such a detailed recombination analysis would exceed the scope of this review and, accordingly, reported recombinants were preliminarily assumed to be genuine on the strength of their original analysis. Confirmation of the 'recombinant status' was sought via the automated Norovirus Genotyping Tool (Version 2.0), available online from the NoroNet website of the Dutch National Institute for Public Health and the Environment (www.rivm.nl/mpf/norovirus/typingtool). Briefly, the tool employs a typing algorithm on ORF1 and ORF2 sequences of genogroup I and II noroviruses, starting with BLAST analysis of the query sequence against a reference set of *Caliciviridae* sequences. This is followed by phylogenetic analysis of the query sequence and a sub-set of the reference sequences, to assign NoV genotype and/or variant, with profile alignment, construction of phylogenetic trees and bootstrap validation [35]. Interestingly, certain NoroNet analyses yielded divergent results from those published by the original authors, either refuting the submitted sequences' 'recombinant' status entirely or divergently identifying either RdRp- or capsid-sequence affiliation. While this effect probably reflects the regular monitoring and updates of the typing tool's reference set by the 'Norovirus working group' members [this is supported by the fact that most 'mismatches' appear in older publications (before 2012)], it also calls into question whether a shared sequencing protocol and stricter baseline limit of nucleotides should be established for analysing and reporting of NoV recombinants [30].

Intragenotype/intersubtype recombinants – importance in the GII.4 lineage

Considering that recombination at the ORF1/2 boundary region, as mediated by the proposed homologous copy-choice mechanism, has been associated with high similarities of implicated sequences [33], intragenotype/intersubtype recombination should actually occur more frequently than intergenogroup and intergenotype recombination [41]. Reports of intragenotype recombinants are, however, often difficult to confirm, necessitating extremely sensitive methods to avoid confusion of true intragenotype recombinants with strains that have simply undergone genetic drift. Logically, this liability is much increased in intragenotype recombinants as compared to those derived from more distantly related parental strains [22]. Accordingly, in 2007, Bull *et al.* were unable to confirm nine reported intragenotype recombinants [34], amongst these notably Hu/NLV/Saitama U3/02/JP and Hu/NLV/GII/MD145-12/87/US (GenBank accession numbers AB039776 and AY032605, respectively) [51]. Surprisingly, the NoroNet Sequencing Tool today identifies the former as a GII.P7/GII.6 recombinant, while the latter groups as a non-recombinant GII.4 Camberwell_1994 in both polymerase and capsid regions.

Nevertheless, intragenotypic recombination has been increasingly cited as an important means for GII.4 (and other) variant emergence and successful navigation of the fitness landscape [52]. The phenomenon has been discussed exhaustively in recent publications [22, 37, 53, 54], but this review would nevertheless not be complete without a short overview of the issue. Postulated mediators for intragenotype GII.4 recombination include selective advantages and improved adaptation to host receptors via physicochemical P2 changes in the virion of new GII.4 subtypes and the evasion of herd immunity against predominant genotypes [29, 41, 42, 55]. Motomura *et al.* [41] reported evidence of intersubtype genome mosaicism of GII.4 subtypes 2007a, 2007b, 2008a and 2008b, showing them to be ORF1/2 recombinants with distinct evolutionary lineages for capsid- and non-structural proteins of previously co-circulating GII.4 subtypes [40, 41]. NoroNet Sequencing Tool analysis revealed the sequences to be recombinants of GII.Pe/GII.4 Osaka_2004 (GenBank accession number AB541190.1) for subtype 2007a and GII.P4 Apeldoorn_2007/GII.4 Den_Haag_2006b for the remaining three strains (2007b: AB541193.1 and AB541192.1; 2008a: AB541196.1 and AB541195.1; 2008b: AB541200.1).

Complex patterns of intragenotypic recombination within the GII.4 lineage were revealed with the identification of double and triple recombinant forms involving the P2 antigenic domain of GII.4/2008 variants [42]. The pattern was backed up by detection of interpandemic recombinants between GII.4 New_Orleans_2009 and GII.4 Sydney_2012, in itself a recombinant variant (representative GenBank accession number of a GII.P4 New_Orleans_2009/GII.4

Table 1. Norovirus intergenotype and intergenogroup recombinant types

Recombinant types, as reported by Bull *et al.* [34] and newly assembled following a systematic PubMed and GenBank search, including literature and database hits published from July 2007 to February 2018. Wherever available, sequences were submitted to the RIVM (NoroNet) Sequence Typing Tool [35] for verification. All recombination breakpoints of recombinants listed in this table are typically located in the vicinity of the OFR1/2 overlap/boundary region, if not otherwise indicated.

NoV genogroup		Prototype strain	RdRp genotype – as first published	Capsid genotype – as first published	GenBank accession no.	RdRp genotype – RIVM (NoroNet) Typing Tool	Capsid genotype – RIVM (NoroNet) Typing Tool	First published	Related strains reported in the literature
Intergenotype									
I	1	Hu/GII.4/Beijing/55042/2007/CHN	GI.a	GI.3	GQ856473.1	GI.Pa	GI.3	[95]	[59]
	2	Hu/GII.4/Beijing/53997/2007/CHN	GI.b	GI.6	GQ856463.1	GI.Pb	GI.6	[95]	[59]
	3	Hu/GII.4/Beijing/54108/2007/CHN	GI.d	GI.3	GQ856470.1	GI.Pd	GI.3	[95]	–
	4	Norovirus Hu/GI/Otofuke/1979/JP	GI.f	GI.3	AB187514.1	GI.Pf	GI.3	Direct submission (Wakuda, *et al.*)	[59, 95]
	5	WUGI/01/JP	GI.2	GI.6	AB081723	GI.Pb	GI.6	[96]	[97–99]
	6	Hiroshima/48-938/04/JP	GI.2	GI.8	AB354289.1	GI.Pb	GI.6	[96]	[97, 100]
	7	Hu/GII.4/Beijing/53671/2007/CHN	GI.3	GI.8	GQ856462.1	GI.P8	GI.8	[98]	–
	8	SaitamaT25	GI.11	GI.14	AB112100.1	GI.Pd	GI.3	[101]	–
	9	Sydney2212/98/AU	GII.a	GII.3	AF190817.1	GII.Pa	GII.3	[102]	[33, 103–106]
	10	Picton/03/AU	GII.b/GII.21*	GII.1	AY580335	GII.P21	GII.1	[33]	[61, 62, 95, 107–111]
	11	Pont de Roide 673/04/Fr	GII.b/GII.21	GII.2	AY682549	GII.P21	GII.2	[108]	[58, 110]
	12	SydneyC14/02/AU	GII.b/GII.21	GII.3	AH011002 (formerly AF409067)	GII.P21	GII.3	[112]	[33, 39, 43, 56–59, 61, 62, 95, 107–110, 113–123]
	13	Hu/Hiroshima/32-754/2003/JP	GII.b/GII.21	GII.3	AB354294.1	GII.P12	GII.13	[97]	–
	14	Nyiregyhaza/1057/02/HUN	GII.b/GII.21	GII.4	unpublished	N/A	N/A	[113]	[108, 110, 124]
	15	Hu/V1628/06/IND†	GII.b/GII.21	GII.7	AB453773.1	GII.P21	GII.7	[125]	–
	16	Hu/GIIb/04150102/2004/AUS (RdRp) and Hu/GII.13/04150102/2004/AUS	GII.b/GII.21	GII.13	FJ972707.1 (RdRp) and FJ986385.1 (capsid)	GII.P21	GII.13	[61]	[62, 98, 121, 123, 126]
	17	Hu/GII.4/Seoul/0093/2008/KOR	GII.b/GII.21	GII.16	FJ595913.1	GII.P21	GII.21	[127]	–
	18	Hu/Aur/A832/2006/India; Hu/Ahm/PC03/2006/India	GII.b/GII.21	GII.18	EU921356.1; EU019230	N/A (EU921356.1)/ GII.P21 (EU019230)	GII.21	[124]	[128, 129]
	19	Snow Mountain2/76/US	GII.c	GII.2	AY134748.1	GII.Pc	GII.2	[50]	–
	20	SaitamaT66e/02/JP	GII.d/GII.22	GII.3	AB112321	GII.P22	GII.3	[101]	[124]
	21	Hokkaido133/03/JP	GII.d/GII.22	GII.5	AB212306.1	GII.P22	GII.5	[101]	[59, 130, 131]
	22	Hu/GII.6/NZ13563/2013/NZ	GII.d/GII.P22*	GII.6	KT151023.1 (capsid)	N/A	GII.6	[59]	–
	23	Hu/Pune/PC51/2007/India	GII.e	GII.b	EU921388.2	GII.Pe	GII.4 Osaka_2007	[128]	[14]‡
	24	Norovirus GII isolate 15120271	GII.e	GII.2	KX064759.1	GII.Pe	GII.2	[56]	[132]

Table 1. cont.

NoV genogroup		Prototype strain	RdRp genotype – as first published	Capsid genotype – as first published	GenBank accession no.	RdRp genotype – RIVM (NoroNet) Typing Tool	Capsid genotype – RIVM (NoroNet) Typing Tool	First published	Related strains reported in the literature
	25	Hu/GII/IPH217-09VG4/2009/BE	GII.e	GII.3	JF697282.1	GII.Pe	GII.3	[64]	–
	26	Hu/OC07138/07/JP	GII.e	GII.4 Osaka_2007	AB434770.1	GII.Pe	GII.4 Osaka_2007	Only in GenBank (2008; Iritani)	[57, 58, 64, 99]
	27	Hu/GII.4/Sydney/NSW0514/2012/AU	GII.e	GII.4 Sydney_2012	JX459908.1	GII.Pe	GII.4 Sydney_2012	[22]	[27, 43, 56, 58, 75, 99, 130, 133]
	28	GII/Hu/BRA/2005/GII.Pe-GII.17/RS11178	GII.Pe	GII.17	KR074152.1	GII.Pe	GII.17	[123]	–
	29	Hu/GII.g-GII.12/StGeorge/NSW199U/2008/AU	GII.e (as published) GII.g (as in GenBank)	GII.12	GQ845370.2	GII.Pg	GII.12	[63]	[134]
	30	Hu/GII/IPH163-LIM001/2010/BE	GII.g	GII.1	JF697289.1	GII.Pg	GII.1	[64]	[28, 29, 43, 56, 58, 59, 75, 131, 133, 135–139]
	31	Hu/US/1972/GII.Pg_GII.3/ShippensburgB24	GII.Pg	GII.3	KY442319.1	GII.Pg	GII.3	[66]	–
	32	Hu/GII.12/Shelby/2009/USA	GII.g	GII.12	HQ688986.1	GII.Pg	GII.12	[139]	[28, 43, 55, 57–59, 95, 123, 130, 135, 138, 140, 141]
	33	Hu/GII/Goulburn Valley G5175 B/1983/AUS	GII.g	GII.13	DQ379714.1	GII.Pg	GII.13	[65]	[28]
	34	Hu/GII.4/Beijing/53931/2007/CHN	GII.n	GII.22	GQ856469.1	GII.Pn	GII.22	[95]	–
	35	Hu/Pune/PC24/2006/India	GII.1	GII.12	EU921353.2	GII.Pm	GII.12	[124]	[128]
	36	Hu/NLV/S63/1999/France	GII.2	GII.5	AY682550.1	GII.pf	GII.5	[33]	[107]
	37	Hu/GII.7/NZ13382/2013/NZ	GII.P2	GII.7	KT151014.1 (capsid)	N/A	GII.7	[59]	–
	38	Hu/Pune/PC25/2006/India	GII.3	GII.13	EU921354.2	GII.P13	GII.13	[124]	[128]
	39	Hu/NLV/E3/1997/Crete	GII.4§	GII.2	AY682552.1	GII.Pj	GII.2	[33]	[97, 107, 142]
	40	Chiba1/04/JP	GII.4	GII.3	DQ372864.1	GII.P12‖	GII.3	[111]	[98, 113, 114, 117, 118, 121, 126, 127, 129, 130, 143–145]
	41	771/05/IRL	GII.4/GII.d	GII.4	EF219487	Could not assign	GII.4 Asia_2003	[36]	–
	42	Hu/GII.4/Wuhan/E856/CHN/2007	GII.4	GII.6	JQ751006.1 (RdRp); JQ750979.1 (capsid)	GII.P4 Den_Haag_2006b	GII.6	[117]	[118]
	43	Hu/V1656/06/IND	GII.4	GII.8	AB453774.1	GII.P4 Apeldoorn_2007	GII.20	[125]	–
	44	Mc37/01/Th	GII.4	GII.10	AY237415.2	GII.P12	GII.10	[146]	[118]
	45	SaitamaU1/02/JP	GII.4	GII.12	AB039775	GII.P12	GII.12	[96]	[33, 41, 51, 57, 97, 118]
	46	Hu/Hiroshima/32-754/2003/JP	GII.4	GII.14	AB354294.1	GII.P12	GII.13	[97]	–
	47	Hu/GII.17/C142/1978/GUF	GII.4	GII.17	KC597139.1	Could not assign	GII.17	[147]	–

Table 1. cont.

NoV genogroup		Prototype strain	RdRp genotype – as first published	Capsid genotype – as first published	GenBank accession no.	RdRp genotype – RIVM (NoroNet) Typing Tool	Capsid genotype – RIVM (NoroNet) Typing Tool	First published	Related strains reported in the literature
	48	Hu/GII/Dhaka85/2011/BGD	GII.4 New_Orleans	GII.21	JX477805	GII.P4 New_Orleans_2009	GII.21	[148]	–
	49	Irl05N771	GII.4	GII.d	EF219487			[36]	
	50	Hu/V1737/07/IND	GII.5	GII.12	AB447418.1	GII.Pn	GII.12	[125]	–
	51	VannesL23/99/US	GII.5	GII.15	AY502010	GII.P16	GII.16	[33]	[107]
	52	GII.4/Jinan/JNHNV1217/2010/CHN	GII.6	GII.4	JX666247.1 (RdRp); JX666317.1 (capsid)	GII.P7	GII.4 Den_Haag_2006b	[144]	–
	53	146/Kunming/04/Ch	GII.6	GII.7	DQ304651	GII.P7	GII.7	[149]	[126]
	54	Hu/Shanxi/50106/2006/CHN	GII.6	GII.14	EF670650.2	GII.P7	GII.14	[143]	[59, 126]
	55	Hu/Minato/N1/14/99/JP	GII.6	GII.15	AB233474.1	GII.P7	GII.16	[150]	–
	56	Hu/GII.5/NZ13580/2013/NZ	GII.P7	GII.5	KT151019.1 (capsid)	N/A	GII.5	[59]	–
	57	Hu/GII/L18/Beijing/2011	GII.7	GII.6	JQ889816.1	GII.P7	GII.6	[95]	[38, 43, 57–59, 123, 131, 151, 152]
	58	Hu/GII.P7_GII.9/NSW591P/2013/AU	GII.P7	GII.9	KT239631.1	GII.P7	GII.9	[59]	
	59	sewage/GII.13/Toyama/SW0709-11/2007/JP	GII.7	GII.13	AB504710.1	GII.P7	GII.14	[100]	–
	60	Hu/GII.4/Beijing/55028/2007/CHN	GII.7	GII.14	GQ856465.1	GII.P7	GII.14	[95]	[43, 123]
	61	Hu/GII/PA/QU1-38F1/BR/08–2008	GII.7	GII.20	JX047023	GII.P7	GII.20	[153]	–
	62	GII/LR13/Greece/2012; GII/LR15/Greece/2012	GII.9	GII.4	KF848658 (LR13); KF848659 (LR15)	GII.P7 (both strains)	GII.4 (LR13); GII.4 Hunter_2004 (LR15)	[154]	–
	63	Hu/GII/A6/Greece	GII.9	GII.6	HM172493 (ORF1); HM172494.1 (ORF2)	GII.P7	GII.6	[155]	[156]
	64	pig/GII/Ch6/China/2009	GII.11	GII.19	HQ392821.1	GII.P11	GII.11	[157]	–
	65	Hu/GII.1/Ascension208/2010/USA	GII.12	GII.1	JN797508.1	GII.Pg	GII.1	Direct submission (Barclay, L., Vega, E. and Vinjé, J.)	[120]
	66	Hu/77/b3/2007/AU	GII.12	GII.3	JN602260.1	GII.P12	GII.3	[116]	[56, 59, 95, 117, 119–121, 130, 140]
	67	Hu/GII.4/Hong Kong/CU041206/2004/CHN	GII.12	GII.4	HM802546.1	GII.12	GII.4 Asia_2003	[95]	[43]
	68	Hu/GII.a/b/C9-439/KOR	GII.12	GII.13	KJ742435.1 (RdRp); KF289337.1 (capsid)	GII.P3	GII.13	[158]	–

Table 1. cont.

NoV genogroup		Prototype strain	RdRp genotype – as first published	Capsid genotype – as first published	GenBank accession no.	RdRp genotype – RIVM (NoroNet) Typing Tool	Capsid genotype – RIVM (NoroNet) Typing Tool	First published	Related strains reported in the literature
	69	Hu/GII.17/C15b/Bonaberi/Cameroon	GII.P13	GII.17	JF802507.1	GII.P13	GII.17	[159]	[25, 117, 123]
	70	Hu/Wuhan/E2120/CHN/2010	GII.16	GII.2	JQ751040.1	GII.P16	GII.2	[117]	[26, 43, 59, 95, 118, 119, 132, 160, 161]
	71	Hu/GII.16-GII.3/Dhaka53/BGD/2012	GII.16	GII.3	JX683114; JX683115	GII.P16	GII.3	[162]	[57, 59, 123, 133, 163]
	72	Hu/GII/JP/2016/GII.P16-GII.4_Sydney2012/Kawasaki194	GII.P16	GII.4	LC175468	GII.P16	GII.4 Sydney_2012	[164]	[133, 161, 165]
	73	Hu/GII.10/NZ14608/2014/NZ	GII.P16	GII.10	KT151035.1 (capsid)	N/A	GII.10	[59]	–
	74	Norovirus strawberry/II.16/95–12/DEU/2012	GII.16	GII.13	KC207117 (RdRp) KC207118.1 (capsid)	GII.P16	GII.13	[166]	[43, 57, 119, 163]
	75	Hu/7299/2011/ZAF	GII.P16	GII.17	KC962460	GII.P16	GII.17	[58]	–
	76		GII.P17	GII.2	Sample code JP14809	N/A	N/A	[132]	–
	77	Hu/GII/JP/2014/GII.P17_GII.17/Kawasaki323	GII.P17¶	GII.17	AB983218.1	GII.P17	GII.17	[167]	[133]
	78	GII/Hu/ZA/2010/GII.P unassigned_GII.3/Bushbuckridge6387	GII.P not assigned	GII.3	KC962458.2	N/A	GII.3	[58]	–
	79	Bo/Thirsk10/00/UK	GIII.1	GIII.2	AY549161	- GIII, Genotype N/A	- GIII, Genotype N/A	[168, 169]	–
	80	B-1SVD/03/US	GIII.2	GIII.1	AY274819.1	- GIII, Genotype N/A	- GIII, Genotype N/A	[34]	[170, 171]
Intergenogroup									
I/II	1	Hu/Kolkata/L8775/2006/IND	GI.3	GII.4	AB290150.1	GI.P8	Could not assign	[172]	–
II/I	2	GII.9/GI isolate AG4	GII.9	GI.7	KC662401 (RdRp); KF369986 (capsid)	GII.P7	GII.6	[154]	–
IV/VI	3	GVI.1/Ca/JPN/2012/M49-1	GII.IV.2	GII.VI.1	LC011951.1 (RdRp); LC011950.1 (capsid)	GII.IV	GII	[94]	–

N/A: sequence not reported.

Unshaded: novel recombinant types published between July 2007 and February 2018 and verified via the NoroNet Sequence Typing Tool.

Shaded light grey: recombinant types, as reported by Bull *et al.* and verified via the NoroNet Sequence Typing Tool.

Shaded dark grey: reported (either by Bull *et al.* or original publication) as recombinant NoV strain but *not* recombinant according to the NoroNet Sequence Typing Tool.

Shaded blue: sequence confirmed as recombinant but exact ID *divergent* from initial report; this colour coding does *not* imply that successively reported sequences do not correspond to their respective recombinant type.

Shaded orange: sequence reported as one of the recombinant types already established by [34], but identified as novel recombinant type via NoroNet Sequence Typing Tool.

Shaded green: sequence (part) not sorted into a defined cluster/recombinant type upon initial reporting but retrospectively identified as constituting a recombinant type.

Sydney_2012 recombinant: KF378731.1) [23, 24, 56–59]. The GII.4 New_Orleans_2009/GII.4 Sydney_2012 strain, reported to have undergone genetic changes (to its hypervariable antigenic epitopes) between its first emergence in 2015 and re-emergence in June 2016, has recently been proposed as a candidate new epidemic strain [23].

GII.4 recombinant sequences with typical ORF1/2 breakpoints thus include the strains Asia_2003, Osaka_2007, Japan_2008, Randwick_2011, New_Orleans_2009, Sydney_2012, Seoul/1071/2010/KR (GenBank accession number JX448566.1), LC31912/2012/US (GenBank accession number KF429777.1), GII.Pe/GII.4 Sydney_2012 [60]. Furthermore, minor ORF1/2 recombinant variants GII.P4 New_Orleans_2009/GII.4 Hunter_2004 and GII.P4 Yerseke_2006a/GII.4 Apeldoorn_2007 were recently reported in South Africa [54]. The recombinant GII.4 strain Cape Town 6745 (GenBank accession number KJ710245.2) [58], with a New_Orleans_2009 polymerase, still remains unassigned in its capsid sequence.

A putative GII.4 intragenotype recombinant, sporting an atypical recombination event between ORFs 2 (new GII.4 variant) and 3 (Den Haag subcluster) (GenBank accession number EU921388) was identified in 2010 via full-genome sequencing using overlapping primer sets [39]. The Noro-Net Sequencing Tool, however, which does not use ORF3 sequences, identifies the sequence as GII.Pe/GII.4 Osaka_2007 recombinant (supported with phylogenetic analysis and bootstrap 100.0 (≥70.0)). Intragenotype recombinants, GII.4 US95_96/GII.4 Kaiso_2003 (GenBank accession numbers KU756290–KU756293) and GII.4 Den_-Haag-2006b/GII.4 Yerseke_2006a (GenBank accession numbers KU756294 and KU756295) sporting another atypical breakpoint within the RdRp, were recently identified [37]. The strains Hunter_2004 and Apeldoorn_2007 possess breakpoints in the S (5318, 5326) and P domains (5828) of ORF2 [54].

Both typical and atypical intragenotype recombination thus contribute to the growing complexity of the GII.4 lineage, furthering GII.4 variant emergence and spread.

Obligatory norovirus recombinants – the 'odd ones out' in an unsolved and never-ending Rubik's cube?

While a NoV recombinant is typically comprised of two (or more) sections of different ORF1 and ORF2 genotypes which are paired in the respective parental strains at either side of the typical recombination breakpoint, several exceptions exist. A number of polymerase types, such as GII.a, c, e and n, have been assigned preliminary letter-based names rather than genotype numbers to signify that only their ORF1 sequences have been detected with no known 'own' capsid sequences [14]. Such 'orphan' ORF1 genotypes can be promiscuously associated with capsids of different genotypes. As such, GII.b (now known as GII.P21) was initially described as an obligatory recombinant owing to its typical pairing of a phylogenetically unique ORF1 region with one of a number of genotypes (GII.1, GII.2, GII.3 and GII.4

*GII.b (now known as GII.21) was initially described as an obligatory recombinant owing to its typical pairing of a phylogenetically unique ORF1 region with one of a number of genotypes (GII.1, GII.2, GII.3 and GII.4 amongst others) in the absence of a ORF2 GII.b phylogenetic cluster [61, 62]. When the Indian strain Hu/NoV/Ahm PC03/2006/India (GenBank accession number EU019230) was identified as present in the pORF GII.21 cluster together with representatives of the pORF1 type II.b, the orphan GII.b cluster was renamed GII.21 [14]. The strain Hu/Ahm/PC03/2006/India was identified as GII.21 by [173] and in this study. The sole pORF1 orphan type GII.d was grouped in the GII.22 cluster [14].

†The strain V1628 (AB453773.1) showed 96 % identity in its RdRp region with Pont de Roide 673/FRN (GenBank accession number AY682549), in itself identified as a GII.P21/GII.2 recombinant, whereas V1628 capsid region resembled GII.7/Osaka F140/JPN strain (98 %) [125].

‡Interestingly, when Kroneman *et al.* [14] typed the strain Hu/Pune/PC51/2007/India (GenBank accession number EU921388.2) the recombinant sequence was assigned as GII.e/GII.b [14], whereas the group's own Sequence Typing Tool (introduced in the cited publication) called a GII.Pe/GII.4 Osaka_2007 recombinant upon analysis in January 2018 (10/01/2018). This result probably reflects the regular monitoring and updates of the typing tool's reference set by the norovirus working group members.

§Unless otherwise specified, 'GII.4' can be understood to refer to the GII.4 genotype in general in that either several GII.4 subtypes were detected to be represented in a GII.4 recombinant type or further subtyping of a recombinant involving GII.4 was not performed. Thus, e.g. the recombinant type GII.4/GII.3 includes the strain Norovirus Hu/SZ-2011–8/CHN (GenBank accession number KR093991.1), a GII.4 2006b/GII.3 NoV [121].

‖GII.12 sequences (reference Saitama U1/JP), especially of ORF1, have been known to branch inside GII.4 clusters [41] and similarities between the two genotypes have also been suggested by their capsid domain crystal structures and capacity for HBGA recognition [55]. The 'confusion' of GII.4 and GII.12 strains in earlier publications seems correspondingly to be a common problem. Thus, e.g. [111] report the Chiba1/04/JP (GenBank accession number DQ372864.1) as a GII.4/GII.3 recombinant. The NoroNet sequencing tool, however, identifies it as norovirus GII.P12/GII.3. Equally, [98] report a GII.4 Sakai/GII.3 strain (accession number GQ856467.1) which is identified as GII.P12/GII.3 by NoroNet. Nevertheless, the strain Hu/Tokyo/7882/2007/JPN, a GII.4 2006b/GII.3 NoV recombinant (accession number KR093991.1) reported by [121], is confirmed as such by NoroNet analysis. Hu/Hiroshima/60-1015/2005/JP (GenBank accession number AB354299.1) was reported as a GII.4/GII.2 NoV by [97], but is identified as a GII.P12/GII.2 NoV recombinant by the NoroNet Sequencing Tool. Inversely, Hu/Tokyo/7882/2007/JPN (GenBank accession number FJ875971.1) reported as a GII.P4 Den_Haag_2006b/GII.2 [142] is correctly identified by the NoroNet Sequencing Tool.

¶Viruses with a GII.17 VP1 genotype typically contain various ORF1 genotypes (ORF1 GII.P16, GII.P3 and GII.P4). Sequence comparison showed that the ORF1 region of the novel GII.17 viruses had not previously been detected [167]. As the first orphan ORF1 sequence associated with GII.17, it was designated GII.P17 according to the criteria of the proposal for unified norovirus nomenclature and genotyping [14, 25].

amongst others) in the absence of an ORF2 GII.b phylogenetic cluster [61, 62]. Its status was only changed when the Indian strain Hu/NoV/Ahm PC03/2006/India (GenBank accession number EU019230) was identified as present in the partial ORF (pORF) GII.21 cluster together with representatives of the pORF1 type II.b. The orphan GII.b cluster was consequently renamed GII.21 [14]. First emerging in 2008 in Victoria, Australia, the obligatory recombinant GII.e (identified by ORF1 nucleotide sequencing) became the prominent ORF1 genotype in 2012, superseding GII.4 [27]. No unique GII.e ORF2 genotype has been identified and GII.e continues to cluster with a multitude of ORF2 sequences (amongst these GII.3, GII.12 and various GII.4 variants) [27, 56, 63, 64]. The obligatory NoV recombinant GII.Pg, already present in 1989 [65], re-emerged clinically around the same time as GII.Pe, and has so far been confidently associated with the four different OFR2 genotypes, GII.I, GII.3, GII.12 and GII.13 [28, 64, 66].

Similarly to the obligatory recombinant ORF1 genotypes, other polymerase genotypes, notably GII.4, GII.7, GII.12, GII.16 and GII.21 have been reported to be associated to more than one capsid genotype, supporting the polymerase as the driving factor in recombination [34, 39, 57, 64]. While the reasons for this are unclear, poor processivity of certain polymerases, rendering an ORF1/2 template switch more likely, has been advanced as a possible factor [34]. Polymerase fidelity has been identified as the determining factor in driving recombination in other RNA viruses [67] and should also be considered in this context. Inversely, but less frequently, certain capsid types (e.g. GII.3) have been associated with multiple polymerases. A quantitative representation, showing intragenogroup association of NoV polymerases to genetically diverse capsid types, is provided in Fig. 2.

It has been noted that 'the fact that a virus has an identical VP1 and pORF2 type does not necessarily mean that it is not a recombinant' [14]. It seems, that in the ever-changing, ever-shifting association of NoV capsid and polymerase types, it is near-impossible to clearly say 'who originally belonged to whom'. This begs the question whether NoV classification, necessarily based on a transversal, arbitrary cut-off, indeed correctly associates capsids and polymerases, or whether 'parental' strains are in themselves recombinants. We can liken this to an unsolvable Rubik's cube, in which matching colour codes do not necessarily signify togetherness and in which each rotatable single square may be pivoted to partner with any of the other squares.

NOROVIRUS RECOMBINATION IN THE LAB – A RECAP OF *IN VITRO* AND *IN VIVO* STUDIES

Few experimental data are available concerning NoV recombination under laboratory conditions. The first *in vitro* experimental evidence of NoV recombination, was obtained for MuNoV (GV), using a PCR-based discriminating tool, to demonstrate a homologous recombination event at the ORF1/ORF2 overlap of the MuNoV genome [68]. In this study, cell cultures were co-infected with two parental homologous MuNoV strains CW1 and WU20, sharing an 87 % nucleotide sequence similarity in their complete genomes, and a single viable recombinant virus was detected and isolated from an infectious centre assay. This recombinant has since been shown to display similar biological properties to its parental strains *in vivo*, albeit with a slight reduction in replicative fitness [69]. The first artefact-free estimate of *in vitro* recombination rates (P_{rec}) between these same two strains co-infecting a murine macrophage culture (at high m.o.i.:2) was obtained via use of drop-based microfluidics [70]. The P_{rec} of co-infecting progeny viruses was measured as $3.3\times10^{-4}\pm2\times10^{-5}$; however, it remains undetermined whether the rare RNA recombinants identified were indeed viable infectious MuNoV recombinants. Recently, we examined whether different parameters of co- and superinfection, prerequisites for recombination events, influence the frequency of recombination *in vitro*. No viable recombinants could be detected after synchronous and asynchronous infections of cultured murine macrophagic cells with the two homologous MuNoV strains WU20 and CW1 using different multiplicities and different times of infection (as yet unpublished preliminary results). The phenomenon of NoV recombination is not easily reproducible in laboratory conditions, and has been shown to be apparently rare both *in vitro* [68, 70] and in a recent *in vivo* study [44], where MuNoV recombinants were isolated from CW1- and WU20 co-infected mice. Interestingly, in addition to the typical ORF1/ORF2 breakpoint, Zhang *et al.* [44] also detected recombination events with low frequencies in the VPg, protease and 3′end of the RdRp coding region as well as the VP1 S domain of MuNoVs. These newly detected recombinants were, to our knowledge, again not tested for infectivity and it is uncertain, whether the identified additional breakpoints would generate replication-effective recombinants, as virus-amplification steps did not succeed their identification. This somewhat limits the extent to which reliability can be placed upon the occurrence of such a recombination event in a replication-effective MuNoV.

Compared to the sheer quantity of reports of HuNoV recombinants detected in the field, the paucity of information regarding MuNoV recombination as studied in the lab either *in vitro* or even *in vivo*, is evident. Equally, the difficulties accompanying the generation of a recombinant MuNoV *in vitro* [68], stand in stark contrast to the comparable ease of generating recombinants of other more distantly related [71] or even very similar [72] viruses under comparable circumstances. The only epidemiological data available for MuNoVs are the early screening results of 76 faecal samples from 28 different SPF mouse lines which suggested intergenotypic recombination events for MuNoVs [73]. Comparisons of the vastly different-sized datasets for HuNoVs and MuNoVs and their corresponding denominators allow only very limited inferences and, due to a lack of data, no conclusions can be drawn at this time regarding a comparison of recombination frequencies in the field and under laboratory conditions.

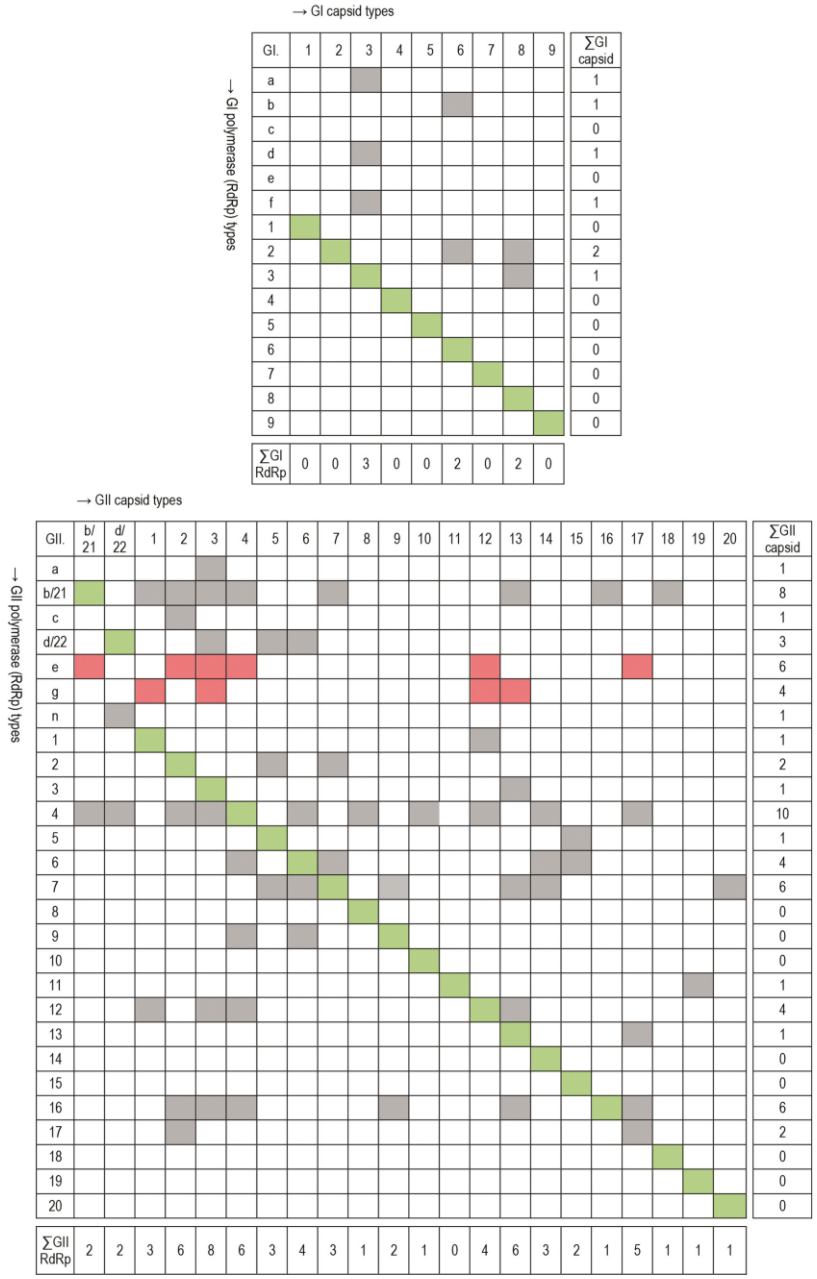

Fig. 2. Intragenogroup association of norovirus polymerases to genetically diverse capsid types. The figure shows the association of norovirus polymerase types (far left column) with different capsid types (top row) and vice versa for genogroups GI (above) and GII (below). The total number of genetically diverse capsid types (∑capsid) to which a polymerase type within the same genogroup is associated, is given in the far right column. The total number of genetically diverse polymerase (RdRp) types (∑RdRp) to which a capsid type within the same genogroup is associated, is given in the bottom row. Known 'obligatory recombinants' GII.Pe and GII.Pg are shaded in red tones. Green squares mark spatial positions of strains with 'matching' polymerase and capsid types (e.g. GII.P4/GII.4) as reported by Vinjé [13].

DRIVERS AND CONSTRAINTS OF NOROVIRUS RECOMBINATION

Loosely based on a pre-existing model for the production of a viable recombinant RNA virus [74], the necessary steps, including their respective drivers and constraints (predictive risk factors), for recombination of NoVs are considered (Fig. 3). Five steps must be successfully passed to generate a viable, replicating recombinant NoV following the classical copy-choice model of replicative NoV recombination. We suggest four steps to obtain the same result via non-replicative recombination, the main difference being the process (and enzymes) needed for joining partial parental sequences, once in close proximity within a cell.

The first (simplified) step necessarily preceding any recombination event is the simultaneous infection (co-infection) of a host with at least two parental strains (Fig. 3a). The ability of different NoV strains to overlap sufficiently in space and time to effect co-infection of a host is undoubted. Not only are several strains frequently detected to be co-circulating within the context of a single outbreak but also within a single patient. Only recently, e.g. circulation of GII.Pg/GII.1 and GII.Pe/GII.4 Sydney 2012 recombinant variants was detected in an asymptomatic population in Indonesia. Of seven positive individuals, two were repeatedly infected with the same strain and heterogenous strains [75]. Both the noroviruses' notorious low-level antigenicity between strains [76] as well as the well-documented delayed immune clearance, genetic diversity and continuing quasispecies evolution in immunocompromised patients [77–79] facilitate host co-infection. Further opportunities for human co-infection present themselves via a typical mode of NoV infection, food- or waterborne outbreaks, and in particular the consumption of bivalve molluscs, which have been known to accumulate several different strains in their intestines [80, 81]. An overview of NoV transmission routes, highlighting the three above-mentioned 'hot-spots' for accumulation of multiple NoV strains and increased risk of viral recombination is presented in Fig. 4.

Once a host has been successfully co-infected, the second step is co-infection of a single cell. While factors such as a strong host immune response or virus-mediated superinfection exclusion might prevent this, novel models of NoV pathology and cell-tropism present intriguing mechanisms for bypassing these barriers. Karst and Wobus compellingly suggest that NoVs, bound to motile bacteria and/or host carbohydrates in the gut lumen, could be taken up via Peyer's patch-associated M-cells to then be delivered to permissive immune cells in the underlying lamina propria [82]. Not only may the simultaneous uptake of multiple viruses into a single cell be possible via this route, but also a putative subsequent persistent infection of target cell immune cells could be seen to heighten chances of co-infection at a later date.

The third step to obtaining a recombinant virus can either be a step of non-replicative recombination in which viral genome fragments are ligated by host factors (Fig. 3b) [46] or, as described for NoVs above, a combination of replication and template switch (step four), between two co-infecting viruses within a cell (Fig. 3c).

By whichever way a recombinant NoV genome is created, it does not necessarily follow that this will yield a replicating recombinant NoV. Any given recombination event, switching out large genome parts in a nascent virus, presents a significant modification. It follows, that initial imprecise recombination events (e.g. introduction of mutations or faulty epistatic interrelationships between the two parts of a novel recombinant) present an evolutionary bottle-neck which can either result in non-functional genome recombinants or in reduced-fitness recombinants. The droplet or fomite transmission of NoVs, their low infectious dose [83] and typically observed high viral loads [80] entail that even poorly performing recombinants may get an opportunity to survive, to resolve and then to proliferate in the viral population at a between-host level. If recombinants do not possess selective advantages over their parental strains, it is unlikely that they will be maintained in a viral population within the original host before they can undergo a stage of resolution optimizing their replicative fitness [45, 84]. The typical ORF1/2 recombination breakpoint of NoVs indicates how recombinants may 'survive' this fifth step, selection. Such a recombination event may confer advantages under host- or population-level immune pressure that can outweigh initial detriments to replicative fitness. Putative replicative disadvantages, it seems, are more than compensated for by other advantages at the level of competitive or transmissive fitness, when a 'coat switching' event occurs in which a novel recombinant couples non-structural proteins from one and structural proteins carrying antigenic determinants from the other parental virus [33, 39, 85, 86].

TRUE RECOMBINATION OR RAPID GENETIC DRIFT?

Detection of viral recombination events at a population level in the field is traditionally based on bioinformatical analyses, implementing similarity, distance, phylogenetic and compatibility methods and/or substitution distribution [17]. When applied to positive-sense RNA viruses with high mutation rates [87], a pitfall of all recombination detection programmes is the possibility of overestimating the frequency of genetic shift. In other words, phylogenetic-based analyses of recombination can be affected by convergent evolution leading to similar sets of nucleotide and amino acid substitutions in independent lineages [42]. For NoVs, mutation rates have been inferred to correspond to a rate of $2-9\times10^{-3}$ substitutions per nucleotide per year [88]. Hoffmann *et al.* [89] demonstrated that GII.4 and GII.7 strains underwent positive selection during chronic infection of immunocompromised patients at an even more elevated evolutionary rate as compared to that found at an inter-host population level (owing to constant intra-individual selection pressure) [89]. It seems probable that this

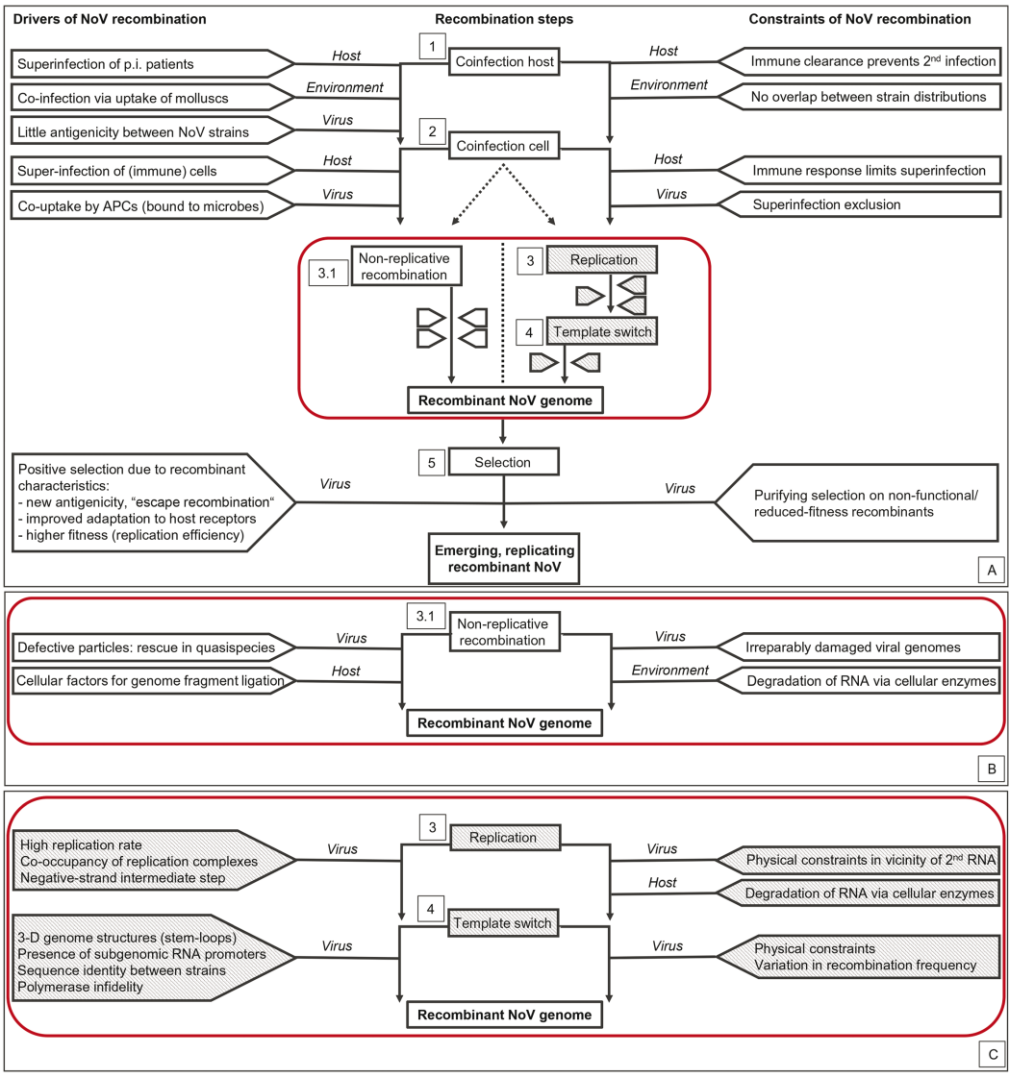

Fig. 3. Steps, drivers and constraints of NoV recombination as adapted from Worobey and Holmes [74]. (a) gives an overview of the different recombination steps and accompanying host, virus or environmental drivers and constraints (predictive risk factors). (b) and (c) focus on putative drivers and constraints of both non-replicative recombination and template-switch-mediated recombination [replication and template switch via the RdRp (NS7)], respectively. APC: antigen-presenting cells; p.i.: persistently infected.

process, whereby NoV strains can acquire enough mutations to constitute novel epidemic subtypes within weeks to months (on a global scale this would normally take years), might contribute to the overestimation of recombination ('false positive' identification of NoV recombinants), when a fast genetic drift is mistaken for recombination. The solving of the Rubik's cube, it seems, can be further muddled by squares changing their colours while the puzzle is being pivoted.

NOROVIRUS RECOMBINATION, INTERSPECIES TRANSMISSION AND ZOONOSIS RISK

The as yet unproven existence of a zoonotic potential for NoVs has long been discussed, potential interfaces of shared species exposure being food, water or animal contact. The discussion about interspecies and/or zoonotic transmission is fuelled by the close relationship of certain animal and human NoV strains, detection of HuNoVs in animal faeces, detection of antibodies against HuNoVs in

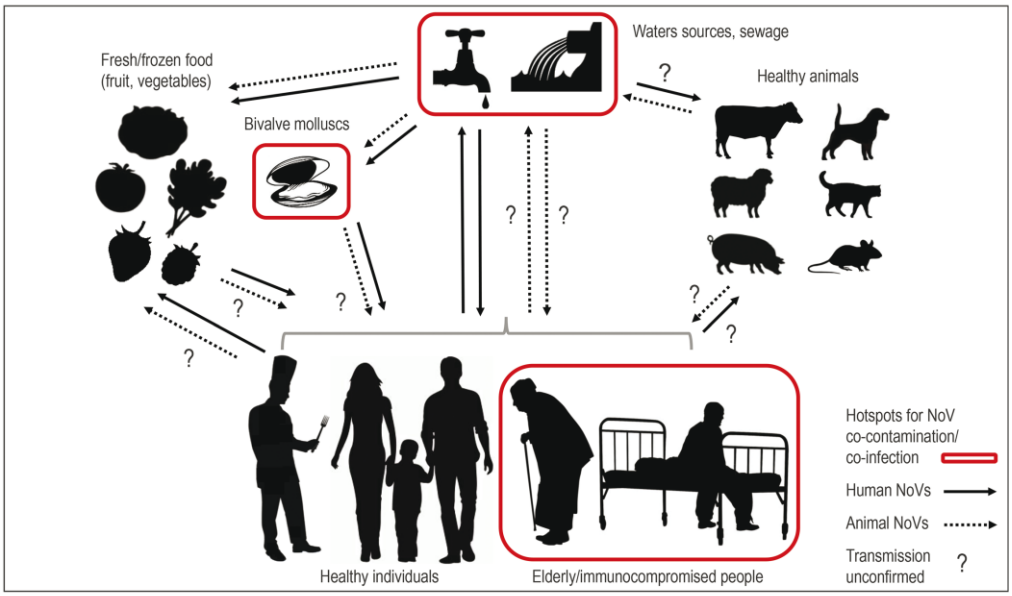

Fig. 4. Norovirus transmission and dispersion routes and hotspots for co-contamination and co-infection with multiple strains. 'Hotspots' for the accumulation of multiple norovirus strains are ringed in red, highlighting stagnant waters, tap waters, sewage and bivalve molluscs that can be contaminated with multiple norovirus strains, as well as immunocompromised persons, which typically harbour a diverse norovirus quasispecies. Immunocompromised animals should be viewed in the same light as immunocompromised people but have not been included to maintain simplicity of the figure.

swine and the demonstration of experimental HuNoV GII infection in gnotobiotic pigs [2, 90–92]. Questions concerning species barrier determinants preventing HuNoV infection of murine cells were recently resolved with the identification of a CD300If proteinaceous receptor as the primary determinant of MuNoV species tropism, showing other components of cellular machinery required for NoV replication to be conserved between humans and mice [93]. If we assume the key to cross-species transmission to be located only at a structural virus-host receptor level, this presents ORF1/2 NoV recombination, by which a nascent recombinant virus gains a complete novel capsid protein set, in an interesting light, in that a 'lucky' intragenogroup recombination event might tender a zoonotic/interspecies recombinant. Indeed, putative GIV.2_GVI.I interspecies recombinant FNoVM49 isolated from a cat captured near a Japanese oyster farm in 2015 [94], may have originated via a similar mechanism.

CONCLUSION AND UNANSWERED QUESTIONS

Recombination, shifting the 'Rubik's cube's building blocks' of NoV classification, remains a significant factor influencing NoV molecular evolution and diversity. The enormous scope of intragenotype, intergenotype and even intergenogroup NoV recombinants and their recurrent implication in reported outbreaks highlight the continued importance of standardized monitoring (via shared sequencing protocols or implementation of whole-genome next-generation sequencing) and reporting of novel NoV recombinant types. With respect to their potential to emerge and re-emerge as dominant NoV strains, early detection of NoV recombinants and an understanding of the possible impact of recombination on (future) vaccine usage must be furthered. Special attention should be paid to recombination between genetically distant NoVs, which may generate novel NoV variants with altered pathogenesis and modified host tropism.

Despite the abundance of epidemiological data recording different, mainly HuNoV, recombinant types, evidence for MuNoV recombinants generated *in vitro* is scarce and the mechanism(s) involved are poorly characterized. It remains to be seen whether there is a true disconnect between NoV recombination frequency in the field and its apparent rarity under laboratory conditions. Since the MuNoV model allows only limited inferences regarding NoV recombination, *in vitro* HuNoV recombination studies in robust cell culture systems, the development of novel tools (NGS analysis of RNA within the cell and improved reverse genetic systems) to allow generation and detection of recombinants, as well as co-infection studies with other animal models, will

help resolve as yet unanswered questions in this area. In this context, drivers and constraints of NoV recombination must be investigated.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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Objectives

Recombination and the accumulation of point mutations are key mechanisms in the evolution and diversity of NoVs. Increasing evidence indicates that recombination shapes NoV pathogenesis and fitness and drives the evolution of emerging HuNoV strains; new recombinant NoV types are continuously described in the context of sporadic cases and field outbreaks (Ludwig-Begall et al., 2018).

The publication "Experimental evidence of recombination in murine noroviruses" (Mathijs et al., 2010), described the *in vitro* isolation of an infectious recombinant NoV. Recombinant MuNoV RecMNV was isolated following co-infection of RAW264.7 cells with two parental homologous MuNoV strains CW1 and WU20 in an infectious centre assay. While demonstrating a significantly lower *in vitro* replicative fitness than either of its parental strains, RecMNV remains one of its kind, to date constituting the only proven infectious experimental NoV recombinant (Mathijs et al., 2010, 2016).

The 2018 *Journal of General Virology* review, "Norovirus recombinants: recurrent in the field, recalcitrant in the lab" (Ludwig-Begall et al., 2018), which compounds chapter 2.3.1 of this thesis, provides an overview of advances on the subject of NoV recombination and outlines the seeming discrepancy between the sheer quantity of naturally occurring NoV recombinants and the paucity of information and difficulties associated to NoV recombination as studied in the lab. Several putative drivers and constraints at various checkpoints of NoV recombination are identified in a conceptual model (see also Figure 10). Following this, host coinfection, single cell coinfection, and recombination must be accomplished to generate a recombinant NoV RNA; incipient recombinant viruses must then survive a process of functional selection to be maintained in the viral population. Figure 8 of this thesis recapitulates the NoV recombination checkpoints and attributes a colour code to indicate the level of confidence associated with their drivers and constraints; the particular drivers and constraints of NoV recombination investigated in the context of experimental sections 1 and 2 of this thesis are highlighted.

Host coinfection may be dependent on spatial and temporal overlap of strain-distributions; cell coinfection, the ultimate prerequisite to viral recombination (Worobey and Holmes, 1999), depends on factors influencing the within-host distribution of viruses to target cells. True coinfection of cells is likely to be a rare event under natural conditions and delayed secondary infections are a more probable occurrence. In the event of an asynchronous infection, the uptake of multiple viruses into a single cell is dependent on factors that may limit consecutive entry of more than one virus particle per cell in a process known as superinfection exclusion. Superinfection exclusion is defined as the ability of an established virus to prevent a secondary infection by the same or a closely related virus (Folimonova, 2012); the primary infecting virus may render cells refractory to subsequent infection through interference at various stages of the replicative cycle of the secondary invader in a time-dependent manner. Viral pre-and post-entry blocks have been described for a number of RNA viruses (Adams and Brown, 1985; Bergua et al., 2014; Bratt and Rubin, 1968; Claus et al., 2007; Huang et al., 2008; Johnson, 2019; Lee et al., 2005; Tscherne et al., 2007; Zhou et al., 2019).

The first part of this thesis (Study 1) is dedicated to examining how different parameters of co- and superinfection may influence the composition of a nascent mixed viral quasispecies and investigates whether superinfection exclusion between two homologous MuNoV strains may play a role in preventing NoV co-infection *in vitro*; importantly, superinfection exclusion has remained hitherto unexplored in NoV biology.

Recombination, while conferring selective advantages to a nascent recombinant virus on a population level under *in vivo* immune pressures, can entail great modifications in a single viral genome, potentially eliciting a replicative fitness cost, which must be compensated via the adaptive capacity of a recombinant virus.

The second part of this thesis (Study 2) aims to characterise the adaptive capacity of *in vitro* generated RecMNV, thus investigating how the accumulation of point mutations through successive viral passaging can compensate for replicative fitness losses. The work, entitled "Replicative fitness recuperation of a recombinant murine norovirus – *in vitro* reciprocity of genetic shift and drift" (Ludwig-Begall et al., 2020), has been published in *Journal of General Virology*.

The aim of this thesis is to evaluate experimental conditions for and implications of MuNoV *in vitro* recombination. The insights thus gained will further a deeper understanding of the drivers and constraints of NoV recombination.

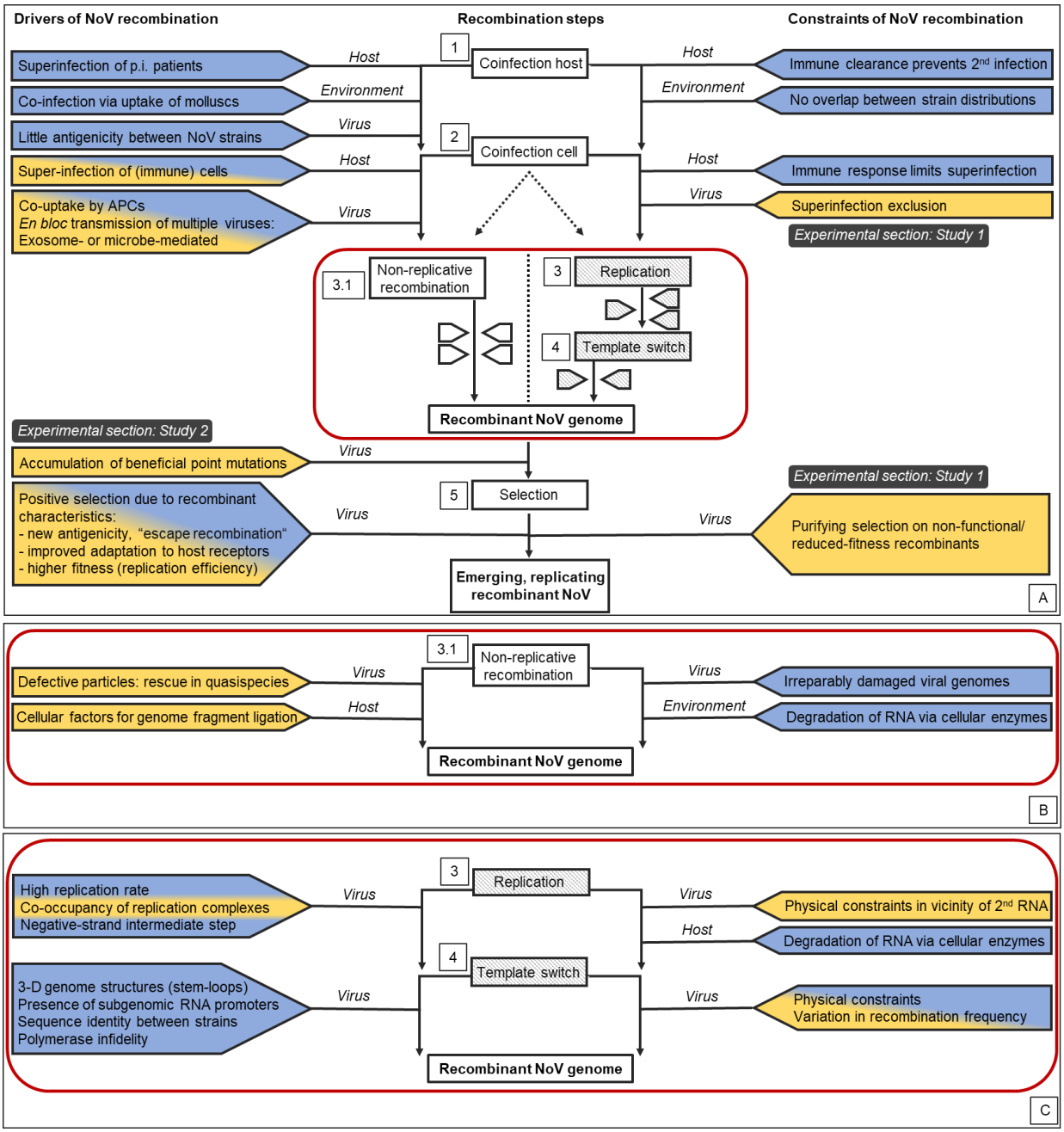

Figure 10. Conceptual model of steps, drivers and constraints of norovirus recombination. (A) gives an overview of the different recombination steps and accompanying host, virus, or environmental drivers and constraints (predictive risk factors). (B) and (C) focus on putative drivers and constraints of both non-replicative recombination and template-switch-mediated recombination, respectively. Blue shading indicates that putative drivers and constraints represent confirmed aspects of norovirus biology (according to pertinent literature). Yellow shading indicates a degree of uncertainty pertaining to the state of the art. The particular drivers and constraints of norovirus recombination investigated in the context of experimental sections 1 and 2 of this thesis are correspondingly annotated. APC: antigen-presenting cells; p.i.: persistently infected; NoV: norovirus

Experimental section

Experimental section

Study 1:

Analysis of synchronous and asynchronous *in vitro* infections with homologous murine norovirus strains reveals time-dependent viral interference effects

Preamble

Viral recombination is a key mechanism in the evolution and diversity of noroviruses. *In vivo*, synchronous single-cell coinfection by multiple viruses, the ultimate prerequisite to viral recombination, is likely to be a rare event and delayed secondary infections are a more probable occurrence. Here, we determine the effect of a temporal separation of *in vitro* infections with the two homologous murine norovirus strains MNV-1 WU20 and CW1 on the composition of nascent viral populations. WU20 and CW1 were either synchronously inoculated onto murine macrophage cell monolayers (coinfection) or asynchronously applied (superinfection with varying titres of CW1 at half-hour to 24-hour delays). 24 hours after initial co-or superinfection, quantification of genomic copy numbers and discriminative screening of plaque picked infectious progeny viruses demonstrated a time-dependent predominance of primary infecting WU20 in the majority of viral progenies. Our results indicate that a time interval from one to two hours onwards between two consecutive norovirus infections allows establishment of a barrier that reduces or prevents super-infection; this first demonstration of time-dependent viral interference for NoVs has clear implications for NoV epidemiology, risk assessment, and potentially treatment.

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Experimental section

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viruses

MDPI

Communication

Analysis of Synchronous and Asynchronous In Vitro Infections with Homologous Murine Norovirus Strains Reveals Time-Dependent Viral Interference Effects

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Abstract: Viral recombination is a key mechanism in the evolution and diversity of noroviruses. In vivo, synchronous single-cell coinfection by multiple viruses, the ultimate prerequisite to viral recombination, is likely to be a rare event and delayed secondary infections are a more probable occurrence. Here, we determine the effect of a temporal separation of in vitro infections with the two homologous murine norovirus strains MNV-1 WU20 and CW1 on the composition of nascent viral populations. WU20 and CW1 were either synchronously inoculated onto murine macrophage cell monolayers (coinfection) or asynchronously applied (superinfection with varying titres of CW1 at half-hour to 24-h delays). Then, 24 h after initial co-or superinfection, quantification of genomic copy numbers and discriminative screening of plaque picked infectious progeny viruses demonstrated a time-dependent predominance of primary infecting WU20 in the majority of viral progenies. Our results indicate that a time interval from one to two hours onwards between two consecutive norovirus infections allows for the establishment of a barrier that reduces or prevents superinfection.

Keywords: norovirus; murine norovirus; coinfection; superinfection; superinfection exclusion; interference

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1. Introduction

Human noroviruses (HuNoVs) are recognised as a leading global cause of sporadic and epidemic viral gastroenteritis [1] and account for a global economic burden of $60 billion, over one million hospitalisations, and 200,000 deaths per annum [2,3]. Customarily an acute and self-limiting illness, HuNoV infection can become chronic in the elderly, malnourished, and/or immunocompromised; such patients may experience protracted severe, even lethal, NoV infections and superinfections [4–8].

Various HuNoV infection models have yielded valuable insights into the NoV life cycle in recent years [9–12]. However, many of these experimental systems are technically challenging and as yet lack the degree of robustness required for detailed decipherment. The genetically and biologically closely related murine norovirus (MuNoV), which combines the advantages of available tools for genetic manipulation [13,14], easy in vivo infection of a genetically tractable native host [15], and efficient in vitro propagation [15–17], thus remains the main model for NoV in vitro studies.

Human noroviruses and MuNoVs belong to the *Norovirus* genus within the *Caliciviridae* family of small, non-enveloped, positive sense, single-stranded RNA viruses [18,19]. The linear, polyadenlyated 7.4–7.7 kb long HuNoV genome is organised into three open reading

frames (ORFs); an additional fourth ORF is described for MuNoVs [20,21]. The 5′ proximal NoV ORF1 encodes a large polyprotein that is co-and post-translationally cleaved into six non-structural viral proteins [22]. ORF2 and ORF3 encode the structural virion components, major and minor capsid proteins, VP1 and VP2, respectively. ORF4, which entirely overlaps the 5′end of ORF2, encodes virulence factor (VF1) [23].

Viral recombination is a key mechanism in the evolution and diversity of NoVs; increasing evidence indicates that recombination shapes NoV pathogenesis and fitness and drives the evolution of emerging strains [24]. Numerous field recombination events, predominantly at a typical ORF1/2 recombination breakpoint [25], have been detected in silico in the *Norovirus* genus [26,27]. In contrast, few experimental data are available concerning NoV recombination under laboratory conditions and the mechanism(s) involved are poorly characterised [26,28–30].

We recently identified a set of checkpoints, including their respective drivers and constraints, that must be successfully bypassed for the generation of a viable recombinant NoV [26,31]. Following this, host coinfection, single cell coinfection, and recombination must be accomplished to generate a recombinant NoV RNA. An incipient recombinant viruses must then survive a process of functional selection to be maintained in the viral population [32–35]. The rise of recombinant viruses resulting from this process is influenced by different factors. In vivo, host coinfection may be dependent on spatial and temporal overlap of strain-distributions. Cell coinfection, the ultimate prerequisite to viral recombination, depends on factors influencing the within-host distribution of viruses to target cells, thereby limiting or increasing the likelihood of cellular coinfections. True coinfection of cells is likely to be a rare event (unless mediated by factors directing synchronous uptake of diverse viruses into both host and cell [36] under natural conditions and delayed secondary infections are a more probable occurrence.

In the event of an asynchronous infection, the uptake of multiple viruses into a single cell is dependent on factors that may limit consecutive entry of more than one virus particle per cell in a process known as superinfection exclusion. Superinfection exclusion is defined as the ability of an established virus to prevent a secondary infection by the same or a closely related virus [37]. The primary infecting virus may render cells refractory to subsequent infection through interference at various stages of the replicative cycle of the secondary invader in a time-dependent manner. Viral pre-and post-entry blocks have been described for a number of RNA viruses [38–46]. However, hitherto, NoVs have not been listed amongst them.

Here, we determine the effect of a temporal separation of in vitro infections with the two homologous parental MuNoV strains MNV-1 WU20 and CW1 on the composition of MuNoV populations. A clear advantage of in vitro systems to study viral population dynamics is that they present a well-defined entity containing only viruses and cells. Effects of other factors interfering with cell coinfection (such as the host immune response or microbiome) may thus be discounted.

Our results demonstrate that a time interval from one to two hours onwards between two consecutive NoV infections allows establishment of a barrier that reduces or prevents superinfection; this first demonstration of time-dependent viral interference for NoVs has clear implications for NoV epidemiology, risk assessment, and potentially treatment.

2. Materials and Methods

A graphical overview of all assays is provided in Figure 1.

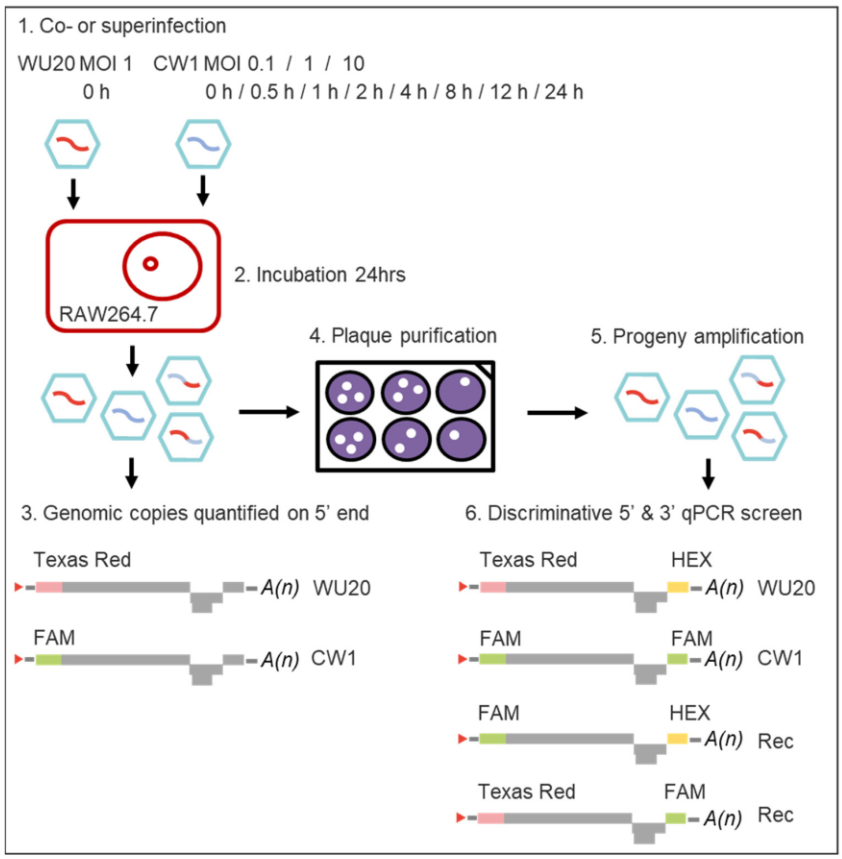

Figure 1. Workflow of the experimental set-up to analyse synchronous and asynchronous in vitro infections with homologous murine norovirus strains MNV-1 WU20 and CW1. MOI = Multiplicity of infection; ORF = Open Reading Frame; qPCR = quantitative polymerase chain reaction.

2.1. Viruses and Cells

The murine macrophage cell line RAW264.7 (ATCC TIB-71) was maintained in Dulbecco's modified Eagle's medium (DMEMc) (Invitrogen, San Diego, CA, USA, Thermo Fisher Scientific, Waltham, MA, USA) containing 10% heat inactivated foetal calf serum (FCS) (BioWhittaker), 2% of an association of penicillin (5000 SI units mL^{-1}) and streptomycin (5 mg ml^{-1}) (PS, Invitrogen), and 1% 1 M HEPES buffer (pH 7.6) (Invitrogen) at 37 °C with 5% CO_2.

Murine NoV isolates MNV-1 CW1 and WU20 (GenBank accession numbers DQ285629 and EU004665.1; 87% nucleotide sequence similarity; previously shown to exhibit highly similar replication kinetics [28,47] were plaque purified and propagated in RAW 264.7 cells as described by Mathijs et al., 2010 [28]. Virus stocks were produced by infection of RAW 264.7 cells at a multiplicity of infection (MOI, expressed as plaque forming units per cell) of 0.05. Two days post-infection, cells and supernatants were harvested and clarified by centrifugation for 20 min at 1000× *g* after three freeze/thaw cycles (−80 °C alternating with 37 °C). Supernatants were purified by ultracentrifugation on a 30% sucrose cushion in a SW28 rotor (Beckman Coulter, Indianapolis, IN, USA) at 23,000 rounds per min for 2 h at 4 °C. Pellets were suspended in 500 µL phosphate-buffered saline (PBS), aliquoted, and frozen at −80 °C. Viral titres were determined via plaque assay for the seventh passage of WU20 and the eighth of CW1 (WU20 P7 and CW1 P8), as described by Hyde et al., 2009 [48]. WU20 P7 and CW1 P8 single-step and multi-step growth curves, performed prior to

launching the co-and superinfection experiments described below, exhibited no significant differences in the replication kinetics of the two virus stocks (Supplementary Figure S1).

2.2. Coinfection and Superinfection of RAW264.7 Cells with Murine Noroviruses WU20 and CW1

Monolayers of RAW 264.7 cells were prepared in 24-well plates at a density of 5×10^4 cells per well. Working on ice, each well was infected with WU20 (MOI = 1; confirmed via back-titration). After 1 h, the WU20 inoculums (300 µL) were removed and stored at −80 °C. The cells were washed twice with PBS and were infected with CW1 at various MOIs (0.1; 1; 10; confirmed via back-titration) at delays of 0 min (coinfection), 30 min, and 1, 2, 4, 8, 12, and 24 h (superinfections). For coinfections, CW1 and WU20 inoculums in a final volume of 300 µL were simultaneously added to cells. Cells and virus then remained on ice for 1 h, whereupon the inoculum was removed. For superinfections, CW1 inoculums were asynchronously dispensed onto cells at the appropriate delays, whereupon cells and virus remained on ice for 1 h until removal of the inoculums; the cells were then washed twice with PBS and 300 µL DMEMc were added. Twenty-four hours post co-or superinfection, both cells and supernatants were frozen and stored at −80 °C until further analysis.

2.3. Quantification of WU20 and CW1 Genomic Copies in Viral Progenies 24 h Post Co- or Superinfection

RNA extractions were performed with Tri Reagent solution (Ambion, Austin, TX, USA) on 120 µL of co-and superinfection supernatants. Extracted RNA was reverse-transcribed into complementary DNA (cDNA) using an iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA). The extracted and reverse-transcribed cDNA was quantitatively analysed via real time quantitative PCR (qPCR), employing primers to allow discrimination between CW1 and WU20 based upon single nucleotide polymorphisms (SNPs) at the 5′ genomic extremity (amplicon in ORF1, dubbed region 1), as described by Mathijs et al. 2010. Primers and probes used in the quantification of genomic copies correspond to those listed in Supplementary Table S1 as published by Mathijs et al., 2010 [28].

Quantifications were performed as previously described by Mauroy et al. (2012) [49]; for generation of the standard curve, region 1 amplicons were amplified for both CW1 and WU20, then cloned into a pGEMt-Easy vector (Promega, Madison, WI, USA) and sequenced. Both CW1-region 1 and WU20-region 1 plasmids were in vitro transcribed with the Ribomax kit (Promega) following manufacturer's instructions. Briefly, *Spe*I-linearised and purified plasmids were transcribed with T7 RNA polymerase, treated with DNAse, and quantified via spectrophotometer. Genomic copy numbers of transcribed RNA were deduced and serial ten-fold dilutions were prepared with ultrapure RNAse free water (Invitrogen). Aliquots of the master stock were stored at −80 °C and measured before dilution and use. Final results were normalised using transcripts of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Barber et al. 2005). A 5 µL qPCR mix (technical duplicates) was set up by adding 1 µL of cDNA to 2.50 µL of iQ supermix, 0.1 µL of both GAPDH-forward and -reverse primers (100 nM final concentration), 0.2 µL of the GAPDH-probe (200 nM final concentration) and 1.1 µL of nuclease free water. Cycling conditions included an initial 5-min denaturation at 95 °C followed by 38 cycles of 10 s at 95 °C and 40 s at 60 °C.

2.4. Isolation and Screening of Infectious Progeny Viruses

Cells and supernatants from the co- and superinfection step were frozen and thawed once and then utilised as inoculums in a plaque assay for purification of infectious progeny viruses following the method described by Hyde et al. 2009 with slight modifications [48]. Briefly, RAW 264.7 monolayers, cultured in six-well plates (2×10^6 RAW264.7 cells/well) were inoculated at room temperature with 1 mL of serial dilutions of virus-containing culture fluids of the co- and superinfection assays. After 1 h, inoculums were removed and cells were overlaid with 2 mL of medium containing 70% DMEM-Glutamax (4.5 g glucose l-1 and 15 mM sodium hydrogen carbonate), 2.5% FCS, 2% PS, 1% HEPES and 0.7%

SeaPlaque agarose (Lonza, Basel, Switzerland) per well. After 48 h of incubation (37 °C, 5% CO_2), 36 individual plaques were randomly selected per condition. Infected cells from the plaque margins were picked with a needle under a microscope and were diluted into fresh DMEMc before propagation by inoculation onto RAW 264.7 cells grown in 24-well plates. After 72 h, supernatants were collected and frozen at −80 °C until further analysis.

Following RNA extraction and reverse transcription, cDNAs of individual plaque-purified virus progenies were analysed via two parallel real time PCR runs employing two pairs of primers to allow discrimination between CW1, WU20 (and recombinant) signals based upon single nucleotide polymorphisms (SNPs) at both genomic extremities (ORF1 and ORF3, dubbed regions 1 and 5, respectively) as described by Mathijs et al. 2010 [28]. Five µl reactions were carried out with iQ supermix. Primers and probes for this TaqMan-based discriminative qPCR correspond to those listed in Supplementary Table S1 as published by Mathijs et al. 2010 [28].

In the case of ambiguous signals originating from mixed virus populations, an additional quick screen was performed via Sanger sequencing of the ORF1/2 overlap (base pairs 4864 to 5298 in MNV-1 CW1), this to exclude the presence of potential recombinants or PCR chimeras from interfering with later calculations of WU20 to CW1 infectious virus ratios.

3. Results

3.1. Absolute and Relative Quantification of Genomic Copies Reveals Skewed WU20 and CW1 Distributions and a WU20 Dominance in Most Viral Progenies 24 h Post Co- or Superinfection

To quantitatively assess viral progeny distributions 24 h after initial co-or superinfection, MNV-1 WU20 and CW1 genomic copy numbers were inferred from the cycle threshold (Ct) values of the qPCR reactions and normalised against GAPDH Ct values. This genomic quantification (5′ region 1 amplicon) revealed WU20 absolute genomic copy numbers, averaging 3.55 (±0.57) $\log_{10}$ genomic copies over all measured time points, to be higher than those of CW1 in all but four of the resulting 24 viral progenies. Only short superinfection delays (t0 h, 0.5 h, 1 h, 2 h) with a WU20 to CW1 starting ratio of one to ten resulted in CW1 genomic copy numbers significantly higher than or equal to those of WU20 24 h post co- or superinfection at 3.23 (±0.96), 3.44 (±0.48), 3.74 (±0.09), and 3.93 (±0.06) $\log_{10}$, respectively (Figure 2, top panels).

These absolute genomic copy numbers translate into relative ratios of genomic copies that reflect a disproportionate WU20 dominance within the majority of viral populations (Figure 2, bottom panels). A WU20 to CW1 starting MOI ratio of 1 to 0.1 (expected to yield 90% WU20 and 10% CW1 genome copies upon qPCR analysis of the viral population) yielded mean WU20 genomic copy numbers of 3.40 (±0.43), 3.17 (±0.34), 3.33 (±0.40), and 4.27 (±0.05) $\log_{10}$ (accounting for 95.98%, 92.46%, 91.74%, and 95.73% of the population) 24 h after either coinfection (t0) or superinfections with delays of half an hour (t0.5) to two hours (t2). From a superinfection delay of four hours (t4) onwards, WU20 mean genomic copy numbers ranging from 3.28 (±0.56) to 4.24 (±0.01) $\log_{10}$ (99.43 to 99.96% of the population) are juxtaposed against CW1 values of 1.43 (±0.81) to 0.07 (±0.32) $\log_{10}$.

At an equal WU20 to CW1 starting MOI of 1 (expected yield to 50% WU20 and 50% CW1 genome copies), 3.17 (±0.33) $\log_{10}$ (77.30%) WU20 to 2.49 (±0.54) $\log_{10}$ (22.69%) CW1 (t0) and 2.98 (±0.78) $\log_{10}$ (62.64%) WU20 to 2.89 (±0.59) $\log_{10}$ (37.36%) CW1 ratios (t0.5), are succeeded by a marked increase of the WU20 proportion, covering 4.27 (±0.11) (77.63%) (t1), 4.17 (±0.18) (89.26%) (t2), and 3.37 (±0.04) $\log_{10}$ (86.01%) (t4), and then reaching values of over 3.16 (±0.47) $\log_{10}$ (95%) from t8 onwards, while CW1 values are consistently at least one order of magnitude lower and never surpass 2.67 (±0.02) $\log_{10}$ from t4 onwards.

A WU20 to CW1 starting MOI ratio of 1 to 10 (expected to yield 10% WU20 and 90% CW1 genome copies) resulted in 3.66 (±0.24) $\log_{10}$ to 3.23 (±0.96) $\log_{10}$ and 3.88 (±0.09) to 3.44 (±0.48) $\log_{10}$ WU20 to CW1 genome copies at t0 and t0.5, respectively (roughly 50–50 ratios), fulfilled expectations with 2.2 (±0.59) $\log_{10}$ WU20 to 3.74 (±0.09) $\log_{10}$ CW1 genome copies (6.16% WU20 to 93.84% CW1) at t1, after which WU20 genome copy numbers progressively increased to 3.76 (±0.01), 3.68 (±0.29), 4.16 (±0.3), 3.09 (±0.63), and 3.19 (±0.32) $\log_{10}$ (accounting for 37.85%, 61.66%, 81.67%, 71.20%, and 74.58% of

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the population) at t2, t4, t8, t12, and t24, respectively. CW1 genome copy numbers correspondingly decreased.

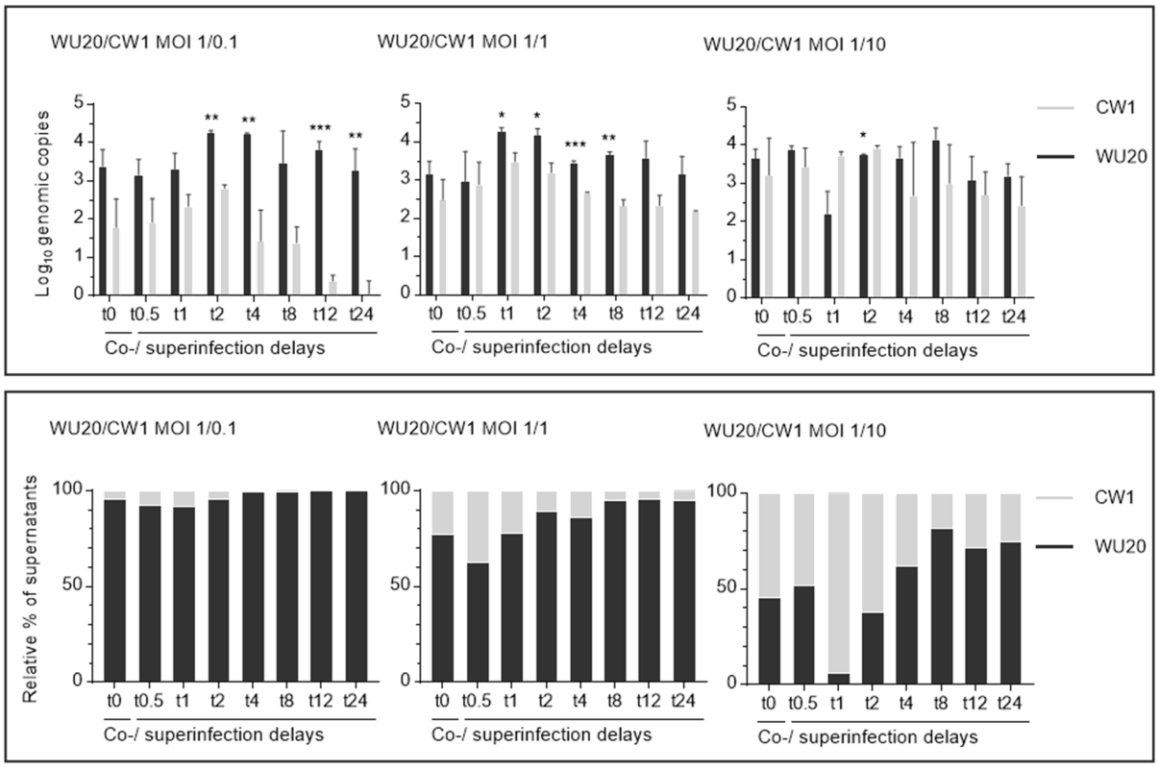

Figure 2. Genomic quantification on 5′ genome ends establishing raw genomic copy numbers (top) and relative proportions of mean genomic copies (below) of co- or superinfecting murine noroviruses MNV-1 WU20 and CW1 in viral progenies 24 h post co- or superinfection. Genomic copy numbers and their relative proportions resulting from one co-infection (t0) and seven asynchronous infections (primary infection: WU20; superinfection at half-hour to 24-h delays (t0.5 to t24): CW1) are shown. Varying multiplicities of infection (MOI) were analysed; the MOI of primary infecting WU20 remained stable at 1 throughout all assays while the MOI of superinfecting CW1 varied between 0.1 (left panels), 1 (middle panels), and 10 (right panels). Black bars represent WU20, grey bars represent CW1. Differences in yield between mean WU20 and CW1 genome copies were analysed using GraphPad Prism 7 (Graph-Pad Software) and p values were determined using two-sided unpaired-sample t tests, where *** $p \leq 0.001$, ** $p \leq 0.01$, and * $p \leq 0.05$.

3.2. Molecular Screening on Picked Lysis Plaques Demonstrates a WU20 Predominance in the Majority of Infectious Viral Progenies

To isolate and screen infectious progeny viruses present within the various viral populations 24 h after initial co-or superinfection, 36 viral plaques per condition were picked from a plaque assay, further propagated in RAW246.7 cells, and then analysed in parallel duplex qPCR runs to discriminate between MNV-1 CW1 and WU20 (as well as possible recombinant viruses) based on 5′ and 3′ SNPs. In three cases, additional ORF1/2 screening confirmed sequence kinship to either WU20 or CW1.

Overall, the previously observed WU20 dominance, particularly following longer CW1 superinfection delays, is mirrored in the proportions of plaque picked infectious viruses (Figure 3). Thus, a WU20 to CW1 starting MOI ratio of 1 to 0.1 (expected to yield infectious progeny virus proportions of 90% WU20 to 10% CW1) yielded 94%, 68%, 83%, 84%, 100%, 88%, 100%, and 100% WU20 24 h after coinfection (t0) or superinfection delays of half an hour (t0.5) to 24 h (t24), respectively. Pure CW1 fractions are seen to account

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for 3%, 16%, and 6% of infectious viral populations at t0, t0.5, and t1. However, with the exception of the eight-hour superinfection delay (3% CW1 at t8), CW1 is not represented in infectious virus progenies from t2 onwards. Mixed WU20 and CW1 progenies make up the remaining fractions of the various populations.

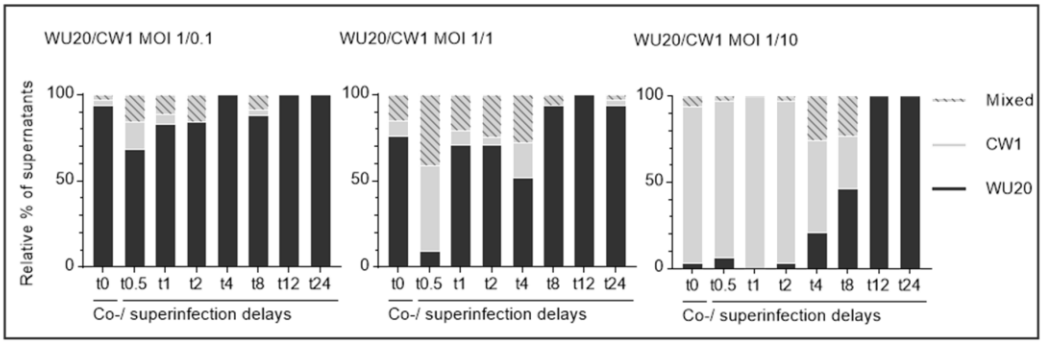

Figure 3. Relative proportions of viable co- or superinfecting murine noroviruses MNV-1 WU20 and CW1 after plaque purification and amplification. Black bars show the proportion of WU20, grey bars show the proportion of CW1, and striped bars indicate mixed signals of both WU20 and CW1 in viral progenies amplified from 36 plaques per condition. One coinfection (t0) and seven asynchronous infections (primary infection: WU20; superinfection at half-hour to 24-h delays (t0.5 to t24): CW1) and varying multiplicities of infection (MOI) were analysed; the MOI of primary infecting WU20 remained stable at 1 throughout all assays while the MOI of superinfecting CW1 varied between 0.1 (left panel), 1 (middle panel), and 10 (right panel)).

At an equal WU20 to CW1 starting ratio (expected to yield balanced infectious WU20 and CW1 proportions), initial 76% WU20 to 9% CW1 (plus 15% mixed) and 9% WU20 to 50% CW1 (plus 41% mixed) ratios at t0 and t0.5 are succeeded by a marked increase of the WU20 proportion. WU20 thus accounts for 67%, 71%, and 52% of infectious viral progenies at t1, t2, and t4, and consistently reaches values of over 94% from an eight-hour superinfection delay (t8) onwards. CW1 and mixed progeny proportions correspondingly decrease following the one-hour superinfection delay (t1).

A WU20 to CW1 starting MOI ratio of 1 to 10 (expected to yield infectious progeny virus proportions of 10% WU20 to 90% CW1) resulted in WU20 proportions of 3% following coinfection (t0) and 6%, 0%, and 3% following early superinfection delays (t0.5 to t2). From t4 onwards, WU20 quantities are seen to progressively increase, accounting for 21% (t4), 46% (t8), and 100% (t12 and t24) of infectious virus progenies.

4. Discussion

Viral recombination has been identified as a key mechanism shaping the evolution and diversity of NoVs [24,26,27]. In contrast to an abundance of field data, few experimental data are available concerning NoV recombination and the mechanism(s) involved remain poorly characterised [26,28–30,50]. An incremental step in the generation of any recombinant viral RNA and consequently any viable recombinant virus is the successful simultaneous infection of a single cell by (a minimum of) two viruses [32–35]. Under natural conditions, various environmental, host, and virus factors may influence the probability of synchronous coinfections and may determine the delay or even the absolute achievability of asynchronous cellular superinfections. Superinfection exclusion, whereby a primary infecting virus may render cells refractory to subsequent infection through interference at various stages of the replicative cycle of the secondary invader [37], is a typically virus-mediated process. Viral pre-and post-entry blocks have been described for a number of RNA viruses [38–45]. Hitherto, NoVs have not been listed amongst them.

Here, we determined the effect of a temporal separation of in vitro infections with the two homologous MuNoV strains MNV-1 WU20 and CW1 on the composition of nascent MuNoV populations. In utilising an in vitro system, we excluded both environmental and host influences and were thus able to examine only those effects mediated by the viruses themselves.

Subsequent to initial WU20 and CW1 cell coinfections or superinfections with half-hour- to 24 h-delays and varying input MOIs (1:0.1; 1:1; 1:10), followed by a 24-h propagation step, individual viral progeny distributions were analysed via qPCR. This quantitative analysis revealed a disproportionate dominance of primary infecting WU20 genomic copies in the majority of resulting viral progenies. While the WU20 dominance appeared to be near-independent of the input MOI ratios of the two viruses (and indeed skewed expected genomic copy ratios throughout), it was markedly time-dependent; increasing CW1 superinfection delays from one to two hours onwards were associated directly with increasing WU20 genome copy fractions. While primary infecting WU20 is expected to have undergone a round of replication before addition of CW1 at superinfection delays of more than eight hours (thus inherently tipping the balance of virus ratios in favour of WU20), input and expected ratios deviate significantly even at earlier time points where this effect cannot serve to explain the observed WU20 dominance.

Interestingly, the way in which higher-than-expected WU20 genomic copy numbers skewed expected genomic copy ratios even after coinfections or short superinfection delays may hint at the mechanism of the pronounced dominance following longer delays. Where input MOIs of 1:0.1, 1:1, and 1:10 were expected to yield WU20 to CW1 genomic copy ratios of 90% to 10%, 50% to 50%, and 10% to 90% following coinfection, these expectations were frustrated in the face of 3.40 ($\pm$0.43) to 1.79 ($\pm$0.75), 3.17 ($\pm$0.33) to 2.49 ($\pm$0.54), and 3.66 ($\pm$0.24) to 3.23 ($\pm$0.96) $\log_{10}$ WU20 to CW1 genomic copy proportions. Vacillating levels of infectious virus and genomic copies have previously been associated with the presence of defective interfering (DI) RNAs or DI particles within NoV populations [51]. DI RNAs or particles, deleterious virus-like by-products of error-prone RNA virus replication, are known interfere with standard virus particles by competing for resources [52,53]. DI RNAs may also play a role in mediating superinfection exclusion by induction of RNA silencing and the homology-dependent degradation of incoming RNA molecules [54]. In this context, it is conceivable that WU20 DI RNAs within the population (necessarily included in the quantitative analysis of genome copies since the qPCR assay does not distinguish between DI RNAs or DI particles and whole (infectious) viral genomes) were recognised by the cellular RNA silencing machinery and served to guide degradation of incoming CW1 RNA sequences, this particularly following longer superinfection delays.

Relative proportions of infectious viruses isolated from viral progenies following coinfection (t0) or short superinfection delays of up to two hours (t0.5 to t2), support a possible role of WU20 DI RNAs. Thus, e.g., coinfection with a one to ten WU20 to CW1 MOI ratio resulted in skewed genomic copy ratios of 45.57% WU20 to 54.43% CW1, but translated into infectious virus ratios of 3% WU20 and 91% CW1 (plus 6% mixed). Following asynchronous infection with longer delays (t4 to t24), a time-dependent WU20 dominance and corresponding CW1 decrease is evident within infectious virus progenies. Mixed populations registered subsequent to two-hour superinfection delays may indicate that that the barrier is established progressively and is, initially, not strong enough to completely repel superinfecting CW1, especially in the face of high input titres.

Taken together, these results demonstrate that a time interval from one to two hours onwards between two consecutive in vitro MuNoV infections allows establishment of a barrier that progressively reduces or prevents superinfection. While viral interference, or superinfection exclusion, has hitherto not been described for NoVs, it is well documented for other positive sense, single-stranded RNA viruses, such as hepatitis C-, bovine viral diarrhoea-, and West Nile virus and may be established within 30 min to several hours of primary infection [43,44,55–57].

In future investigations it will be interesting to leverage population-level deep sequencing to analyse how the viral interference effects pinpointed here may influence the generation of NoV RNA recombinants (and thus ultimately influence the chances of recombinant virus generation under the application of selective pressures). Further work should also focus on the mechanism of NoV interference (pre-or post-entry mode of action analysis) and will investigate whether the observed block can be overcome by superinfecting viruses.

Understanding the influence that viral interference may have on NoV population dynamics has clear implications for NoV epidemiology and risk assessment. The phenomenon is thought to decrease the evolution of drug resistance and immune escape by limiting population variability and virus recombination [55]. Identifying where it plays a role and also where and how it may be overcome in the field by superinfecting variants are also important in the context of treating NoV infections.

Supplementary Materials: The following data are available online at https://www.mdpi.com/article/10.3390/v13050823/s1, Figure S1: Growth curves for MNV-1 WU20 and CW1 at low and high multiplicities of infection (MOI).

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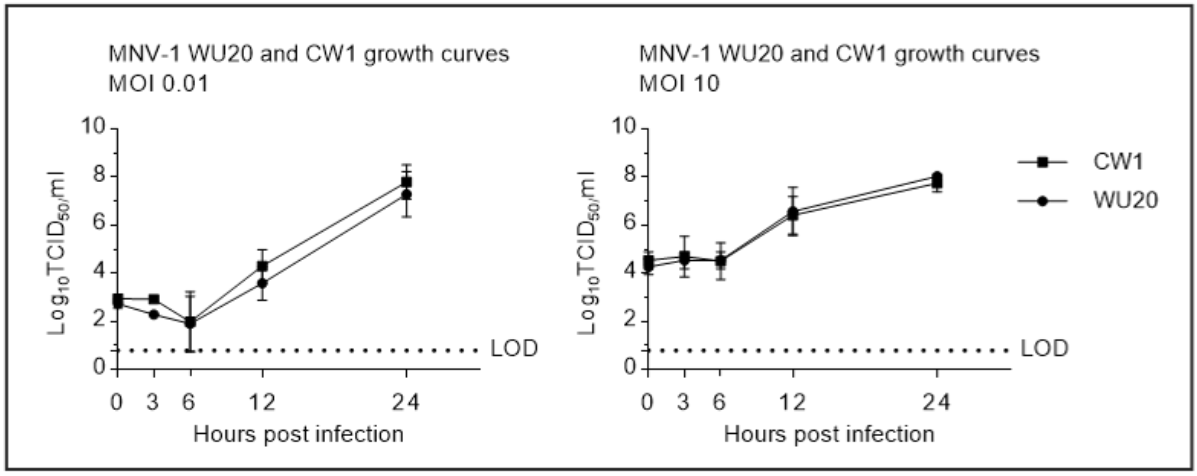

Supplementary Figure 1. Growth curves for MNV-1 WU20 and CW1 at low and high multiplicities of infection (MOI). Data for total, intracellular and extracellular virions were obtained after infection of RAW 264.7 cells as described by Mathijs et al., 2010 (28). Virus titres are expressed as means +SD of cells and supernatant analyses (technical duplicates).

Experimental section

Study 2:

Replicative fitness recuperation of a recombinant murine norovirus – *in vitro* reciprocity of genetic shift and drift

Preamble

Noroviruses are recognised as the major cause of non-bacterial gastroenteritis in humans. Molecular mechanisms driving norovirus evolution are the accumulation of point mutations and recombination. Increasing evidence indicates that recombination influences NoV pathogenesis and fitness and contributes to the evolution of emerging HuNoV strains. For the generation of a viable recombinant NoV, several steps, namely host coinfection, single cell coinfection, RNA recombination, and functional selection, must be accomplished. Study 1 demonstrated how superinfection exclusion may interfere with the generation of recombinant NoV RNA by preventing cell-coinfection; Study 2 now examines the next recombination checkpoint and addresses the issue of how incipient recombinant NoVs may survive a process of functional selection. Recombination can create considerable changes in a viral genome, potentially eliciting a fitness cost, which must be compensated via the adaptive capacity of a nascent recombinant NoV. A replicative fitness cost of the first *in vitro* generated WU20-CW1 recombinant MuNoV, RecMNV, was reported by Mathijs et al., 2010. In this follow-up study, RecMNV's capability of replicative fitness recuperation and genetic characteristics of RecMNV progenies at early and late stages of an adaptation experiment were evaluated. Replicative fitness regain of the recombinant was demonstrated via growth kinetics and plaque sizes differences between viral progenies prior to and post serial *in vitro* passaging. Point mutations at consensus and sub-consensus population levels of early and late viral progenies were characterised via next generation sequencing and putatively associated to fitness changes. To investigate the effect of genomic changes separately and in combination in the context of a lab generated inter-MNV infectious virus, mutations were introduced into a recombinant WU20-CW1 cDNA for subsequent DNA-based reverse genetics recovery (see Figure 11 for an overview of the experimental workflow). We thus associated fitness loss of RecMNV to a C7245T mutation and functional VP2 (ORF3) truncation and demonstrated individual and cumulative compensatory effects of one non-synonymous OFR2 and two synonymous ORF1 consensus level mutations acquired during successive rounds of *in vitro* replication. Our data provide evidence of viral adaptation in a controlled environment via genetic drift after genetic shift induced a fitness cost of an infectious recombinant NoV.

An article describing the work presented in this chapter was published in the *Journal of General Virology* in February 2020 and is reproduced below.

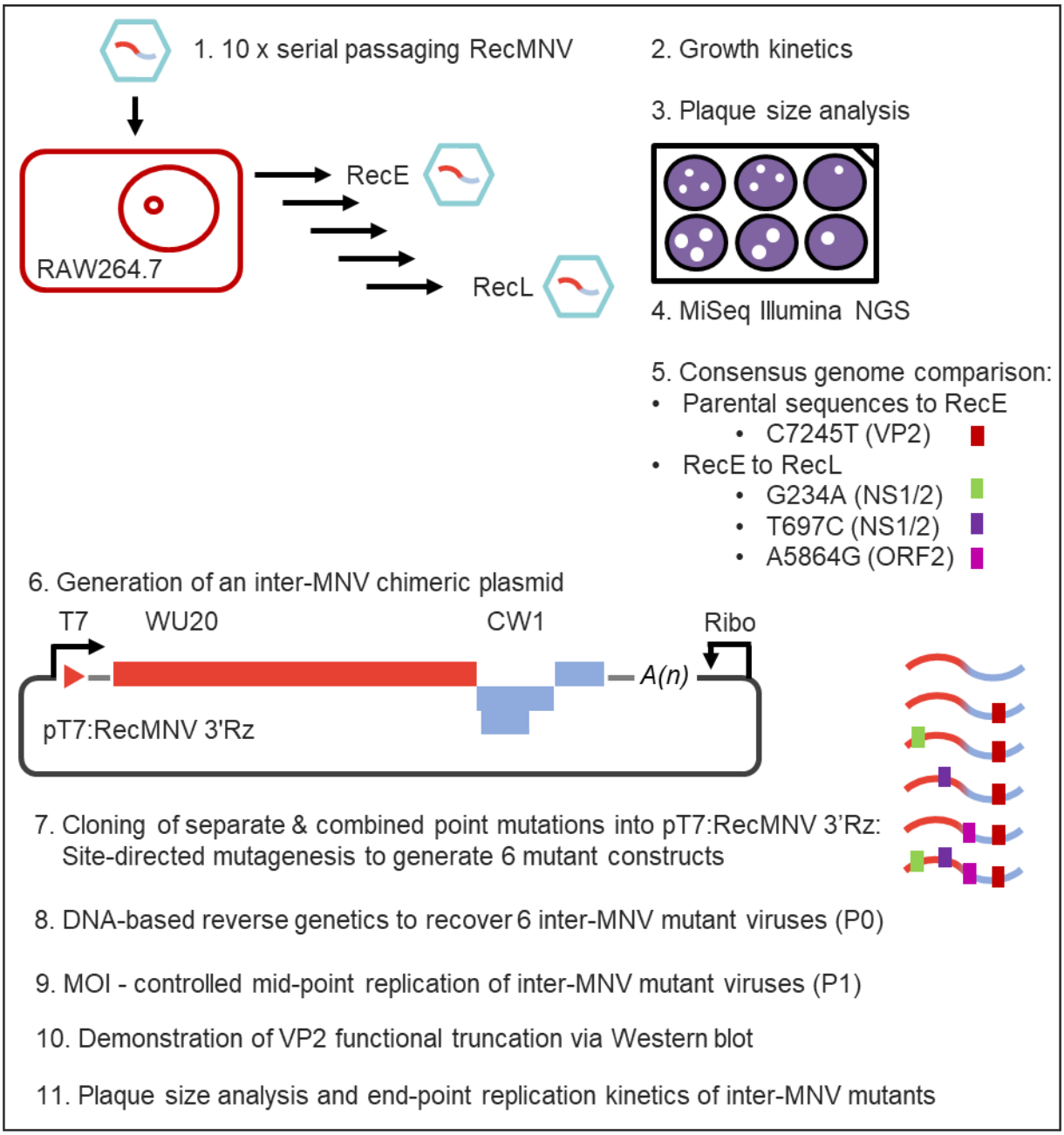

Figure 11. Workflow Study 2. Replicative fitness recuperation of a recombinant murine norovirus (RecMNV)– *in vitro* reciprocity of genetic shift and drift. RecE = early RecMNV passage; RecL = late RecMNV passage; NGS = Next generation sequencing; ORF = Open reading frame; NS = non-structural protein; VP2 = Viral protein 2; Ribo = self-cleaving δ-Ribozyme sequence

Experimental section

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Replicative fitness recuperation of a recombinant murine norovirus – *in vitro* reciprocity of genetic shift and drift

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MICROBIOLOGY SOCIETY

Replicative fitness recuperation of a recombinant murine norovirus – *in vitro* reciprocity of genetic shift and drift

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Abstract

Noroviruses are recognized as the major cause of non-bacterial gastroenteritis in humans. Molecular mechanisms driving norovirus evolution are the accumulation of point mutations and recombination. Recombination can create considerable changes in a viral genome, potentially eliciting a fitness cost, which must be compensated via the adaptive capacity of a recombinant virus. We previously described replicative fitness reduction of the first *in vitro* generated WU20-CW1 recombinant murine norovirus, RecMNV. In this follow-up study, RecMNV's capability of replicative fitness recuperation and genetic characteristics of RecMNV progenies at early and late stages of an adaptation experiment were evaluated. Replicative fitness regain of the recombinant was demonstrated via growth kinetics and plaque size differences between viral progenies prior to and post serial *in vitro* passaging. Point mutations at consensus and sub-consensus population levels of early and late viral progenies were characterized via next-generation sequencing and putatively associated to fitness changes. To investigate the effect of genomic changes separately and in combination in the context of a lab-generated inter-MNV infectious virus, mutations were introduced into a recombinant WU20-CW1 cDNA for subsequent DNA-based reverse genetics recovery. We thus associated fitness loss of RecMNV to a C7245T mutation and functional VP2 (ORF3) truncation and demonstrated individual and cumulative compensatory effects of one synonymous OFR2 and two non-synonymous ORF1 consensus-level mutations acquired during successive rounds of *in vitro* replication. Our data provide evidence of viral adaptation in a controlled environment via genetic drift after genetic shift induced a fitness cost of an infectious recombinant norovirus.

INTRODUCTION

Human noroviruses (HuNoVs) are recognized as major aetiologic agents of global sporadic and epidemic non-bacterial gastroenteritis [1], causing significant morbidity and mortality in developing countries [2] and high economic losses in developed countries [1, 3]. The development of HuNoV replicon bearing cells in a human hepatoma cell line [4], the B-cell culture system [5], the stem-cell-derived intestinal organoid system [6] and zebrafish larvae infection models [7] have all provided invaluable tools to dissect the NoV life cycle. However, there is still a lack of detailed understanding of HuNoV replication and significant questions remain unanswered due to the technical limitations of many of these experimental systems. The genetically and biologically closely related murine norovirus (MuNoV) combines the advantages of efficient *in vitro* culture systems [8, 9], availability of tools for genetic manipulation [10, 11] and easy *in vivo* infection of a genetically tractable native host [12] and thus remains the model of choice for NoV studies. Human noroviruses and MuNoVs [13] belong to the *Norovirus* genus within the *Caliciviridae* family of small, non-enveloped, positive sense,

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Keywords: Norovirus; murine; recombination; evolution; fitness; reverse genetics.
Abbreviations: HuNoV, Human norovirus; MuNoV, Murine norovirus; NoV, Norovirus; RecE, Virus progenies resulting from the first passage of RecMNV; RecL, Virus progenies resulting from the tenth passage of RecMNV; RecMNV, Viable recombinant MuNoV isolated by Mathijs *et al*, 2010 (29).
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GenBank accession numbers for new sequence data: RecE: KU743153 and RecL: KU743152.
One supplememtary figure and two supplementary tables are available with the online version of this article.

single-stranded RNA viruses [14]. The linear, polyadenlyated 7.4–7.7 kb long HuNoV genome is classically organized into three ORFs, while MuNoV genomes are described to additionally harbour a fourth ORF [15, 16]. The 5′ proximal ORF1 encodes a large polyprotein that is co- and post-translationally cleaved into six non-structural viral proteins (NS1/2 to NS7) [17]. ORF2 and ORF3 encode the structural components of the virion, major and minor capsid protein, VP1 and VP2, respectively. ORF4, entirely overlapping the 5′end of ORF2, encodes a virulence factor (VF1) [18].

Replicative, transmissive, competitive and epidemiological fitness are key elements of the overall viral fitness [19], which conceptually determines how well a virus 'fits' into its environment [20]. Viral ecology is based on complex epigenetic and genetic interactions within the common triad of environment, host and virus. A given virus's ecology is thus governed in part by the particularities of its genetic evolution as it attempts to comply with the biological imperatives of genetic survival and replication [21]. Replicative fitness, defined as 'the capacity of a virus to produce infectious progeny in a given environment', can be investigated by either in *silico*, *in vitro*, *ex vivo* or *in vivo* experiments [19].

Molecular mechanisms mediating viral evolution are the accumulation of point mutations and recombination. While an accumulation of point mutations by virtue of the error-prone RNA-dependent RNA polymerase (RdRp) generally leads more gradually to the generation of quasispecies in RNA viruses [22–25], recombination can quickly create considerable changes in a viral genome, allowing for complete antigenic shifts, host jumps and both pathogenesis and fitness modifications [26]. A change of large genomic regions can highly impact the fitness of a novel recombinant virus, but can also provide the virus with new arms regarding its transmissive, competitive and epidemiological fitness [27].

While many field recombination events, predominantly at a typical ORF1/2 recombination breakpoint [28], have been detected *in silico* in the *Norovirus* genus [29, 30], few experimental data are available concerning NoV recombination under laboratory conditions and the mechanism(s) involved are poorly characterized [29]. The first *in vitro* experimental evidence of NoV recombination was provided by Mathijs *et al.* [31], describing the detection and isolation of a single viable recombinant virus from an infectious centre assay following coinfections of mouse leukaemic monocyte-macrophage cells (RAW264.7) with the two homologous parental MuNoV strains MNV1-CW1 and WU20 (87% nucleotide sequence similarity). The ensuing recombinant, RecMNV, composed of a WU20-related ORF1 and CW1-related ORFs 2, 3 and 4, was shown to exhibit reduced *in vitro* fitness compared to its parental strains [31], while nevertheless retaining *in vivo* infectivity (albeit also with a slight reduction of infectivity as measured by comparing weight loss, viral loads in faeces, blood and various organs of RecMNV infected mice) [32].

In the present study, we evaluated the replication capability of previously *in vitro*-generated recombinant MuNoV RecMNV at early (RecE) and late (RecL) stages of an *in vitro* replicative fitness adaptation experiment. We associated population-level genetic modifications to observable phenotypic profiles of viral fitness. Fitness loss of RecMNV was thus linked to a C7245T mutation and functional VP2 (ORF3) truncation; individual and cumulative compensatory effects of one non-synonymous VP1 (OFR2) and two NS1/2 synonymous ORF1 consensus level mutations acquired during successive rounds of *in vitro* replication were demonstrated, suggesting that interactions of viral proteins and/or RNA secondary structures of different ORFs may play a role in the regulation of replicative fitness post recombination. This *in vitro* model simulates the adaptation process (genetic drift) of NoVs after a recombination event (genetic shift); it supplements the scarce experimental data available concerning MuNoV recombination and may also further a conceptual understanding of the mechanisms behind HuNoV evolution.

METHODS

Viruses and cells

The murine macrophage cell line RAW264.7 (ATCC TIB-71) was maintained in Dulbecco's modified Eagle's medium (Invitrogen) containing 10% heat inactivated foetal calf serum (FCS) (BioWhittaker), 2% of an association of penicillin (5000 SI units ml^{-1}) and streptomycin (5 mg ml^{-1}) (PS, Invitrogen) and 1% 1 M HEPES buffer (pH 7.6) (Invitrogen) at 37 °C with 5% CO_2.

BHK cells engineered to express T7 RNA polymerase (BSR-T7 cells, obtained from Karl-Klaus Conzelmann, Ludwig Maximilian University, Munich, Germany) were maintained in DMEM containing 10% FCS, penicillin (100 SI units ml^{-1}) and streptomycin (100 µg ml^{-1}), and 0.5 mg ml^{-1} G418.

Murine NoV isolate RecMNV [31] was propagated in RAW264.7 cells as described by Mathijs *et al.* Initial RecMNV progeny was produced by infection of RAW264.7 cells at a m.o.i. (expressed as plaque-forming units per cell) of 0.05. Two days post-infection, cells and supernatant were harvested and clarified by centrifugation for 20 min at 1000 ***g*** after three freeze/thaw cycles (–80 °C alternating with 37 °C). Supernatants were purified by ultracentrifugation on a 30% sucrose cushion in a SW28 rotor (Beckman Coulter) at 23000 rounds per min for 2 h at 4 °C. Pellets were suspended in 500 µl PBS, aliquoted and frozen at –80 °C. Titres were determined via the $TCID_{50}$ method. For this, RAW 264.7 cells were seeded in 96-well plates, infected with tenfold serial dilutions of MuNoV, incubated for 4 days at 37 °C with 5% CO_2 and finally stained with 0.2% crystal violet for 30 min. The titres, expressed as $TCID_{50}$ ml^{-1}, were calculated according to the Reed and Muench transformation [33].

RecMNV *in vitro* serial replication

To evaluate the capability of replicative fitness adaptation of *in vitro*-generated recombinant MuNoV RecMNV in cell culture, RecMNV was serially replicated in RAW264.7 cells over nine passages. Briefly, monolayers of 5×10^6 RAW264.7 cells were initially infected with RecMNV at a m.o.i. of 0.05

and were incubated for 72 h. Following this, fresh RAW264.7 cell layers were infected with 100 µl supernatant from the preceding passage. The procedure was repeated eight times. The remaining supernatants were centrifuged at 1000 ***g*** for 20 min to remove cell debris and were stored at −80 °C until further analyses. Virus progenies resulting from the initial RecMNV production and those generated following the ninth passage of RecMNV are henceforth referred to as early (RecE) and late (RecL) recombinant progenies.

Plaque size analysis and replication kinetics of early (RecE) and late (RecL) RecMNV progenies

Two independent lysis plaque assays were performed in triplicate in RAW264.7 cells with RecE and RecL. Viral plaque sizes (15 discrete and well-isolated plaques were randomly selected per virus and per triplicate) of RecE and RecL were measured at 48 h p.i. with the open source image processing program ImageJ [34].

To compare infectivity between the progenies, a standardized production of RecE and RecL was performed. Per progeny, triplicate RAW264.7 monolayers in six-well plates were infected at a m.o.i. of 0.01 ($TCID_{50}$/cell). After 24 h p.i., total virus was released by three freeze/thaw cycles, clarified at 3000 r.p.m. for 20 min, and total viral progeny titres were analysed via $TCID_{50}$ (biological and technical triplicates).

Early (RecE) and late (RecL) RecMNV progenies sequence analysis

RNA was extracted from 150 µl of viral suspensions using the NucleoSpin RNA virus kit (Macherey-Nagel) according to the manufacturer's instructions. For genomic DNA depletion, the total RNA was treated with 4 MBU of Baseline-ZERO DNase (Epicentre) in a total volume of 60 µl. The reaction was incubated 15 min at 37 °C and inactivated by a bead-based purification step using the Agencourt AMPure XP (Beckman-Coulter). First-strand cDNA synthesis was performed using SuperScript IV reverse transcriptase (Thermo Fisher Scientific) according to the manufacturer's protocol. Briefly, 10 µl of DNase-treated total RNA was combined with oligonucleotide primers MNV-tail: TTTTTTTTTAAAATGC ATCTAACTACCAC (2.5 µM) and MNV-2745: CTCACGAT CAGCGAGGTAGTC (0.1 µM), dNTPs (10 mM: Promega) and nuclease-free water. Reactions were incubated at 65 °C for 10 min and cooled on ice for 5 min. A second reagent mix was added containing SuperScript IV enzyme (200 U: Thermo Fisher Scientific), RNasin Plus RNase Inhibitor (40 U: Promega), 0.1 M dTT (Thermo Fisher Scientific), before incubating at 50, 55 and 60 °C for 30 min, successively. A final incubation at 80 °C for 10 min was performed for inactivation. Second-strand synthesis was performed using NEBNext mRNA second-strand synthesis module (New England Biolabs) as per the manufacturer's instructions. The resulting dsDNA was purified using Agencourt AMPure XP (Beckman-Coulter) beads according to the manufacturer's instructions and samples eluted in 45 µl of nuclease-free water. Double-stranded cDNA samples were quantified using the Quantifluor dsDNA system (Promega) with the Quantus Fluorometer (Promega). One nanogram of each dsDNA sample was used to prepare sequencing libraries using the Nextera XT DNA Sample Preparation Kit (Illumina) according to the manufacturer's instructions. The libraries were quantified with the Kapa library quantification kit Illumina platforms (Kapa Biosystems) and the insertion size was verified using the Agilent Bioanalyzer with the high-sensitivity DNA kit (Agilent Technologies). Twenty-two libraries were multiplexed using standard Illumina indexing primers. Sequencing was performed using a MiSeq reagent kit version 3 (Illumina) with 2×300 bp paired-end sequencing on a MiSeq Benchtop Sequencer (Illumina).

Bioinformatics

The quality of the raw MiSeq sequence data of each library was assessed using FastQC v0.11.3 (http://www.bioinformatics.babraham.ac.uk/projects/). Trimming was performed using Trim galore! v0.3.8 (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) based on quality (*Q* score >30) and length (length >80 bp, 5′ clip for R1 and R2=20). To obtain near full-length genomes for RecE and RecL, the trimmed reads were assembled *de novo* using SPAdes v3.9.0 with *k* values 21, 33, 55 and a subsample of 10000 or 13, 200 paired-end reads, respectively [35]. Reads from RecE and RecL were aligned to either the parental sequences MNV CW1 (GenBank accession number DQ285629) and WU20 (GenBank accession number EU004665.1) or each other using the Burrows–Wheeler Alignment tool (BWA) [36]. The alignments were sorted using Samtools v1.2 [37], converted using GATK v3.6.0 [38], and PCR duplicates were removed using Picard (http://broadinstitute.github.io/picard/). Subsequently variants were called using LoFreq [39].

Statistics

Statistical analyses of plaque size diameters determined with the Image J software were performed with SAS edition studio (SAS, Institute, Cary, NC, USA) using the NPAR1WAY procedure (non-parametric tests) or analysed using GraphPad Prism 7 (Graph-Pad Software) and *P*-values were determined with the non-parametric Mann–Whitney test, where $^{****}P<0.0001$, $^{***}P<0.001$, $^{**}P<0.01$, $^{*}P<0.05$, and ns is $P\geq0.05$.

GenBank accession numbers

The consensus nucleotide sequences of the near full genomes of RecE and RecL were deposited in GenBank/EMBL/DDBJ under the accession no. KU743153 and KU743152, respectively.

Generation of an inter-MNV chimeric plasmid

An inter-MNV chimeric cDNA was generated to contain a recombinant 'carbon copy' genome sequence ($RecMNV_{cc}$) of parental strains WU20 (before the recombination breakpoint) and CW1 (after the recombination breakpoint) under the control of a truncated T7 RNA polymerase promoter. Wherever SNPs were identified between RecE and its respective parental strains as encoded in GenBank, the position was

sequenced (post reverse transcription-PCR amplification) in the true, biological parental virus population to verify its presence or absence therein. If already present in a parental population, the mutation was considered to have been acquired prior to the recombination event in question (via genetic drift during generation of virus stocks) and was included in $RecMNV_{cc}$.

To build RecMNVcc, a WU20 insert obtained by PCR amplifying the WU20 ORF1 region from infectious virus stock was embedded into a pT7: MNV 3′Rz CW1 infectious clone backbone [40] containing a NotI restriction site in the m53 stem loop via Gibson assembly.

Generation of pT7: MNV 3′Rz M53 NotI

Briefly, to insert a *Not*I restriction site into pT7: MNV 3′Rz at the site of the m53 stem loop (GACCCCGC to GCGGCCGC at nt position 5024–5031), site-directed mutagenesis was performed by overlap mutagenic PCR with KOD Hot Start polymerase (Novagen) using primers IGUC3715 and 6042R (PCR1) and IGUC3716 and 3848F (PCR2) (see Table S1, available in the online version of this article). The resultant PCR products were used as templates for a third PCR with primers 3848F and 6042R to generate an amplicon containing the inserted *Not*I restriction site and flanked by *Afe*I and *Sac*II restriction sites. After *Afe*I and *Sac*II (New England Biolabs) digestion, the PCR3 product was ligated into *Afe*I- and *Sac*II-digested and dephosphorylated (Antarctic Phosphatase, New England Biolabs) pT7: MNV 3′Rz. The sequence of pT7: MNV 3′Rz N53NotI was confirmed using primers 3848F, 4450F and 6042R.

PCR amplification of WU20 ORF1

To enable the generation of an ORF1 WU20 cDNA, RNA was extracted from infectious virus stock (Epoch Life Science, EconoSpin All-in-One Mini Spin Columns), DNAse purified, and copied into oligo (d)T- and random hexamer-primed cDNA using SuperScript III (Invitrogen). Phusion high fidelity polymerase (New England Biolabs) was used to amplify a 5 kb region of WU20 cDNA, using 5′ primer IGUC3720 (containing the truncated T7 polymerase promoter sequence (of the pT7: MNV 3′Rz plasmid) and partial 5′ sequence of WU20) and 3′ primer IGUC3721 [containing the recombination site (sequence identity between WU20 and CW1)] (see Table S1).

Gibson assembly of pT7: MNV 3′Rz M53 NotI and WU20 ORF1

The pT7: MNV 3′Rz M53 NotI vector was cut with restriction enzymes *Ale*I and *Not*I-HF. A Gibson assembly (New England Biolabs) was set up with 100 ng gel purified vector and 200–300 ng column purified WU20 insert, according to the manufacturer's instructions. During the Gibson assembly process, the NotI site, previously inserted for cloning purposes, was removed. Following transformation into and recovery from NEB 5-alpha Gold Competent Cells (New England Biolabs), the identity of RecMNV was confirmed by sequencing with ten overlapping primer pairs covering the entire recombinant NoV genome (see Table S1). Three single nucleotide polymorphisms (SNPs) attributable either to PCR or cloning errors were corrected via site-directed mutagenesis to generate a perfect 'carbon copy' recombinant of parental strains WU20 and CW1.

Cloning of point mutations into a lab-generated inter-MNV chimeric plasmid

Following the generation of RecMNVcc, one consensus-level synonymous and three non-synonymous point mutations identified as entirely novel either to RecE or RecL populations (Table 1a, b, Fig. S1), were cloned into $RecMNV_{cc}$ via site-directed mutagenesis to generate five different mutant constructs, $RecE_{(C7245T)}$ (C7245T present in RecE and RecL), $RecMNV_{(C7245T\_T697C)}$ (T697C present in RecL), $RecMNV_{C7245T\_G234A}$ (G234A present in RecL), $RecMNV_{C7245T\_A5864G}$ (A5864G present in RecL), $RecL_{(C7245T\_G234A\_T697C\_A5864G)}$, in which unique RecE or RecL mutations were either isolated or combined. Insertion of the desired mutation was confirmed by sequencing. Details on the cloning strategy and primers used for the generation of the five different mutant constructs may be found in Table S2.

DNA-based reverse genetics to recover inter-MNV mutant viruses

Virus was rescued from the six RecMNV cDNA clones, wild-type pT7: MNV 3′Rz CW1 (as positive control) and the full-length cDNA clone of polymerase active site mutant pT7: MNV POL-3′Rz in which the NS7 active site is mutated from YGDD to YGGG (as replication-defective control) [41] by using the reverse genetics system based on recombinant fowlpox virus expressing T7 RNA polymerase, as previously described [10, 40]. Briefly, 1 µg of each cDNA expression construct was transfected, using Lipofectamine 2000 transfection reagent (Invitrogen), into BSR-T7 cells previously infected with recombinant fowlpox virus expressing T7 RNA polymerase at a m.o.i. of approximately 0.5 p.f.u. per cell (based on the virus titre in chick embryo fibroblasts). At 48 h post cDNA transfection, three freeze/thaw cycles at −80 °C/37 °C were performed to release virus particles from cells and infectious virus titres were determined as $TCID_{50}$ in RAW 264.7 cells using tenfold serial dilutions typically over a range of undiluted neat virus to 10^{-7}. The viral $TCID_{50}$ ml^{-1} of biological triplicates was determined by scoring signs of cytopathic effect (CPE) using microscopic visualization and crystal violet staining at 4 days post infection.

M.o.i.-controlled mid-point replication (P1) of inter-MNV mutant constructs

Mid-point passaging under standardized conditions [10 h infection, m.o.i. of 0.01 ($TCID_{50}$/cell)] of all six infectious recombinant constructs was carried out in RAW264.7 monolayers. To release infectious viruses of this first passage (P1) from cells, three freeze/thaw cycles were subsequently performed.

Table 1. Nucleotide changes present at consensus and sub-consensus level (under 50%) of the viral population of RecE (left), and RecL (right), as called using LoFreq and mapped against the parental strains, WU20 (GenBank: EU004665.1) and CW1 (GenBank: DQ285629.1), in ORF1/2 and ORF3, respectively. Positions corresponding to WU20 (left: nt 112 to 4865; right: nt 5290 to 7245) are shaded light grey; positions corresponding to CW1 (left: nt 5166 to 7354; right: nt 61 to 4961) are shaded dark grey

nt position	Parental strain	RecE	Raw depth	Frequency (%)
112	T	A	4126	99.95
193	T	A	7933	1.09
197	C	G	7996	1.06
198	G	C	8047	1.06
199	C	G	8020	1.05
203	T	A	8323	1.21
360	A	G	12743	1.08
697	T	C	14431	3.39
711	A	G	14072	1.94
829	C	T	14909	0.30
1039	A	G	14296	0.51
1503	A	G	16768	0.54
1683	A	G	17706	1.32
2030	C	A	18558	0.66
2269	A	G	20062	99.88
2532	T	C	18825	99.90
2804	C	G	12273	3.22
2978	C	T	12001	99.86
3164	C	T	12563	0.93
4607	T	C	18390	98.92
4865	A	G	18337	99.95
5166	C	T	16582	0.34
5461	C	T	16394	0.62
5484	C	T	15954	1.45
5502	T	G	15622	0.56
5613	C	T	15629	0.19
5664	C	T	15982	0.25
5864	A	G	11687	1.54
6089	A	G	16096	0.31
6117	T	C	15917	0.54
6458	T	C	19083	0.52
6534	C	A	20580	0.21
6610	A	G	21335	2.04

Continued

Table 1. Continued

nt position	Parental strain	RecE	Raw depth	Frequency (%)
6625	C	T	21262	0.57
6630	C	T	21366	0.30
6676	C	T	21467	0.40
6677	A	G	21401	0.53
7245	C	T	9718	99.73
7354	C	T	1619	1.23
61	A	G	62	9.67
112	T	A	145	99.31
134	T	C	182	10.43
234	G	A	339	99.70
641	T	A	532	1.31
697	T	C	499	99.59
716	T	C	462	1.51
761	C	T	468	13.88
824	C	T	509	1.37
1077	A	G	565	1.76
1107	A	G	567	2.99
1540	A	G	708	1.55
1727	A	G	636	1.25
1995	G	T	710	2.53
2057	C	T	721	2.63
2211	A	G	814	2.08
2269	A	G	850	99.76
2498	C	T	106	1.32
2532	T	C	976	99.48
2643	C	T	658	2.73
2741	T	C	376	7.44
2978	C	T	376	100.00
2993	G	A	375	1.33
4097	C	G	538	8.55
4607	T	C	691	100.00
4727	A	G	742	1.48
4742	G	A	736	6.52
4865	A	G	657	99.84
4961	A	G	613	6.19
5290	C	T	542	1.84
5631	C	T	626	1.43

Continued

Table 1. Continued

nt position	Parental strain	RecE	Raw depth	Frequency (%)
5703	C	T	610	4.91
5864	A	G	432	72.22
6372	C	T	613	2.28
6510	G	A	703	2.27
6531	C	T	692	3.75
6534	C	A	697	4.87
6534	C	G	697	12.19
6657	A	G	720	1.52
7215	T	C	381	2.09
7245	C	T	330	99.69

Frequency: count (the number of times a particular nt occurs)/coverage.
nt: nucleotide.

Demonstration of VP2 functional truncation via Western blot

To analyse protein expression and specifically reveal VP2 functional truncation generated as a result of the C7245T mutation, BSR-T7 cells were harvested for Western blot analysis 48 h post-transfection with C7245T-mutated wild-type cDNA. Briefly, cells were lysed in RIPA buffer [50 mM Tris/HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS] and analysed subsequently by Western blot using a rabbit polyclonal antiserum to the minor capsid protein VP2 as described in [40].

Plaque size analysis and end-point replication kinetics of inter-MNV mutant constructs

To compare infectious virus titres of the six P1 inter-MNV constructs, $TCID_{50}$ assays were performed in RAW 264.7 cells using tenfold serial dilutions (as described above). Plaque assays were performed and analysed in RAW264.7 cells for each of the six constructs. Viral plaque sizes (25 discrete and well-isolated plaques were randomly selected per virus and per triplicate; i.e. n=75 plaques/virus) of each inter-MNV construct were measured at 48 h p.i. with the open source image processing program ImageJ.

RESULTS

Early and *in vitro* serially replicated late recombinant murine norovirus RecMNV progenies display differences in plaque sizes and replication kinetics

Differences of *in vitro* replicative fitness of RecMNV progeny RecE ('early'; prior to *in vitro* replication) and serially replicated, 'late' recombinant murine norovirus progeny RecL were analysed by comparing plaque sizes and replication kinetics. Plaque phenotypes showed that diameters of RecL were significantly larger (0.5 mm^2) than those of RecE (0.1 mm^2) (Fig. 1a). Standardized single-step replication of RecE and RecL and analysis of viral progenies via $TCID_{50}$ showed viral titres to differ by two orders of magnitude (2 log10) with mean values of $2.58\pm0.44\times10^5$ $TCID_{50}$ ml^{-1} for RecE and $1.00\pm0.55\times10^7$ $TCID_{50}$ ml^{-1} for RecL (Fig. 1b). Thus, both the plaque size analysis and standardized production of RecE and RecL progenies indicated a replicative fitness adaptation of RecMNV over intervening steps of viral amplification.

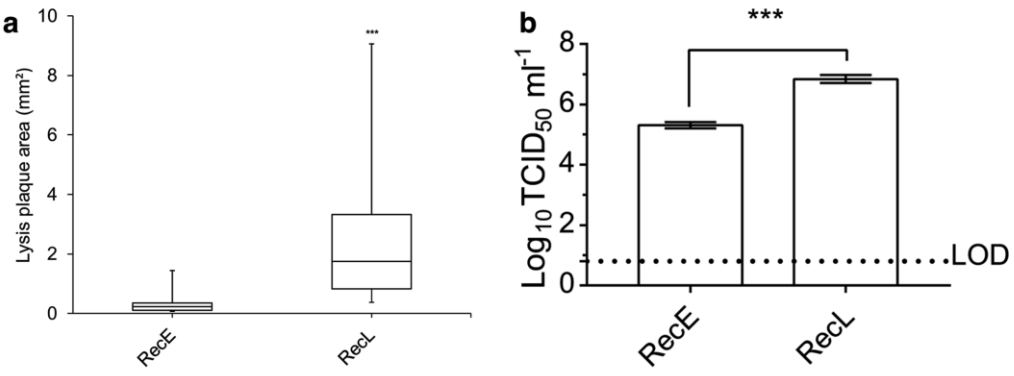

Fig. 1. Lysis plaque size comparison and analysis of viral progeny titres of RecE (a) and RecL (b). Plaque size quantification was performed on discrete, well-isolated plaques. The increase of mean surface area of the plaques from 0.1 to 0.5 mm^2, as determined with the Image J software and statistically analysed with procedure NPAR1WAY of SAS edition studio (SAS, Institute, Cary, NC, USA), is shown in (a). ***: P<0.001. Standardized production of RecE and RecL (infection of six-well plates at m.o.i. 0.01, 24 h incubation) and analysis of viral progeny titres via $TCID_{50}$ (biological and technical triplicates) shows viral titres differing by two orders of magnitude (2 log10) with mean values of $2.58\pm0.44\times10^5$ $TCID_{50}$ ml^{-1} reported for RecE and $1.00\pm0.55\times10^7$ $TCID_{50}$ ml^{-1} for RecL (b). P-values were computed by using a two-sided independent sample t-test. ***: P<0.001.

Next-generation sequencing (NGS) of early and late RecMNV progenies reveals a C7245T mutated VP2 in both populations and three further point mutations in the late RecMNV consensus sequence.

To associate differences in replicative fitness between RecE and RecL populations to changing molecular characteristics within the respective viral populations, MiSeq Illumina NGS was performed for RecE and RecL. Near-full-length, 7362 nucleotide-long consensus genomes were obtained by *de novo* assembly for RecE and RecL. The median read depth for both samples was at least 583 with 99.6% of the bases covered at least 20 times for RecE and 98.5% for RecL. These coverage rates allowed the confident detection of low-frequency single nucleotide variants [42]. The consensus sequence of RecE (GenBank accession number: KU743153) was compared to the corresponding sequences of its parental strains as encoded in GenBank. The recombination breakpoint was confirmed to be located at the ORF1/ORF2 junction in a 123 base pair large region of complete sequence identity (nucleotides 4968 to 5090) between the parental isolates. Thus, the complete RecE ORF1 sequences were matched against WU20 (GenBank: EU004665.1), while ORF2 and ORF3 sequences were compared to the corresponding regions of CW1 (GenBank: DQ285629.1) (Table 1). Wherever SNPs were identified at consensus level (over 50%) within the RecE population and its respective parental strains as encoded in GenBank, the position was sequenced in the original parental WU20 or CW1 virus population to verify its presence or absence therein. If already present in a parental population, the mutation must have been acquired prior to the recombination event in question (via genetic drift during generation of virus stocks) and was thus not included in further investigations. Accordingly, a single nucleotide transition from C (CW1) to T (RecE) at position 7245 was identified to have introduced a stop codon (Gln→Stop187) in ORF3, resulting in a 20 amino acid truncated VP2 in RecE (Table 2a, b).

The RecE consensus sequence was then mapped against that of RecL to identify mutations appearing between the two populations (and potentially associated the observed differences in replicative fitness) (Table 2a). A comparison of the consensus genome sequences of RecE and RecL (GenBank accession number: KU743152) revealed three nucleotide changes in total. Within NS1/2 (ORF1), two changes at positions 234 (G to A) and 697 (T to C) occurred, both of which resulted in amino acid mutations at positions 77 (Gly→Ser77) and 231 (Leu→Pro231), respectively (Table 2b). Interestingly, due to the non-silent mutation at nucleotide position 234, the RecL sequence corresponded to that of CW1 at the same position in both its nucleotide and amino acid sequence, reflecting the WU20 non-structural region 'picking up' a codon present in the corresponding region of CW1. ORF2 of RecL harboured a novel synonymous mutation at position 5864 (A to G), while the previous change at position 7245 was maintained in ORF3 of RecL.

The relative percentages of mutations (variants) within the population were determined after mapping the processed MiSeq Illumina sequencing reads to the respective reference sequence. Except at nucleotide positions 697 and 5864, variants hitherto reported were present at >98% within both populations and can confidently be viewed as stably established within the population. At position 697, 3.39% cytosine (C) and at nucleotide position 5864, 1.54% adenine (A) were present within the RecE population. Indicating a positive selection over the interim passages, these values mounted to 99.59 and 72.22%, respectively in RecL.

Table 2. Nucleotide changes (a) and non-synonymous mutations (b) between the consensus sequences of RecE, RecL and the parental strains WU20 and CW1. The relative percentages of mutations (variants) within the population were determined after mapping the processed MiSeq Illumina sequencing reads to the respective reference sequence, WU20 (GenBank: EU004665.1) in ORF1, CW1 (GenBank: DQ285629.1) in ORF2 and 3. Wherever deviating from the reference sequence, positions were sequenced in the respective WU20 or CW1 parental virus population. If already present in a parental population, the mutation was considered to have been acquired prior to the recombination event in question (via genetic drift during generation of virus stocks) and was not included in this table

	ORF1		ORF2	ORF3
	NS1/2			
nt position	234	697	5864	7245
WU20	G	T	A	C
CW1	A	T	A	C
RecE	**G**	**T**	A	**T**
RecL	**A**	**C**	G	**T**

	ORF1		ORF3
	NS1/2		
AA	77	231	190
WU20	Gly	Leu	Gln
CW1	Ser	Leu	Gln
RecE	Gly	Leu	Stop
RecL	Ser	Pro	Stop

Those changes resulting in a non-synonymous mutation (amino acid change) are marked in bold. The parental strain for the respective ORF is shaded in grey. Dotted underlining of nucleotides or amino acids signals changes appearing between the parental strain and RecE. Solid underlining of nucleotides or amino acids signals changes appearing between RecE and RecL. ORF: open reading frame; nt: nucleotide; AA: Amino acid.

Introduction of separate and combined RecMNV point mutations into an inter-MNV chimeric plasmid backbone via site-directed mutagenesis generates six chimeric plasmids

To investigate the effect of individual observed genomic changes within the RecE and RecL populations, an inter-MNV chimeric plasmid was generated by replacing the

ORF1 region of a CW1 cDNA (pT7: MNV 3'Rz [40]) with a PCR-amplified WU20 ORF1 to represent a recombinant 'carbon copy' ($RecMNV_{cc}$) of the parental strains. The unique consensus-level synonymous and three non-synonymous RecE or RecL point mutations identified in previous steps were introduced separately and in combination into $RecMNV_{cc}$ via site-directed mutagenesis, generating five different mutant constructs, $RecE_{(C7245T)}$ (C7245T present in RecE and RecL), $RecMNV_{(C7245T\_T697C)}$ (T697C present in RecL), $RecMNV_{C7245T\_G234A}$ (G234A present in RecL), $RecMNV_{C7245T\_A5864G}$ (A5864G present in RecL), $RecL_{(C7245T\_G234A\_T697C\_A5864G)}$. Insertion of the desired mutation was confirmed by sequencing.

DNA-based reverse genetics allows recovery of six infectious inter-MNV chimeric viruses

A DNA-based reverse genetics system allowed recovery of infectious virus (P0) at similar titres for all six recombinant constructs $RecMNV_{cc}$, $RecE_{(C7245T)}$ (C7245T present in RecE and RecL), $RecMNV_{(C7245T\_T697C)}$ (T697C present in RecL), $RecMNV_{C7245T\_G234A}$ (G234A present in RecL), $RecMNV_{C7245T\_A5864G}$ (A5864G present in RecL), $RecL_{(C7245T\_G234A\_T697C\_A5864G)}$ and wild-type MNV (Fig. 2) demonstrating that no mutation was so deleterious as to impair virus rescue.

A C7245T mutation results in functional truncation of VP2

The presumptive functional truncation of VP2 caused by the C7245T mutation in infectious viral progeny (passage 1) was confirmed via Western blot analysis using a rabbit polyclonal antiserum to the minor capsid protein VP2 as described in [40] (Fig. 3).

A replicative fitness cost of the C7245T VP2 truncation is compensated by separate and cumulative point mutations associated to late RecMNV

Mid-point passaging in RAW264.7 cells at low m.o.i. (0.01) of all six infectious recombinant constructs yielded a standardized passage 1 (P1) stock. Differences in *in vitro* replicative fitness of inter-MNV recombinant P1 progenies were compared using end-point replication kinetics and plaque size comparison and as proxy measurements. Titres of inter-MNV P1 viruses $RecMNV_{cc}$ ($1.36\pm0.08\times10^5$ $TCID_{50}$ ml^{-1}), $RecE_{(C7245T)}$ ($2.42\pm0.17\times10^4$ $TCID_{50}$ ml^{-1}) and $RecMNV_{C7245T\_T697C}$ ($2.42\pm0.08\times10^4$ $TCID_{50}$ ml^{-1}) differed by approximatively one order of magnitude (1 log10). Titres for $RecMNV_{C7245T\_G234A}$ ($7.65\pm0.17\times10^4$ $TCID_{50}$ ml^{-1}) and $RecMNV_{C7245T\_A5864G}$ ($1.36\times10^5\pm0.14$ $TCID_{50}$ ml^{-1}) were similar to that of $RecMNV_{cc}$, while the titre of $RecL_{(C7245T\_G234A\_T697C\_A5864G)}$ was slightly higher at $3.55\pm0.14\times10^5$ $TCID_{50}$ ml^{-1} (Fig. 4c).

The mean surface area of plaques [n=75 (3×25), biological triplicates; mm^2] was shown to differ significantly between the six constructs (Fig. 4b). $RecMNV_{cc}$ plaques were shown to have a mean surface area of $1.821\pm0.1708\,mm^2$, whilst $RecE_{(C7245T)}$ plaques were smaller by a factor of 3.9 ($0.465\pm0.08285\,mm^2$). Plaque sizes of $RecMNV_{C7245T\_G234A}$ (mean surface area $2.261\pm0.2197\,mm^2$; augmentation of plaque size by factor 1.2 as compared to $RecMNV_{cc}$) and $RecMNV_{C7245T\_A5864G}$ ($1.912\pm0.1903\,mm^2$) were shown to be similar to those of $RecMNV_{cc}$, and $RecMNV_{(C7245T\_T697C)}$ plaques with a mean surface area of $0.670\pm0.105\,mm^2$ displayed a factor 2.7 reduction of plaque size as compared to $RecMNV_{cc}$. The surface area of $RecL_{(C7245T\_G234A\_T697C\_A5864G)}$ plaques ($3.866\pm0.2482\,mm^2$) was shown to be 2.1 times larger than that of $RecMNV_{cc}$ plaques indicating a cumulative effect of three mutations in two different ORFs in RecL.

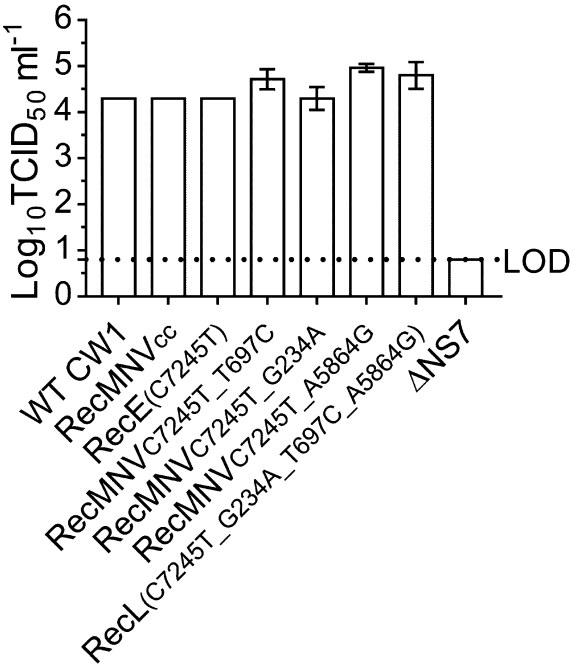

Fig. 2. A DNA-based reverse genetics system allowed recovery of infectious virus (P0) at similar titres for all six recombinant constructs, $RecMNV_{cc}$, $RecE_{(C7245T)}$, $RecMNV_{(C7245T\_T697C)}$, $RecMNV_{C7245T\_G234A}$, $RecMNV_{C7245T\_A5864G}$ and $RecL_{(C7245T\_G234A\_T697C\_A5864G)}$ and wild-type CW1. The mean log10 $TCID_{50}$ ml^{-1} and the standard error of the mean for each of the viruses are determined; formal statistical hypothesis testing, assuming independence between measurements of the infectivity of the wild-type and mutant viruses and computed by using two-sided independent sample *t*-tests showed no statistical differences between RecMNV mutants and WTCW1. WTCW1: plasmid pT7: MNV 3'Rz; ΔNS7: polymerase active site mutant pT7: MNV POL-3'Rz in which the NS7 active site is mutated from YGDD to YGGG.

DISCUSSION

This is the first study in which the capability of replicative fitness adaptation and associated genetic characteristics of a previously *in vitro*-generated recombinant MuNoV were evaluated at early and late time points of serial *in vitro* passaging. Our data provide evidence of viral adaptation to a controlled environment (here a cell-culture system) after a

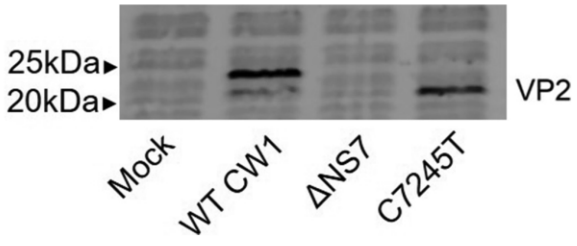

Fig. 3. The presumptive functional truncation of VP2 caused by the C7245T mutation in infectious viral progeny was confirmed via Western blot analysis using a rabbit polyclonal antiserum to the minor capsid protein VP2. The molecular ladder used was BioRad PrecisionPlus Dual colour. Mock: mock infection; WTCW1: plasmid pT7: MNV 3′Rz; ΔNS7: polymerase active site mutant pT7: MNV POL-3′Rz in which the NS7 active site is mutated from YGDD to YGGG.

recombination event, which initially induced a steep reduction of replication capacity [31, 32].

Recombination has previously been shown to incur fitness costs in viruses either as a result of disrupted epistatic interrelationships between the genetic loci of a novel recombinant (this typically between highly divergent viruses) [43] or as a result of point mutations acquired while bypassing the evolutionary bottleneck that is recombination. The recombinant strain RecMNV has been demonstrated to generate smaller plaques and have slower replication kinetics than its parental strains. Its lower replicative fitness was previously putatively associated to a longer cell sequestration before release [31].

Within compact viral genomes that encode only a few proteins, even single non-synonymous mutations can be sufficient to alter the structure or function of virus-encoded proteins to mediate fitness modifications [44, 45]. To investigate the origin of the reduced replicative fitness of RecMNV, i.e. RecE in this study, we investigated its genetic variant spectrum via MiSeq Illumina sequencing and matched its consensus sequence against those of its parental strains. The sole ORF1/2 recombination breakpoint [31] was confirmed, with ORF1 (NS1/2 to NS7) of RecE mapping against WU20, while ORFs 2 (VP1), 3 (VP2) and 4 (VF1) aligned homologously with CW1 sequences. A single C7245T point mutation and consequent introduction of a stop codon (Gln→Stop187) in ORF3 of RecE was shown to have caused a substantial functional truncation in the middle of a predicted VP2 stem-loop structure [46]. Minor capsid protein VP2, encoded by all caliciviruses, is located at the interior of the viral capsid and bound to a conserved motif in the shell domain of major capsid protein VP1. It is postulated to be involved in MuNoV encapsidation via an interaction with viral genomic RNA [17] and acidic regions of VP1 [47] and is held to regulate expression and stability of VP1 in HuNoVs [48]. Feline calicivirus VP2 forms a portal-like assembly following host cell receptor engagement [49]. VP2 integrity has previously been shown to be essential for productive replication of infectious feline calicivirus and attempts at producing infectious viruses after adding stop codons were previously unsuccessful [50]. To confirm the putative deleterious effect of point mutation C7245T in the context of a homologous recombinant background, albeit non-lethal, we implemented DNA-based reverse genetics to rescue both $RecMNV_{cc}$, an inter-MNV chimeric virus representing a perfect 'carbon copy' recombinant of parental WU20 and CW1 sequences, and $RecE_{(C7245T)}$, a $RecMNV_{cc}$ C7245T mutant. Plaque size comparison of inter-MNV chimeric viruses $RecMNV_{cc}$ and $RecE_{(C7245T)}$ indicated a deleterious effect of the C7245T mutation on replicative fitness of $RecE_{(C7245T)}$ viral progenies by a near factor four diminution of viral plaque sizes. Plaque size diameters are proportional to the number of cells that a virus lyses in a given time period. Their size is therefore related to virus productivity and cell-to-cell spread and their analysis is a well-established measure of viral fitness [19, 51–55]. The smaller lysis plaques of VP2 truncated RecE, indicating an inhibition of viral spread, are in line with the recent hypothesis that intact calicivirus VP2 functions as a channel for viral genome release from the endosome into the cytoplasm of a host cell [49]. A viral fitness cost was further confirmed by lower infectious titres of $RecE_{(C7245T)}$.

While initial imprecise recombination events present an evolutionary bottleneck and can induce a fitness cost, they may be followed by a stage of resolution optimizing viral fitness [43, 56]. We here report on a significant increase of plaque size between early and late progenies of recombinant MuNoV RecMNV, demonstrating a replicative fitness regain of the initially disadvantaged RecMNV after successive *in vitro* passaging. The fitness regain was additionally confirmed by differences in the kinetics of viral replication between RecE and RecL shown via a standardized virus production and supported by one-step and multi-step growth curve analyses. Titres of RecL at 24 h p.i. were two orders of magnitude (2 log10) higher than those of RecE and approached those of the parental strains at high m.o.i. To investigate the genetic changes that favoured the selection of viruses with faster replication kinetics and a large plaque phenotype, we obtained the near-complete genomic sequence of RecL. Comparison of RecE and RecL showed the C7245T mutation previously identified in RecE to be maintained in RecL. In addition, two non-synonymous nucleotide changes occurred within NS1/2 and a non-synonymous one in ORF2. NGS analysis allowed us to follow the evolution of RecMNV on a population level, showing not only the apparition of two of the variants at consensus level but also suggesting the positive selection and ultimate establishment of mutations initially represented at sub-consensus level within the population (T697C present at 3.39% in RecE and at 99.59% in RecL; A586G present at 1.54% in RecE and at 72.22% in RecL).

NS1/2, the least conserved NoV NS protein [47, 57], is involved in replication complex formation by associating with components of the endocytic and secretory pathway together with co-localizing NS4 [47, 58–60]. For MuNoVs, a single amino acid change in NS1/2 has been shown to induce a fitness gain in form of colonic tropism and persistence [61]. Here we demonstrate gain of replicative fitness in cell culture

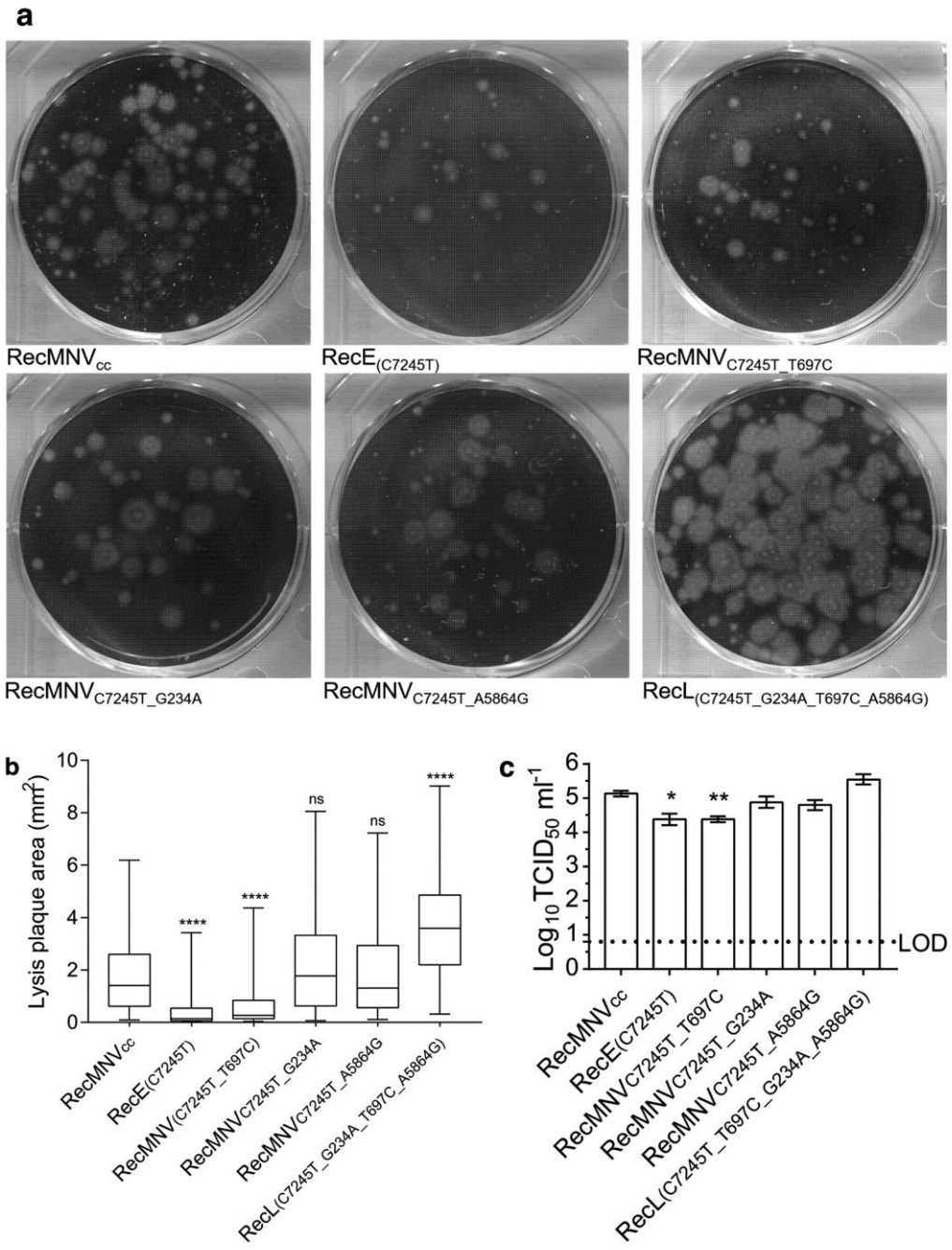

Fig. 4. Lysis plaque analysis, plaque size comparison and analysis of viral progeny titres of infectious inter-MNV chimeric viruses RecMNV$_{cc}$, RecE$_{(C7245T)}$, RecMNV$_{(C7245T_T697C)}$, RecMNV$_{C7245T_G234A}$, RecMNV$_{C7245T_A5864G}$ and RecL$_{(C7245T_G234A_T697C_A5864G)}$.

via acquisition of two non-synonymous NS1/2 mutations. Interestingly the 5′-proximal G234A mutation rescued the fitness cost mediated by 3′-proximal C7245T to higher levels than T697C. It has previously been demonstrated that physical interactions between the 5′ and 3′ ends of the NoV genomic RNA, which are sequence-mediated and further stabilized by cellular proteins, contribute to RNA circularisation and play a role in viral replication [62]. Sequence complementarity

has been shown to direct 5′–3′ end contacts; it is therefore intriguing that the C7245T mutation was followed by G234A, restoring complementarity (A-T to G-C) to a putative pairing.

In addition to non-synonymous mutations, synonymous mutations may also substantially impact viral fitness via non-neutral epistatic effects influencing RNA stability and splicing [20, 63] and silent tuning for increased adaptability [44, 64–66]. Since VP2 has been suggested to interact not only with the internal acidic domains of the calicivirus virion, but also with viral RNA [17, 50], the synonymous A5864G VP1 mutation is interesting in that it might have facilitated interactions between C7245T mutated VP2 and the viral genome.

To investigate the effect of the two non-synonymous NS1/2 mutations as well as the synonymous A5864G ORF2 (VP1) mutation on replication deficient $RecE_{(C7245T)}$, thus mimicking the natural genetic shift of RecMNV populations during serial passaging, inter-MNV chimeric viruses, $RecMNV_{C7245T\_G234A}$, $RecMNV_{(C7245T\_T697C)}$, $RecMNV_{C7245T\_A5864G}$ and $RecL_{(C7245T\_G234A\_T697C\_A5864G)}$, carrying individual and combined mutations were rescued via reverse genetics; the two previously described proxy measurements for replicative viral fitness indicated not only an augmentation of fitness for all three individual mutants but also a cumulative beneficial effect in which the replicative fitness of $RecMNV_{cc}$ was not only matched by $RecL_{(C7245T\_G234A\_T697C\_A5864G)}$ (as indicated by similar viral titres) but surpassed as regards lysis plaque size.

Additional factors such as the presence of different numbers of defective interfering particles can influence the fitness of different virus populations [67] and might have mediated differences between the RecE and RecL populations. This hypothesis was however not supported as Ct values for virus samples of similar titres obtained from a two-step RT qPCR analysis targeting the 5′-end of the MuNoV genome [31] were identical (results not shown).

Conclusion

Our results show that when a recombination event initially disadvantages a nascent chimeric NoV, an initial fitness cost precipitated by this genetic shift can be regained *in vitro* via genetic drift. Sporadic but regular emergence of HuNoV recombinant field strains may be explained with the help of this *in vitro* proof-of-concept model. *In vivo*, putative replicative disadvantages mediated by recombination events, can be compensated by other advantages at the level of competitive or the transmissive fitness (e.g. 'coat switching'), giving the virus time to regain its replicative fitness and even become dominant over its parental strains [29]. Indeed, for NoVs in the field the ability of the viral polymerase to switch templates at the start of ORF2 is considered advantageous, helping viruses to escape the evolutionary bottlenecks of host immune responses by the acquisition of a novel antigenic VP1 [30]; these recombinant viruses probably represent only subset of those that are actually generated, and are the ones that are maintained in the viral population after a rigorous functional selection and accumulation of adaptive point mutations. It is important to identify which parts of the genome are specifically prone to mediate fitness adaptation and to provide information for the production of effective detection and surveillance tools for the screening of emerging NoV strains, including recombinant ones. This study may serve as a starting point for the further development of *in vitro* HuNoV recombination studies in robust cell-culture systems to allow generation and detection of recombinants and elucidation of as yet unresolved mechanics of NoV recombination.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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Supplementary material

Supplementary Table S1

Primers used in the construction of inter-MNV chimeric plasmid RecMNVcc

A) Insertion of NotI restriction site into pT7: MNV 3'Rz for pT7: MNV 3'Rz M53 NotI generation

Name	Sequence	Length (nt)	Position in pT7: MNV 3'Rz
3848F	5' GAAGCCCTGGACATTTAAGAAGGCTTG 3'	27	3848 - 3874
IGUC3716	5' CTGCTGAGCGTTCCT GCGG**CCG**CTCAGCATCCATTGTTC 3'	36	5008 – 5046
IGUC3715	5' GAACAATGGATGCTGAG**CGG**CCGCAGGAACGCTCAGCAG 3'	36	5008 – 5064
6042R	3' CGATCTCCAGTTGCCCAGAGAAATCG 5'	26	6042 – 6067

NotI restriction site marked in bold and underlined.

B) Sequencing of pT7: MNV 3'Rz M53 NotI

Name	Sequence	Length (nt)	Position in pT7: MNV 3'Rz
4450F	5' GCCCTTGCACCACACAGCTGAATAGTTTGG 3'	30	4450 - 4479

C) PCR amplification of WU20 ORF1

Name	Sequence	Length (nt)	Position in pT7: MNV 3'Rz
IGUC3720	5' GCTCACTAGTTAATACGACTCACTATA **GTGAAATGAGGATGGCAACGCC ATCTTCTGCGTCC** 3'	62	11784 – 11810/*35*
IGUC3721	5' GACTGCTGAGCGTTCCTGC**GGGGTCTCAGCATCC** 3'	34	5015/*30* – 5048

WU20 sequences marked in bold and underlined. Overlap with pT7: MNV 3'Rz in Roman script.

D) Sequencing of lab-generated inter-MNV chimeric plasmid RecMNV$_{cc}$

Name	Sequence	Length (nt)	Position in pT7: RecMNV$_{cc}$ 3'Rz
IGIC164	5'ATGACCATGATTACGCCAAGCTCCCCAATACGC 3'	33	11491 - 11523
LLBP1R	3' GCAGACGGCGGCGCCAGGA 5'	19	500 – 5018
LLBP2F	5' CAAAGGAGCCCGTAGTGGGGT 3'	21	400 – 420
LLB2R	3' AGTGCCTTCATAAATTCGGCCCC 5'	23	1323 – 1345
LLBP3F	5' AGACCCAGTGCCCGCCCTG 3'	19	1220 – 1238
LLB3R	3' GGCGGTCACCTTGCCAGCGT 5'	20	2140 – 2159
LLBP4F	5' CACCAAAACCATTGGCGCCACT 3'	22	2039 – 2060
LLB4R	3' GGAAACCGGGGCCTCAAAGCT 5'	21	2979 – 2999
LLBP5F	5' TTCAGGTGGCGACATCCGCG 3'	20	2879 – 2898
LLB5R	3' CGGTTCTCGATGGCATCGCAAACC 5'	24	3800 – 3823
LLBP6F	5' AGAGAGTGGATGGCCCCTCCTTG 3'	23	3700 – 3722
LLB6R	3' CCGTAGCGCTTCAGTACCATAGTG 5'	24	4619 – 4642
LLBP7F	5' CAGGAACCGCCTTCATCGGTG 3'	21	3940 – 3960
LLB7R	3' AGGGTGGTACAAGGGCAACAACC 5'	23	5421 – 5443
LLBP8F	5' CCCTACCTTGCCCACCTCTCAG 3'	22	5323 – 5344
LLB8R	3' TGGTGTCCTGAAAACCGTAGATGG 5'	24	6245 – 6268
LLBP9F	5' AACGCGGACCAGGCCCCCTA 3'	20	6145 – 6164
LLB9R	3' TGGTTAGCGGTGTAGTACCGC 5'	21	7064 – 7084
LLBP10F	5' TCAAAACGGCGCAGCTCCAGG 3'	21	6964 – 6984
LLB410R	3' CCATTCGCCATTCAGGCTGCG 5'	21	7700 – 7720

Supplementary Table S2

Primers used for site-directed mutagenesis of inter-MNV chimeric plasmid RecMNV$_{cc}$

Name	Sequence	Length (nt)	Position in pT7: RecMNV$_{cc}$ 3'Rz
7227F	5'GACCATACGCCGGCGACT**T**AAGGCACCTACACGAACG 3'	37	7227 – 7263
7487R	3' GCCCTCGAGGGTCCCATTCGCC 5'	21	7487 – 7507
LLB11755F	5' GTACCGGTCCGGAATTCCCG 3'	20	11755 – 11774
LLB697R	3' GAGATCTTCGCCCTCTTC**A**GCCAAGTGTCTTTAATCC 5'	37	679 – 715
LLB697F	5'GGATTAAAGACACTTGGC**T**GAAGAGGGCGAAGATCTC 3'	37	679 – 715
LLB1894R	3'GATCTGGCCCGGGCAGCATCCACTACAGGGCTCTCAGC 5'	38	1881 – 1918
LLB11755F	5' GTACCGGTCCGGAATTCCCG 3'	20	11755 – 11774
LLB234R	3' TCAGCACGCGTGCATCAC**T**TCGCGTCACGGGGAGCCC 5'	36	216 – 252
LLB234F	5' GGGCTCCCCGTGACGCGA**A**GTGATGCACGCGTGCTGA 3'	36	216 – 252
LLB1894R	3'GATCTGGCCCGGGCAGCATCCACTACAGGGCTCTCAGC 5'	38	1881 – 1918
LLB5744F	5' TCGACTTGCCCGTGATACAGCCGCGGCTGTGCACG 3'	35	5744 – 5778
LLB5864R	3'AGCAGGGTCCCATCAACG**C**GCACCCTTCCATTCTGCC 5'	36	5846 – 5882
LLB5864F	5' GGCAGAATGGAAGGGTGC**G**CGTTGATGGGACCCTGCT 3'	36	5846 – 5882
LLB6699R	3'CCCATCAGGCCACCTCCAATCGCTCC 5'	26	6699 – 6724

Site of inserted mutation marked in bold and underlined.

Strategy for site-directed mutagenesis of inter-MNV chimeric plasmid RecMNV$_{cc}$:

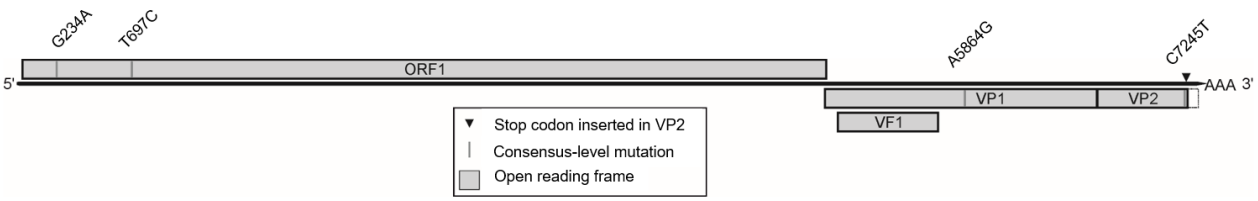
To engineer an ORF3 insert with C→T mutation at nt 7245 by overlap mutagenic PCR, PCR1 used plasmid pT7: RecMNV$_{cc}$ 3'Rz as template, with 7277F (mutagenesis primer containing a unique *Mre*I restriction site) and 7487R as primers. The resulting product contained *Mre*I and *Nhe*HI restriction sites flanking the mutation at position 7245.

To engineer an ORF1 insert with C→T mutation at nt 697 by overlap mutagenic PCR, PCR1 and PCR2 used plasmid pT7: RecMNV$_{cc}$ 3'Rz as template, with either LLB11755Fw and LL697Rev, or LL697Fw and LLB1894Rev as primers. The resulting products, named LLB11755Fw/LL697Rev and LL697Fw/LLB1894Rev, respectively, were purified and used as the template for the PCR3 with LLB11755Fw and LLB1894Rev as primers. Since *Rsr*II and *Srf*I restriction sites were within the LLB11755Fw and LLB1894Rev region, the product of PCR3 contained these two sites as well, flanking the inserted mutation.

To engineer an ORF1 insert with G→A mutation at nt 234 by overlap mutagenic PCR, PCR1 and PCR2 used plasmid pT7: RecMNV$_{cc}$ 3'Rz as template, with either LLB11755Fw and LL234Rev, or LL234Fw and LLB1894Rev as primers. The resulting products, named LLB11755Fw/ LL234Rev and LL234Fw/LLB1894Rev, respectively, were purified and used as the template for the PCR3 with LLB11755Fw and LLB1894Rev as primers. Since *Rsr*II and *Srf*I restriction sites were within the LLB11755Fw and LLB1894Rev region, the product of PCR3 contained these two sites as well, flanking the inserted mutation.

To engineer an insert with A→G mutation at nt 5864 by overlap mutagenic PCR, PCR1 and PCR2 used plasmid pT7: RecMNV$_{cc}$ 3'Rz as template, with either LL5744Fw and LL5864Rev, or LL5864Fw and LL6699Rev as primers. The resulting products, named LL5744Fw/LL5864Rev and LL5864Fw/LL6699Rev, respectively, were purified and used as the template for the PCR3 with LL5744Fw and LL6699Rev as primers. Since *Sac*II and *Bsm*I restriction sites were within the LL5744Fw and LL6699Rev region, the product of PCR3 contained these two sites as well, flanking the mutation.

Supplementary Figure F1



Graphical representation of the murine norovirus genome in which the positions of the four consensus-level point mutations present in early and late recombinant murine norovirus progenies, RecE and RecL (GenBank accession numbers KU743153 and KU743152), are indicated.

Discussion - Perspectives

The accumulation of point mutations (genetic drift) and viral recombination (genetic shift), and the interplay of these two pivotal evolutionary processes, are key mechanisms shaping the evolutionary dynamics and diversity of NoVs.

Increasing evidence indicates that recombination modifies NoV pathogenesis and fitness and contributes to the evolution of emerging HuNoV strains (Ludwig-Begall et al., 2018). The emergence (or re-emergence) of NoV strains may have far-reaching practical consequences for routine diagnostics, typing, and epidemiological surveillance (difficulties generating sequence data from certain recombinant NoV strains were recently reported (Bonura et al., 2021)). NoV evolution in general, and recombination in particular, may further impact the development of vaccines (putatively necessitating regular updates of vaccine valencies) and the administration of antivirals (escape recombination may rescue virus populations from artificially-induced error catastrophe scenarios). It may also have considerable clinical implications should nascent strains display increased morbidity or be responsible for changes in disease severity.

Despite its importance, the mechanisms involved in NoV recombination remain relatively understudied. The conceptual model presented in Chapter 1 (page 67) and in the Objectives of this thesis (page 77) outline the various steps, including their respective putative drivers and constraints, to be successfully bypassed for the generation of a viable recombinant NoV.

While many predictive risk factors constitute confirmed aspects of NoV biology and their role in the context of recombination may thus reliably be inferred, others represent unknown variables that remain to be elucidated. In an update of the previous conceptual model, Figure 12 recapitulates the NoV recombination checkpoints, host coinfection, single cell coinfection, recombination, and functional selection, and attributes a colour code to indicate the level of confidence associated with their drivers and constraints based on perusal of pertinent literature and the experimental *in vitro* results obtained in this thesis.

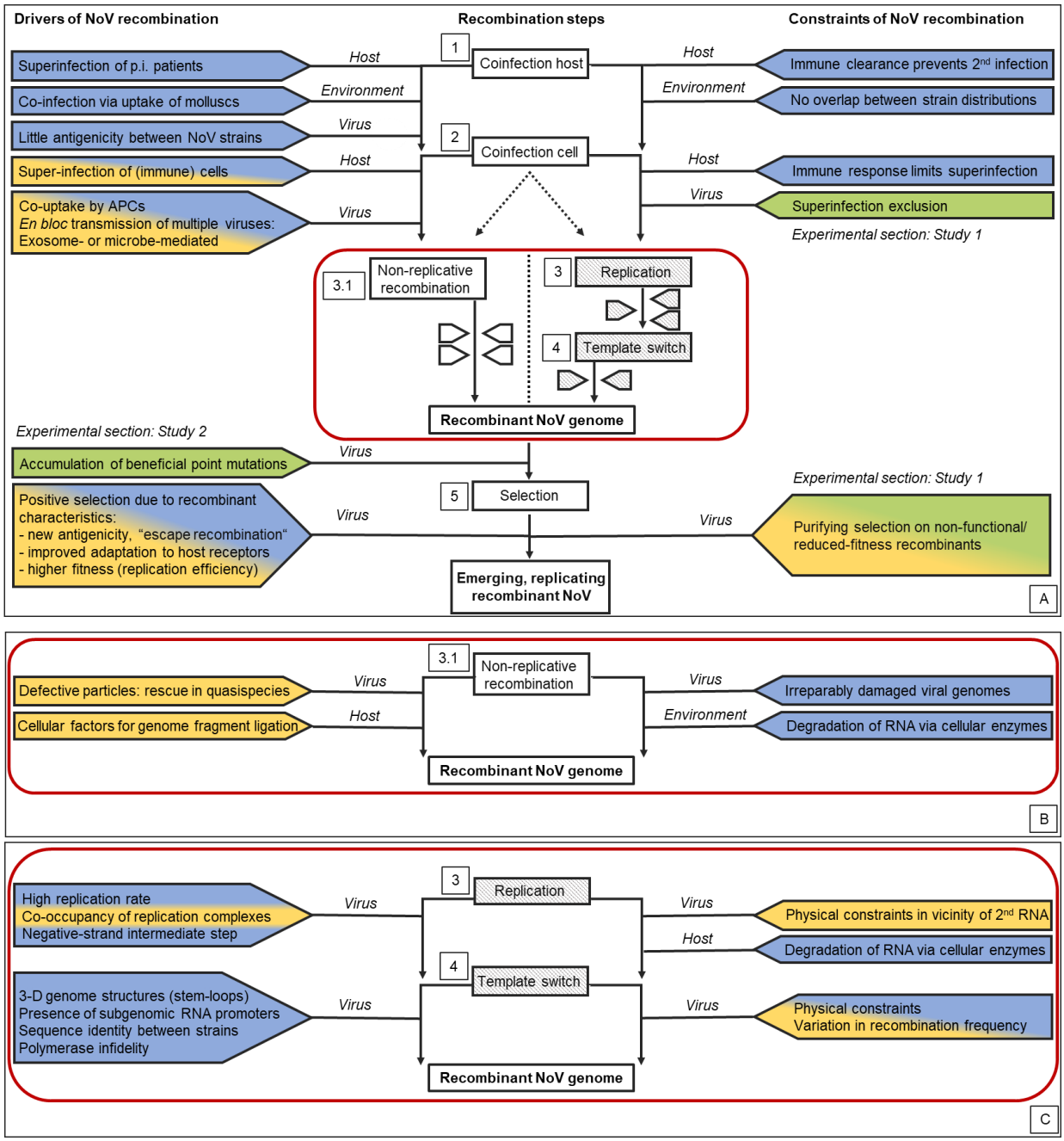


Figure 12. Conceptual model of steps, drivers and constraints of norovirus recombination. (A) gives an overview of the different recombination steps and accompanying host, virus, or environmental drivers and constraints (predictive risk factors). (B) and (C) focus on putative drivers and constraints of both non-replicative recombination and template-switch-mediated recombination, respectively. Blue shading indicates that putative drivers and constraints represent confirmed aspects of norovirus biology (according to pertinent literature). Green shading indicates confirmation of putative drivers and constraints as obtained in the context of this thesis. Yellow shading indicates a degree of uncertainty either as regards the current state of the art or the interpretation of experimental results of the thesis. APC: antigen-presenting cells; p.i.: persistently infected; NoV: norovirus

Host coinfection may be dependent on spatial and temporal overlap of strain-distributions and host immune responses; Chapter 1.6.3 of this thesis describes how epidemiological analyses of contaminated foodstuffs (Mäde et al., 2013), waste-water treatment plants (Blanco Fernández et al., 2011), environmental waters (da Silva Polo et al., 2016), and filter-feeding molluscs grown in effluent-contaminated breeding grounds (Campos et al., 2017; Razafimahefa et al., 2019) have demonstrated overlapping NoV strain-distributions. The strain dependent differences in the induction of protective immune responses (Zhu et al., 2013), antigenic diversity and known lack of heterotypic cross-protection between certain NoV genogroups, genotypes and strains (Rockx et al., 2005a) that confound the determination of immunity duration (Cates et al., 2020) are discussed in chapter 1.6.4; chapter 1.6.6 discussed the clinical analyses that have shown that patients can be infected by more than one NoV strain and that this is indeed a common occurrence in persistently infected individuals (Brown et al., 2017). It appears that, owing to the particularities of NoV epidemiology and transmission, host coinfection thus presents a relatively easily surmountable barrier to NoV recombination.

Cell coinfection, the ultimate prerequisite to viral recombination, depends on factors influencing the within-host distribution of viruses to target cells, thereby limiting or increasing the likelihood of cellular coinfections. In the case of NoVs, true coinfection may be facilitated either by synchronous uptake through consumption of contaminated food or drink (specifically bivalve molluscs carrying mixed virus loads) and/or promoted by other factors directing synchronous uptake of enteric viruses into both host and cell, such as multi-virion binding to intestinal bacteria (Erickson et al., 2018; Jones and Karst, 2018) (see also chapter 1.9.2). However, true coinfection of cells is likely to be a rare event and delayed secondary infections are typically a more probable occurrence.

In the event of an asynchronous infection, the uptake of multiple viruses into a single cell is dependent on factors that may limit consecutive entry of more than one virus particle per cell in a process known as superinfection exclusion (chapter 2.1.6). Superinfection exclusion is defined as the ability of an established virus to prevent a secondary infection by the same or a closely related virus (Folimonova, 2012); the primary infecting virus may render cells refractory to subsequent infection through interference at various stages of the replicative cycle of the secondary invader in a time-dependent manner. Viral pre-and post-entry blocks have been described for a number of RNA viruses (Adams and Brown, 1985; Bergua et al., 2014; Bratt and Rubin, 1968; Claus et al., 2007; Huang et al., 2008; Johnson, 2019; Lee et al., 2005; Tscherne et al., 2007; Zhou et al., 2019); hitherto, NoVs have not been listed amongst them.

In Study 1, we determined the effect of a temporal separation of *in vitro* infections with the two homologous parental MuNoV strains MNV-1 WU20 and CW1 on the composition of MuNoV populations and demonstrated that a time interval from one to two hours onwards between two consecutive NoV infections allows establishment of a barrier that reduces or prevents superinfection; this first demonstration of time-dependent viral interference for NoVs has clear implications for NoV epidemiology, risk assessment, and potentially treatment.

Viral interference has been shown to be an active, virus-controlled process in various RNA virus infections (Bratt and Rubin, 1968; Folimonova, 2012; Huang et al., 2008); examples for superinfection exclusion as directed by positive sense RNA viruses include the cleavage of incoming NS precursors by pre-existing proteases of primary infecting hepatitis C virus (Tscherne et al., 2007) or the dual pre- and post-entry blocks to superinfection launched by bovine viral diarrhoea virus within an hour post primary infection (Lee et al., 2005). Host-cell mediated processes may also intervene in viral interference, this both by induction of the intrinsic intracellular antiviral IFN system (see chapter 1.6.4) and/or activation of cellular RNA silencing. Type I and type II IFNs have been shown to inhibit translation of MuNoV proteins in RAW264.7 macrophage cells (Changotra et al., 2009). In analogy to an IFN pre-treatment of cells, is it possible that the priming of cellular IFN responses via primary infecting WU20 may have initiated interference with superinfecting CW1 in the context of the asynchronous infections performed in Study 1. While skewed input to output ratios of infectious viruses and genomic copies in Study 1 hinted at a role for DIPs or DI RNAs in mediating superinfection exclusion by induction of RNA silencing and the homology-dependent degradation of incoming RNA molecules, these results must be interpreted cautiously since superinfection inhibition may be multifactorial and/or occur at different stages of the viral cycle. Future work will focus on the mechanics and temporal dynamics of NoV interference (pre- or post-entry mode of action analysis), thus aiming to further a deeper understanding of superinfection exclusion and ultimately its influence on NoV recombination both *in vitro* and *in vivo*.

In a follow-up project to Study 1, utilisation of a lab-generated GFP-tagged (or FLAG-tagged) MuNoV infectious clone in co- and superinfection experiments may help elucidate how superinfection exclusion, which has been shown to be overcome by various viral mechanisms after a period of adaptation *in vivo* (Lee et al., 2005; Webster et al., 2013; Zou et al., 2009), plays a role in preventing NoV co-infection *in vitro.* Briefly, a reporter-tagged MuNoV will be generated via insertion of a green fluorescent protein (GFP) reporter gene into a plasmid containing wild type CW1 cDNA under control of a truncated T7 polymerase promoter. Following a construction protocol for gene expression plasmids previously described for HuNoVs (Katayama et al., 2014), the GFP gene will be cloned into ORF1 between NS3 and NS4 of the MuNoV genome (this corresponds to a tolerated insertion site between the NTPase and 3A-like protein in

HuNoVs). The DNA-based reverse genetics system (Arias et al., 2012b; Yunus et al., 2010) described in chapter 1.9.3 of this thesis will be used for recovery of viable infectious viruses carrying the GFP tag. Following virus rescue, cultured murine macrophage cells will be infected synchronously or asynchronously with two homologous MuNoV strains (primary infection: WU20; superinfection: GFP-tagged MNV1-CW1), using staggered superinfection times of 30 minutes to 24 hours to then trace superinfection exclusion by the simple exigency of localising GFP-tagged superinfecting virus via fluorescence microscopy. In circumvention of a possible entry block, synchronous and asynchronous transfection of MuNoV strains may also be investigated.

While mixed populations of co- and superinfecting MuNoVs MNV-1 WU20 and CW1 were identified after plaque picking and amplification of viral progenies, not a single viable recombinant virus was isolated from the molecular screening process performed on a total of 864 plaque-picked infectious progenies (36 plaques x 24 conditions of co- or superinfection). Mathijs et al., 2010, previously demonstrated isolation of MuNoV recombinant RecMNV from an infectious centre assay involving mixed infections of WU20 and CW1 and screening of 332 progeny virions, thus demonstrating that recombination is mechanistically possible between these viruses (Mathijs et al., 2010). Importantly, RecMNV was shown to exhibit a viral fitness loss as evidenced by changed viral replication kinetics and smaller lysis plaque sizes in comparison to its two parental strains (also see Study 2). The absence of viable recombinants in Study 1 does thus not necessarily imply that recombination did not occur when viral coinfection was not impeded by superinfection exclusion. Rather, it may reflect a bias in the methodology where isolation of single viruses relied on plaque picking; if nascent recombinant viruses experienced a loss of replicative fitness similar to that of RecMNV, they may have been “overlooked” in the screening process and/or lost in viral replication steps. To avoid a similar bias in follow-up assays, limiting dilutions may be considered as an alternative for the isolation of single viruses. Future studies could further leverage population-level deep sequencing to analyse how the viral interference effects pinpointed here may influence the generation of non-viable NoV RNA recombinants (and thus ultimately influence the chances of viable recombinant virus generation under the application of selective pressures *in vivo*).

The experimental workflow, notably the order of infection (primary infection: WU20; secondary infection: CW1), was not reversed in the set of experiments presented in Study 1, the assumption being that due to their identical growth curves, high levels of sequence similarity, and the similar input Ct values of the viral progenies used (results not shown), the effects would simply mirror those already observed. However, future confirmation of the reported interference effects might benefit from an inversion of the experimental workflow, the use of input viruses from viral passages with deviating infectious titres to Ct

values (this to account for a putative bias of interfering viral particles), and the use of other MuNoV strains to be juxtaposed against either WU20 or CW1.

Novel *in vitro* systems for HuNoV culture (HIE cultures and BJABs as described in chapter 1.9.2) as well as *in vivo* MuNoV and/or HuNoV platforms (adult or neonatal mice (chapter 1.5.3) and zebrafish larvae (chapter 1.9.1), respectively) which may more closely mirror natural conditions in co- and superinfection assays, may further be utilised to gain a more differentiated picture of how cell coinfection, the second step in the recombination pathway, is accomplished by NoVs. *In vivo* models in particular, involving either the co-infection of mice with MuNoVs or that of zebrafish with HuNoVs, may provide insights into how host immune systems, different subsets of host cells, and also the presence of gut microbiota may positively or negatively impact the occurrence and outcome of NoV recombination.

The third step, generation of a recombinant NoV genome, is typically considered to occur in a replicative process following a framework which combines the copy-choice model of homologous recombination via mid-replication RdRp template switch with an internal initiation mechanism for subgenomic synthesis at the highly conserved ORF1/ORF2 overlap corresponding to the junction of RdRp and capsid sequences (Bull et al., 2007, 2005; Ludwig-Begall et al., 2018) (discussed in chapter 2.3). Sequence analysis of field HuNoV strains has overwhelmingly shown the predominant recombination breakpoint to lie in the highly conserved ORF1/ORF2 overlap (both sequence similarity and the presence of a subgenomic RNA promoter at this locus suggest a similarity-assisted model of NoV recombination; see chapters 2.1.4 and 2.3) and both the standardized NoV nomenclature and current genotyping assays are designed to accommodate this recombination hotspot. However, atypical recombination breakpoints have also been observed (Ludwig-Begall et al., 2018). Recombination in the absence of an obvious RNA promoter or triggering secondary structure has been suggested to indicate that, at atypical recombination sites, recombination may have occurred by other mechanisms than those that induce a breakpoint in or around the ORF1/2 overlap (Bull et al., 2007). The possibility of non-replicative recombination, involving self-ligation or host-factor-mediated joining of randomly cleaved RNA strands, has been demonstrated for other positive-sense single-stranded RNA viruses (Büning et al., 2017; Gallei et al., 2004; Galli and Bukh, 2014; Lowry et al., 2014), and may be considered in this context. An RdRp-independent mechanism of RNA recombination remains unproven for NoVs and was not examined in the context of this thesis. Future assays to elucidate the possibility of non-replicative NoV recombination may follow an experimental design used to prove RNA recombination in the absence of viral replication of pestiviruses, which allowed the generation of recombinant viral genomes following cotransfection of noninfected cells with various pairs of mutagenised nonreplicable RNA derivatives (Gallei et al., 2004).

By whichever way a recombinant NoV genome is ultimately generated, it is by no means a foregone conclusion that the process will result in a replicating recombinant NoV; recombination typically entails significant modifications to a single viral genome and may thus elicit a replicative fitness cost which must be compensated via the adaptive capacity of a nascent recombinant virus for it to survive in a viral population. Indeed, studies in various RNA viruses have shown that circulating recombinants probably only represent a subset of those that are actually generated, and are the ones that are maintained in the viral population after a rigorous functional selection, having bypassed this fifth and final step of successful RNA virus recombination (Bagaya et al., 2017; Banner and Mc Lai, 1991; Lowry et al., 2014).

Study 2 aimed to characterise the adaptive capacity of previously *in vitro* generated WU20-CW1 recombinant MuNoV RecMNV, thus investigating how the accumulation of point mutations through successive viral passaging may compensate for initial replicative fitness losses incurred during recombination processes. By comparing the replicative fitness and genetic characteristics of RecMNV progenies at early and late stages of an adaptation experiment, replicative fitness regain of the recombinant was demonstrated between viral progenies prior to and post serial *in vitro* passaging. Observable phenotypic profiles of viral fitness were associated to population-level genetic modifications. Fitness loss of RecMNV was thus linked to a C7245T mutation and functional VP2 (ORF3) truncation; individual and cumulative compensatory effects of one synonymous VP1 (OFR2) and two non-synonymous NS1/2 (ORF1) consensus-level mutations acquired during successive rounds of *in vitro* replication were demonstrated, suggesting that interactions of viral proteins and/or RNA secondary structures of different ORFs may play a role in the regulation of replicative fitness after a recombination event.

A caveat of the Study 2 NGS approach, whereby whole consensus genome sequences of RecE and RecL were derived from the alignment of fragmented and trimmed MiSeq reads (circa 300 bp read length) via *de novo* assembly, is the underlying assumption for all consensus-level mutations to be present on the same viral genome. While viral populations (or quasispecies) typically cluster around a modal master sequence, it is not necessarily a given that consensus-level SNPs actually accumulate on a single viral genome rather than being dispersed amongst the members of the viral population. To investigate linkage or dispersion of consensus-level SNPs within RecE and RecL populations, it would thus be interesting to apply a nanopore-based sequencing approach that allows analysis of complete viral genomes and differentiation of viral variants (with respect to both consensus- and subconsensus level SNPs) (Reuter et al., 2015; Riaz et al., 2021).

Irrespective of whether mutations are coupled on a single viral genome or are dispersed amongst the viral progeny, this *in vitro* proof-of-concept study simulated successful adaptation (genetic drift) of a nascent NoV population after recombination (genetic shift).

The model demonstrates that an initial fitness cost precipitated by genetic shift can be regained via genetic drift of a recombinant NoV. It serves to conceptualise how the emergence of recombinant HuNoV field strains, held to represent an adapted and functionally selected subset of all generated NoV recombinants, may be regulated by an interplay between the two evolutionary processes of recombination and point mutation accumulation. *In vivo*, putative replicative fitness costs of nascent HuNoV recombinants may be temporarily compensated by other advantages at the level of competitive or transmissive fitness; a nascent virus may regain its replicative fitness via point mutation accumulation and, having undergone a process of functional selection, become dominant within a viral population.

This study may serve as a starting point for the development of *in vitro* or *in vivo* HuNoV recombination studies in robust culture systems and will further the identification of NoV genome segments specifically prone to fitness adaptation mediation. HuNoV *in vivo* models to study NoV recombination and adaptation following recombination may involve infection of zebrafish (as described above). Further studies concerning the adaptation of a MuNoV recombinant to *in vivo* conditions, may feasibly build on prior work performed by Mathijs et al., involving the infection of Balb/cByJ mice with RecMNV; the RecMNV populations resulting from the 48- and 72-hour *in vivo* infections (Mathijs et al., 2016) merit attention with regard to population-level genomic changes putatively incurred during several rounds of viral replication in an immunocompetent host.

The knowledge gained via *in vitro* and *in vivo* studies involving various model systems will provide a more complete picture of the interplay between NoV genetic shift and drift and will provide information for the effective detection and screening of emerging recombinant NoV strains.

In conclusion, this thesis aimed to provide a deeper understanding of the steps, drivers and constraints of NoV recombination via implementation of the *in vitro* MuNoV model. It served to provide a comprehensive overview of the recombination checkpoints to be bypassed and, in investigating both superinfection exclusion as well as functional selection, provided novel insights into prerequisite processes both before and after the generation of a recombinant NoV genome.

It would seem remiss to end this thesis without at least a nod to the father of evolution and I close with a quote by Charles Darwin who wrote, in a statement particularly apt to NoVs, that:

"It is not the strongest of the species that survives, nor the most intelligent that survives. It is the one that is the most adaptable to change."

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