

1 Polyprotein driven formation of two interdependent sets  
2 of complexes supporting hepatitis C virus genome  
3 replication

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5 Running title: Polyprotein-driven protein complex formation  
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17 Abstract: 249 words

18 Importance: 132 words

19 Main body: 8033

## Abstract

Hepatitis C virus (HCV) requires proteins from the NS3-NS5B polyprotein to create a replicase unit for replication of its genome. The replicase proteins form membranous compartments in cells to facilitate replication, but little is known about their functional organization within these structures. We recently reported on intragenomic replicons, bicistronic viral transcripts expressing an authentic replicase from ORF2 and a second duplicate NS polyprotein from ORF1. Using these constructs and other methods, we have assessed polyprotein requirements needed for rescue of different lethal point mutations across NS3-5B. Mutations readily tractable to rescue broadly fell into two groupings; those requiring expression of a minimum NS3-5A and those requiring expression of a minimum NS3-5B polyprotein. A *cis*-acting mutation that blocked NS3 helicase activity, T1299A, was tolerated when introduced into either ORF within the intragenomic replicon, but unlike many other mutations required the other ORF to express a functional NS3-5B. Three mutations were identified as more refractile to rescue; one that blocked cleavage of the NS4B5A boundary (S1977P), another in the NS3 helicase (K1240N) and a third in NS4A (V1665G). Introduced into ORF1, these exhibited a dominant negative phenotype, but with K1240N inhibiting replication as a minimum NS3-5A polyprotein whereas V1665G and S1977P only impaired replication as a NS3-5B polyprotein. Furthermore, a S1977P mutated NS3-5A polyprotein complemented other defects shown to be dependent on NS3-5A for rescue. Overall, our findings suggest the existence of two inter-dependent sets of protein complexes supporting RNA replication, distinguishable by the minimum polyprotein requirement needed for their formation.

## Importance

Positive strand RNA viruses reshape the intracellular membranes of cells to form a compartment within which to replicate their genome, but little is known about functional organization of viral proteins within this structure. We have complemented protein-encoded defects in HCV by constructing sub-genomic HCV transcripts capable of simultaneously expressing both a mutated and functional polyprotein precursor needed for RNA genome replication (intragenomic replicons). Our results reveal that HCV relies on two interdependent sets of protein complexes to support viral replication. They also show that the intragenomic replicon offers a unique way to study replication complex assembly as it enables improved composite polyprotein complex formation compared to traditional *trans*-complementation systems. Finally, the differential behaviour of distinct NS3 helicase knock-out mutations hints that certain conformations of this enzyme might be particularly deleterious for replication.

## Introduction

Exposure to hepatitis C virus (HCV) often results in chronic infection and can lead to liver-related mortality and morbidity. Intensive research efforts since discovery of the virus have translated into improved patient care, with the recent advent of direct acting antiviral (DAA) therapy now providing high cure rates, particularly in genotype (gt)1-infected patients (1). However, certain aspects of viral replication remain poorly understood, including how viral proteins associate with each other and host proteins to form the platforms that support replication of the viral genome.

HCV has a single-stranded, positive-sense RNA genome of 9.6kb in length that contains a single open reading frame (ORF) flanked by 5' and 3' untranslated regions (UTRs) (2). Translation of the ORF is driven by an internal ribosome entry site (IRES) within the 5'UTR (3), resulting in the production of a polyprotein, which is cleaved co- and post-translationally by host- and virus-encoded proteases to give rise to the mature viral proteins. The latter two thirds of the polyprotein encompass the NS3-5B replicase unit (NS3, NS4A, NS4B, NS5A and NS5B), which provides the viral proteins that are sufficient for genome replication (4). NS3 is the protease responsible for NS3-5B processing and also provides essential helicase activity, encoded within its C-terminal domain (5). It is anchored to the ER membrane by both an amphipathic helix and by its protease co-factor NS4A (5). RNA polymerase activity is provided by NS5B (6). The functions of NS4B and NS5A are less well understood, although both proteins bind RNA (7, 8).

In infected cells, viral RNA synthesis occurs in virus-induced cytoplasmic compartments referred to as membranous webs or membrane-associated foci (MAF) (9). Morphologically, these compartments appear as a collection of ER-derived single- and double-membrane vesicles

(10). Current evidence points to the double-membrane vesicles as the site for polymerase activity and hence RNA replication (11). Expression of polyproteins encoding NS3-5A or NS3-5B, leads to the formation of MAF-like structures in the absence of replication (10). Double-membrane vesicles are also present in cells expressing NS5A alone (10), and both drugs and mutations that target NS4B and NS5A disrupt MAF architecture (12-15); this suggests that NS4B and NS5A are key structural components of these assemblies.

Genetic complementation of mutations that block replication offers a way to gain insight into functions of viral NS proteins in RNA replication. From early studies with gt1b replicons, most mutations in the HCV replicase were refractile to rescue, except for a few mutations in the low complexity sequence 1 (LCS1) region between domains 1 and 2 in NS5A (16). Subsequent analysis using replicons encoding strain JFH-1 (gt2a) sequences, which give higher levels of replication, revealed mutations in other NS5A regions as well as NS4B mutations could be rescued, albeit less efficiently than LCS1 mutations (14, 17). The range of mutations that can be rescued has recently been extended to include NS3, NS4A and NS5B by using Venezuelan Equine Encephalitis Virus (VEEV) replicons to express high levels of NS3-5B (18).

All of the above approaches have relied on supplying functional counterparts of proteins *in trans* that are expressed from separate transcripts to rescue defective NS3-5B replicase units. Recently, we reported an alternative system to complement deleterious mutations using intragenomic HCV replicons that carry two ORFs (ORF1 and ORF2), each driven by separate IRES elements. Thus the complementing viral proteins are provided *in trans* from distinct translation units but encoded *in cis* from the same RNA molecule. Two advantages of our system were that a) complementing proteins were translated to a similar level compared to their non-functional counterpart and b) functional and non-functional polyproteins could be translated in

close apposition. Using this approach, enhanced rescue of defective NS5A containing a S2208I mutation could be achieved by expressing a NS3-5A cassette in ORF1 compared to expressing NS5A alone (19). Enhanced rescue of this defective NS5A also correlated with the ability of NS3-5A to target the protein to MAF. However, despite the efficiency with which NS3-5A targeted NS proteins to the MAF, both its ability and the capacity of helper viruses expressing NS3-5B to complement NS4B defects remained limited.

In this study, we set out to understand why rescue of certain mutations remained limited, despite efficient targeting of NS proteins to the MAF by NS3-5A using the intragenomic replicon system (19). By extending the capacity of ORF1 to express NS3-5B, our data reveal the existence of genetically distinguishable complexes that support HCV RNA replication.

## Methods and Materials

*Cell lines.* Generation of the SGR-JFH1neo replicon cell line has been described, as has propagation of this cell line and naive Huh7.5 cells (19, 20).

*Plasmid vectors* - The production of pFBM-based baculovirus constructs for expression of JFH1 NS34A, GFP-tagged versions of JFH1 NS5A expressed either alone or in the context of NS3-5A and NS3-5B, pSGR-FLAG constructs containing G1911A and S2208I mutations, and intragenomic replicon containing plasmids pRep\_R2NS5A<sup>V5</sup>/NS3-5B<sup>FLAG</sup> and pRep\_R2NS3-5A<sup>V5</sup>/NS3-5B<sup>FLAG</sup> with either a functional ORF2, or an ORF2 containing S2208I and G1911A have been described (19).

To extend intragenomic replicon ORF1 to express a Renilla-Foot and Mouth Disease Virus 2A (FMDV2A)-NS3-NS5B polyprotein containing a V5 epitope tag at the COOH-terminus of NS5A, two 1000 bp DNA Strings (Invitrogen) representing an entire synonymous codon altered NS5B sequence + NS5A5B boundary and flanked at the 3' end by a stop codon, *Pme* I site and M13(-20) primer site were synthesized. These DNA Strings along with LIT\_Ren-2A-based vector containing the codon altered NS3-5A<sup>V5</sup> sequence (LIT\_Ren-2A-NS3-5A<sup>V5</sup>) (19) were used as templates in 3 separate PCR reactions involving primer pairs 1 + 2, 3 + 4 and 5 + 6 (Table S1). All three products were combined in a second PCR reaction with primers 1 and 6, and the resulting DNA cloned back into LIT\_Ren-2A-NS3-5A<sup>V5</sup> via *Sal* I and *Pme* I restriction sites forming LIT\_Ren-2A-NS3-5B<sup>V5</sup> (for the codon altered NS3-5B(5A<sup>V5</sup>) sequence see Genbank, accession number KR140016). The Ren-2A-NS3-5B<sup>V5</sup> expression cassette from this vector was cloned into existing pRep\_R2NS3-5A<sup>V5</sup>/NS3-5B<sup>FLAG</sup> based vectors via *Bgl* II and *Pme* I restriction sites to generate pRep\_R2NS3-5B<sup>V5</sup>/NS3-5B<sup>FLAG</sup> constructs where ORF2 was either functional or contained the ΔGDD, G1911A or S2208I mutations. The terminology in the

136 main body of text used to describe the transcripts generated from these constructs is wt<sup>NS3-</sup>  
137 <sup>5B</sup>/wt<sup>NS3-5B</sup>, wt<sup>NS3-5B</sup>/ΔGDD<sup>NS3-5B</sup>, etc.

138 To produce intragenomic replicons expressing Ren-2A-NS34A, Ren-2A-NS5A5B and  
139 Ren-2A-NS5B cassettes, LIT\_Ren-2A-NS3-5B<sup>V5</sup> was used as a template in a PCR reaction with  
140 primers 7 + 8, 9 + 6 and 10 + 6 respectively. The resulting NS coding regions were transferred to  
141 a LIT\_Ren-2A-based vector using *Rsr* II and *Pme* I restriction sites to produce LIT\_Ren-2A-  
142 NS34A, LIT\_Ren-2A\_NS5A5B and LIT\_Ren-2A-NS5B. The expression cassettes from these  
143 vectors were cloned into existing intragenomic replicon containing plasmids via *Bgl* II and *Pme* I  
144 restriction sites.

145 Introduction of the L1157A mutation into pSGR-FLAG (19) was through exchange of an  
146 *Acc65* I - *Spe* I restriction fragment with that contained within the pCITE-NS3-3'/JFH1 vector  
147 containing this same mutation (2). Introduction of the S1977P mutation into pSGR-FLAG was  
148 through exchange of an *Nsi* I – *Rsr* II restriction fragment with that present in pFBM(NS3-  
149 5A<sup>GFP</sup>)S1977P. Introduction of the other lethal mutations into pSGR-FLAG relied on a two-step  
150 PCR reaction using either JFH1 cDNA or pSGR-FLAG as template. Appropriate flanking  
151 primers were used in combination with internal mutagenic primers 11 + 12 (for PP1220-1GG),  
152 13 + 14 (for K1240N), 15 + 16 (for V1665G), 17 + 18 (for Y1706A), 19 + 20 (for S1977P), 21 +  
153 22 (for P2008A), 23 + 24 (for GDD>GAA), 25 + 26 (T1299A) and 27 + 28 (G2323A). (Note:  
154 for the G2323A mutation, a JFH1 template with a small number of silent substitutions including  
155 a 10 nucleotide poly C tract adjacent to the mutagenized codon was used to allow effective  
156 overlapping primer design. The modified sequence also lacked the *Bam*H I site in NS5A but not  
157 in NS4B - sequence is available on request). Fragments generated were cloned into pSGR-FLAG  
158 via *Cla* I and *Spe* I (K1240N), *Cla* I and *Nsi* I (PP1220-1GG + T1299A), *Nsi* I and *Rsr* II



(V1665G + Y1706A + P2008A + S1977P), *Sfi* I and *SnaB* I (GDD>GAA) or *BamH* I and *Rsr* II (G2323A). Transfer of mutations from pSGR-FLAG into ORF2 of intragenomic replicon containing plasmids, took advantage of a set of unique restriction sites in a region of sequence identity shared by the two constructs, between the start of the EMCV IRES through to the end of the 3'UTR.

Mutations in ORF1 of plasmids containing the intragenomic replicons were introduced by a two-step PCR approach using LIT\_Ren-2A-NS3-5A<sup>V5</sup> and/or LIT\_Ren-2A-NS3-5B<sup>V5</sup> as templates. Appropriate flanking primers were used in combination with internal mutagenic primers 29 + 30 (for K1240N), 31 + 32 (for S1977P), 33 + 34 (for GDD>GAA), 35 + 36 (for T1299A), 37 + 38 (for G1911A) and 39 + 40 (for V1665G). The resultant DNA was cloned back into LIT\_Ren-2A-NS3-5A<sup>V5</sup> and/or LIT\_Ren-2A-NS3-5B<sup>V5</sup> via either *Rsr* II and *Mlu* I (K1240N), *Nde* I and *Sal* I (G1911A + S1977P), *Sal* I and *Pme* I (GDD>GAA) or *Not* I + *Mlu* I (T1299A) restriction sites. The same approach was used to introduce the K1240N mutation into LIT\_Ren-2A-NS34A, but with the PCR product being cloned back into this plasmid via *Rsr* II and *Pme* I sites. The expression cassettes from these vectors were cloned into existing intragenomic replicon containing plasmids via *Bgl* II and *Pme* I restriction sites. In the case of the V1665G mutation, cloning was directly into the intragenomic vector via *Not* I + *Mlu* I.

To generate monocistronic replicons, pSGR-FLAG was used as a template in a PCR reaction with primers 41 and 42, and the resulting product cloned into pRep\_R2NS3-5A<sup>V5</sup>/NS3-5B<sup>FLAG</sup> via the two *Rsr* II sites present within the vector to generate pJFH1-mono (the DNA template for transcription of JFH1-mono). Both pRep\_R2NS3-5B<sup>V5</sup>/NS3-5B<sup>FLAG</sup> and pJFH1-mono were used as templates in a two-step PCR reaction with primer pairs 43 + 44 and 45 + 46.

181 The resultant DNA, produced in the second round reaction with primers 43 and 46, was cloned  
182 into pJFH1-mono via *Asc* I and *Sal* I sites to generate the pDVR/JFH1@7667-mono.

183 *Sequence analysis.* Analysis of the synonymous codon-altered sequence to establish the CpG and  
184 UpA motif frequencies was performed using Simple Sequence Editor software (21).

185 *Transient replication assays* – Generation of T7 RNA transcripts and their electroporation into  
186 Huh7.5 cells or Huh7.5 cells previously transduced with baculovirus has been described (19).  
187 For all assays except those looking at the *trans*-dominant impact of K1240N on replication, cells  
188 were electroporated with 2 µg of RNA transcript. In the case of these latter assays, 1 µg of  
189 replication-competent JFH1-mono was transfected with 4 µg of either yeast tRNA or the  
190 replication-defective bicistronic SGR replicon. Cell lysates were harvested in 1 x passive lysis  
191 buffer (Promega) up to 96 hours post-transfection and luciferase activity measured. Firefly and  
192 Renilla luciferase activities were measured using the Bright-Glo Luciferase Assay system  
193 (Promega) and Renilla Luciferase Assay Kit (Biotium) according to manufacturer's  
194 recommendations. For graphical representation, the background signal from mixing Passive  
195 Lysis Buffer only with luciferase reagent was subtracted from the raw data values. On some  
196 occasions where different experiments containing multiple experimental groups were performed  
197 in parallel, control experimental groups were shared between experiments (indicated in figure  
198 legends).

199 *Western blot analysis* – Transfer of proteins to membranes and subsequent detection has been  
200 described (19). Antibodies used were anti-NS3 (BioFront), anti-GAPDH (Chemicon), anti-V5  
201 (gift from Prof. R. Randall, University of St Andrews), anti-FLAG (Biolegend) and anti-GFP  
202 (Biolegend).

203 *Indirect immunofluorescence* – 72 hours post-transfection cells were fixed with methanol at -  
204 20°C for 30 minutes, rehydrated with PBS, and blocked with PBS containing 2% FCS (PBS/FCS)  
205 for 10 minutes. Cells were then probed with anti-V5 and anti-FLAG antibodies for 1.5 hours at  
206 room temperature, washed extensively with PBS/FCS and finally incubated with anti-mouse-  
207 Alexa594 and anti-rat-Alexa488 for 1 hour at room temperature. All antibody incubations were  
208 carried out in PBS/FCS. Cells were washed with PBS/FCS followed by PBS, rinsed with H<sub>2</sub>O  
209 and then mounted with Vectashield (Vector Laboratories) that contained DAPI to stain nuclei.  
210 Images were captured with a Zeiss LSM 710 confocal microscope.

211 *Northern blot analysis* – Cellular RNA, extracted using TriFAST reagent (Peqlabs), was run on a  
212 formaldehyde-agarose gel and transferred by capillary action to positively charged Nylon  
213 membrane using standard procedures. DNA probes were biotinylated using the PlatinumBright  
214 labelling kit (Kreatech). Hybridization was performed using Ultrahyb (Ambion) according to  
215 manufacturer's instructions and bound probe detected using the Bright Star detection kit  
216 (Ambion) and film-based exposure.

## Results

### *Replication of HCV RNA from two copies of the NS3-5B replicase unit in a single RNA molecule*

We recently described use of intragenomic replicons as a novel system to perform HCV complementation assays (Fig 1) (19). Using these constructs, we showed that the intracellular distribution of a V5-tagged NS5A, expressed from ORF1 as an NS3-5A polyprotein, extensively overlapped with a second copy of a FLAG-tagged NS5A produced from an ORF2-encoded replicase. Along with additional experimental data, the results were consistent with NS3-5A directing mature protein products from the polyprotein to MAF, but did not account for why expression of NS3-5A from ORF1 only allowed rescue of constructs carrying the NS5A S2208I mutation in ORF2, not a NS4B G1911A mutation (numbers refer to position of the residue in the JFH1 polyprotein). One possible explanation was the existence of more than one type of NS protein complex within MAF such that NS3-5A, being an incomplete component of the full NS3-5B replicase, only contributed to the formation of some of these complexes. Two alternative explanations were that linking the ORF1 polyprotein to a Renilla luciferase – Foot and Mouth Disease Virus (FMDV) 2A fusion protein, or use of a recoded NS sequence in ORF1, impacted on polyprotein function. To examine these last two possibilities, monocistronic replicons were generated in which the NS3-5B replicase was expressed as a Renilla-FMDV2A fusion protein (Fig 2a). For one replicon the NS protein coding region was derived from JFH1 (JFH1-mono) but for the other it was chimeric, consisting of the NS3-5A recoded region fused to JFH1 NS5B (JFH1<sup>DVR</sup>-mono). Expressed polyproteins also encoded the same FLAG-tagged (JFH1-mono) and V5-tagged (JFH1<sup>DVR</sup>-mono) versions of NS5A present in ORF2 and ORF1 of the intragenomic replicon construct, the former already having been shown not to affect replicative function (19). Both replicons showed equally robust replication when transfected into

cells whereas a polymerase defective control monocistronic construct containing a GDD>GAA mutation (JFH1-mono(GDD>GAA)) did not replicate (Fig 2a). These data verify that neither linking a luciferase reporter to NS3-5B using FMDV 2A, nor use of recoded NS3-5A sequence, interfere with RNA replication. They also demonstrate that the presence of the V5 tag in recoded NS5A does not impair replicase function.

To determine whether intragenomic replicons could be adapted for identifying distinct replication complexes (RCs), we then extended NS3-5A in ORF1 to encode NS3-5B such that the synthetic NS5B sequence, similar to the upstream NS3-5A segment, also contained synonymous changes across its coding region. The strategy to introduce synonymous changes was aimed at limiting the possibility of recombination with NS3-5B in ORF2, and had the added advantage of preventing duplication of *cis*-acting RNA elements (CREs) present in ORF2 (22, 23). Transfection of the resultant wt<sup>NS3-5B</sup>/wt<sup>NS3-5B</sup> RNA into Huh7.5 cells gave robust replication (Fig 2b). To test whether the codon-modified NS3-5B sequences in ORF1 could direct replication, the GDD motif in the NS5B RNA polymerase in ORF2 was deleted ( $\Delta$ GDD). This resultant intragenomic construct (wt<sup>NS3-5B</sup>/ $\Delta$ GDD<sup>NS3-5B</sup>) gave luciferase activity at 72 hours, which was only about 3-fold lower than the replicon with wt sequences in ORFs1 and 2. Combining another polymerase knock-out mutation (GDD>GAA) in ORF1 with the  $\Delta$ GDD mutation in ORF2 (giving GDD>GAA<sup>NS3-5B</sup>/ $\Delta$ GDD<sup>NS3-5B</sup>) blocked replication (Fig 2b). These data demonstrate that it is possible to express two functional copies of the HCV NS3-5B replicase unit from a single RNA molecule.

#### *Mutations in NS3, NS4B and NS5A that are refractile to rescue with NS3-5A.*

To examine the effect of introducing other lethal mutations into ORF2 of the intragenomic replicon, constructs were made expressing either wt NS3-5A or NS3-5B from

ORF1 and a series of NS3-5B replicases carrying single lethal mutations from ORF2 (Fig 3). The first two of these mutations were the S2208I NS5A and G1911A NS4B mutations from our previous study (19), in which only S2208I could be complemented by NS3-5A expressed from ORF1. The third was another mutation in NS5A (P2008A), previously reported to be rescued by *trans*-complementation and chosen because it belonged to a different complementation group from the S2208I mutation (17). The final mutation was K1240N, located in the Walker A motif of the NS3 helicase and which blocks helicase activity by preventing NTP binding.

This series of replicons, and relevant polymerase-defective control constructs ( $\text{wt}^{\text{NS3-5A/GDD>GAA}^{\text{NS3-5B}}}$ ), were transfected into Huh7.5 cells and luciferase activity was monitored over a 96 hour period (Fig 3). Replication was slightly lower for  $\text{wt}^{\text{NS3-5B}}/\text{wt}^{\text{NS3-5B}}$  compared to  $\text{wt}^{\text{NS3-5A}}/\text{wt}^{\text{NS3-5B}}$ , which may arise because of the increased length of the RNA from incorporating NS5B sequences into ORF1 of  $\text{wt}^{\text{NS3-5A}}/\text{wt}^{\text{NS3-5B}}$ . As predicted,  $\text{wt}^{\text{NS3-5A/GDD>GAA}^{\text{NS3-5B}}}$  did not replicate as the RNA only expresses a non-functional virus-encoded RNA polymerase. Similar to the results from  $\text{wt}^{\text{NS3-5B}}/\Delta\text{GDD}^{\text{NS3-5B}}$  in Fig. 2,  $\text{wt}^{\text{NS3-5B}}/\text{wt}^{\text{NS3-5B}}$  also showed robust replication. Results with the constructs containing other mutations in ORF2 revealed differences in replication between the experimental groups (Fig 3a). For the series of constructs expressing NS3-5A from ORF1, only the one carrying S2208I showed robust replication, whereas replication of constructs carrying all other mutations (G1911A, P2008A and K1240N) was absent. When ORF1 instead expressed NS3-5B, the relative replication of the construct with S2208I in ORF2 was enhanced compared to its wt counterpart, but more noticeable was the differences in replication for those constructs carrying the other mutations in ORF2. Constructs with P2008A and G1911A mutations replicated as well as had been observed for  $\text{wt}^{\text{NS3-5B}}/\text{GDD>GAA}^{\text{NS3-5B}}$ . In theory, this was consistent with the

ability of ORF1 NS3-5B simply substituting for the functional replicase produced from ORF2 NS3-5B. However, the same phenomenon was not observed for the K1240N mutation, as replication of this construct was markedly impaired, with luciferase activity barely detected even at 72 and 96 hours post transfection. Thus while replication of intragenomic constructs carrying functional NS3-5B in ORF1 and defective NS3-5B in ORF2 might in part depend on a substitution of the ORF2 replicase by that encoded in ORF1, polyprotein mixing and composite RC formation appeared to occur also.

To compare how levels of rescue might differ using trans-complementation, a stable replicon cell line expressing functional NS3-5B was transfected with a bicistronic subgenomic replicon encoding a firefly luciferase reporter in ORF1 and a functional or mutated (NS3<sup>K1240N</sup>, NS4B<sup>G1911A</sup>, NS5A<sup>P2008A</sup>, NS5A<sup>S2208I</sup> and NS5B<sup>GDD>GAA</sup>) NS3-5B polyprotein in ORF2. Consistent with reports from other studies, only robust rescue of NS5A<sup>S2208I</sup> was observed, with levels of luciferase activity being approximately 10% of those levels seen for the functional control construct (Fig 3b). Other mutations were either rescued at a low levels (NS4B<sup>G1911A</sup> and NS5A<sup>P2008A</sup>) or not at all (NS3<sup>K1240N</sup> and NS5B<sup>GDD>GAA</sup>). As expected, none of the mutated transcripts replicated when transfected into naive Huh7.5 cells (Fig 3c).

#### *NS3 helicase mutation K1240N is dominant negative*

The inability to rescue the NS3 K1240N mutation in ORF2 using constructs where ORF1 expressed wt NS3-5B was potentially consistent with the formation of composite NS protein complexes where the mutation in the NS3 helicase had a dominant negative effect on replication. To test this the K1240N mutation was introduced into ORF1 of the intragenomic replicon carrying a functional NS3-5B in ORF2 and the impact on replication was assessed (Fig 4a). To maximise the utility of the intragenomic system, the constructs examined included those

expressing NS34A, NS3-5A and NS3-5B from ORF1, with these polyproteins containing both wt sequences and the K1240N mutation.

From monitoring luciferase levels in cells transfected with the various constructs, K1240N<sup>NS34A</sup>/wt<sup>NS3-5B</sup> had comparable levels of replication to its counterpart expressing functional NS34A from ORF1 (Fig 4a). In contrast, expressing the K1240N mutation from NS3-5A ORF1 (K1240N<sup>NS3-5A</sup>/wt<sup>NS3-5B</sup>) gave a noticeable drop in replication of approximately 10-fold in the first 48 hours compared to wt<sup>NS3-5A</sup>/wt<sup>NS3-5B</sup>, although replication recovered to some extent at later time points. The impact of expressing K1240N from the NS3-5B ORF1 was even greater, with replication levels of ~20-30 fold lower than the comparable control construct during the first 48 hours and remaining low over the course of 96 hours.

To establish whether a K1240N mutated polyprotein could suppress replication when expressed from a separate RNA, JFH1-mono was co-transfected into Huh7.5 cells with either yeast tRNA, or bicistronic subgenomic replicons encoding a firefly luciferase reporter gene in ORF1 and K1240N or GDD>GAA mutated NS3-5B in ORF2 (Fig 4b). Monitoring firefly activities in the cells 4 hours and 24 hours post-transfection showed that both bicistronic replication defective transcripts were present at broadly similar levels and exhibited a similar rate of decay. Despite this, the replication of JFH1-mono was significantly inhibited in the presence of K1240N NS3-5B (replication ~30-55% of that of the tRNA group over 24 to 96 hours). In contrast, inhibition was not seen in the presence of GDD>GAA mutated NS3-5B, indicating that reduced replication imposed by K1240N was not due to competition between NS proteins for limited host cell factors (24).

*Intragenomic replicon replication of constructs carrying the T1299A helicase mutation differs from those carrying the K1240N mutation.*



Kazakov et al. (18) recently proposed NS3 helicase activity to be *cis*-acting on the basis that several mutations blocking NS3 helicase activity could not be *trans*-complemented using various systems expressing NS3-5B. However, the fact that K1240N imposed a *trans*-dominant negative phenotype on replication offered a potential alternative explanation as to why rescue of helicase mutations had not been observed. It was therefore important to examine whether another mutation (T1299A), which disrupts helicase binding to the phosphate backbone of nucleic acid and used in the aforementioned study, might also be dominant negative. Hence, T1299A was introduced into ORF1 in a series of intragenomic replicons, and the impact it had on ORF2 replicase activity assessed by comparing replication of these constructs with that of their counterparts expressing functional polyprotein from ORF1. Remarkably, replication was unaffected by the presence of T1299A, irrespective of whether it was expressed from ORF1 NS3-5A or NS3-5B polyproteins (Fig 4c). In contrast, a control transcript expressing K1240N from NS3-5B ORF1 did show suppressed replication.

If T1299A was not dominant negative, as our data suggested, this provided an opportunity to examine the extent to which ORF1 could replace the *cis*-acting role of ORF2. Therefore, T1299A was introduced into ORF2 of an intragenomic replicon expressing functional NS3-5A or NS3-5B from ORF1. Transfection of these transcripts into Huh7.5 cells revealed that while replication was not supported by ORF1 expressing NS3-5A, replication was detected with the construct expressing functional NS3-5B from ORF1 (Fig 4d). We conclude that while NS3 helicase activity is likely to be a *cis*-acting function of the replicase, mutations disrupting this activity can manifest additional phenotypes, as seen with K1240N exerting a dominant negative effect on replication. We further conclude that ORF1 NS3-5B can substitute for *cis*-acting functions normally provided by the authentic NS3-5B replicase expressed from ORF2.

355 *Establishing that NS4B, NS5A and NS5B mutations depend on NS3-5B for rescue.*

356       The data above indicated that NS3-5B expressed from ORF1 of an intragenomic replicon  
357 was capable of replacing the *cis*-acting functions of ORF2. To further exploit the potential of the  
358 intragenomic replicon system, mutations were introduced into wt<sup>NS3-5B</sup>/wt<sup>NS3-5B</sup> in different  
359 combinations and permutations. Initially, we placed the NS4B G1911A mutation in ORF1 and  
360 the NS5A P2008A mutation in ORF2 to create G1911A<sup>NS3-5B</sup>/P2008A<sup>NS3-5B</sup>. RNA from this  
361 construct and other controls was transfected into Huh7.5 cells, and replication was assessed by  
362 measuring luciferase activity (Fig 5a). The pattern of reporter activity for wt<sup>NS3-5B</sup>/wt<sup>NS3-5B</sup>,  
363 wt<sup>NS3-5B</sup>/G1911A<sup>NS3-5B</sup> and wt<sup>NS3-5B</sup>/P2008A<sup>NS3-5B</sup> was identical to that seen in Fig. 3.  
364 Interestingly, G1911A<sup>NS3-5B</sup>/P2008A<sup>NS3-5B</sup> also replicated at levels that were only about 3-fold  
365 less than that for either wt<sup>NS3-5B</sup>/G1911A<sup>NS3-5B</sup> or wt<sup>NS3-5B</sup>/P2008A<sup>NS3-5B</sup> in contrast to  
366 GDD>GAA<sup>NS3-5B</sup>/ΔGDD<sup>NS3-5B</sup> which did not replicate. Northern blot analysis (Fig 5b) revealed  
367 that RNA identical in length to *in vitro* transcribed wt<sup>NS3-5B</sup>/wt<sup>NS3-5B</sup> transcripts was clearly  
368 present in cells 72 hours after transfection with wt<sup>NS3-5B</sup>/wt<sup>NS3-5B</sup>, wt<sup>NS3-5B</sup>/G1911A<sup>NS3-5B</sup> and  
369 wt<sup>NS3-5B</sup>/P2008A<sup>NS3-5B</sup>. A similar sized band, albeit at a much reduced level was also seen in cells  
370 transfected with G1911A<sup>NS3-5B</sup>/P2008A<sup>NS3-5B</sup>. No obvious band of this size was observed in cells  
371 transfected with the polymerase knock-out control intragenomic replicon. As neither G1911A  
372 nor P2008A could be rescued by NS3-5A, we conclude that composite complexes must have  
373 formed from the mixing of two defective NS3-5B polyproteins.

374       Rescue of replication is proposed to occur through exchange of NS proteins between  
375 individual polyprotein-derived complexes (18). However, published data suggest that rescue of  
376 NS5B defects instead depends on strand exchange, a process where the viral RNA transfers from  
377 an NS5B-containing complex derived from one NS3-5B ORF to another NS5B-containing

complex derived from a second NS3-5B ORF (18). Under such circumstances, NS3-5B provided *in trans* is only able to rescue polymerase defects if it itself carries no lethal mutations. Our intragenomic replicon system provided an opportunity to examine rescue of defective NS5B under conditions where two polyproteins were translated from the same RNA. Therefore, constructs were made in which the NS4B mutation G1911A was combined with the polymerase knock out mutation GDD>GAA to give GDD>GAA<sup>NS3-5B</sup>/G1911A<sup>NS3-5B</sup> (Fig 5c). This construct replicated at lower levels than wt<sup>NS3-5B</sup>/wt<sup>NS3-5B</sup> or single mutated control constructs wt<sup>NS3-5B</sup>/G1911A<sup>NS3-5B</sup> and wt<sup>NS3-5B</sup>/GDD>GAA<sup>NS3-5B</sup>, but still generated a luciferase signal 100-fold higher than the replication defective construct GDD>GAA<sup>NS3-5B</sup>/ΔGDD<sup>NS3-5B</sup>. The two mutations were subsequently switched between ORF1 and ORF2, resulting in the generation of G1911A<sup>NS3-5B</sup>/GDD>GAA<sup>NS3-5B</sup>. Again, low but detectable levels of replication were seen in cells transfected with this replicon (Fig. 5d). The fact that both GDD>GAA<sup>NS3-5B</sup>/G1911A<sup>NS3-5B</sup> and G1911A<sup>NS3-5B</sup>/GDD>GAA<sup>NS3-5B</sup> replicate further supports the view that ORF1 and ORF2 within the intragenomic replicon are each capable of contributing replicase *cis*-acting functions. To confirm rescue of a polymerase defect *in cis* required NS5B to be expressed in the context of a polyprotein, intragenomic replicons were made expressing NS5B or NS5A5B in ORF1 and encoding either a functional or polymerase-defective replicase in ORF2 (Fig 5e). Upon transfection into Huh7.5 cells, only those constructs encoding a functional NS3-5B in ORF2 replicated, indicating that rescue of polymerase activity is indeed dependent on expression of functional NS5B in the context of a polyprotein, most probably NS3-5B.

Having established that G1911A and GDD>GAA were dependent on NS3-5B for rescue, we combined these same mutations in ORF2 with the helicase mutation T1299A in ORF1. Interestingly, transfection of the resulting transcripts into Huh7.5 cells revealed that neither

combination of mutations supported intragenomic replicon replication (Fig 5f). Thus despite evidence that composite replication complexes are formed between NS3-5B expressed from both ORFs, it appears that neither G1911A nor GDD>GAA mutated NS3-5B can substitute for the *cis*-acting role of a T1299A mutated polyprotein. The implications of this are discussed later, but suggest that each NS3-5B polyprotein which contributes to functional NS3-5B-dependent complex formation requires helicase activity.

Overall, our results show that complementation of some mutations in the NS3-5A region are indeed dependent on NS3-5B. The data also further supports the notion that ORF1 can substitute for ORF2 *cis*-acting interactions and that helicase activity provided both *in cis* and *in trans* is critical for NS3-5B dependent complex formation.

#### *Lethal mutations in NS3 and NS4A exist that can be rescued in the intragenomic system*

The above data indicated that all NS3 mutations which blocked helicase activity were refractile to complementation. To determine whether this was representative of all mutations in NS3, and its co-factor NS4A, we selected four further mutations in these proteins that were lethal to replication but did not cause defective polyprotein processing (25-28). Two mutations were in NS3, mapping to the protease domain (L1157A) and hinge region between the protease and helicase domains (PP1220-1GG) respectively. The other two mutations were situated at either end of NS4A (V1665G and Y1706A), the former locating to and disrupting NS4A trans-membrane dimerization and the later representing an essential conserved residue within the acidic domain of NS4A.

The mutations were introduced into ORF2 of intragenomic replicons that expressed NS34A, NS3-5A or NS3-5B from ORF1. All constructs expressing NS34A were replication defective (Fig 6a). In contrast, three of the constructs (L1157A, PP1220-1GG and Y1706A)

replicated when ORF1 instead expressed NS3-5A; luciferase values indicated that L1157A was rescued most efficiently, followed by PP1220-1GG and then Y1706A. When ORF1 expressed NS3-5B, replication occurred for all four mutations. For L1157A, PP1220-1GG and Y1706A levels of replication were broadly comparable, while they were lower but still detectable for V1665G. To establish whether this latter mutation was rescuable in an NS3-5B dependent manner, the mutation was introduced into ORF2 of an intragenomic construct expressing an NS4B G1911A mutated NS3-5B from ORF1. For comparison, constructs expressing wt NS3-5B from ORF1 and V1665G or G1911A mutated NS3-5B from ORF2 were included. Transfection into Huh7.5 cells revealed that for those constructs carrying only a single mutation in ORF2, V1665G had a more inhibitory effect on replication compared to G1911A (Fig 6b). The replicon carrying G1911A in ORF1 and V1665G in ORF2 did not replicate. The fact that wt<sup>NS3-5B</sup>/V1665G<sup>NS3-5B</sup> replicated at a noticeably lower level than wt<sup>NS3-5B</sup>/G1911A<sup>NS3-5B</sup> suggested that V1665G might exert a dominant negative phenotype. To establish whether this was the case, the mutation was introduced into NS3-5A or NS3-5B expressed from ORF1 (Fig 6c) and the replication of these intragenomic constructs compared to the equivalent constructs expressing functional NS3-5A and NS3-5B from ORF1. Interestingly, the presence of V1665G in ORF1 suppressed replication compared to equivalent constructs expressing functional polyprotein, but only when ORF1 encoded NS3-5B. It therefore appears that V1665G is dominant negative, but unlike the K1240N NS3 helicase mutation only manifests this phenotype when expressed in the context of a full length replicase.

To compare levels of rescue with that seen using *trans*-complementation, all four mutations were introduced into a bicistronic subgenomic replicon encoding a firefly luciferase reporter in ORF1. A stable cell line expressing a functional replicon was transfected with these

constructs along with control constructs carrying the GDD>GAA, G1911A and S2208I mutations to allow comparisons to be made with earlier experiments (Fig 6d). Other than GDD>GAA and V1665G, all mutations showed some degree of complementation. For the NS3 mutation L1157A and NS4A mutation Y1706A levels of rescue were only slightly less than observed for the NS5A mutation S2208I. In contrast, rescue of the other NS3-5A dependent mutation in NS3, PP1220-1GG, was lower and comparable to that seen for the NS3-5B dependent mutation G1911A in NS4B. None of the mutated constructs replicated when transfected into naive Huh7.5 cells (data not shown). It was interesting that Y1706A was rescued as efficiently as L1157A given the limited extent to which the former mutation was rescued by NS3-5A when expressed from ORF1 of an intragenomic replicon. We therefore transfected the same firefly replicon constructs into Huh7.5 cells transduced with baculovirus expressing NS3-5A (Fig 6e). While the data confirmed that rescue of L1157A, PP1220-1GG and Y1706A required expression of a minimum NS3-5A polyprotein, these levels were reasonably comparable for L1157A and Y1706A, similar to the situation using helper replicons.

We conclude that like NS5A, there are mutations in NS3/4A capable of being rescued by a minimum NS3-5A precursor whereas others are not. Rescue of mutations dependent on a minimum NS3-5A polyprotein is possible when this protein is expressed *in cis* from ORF1 or *in trans* from a separate mRNA.

#### *Blocking NS4B5A cleavage only impacts on functions within complexes dependent on NS3-5B expression*

Since cleavage between NS4B and NS5A by NS3/4A is slower than at other boundary sites in the NS3-5B polyprotein, we and others have proposed that the presence of a NS4B5A precursor within a complex destined to form the RC might serve as a check-point to prevent

premature membrane deformation events linked to RC maturation (29, 30). Given our data suggesting that NS3-5A and NS3-5B provide differential access to different NS protein complexes supporting replication, we were interested to know the impact that blocking cleavage of NS4B5A would have in the context of the intragenomic replicon system.

In the first instance an NS5A mutation, S1977P, designed to block NS4B5A boundary cleavage, was introduced into ORF2 of an intragenomic replicon expressing NS3-5B in ORF1. Transfection of this construct into Huh7.5 cells showed that, compared to a control construct carrying the NS5A P2008A mutation in ORF2, replication was considerably impaired (Fig 7a). S1977P was then introduced into NS3-5A or NS3-5B expressed from ORF1 of an intragenomic replicon (Fig 7b). As was observed for the K1240N and V1665G mutation, NS3-5B expressed from ORF1 carrying the S1977P mutation measurably impaired replication (Fig. 7b). Similar to the V1665G mutation but unlike the K1240N mutation, S1977P failed to inhibit replication when expressed from ORF1 in the context of NS3-5A. Western blot analysis confirmed the presence of uncleaved NS4B5A from ORF1 in cell extracts from cells transfected with S1977P<sup>NS3-5A</sup>/wt<sup>NS3-5B</sup> and S1977P<sup>NS3-5B</sup>/wt<sup>NS3-5B</sup> (Fig. 7c). These data suggested that S1977P had dominant negative activity, albeit only when expressed in the context of NS3-5B. This was verified by comparing the impact that K1240N, S1977P, G1911A and GDD>GAA mutations in ORF1 had on replication (Fig 7d). Critically, only K1240N and S1977P markedly suppressed replication while the other mutations exhibited little or no effect.

Two possible explanations might account for the failure of NS3-5A carrying the NS5A<sup>S1977P</sup> mutation to inhibit replication. The first was that complexes formed by NS3-5A tolerated the presence of an uncleaved NS4B5A precursor, unlike those formed by NS3-5B. The second possibility was that the mutation had prevented integration of NS4B5A into NS3-5A-

dependent complexes. As this latter event might have allowed dissociation of NS4B5A from the MAF, confocal analysis was employed to examine the location of NS4B5A precursor from S1977P<sup>NS3-5A</sup>/wt<sup>NS3-5B</sup> with respect to the functional NS5A expressed from ORF2. Results showed that extensive overlap of signals was evident (Fig 8a).

To further investigate the impact of S1977P on NS3-5A-dependent complex formation we took advantage of our earlier observation that rescue of the NS5A S2208I mutation occurred extremely efficiently using NS3-5A, reasoning that if S1977P NS3-5A still formed NS3-5A dependent complexes it might be able to rescue this latter mutation. Intragenomic constructs carrying S1977P mutated NS3-5A and NS3-5B in ORF1 were therefore further modified to carry the S2208I mutation in ORF2. Remarkably, despite being unable to produce mature NS5A, a NS3-5A polyprotein carrying S1977P expressed from ORF1 could rescue replication when ORF2 contained the S2208I mutation (Fig 8b), albeit 3-4 fold less efficiently than when ORF1 expressed functional NS3-5A. By contrast, the construct expressing S1977P mutated NS3-5B from ORF1 and S2208I mutated NS3-5B in ORF2 barely replicated. To verify this result, *trans*-complementation of the S2208I mutation was examined under conditions where functional NS5A, functional NS3-5A and S1977P mutated NS3-5A were provided by baculovirus transduction (Fig 8c). While wt NS3-5A was most effective at rescuing the S2208I mutation, expression of S1977P mutated NS3-5A also resulted in a substantial level of rescue that was several fold higher than seen using NS5A alone. Western blot analysis (Fig 8d) established that NS5A expression was greatest in NS5A and lowest in NS3-5A<sup>S1977P</sup> transduced cells, indicating that expression levels do not account for the improved rescue of S2208I with NS3-5A<sup>S1977P</sup> versus NS5A alone.



To extend our analysis to another defect in NS5A, we selected a further mutation (G2323A) belonging to a different intragenic complementation group to that of S2208I and P2008A (17). This mutation was introduced into ORF2 of a series of intragenomic constructs expressing either wt NS3-5A, S1977P mutated NS3-5A or wt NS3-5B from ORF1. Transfection of the RNAs into Huh7.5 cells revealed that NS3-5A was able to efficiently rescue G2323A and that this still occurred, albeit at slightly lower levels when ORF1 instead carried a S1977P mutated NS3-5A (Fig 8e). On the basis of these combined results we conclude that at least some NS5A functions operate within an NS3-5A dependent complex that is physically distinct from that formed by NS3-5B/NS3-5B interactions.

## Discussion

Our results demonstrate that the truncated polyprotein NS3-5A, although able to target NS protein components to MAF, can only complement certain replication defects contained in this same coding region. As replication depends on homo and heterotypic interactions between different NS proteins within NS3-5B (18, 31-36), functional NS-NS protein complex formation would explain this observation. Our proposal is that the inability to rescue certain mutations by NS3-5A is due to this precursor participating in the formation of only a subset of complexes formed by NS3-5B/NS3-5B interactions. Evidence supporting this conclusion comes from several observations. Firstly, two mutations in the NS3-5A coding region were not complemented by NS3-5A but were efficiently rescued by NS3-5B. While this rescue, in theory, could be due to NS3-5A being less efficient at forming the same complexes as NS3-5B, experimental data suggests otherwise. We identified one mutation (PP1220-1GG) whose defect was rescued by NS3-5A, yet the same mutation showed comparable rescue to the NS3-5B-dependent defects G1911A and P2008A using helper virus. Secondly, despite the fact that the three dominant mutations, K1240N, V1665G and S1977P, inhibited replication to broadly similar levels when expressed in the context of NS3-5B, only K1240N inhibited replication when expressed as an NS3-5A precursor. Finally, expression of the dominant negative mutation, S1977P, in the context of a NS3-5A precursor enabled rescue of two NS5A mutations belonging to different intragenic groupings. Given that all three mutations are on the same protein, it seems probable that S1977P expressed from NS3-5A must be selectively incorporated into an NS5A-containing complex disabled by either S2208I or G2323A, while being excluded from another complex for which S1977P itself is lethal.

Rescue of replication defective mutations in positive strand RNA viruses has typically

548 employed *trans*-complementation systems, where the functional protein or polyprotein is  
549 expressed from an RNA separate to the one being rescued. While our study is not the first to  
550 produce two copies of an NS protein from a replication competent RNA, it is the first to express  
551 an entire duplicated replicase. Use of similar constructs has proven problematic in the past.  
552 Rescue that relied on the placement of duplicated NS coding regions in poliovirus found the  
553 duplicated sequence was rapidly lost (37), presumably because of selective pressures arising  
554 from use of infectious virus with larger than unit sized genomes and subsequent packaging  
555 constraints which this imposed. In addition, the use of virus rather than replicons meant these  
556 selection pressures operated across an entire cell population rather than within individual  
557 transfected cells. By using replicons carrying the second copy of the NS polyprotein as a re-  
558 coded sequence, we have studied replication under transient conditions where recombination, if  
559 it occurs, makes little or no contribution to the overall assay readout. Supporting this view, we  
560 found that constructs expressing functional NS3-5A from ORF1 and NS3-5B from ORF2 failed  
561 to generate a replication signal when ORF2 carried mutations K1240N, V1665G, G1911A and  
562 P2008A, despite the fact that a single recombination event had the potential to generate  
563 replication competent monomeric transcripts. Northern blot analysis of replication assays 72  
564 hours post transfection also showed that the replicon transcripts present in cells were the same  
565 size as the input transcripts. Tight temporal and spatial control of expression obtained from  
566 producing the two replicases from the same RNA makes the intragenomic replicon systems a  
567 potentially valuable tool for studying replication of other positive strand RNA viruses. That NS3-  
568 5B expressed from either ORF is able to assume *cis*-acting roles in replication adds further value  
569 to the system as it provides opportunities to look at replication complex formation in ways that  
570 have not been possible until now.

RNA replication depends not only on viral polyprotein expression, but also on the interactions between these polyprotein components and RNA elements present in the genome. In HCV, the NS3-5B coding region lies adjacent to the 3'UTR and contains *cis*-acting RNA elements within its 3' end that make essential kissing loop interactions (22, 23, 38). Remarkably, there is no requirement for the NS3-5B coding region to overlap the kissing loop structure (38), although until now it has been unclear whether there is a spatial relationship between the NS3-5B ORF and the 3' end of the genome. By demonstrating that NS3-5B expressed from ORF1 of an intragenomic replicon can support replication when ORF2 carries the *cis*-acting NS3 helicase mutation T1299A (18), our data indicates that a considerable distance between the polyprotein ORF and 3'UTR can be tolerated. This conclusion is further supported by our finding that intragenomic constructs expressing two NS3-5B polyproteins, each with separate mutations in NS4B (G1911A) or NS5B (GDD>GAA), still replicate, irrespective of which ORF the two mutations are placed in. Based on studies by others (18), such combinations would not be tolerated if ORF1 did not assume *cis*-acting functions normally provided by the authentic replicase encoded in ORF2.

As our data showed ORF1 is able to substitute for the *cis*-acting role of ORF2, it was surprising to see that intragenomic constructs carrying the *cis*-acting NS3 helicase mutation T1299A in ORF1 were unable to tolerate the presence of the NS4B G1911A mutation in ORF2. This was despite the fact that neither of these mutations were dominant negative and data from both this study and others show that the function blocked by G1911A is not *cis*-acting (14). Given NS3-5A expressed from ORF1 of an intragenomic replicon cannot rescue T1299A in ORF2, and G1911A requires NS3-5B for rescue, it seems likely that both *cis*-acting and *trans*-acting events blocked by these two mutations localize to an NS3-5B containing complex which

594 NS3-5A is excluded from. Based on this conclusion and other observations, we propose the  
595 following model where formation of this complex involves an association of multiple copies of  
596 an NS3-5B precursor shortly after their translation. Firstly, polyprotein association is catalysed  
597 by an initial binding event between a *cis*-acting replication element (CRE) within the RNA  
598 genome and NS3 helicase, as has been suggested by others (18). However, subsequent assembly  
599 requires the presence of NS5B as a structural component, both in the polyprotein that makes the  
600 *cis*-acting contact with the viral RNA, as well as in the polyproteins that engage *in trans* with this  
601 initial *cis*-acting complex. This requirement for NS5B has similarities to the model put forward  
602 by Kazakov *et al* (18), who suggested the protein provided a structural component for *cis*-  
603 dependent interactions, but did not identify similar role for it *in trans*. A further distinguishing  
604 feature of our model is that incorporation of NS3-5B into this growing complex also requires the  
605 newly arriving polyproteins to display NS3 helicase RNA binding activity. In other words, NS3  
606 helicase has both essential *cis*- and *trans*-acting roles in NS3-5B-dependent complex formation.  
607 Figure 9 illustrates this as well as how assembly of such a complex would be influenced by  
608 different mutations used in this study. Importantly, some of the predictions made by our model  
609 can account for the findings of others. It has been shown that replication of viral transcripts  
610 expressing NS3-5A *in cis* cannot be supported by expression of NS3-5B *in trans* (18), despite the  
611 fact that replication of mini-genomes which fail to express viral proteins can be supported by  
612 expression of a viral replicase (39). Our model offers the possibility that NS3-5A expressed *in*  
613 *cis* is capable of making non-productive interactions with CRE elements in the viral genome,  
614 thus frustrating assembly of the NS3-5B-dependent complex. Perhaps relating to this mode of  
615 action, we identified one mutation, NS4A<sup>Y1706A</sup>, which was rescued far more effectively when  
616 NS3-5A was expressed *in trans* from a separate RNA than *in cis* when expressed from ORF1 of

617 an intragenomic replicon. Identifying mutations in ORF2 NS3-5B that weaken its ability to  
618 outcompete ORF1 expressed NS3-5A for binding to CRE elements could provide useful  
619 information about protein-protein and protein-RNA contacts made during complex assembly.  
620 While the NS4A<sup>Y1706</sup> residue is relatively conserved, it does lie adjacent to another more variable  
621 residue (position 1708 in JFH1) previously implicated in orchestrating physical interactions  
622 between NS4A and all other NS proteins within the NS3-5B polyprotein (32). It would be  
623 interesting to see whether mutations of residues such as those identified by co-variant amino acid  
624 analysis alter the ability of different polyproteins expressed *in cis* to compete for CRE elements.

625         A general feature of the mutations dependent on NS3-5A for rescue compared to those  
626 dependent on NS3-5B was the greater extent with which they were *trans*-complemented by a  
627 helper replicon. Reduced levels of stringency for rescue fit with a proposal by Fridell and  
628 colleagues that NS5A supports functions both inside and outside the replication complex (16).  
629 Importantly, they speculated that mutations blocking NS5A function outside and inside the  
630 replication complex included S2208I and P2008A; these mutations which disrupted NS3-5A and  
631 NS3-5B-dependent complex activity respectively in our hands. Compartmentalization of NS3-  
632 5A and NS3-5B-dependent complex functions is certainly a possibility that warrants  
633 consideration. Assuming that exchange of individual NS proteins within complexes occurs (18),  
634 without compartmentalization it is difficult to envisage why some mutations in the NS3-5A  
635 region show dependence on NS3-5B for rescue whereas others in the same NS protein are  
636 efficiently rescued by NS3-5A. Furthermore, the proposed role that cleavage of the NS4B5A  
637 boundary has in enabling NS5A driven double membrane formation (29, 30), coupled to the fact  
638 that the S1977P mutation which blocks boundary cleavage is only dominant negative as an NS3-  
639 5B precursor, is consistent with NS3-5A-dependent complexes being excluded from the RC. Our

640 attempts to see whether discrete compartmentalization occurs have so far been restricted to  
641 confocal microscopy analysis. Given the size of the double membrane vesicles in MAF, the  
642 structures thought to contain the functional replication complexes, it is perhaps not surprising  
643 that we failed to observe separation of NS3-5A versus NS3-5B dependent complexes (data not  
644 shown). Further physical and electron microscopy studies are planned.

645         An unexpected outcome of our study was the trans-dominant negative phenotype  
646 displayed by one NS3 helicase mutation (K1240N) but not by another (T1299A). While both  
647 mutations block helicase activity, this occurs through disruption of different sites within the  
648 enzyme. NS3<sup>K1240N</sup> disrupts the Walker A motif responsible required for ATP binding and  
649 hydrolysis whereas NS3<sup>T1299A</sup> blocks RNA helicase binding by disrupting the TxGx motif  
650 involved in phosphate backbone interactions (40). Disruption of the Walker A motif would also  
651 have the likely consequence of locking the enzyme in an ATP-free open state, potentially  
652 increasing its propensity to bind RNA compared to wild type NS3 (41). Thus one possible reason  
653 for NS3<sup>K1240N</sup> displaying a dominant negative phenotype is that it displays either a normal or  
654 enhanced ability to bind to both CRE elements and other RNA sites on the RNA genome,  
655 ensuring that defective NS3 populates NS3-5B dependent complexes. However, the fact that  
656 NS3<sup>K1240N</sup> was also dominant negative expressed as an NS3-5A precursor suggests that the  
657 inhibitory effect extends beyond the NS3-5B dependent complex. Perhaps the mutated NS3  
658 helicase ‘padlocks’ itself onto viral RNA present within the membranous web, either  
659 immobilizing it and/or preventing functional helicase molecules translocating along it.  
660 Understanding what mechanisms may be involved is important. Studies have already highlighted  
661 the potential importance of *trans*-dominant mutations in guiding the development of novel viral  
662 inhibitors (42). Should pharmacological recapitulation of this dominant negative phenotype

663 prove possible it would provide added therapeutic benefit to any drug targeting helicase activity.

664 In summary, our work reveals the existence of two functional sets of complexes that  
665 reside within the HCV MAF, both of which are necessary for replication of the viral genome and  
666 each of which performs distinct functions. It seems unlikely that this is a unique mechanism  
667 adopted by HCV. Rescue of defects in pestivirus NS5A show remarkable similarities to that seen  
668 for HCV NS5A, with some but not all mutations being readily rescued by helper virus constructs  
669 (43). Similarly, certain defects in flavivirus NS proteins can be complemented but other defects  
670 in the same protein cannot (44). The intragenomic replicon system represents a potential  
671 powerful approach to investigate the formation and function of complexes, in part due to its  
672 ability to overcome the traditional *cis*-acting barriers encountered by *trans*-complementation.  
673 Understanding the roles different complexes play will inform general aspects of viral RNA  
674 replication.



678    **Funding Information**

679    Funding for this work was provided by MRC grants G0701215 and 4050295596 awarded to  
680    CJM and JM respectively, and by the Brazilian Science without Borders studentship CAPES -  
681    Proc No. 9173/13-7 awarded to RG. The funders had no role in study design, data collection and  
682    interpretation, or the decision to submit the work for publication.

683

684    **Acknowledgements**

685           We wish to thank Prof C. Rice and Dr T. Wakita for the gift of Huh7.5 cells and JFH1  
686    cDNA containing vectors and Colin Loney for expert technical assistance.

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**Figure 1. Schematic representation of the intragenomic HCV replicons used in this study.**

The HCV IRES in the 5'UTR drives translation of ORF1 which encodes for indicated NS polyproteins expressed as part of a Renilla-Foot and Mouth Disease (FMDV) 2A fusion protein. An encephalomyocarditis virus (EMCV) IRES drives translation of the NS3-5B replicase in ORF2. When present, NS5A is expressed from ORF1 as a COOH-terminal V5 epitope tagged protein. Constructs express NS5A from ORF2 as a FLAG tagged protein. Coding regions in ORF1 downstream from Renilla 2A represent recoded NS sequence incorporating synonymous sequence alterations compared to authentic viral counterpart sequence present in ORF2. The table compares the amino acid and nucleotide sequences, as well as the CpG and UpA frequencies in ORF1 (synthetic sequence) and ORF2 (authentic viral sequences).

**Figure 2. Replication of constructs expressing NS3-5B as a Renilla-FMDV 2A fusion**

**protein.** Replication was assessed using both monocistronic (a) and intragenomic replicon constructs (b). The schematic above each graph indicates the regions of NS3-5B encoded by the recoded and authentic JFH1 coding sequences for each construct. Data represents the mean  $\pm$  s.d. from two independent experiments (a) and mean  $\pm$  s.e.m. from three independent experiments (b).

**Figure 3. Assessing the capacity of ORF1 polyproteins to support replication of intragenomic replicons with different defects in ORF2 and rescue of the same defects by**

**trans-complementation.** Replication assay data (a) of intragenomic replicons expressing functional NS3-5A or NS3-5B in ORF1 and expressing NS3-5B carrying a lethal mutation in ORF2 as defined in the schematic above the graph. These same mutations were introduced into



bicistronic replicons expressing firefly luciferase in ORF1 and the capacity of these constructs to replicated assessed in a stable neo<sup>R</sup> helper replicon cell line (b) or in naive Huh7.5 cells (c). Data represents the mean  $\pm$  s.d. from two independent experiments.

**Figure 4. Impact of single helicase point mutations K1240N and T1299A on replicon**

**replication.** Schematics above the graphs provide details of the constructs used and positioning of mutations within them. Replication assays either employed a single intragenomic replicon construct per experimental group transfected into Huh7.5 cells (a, c, d) or involved co-transfection of two separate RNAs into naive Huh7.5 cells to assess whether K1240N was dominant negative *in trans* (b). Results shown from this latter assay also include the firefly signal at 4 and 24 hours derived from the replication-defective constructs used. Data represent the mean  $\pm$  s.d. (a, c, d) or mean  $\pm$  s.e.m. (b) from 2 and 3 independent experiments respectively. Where n=3, values significantly different from the control group (mono + tRNA) are highlighted with an asterisk (p<0.05; 2-tailed t-test). Note: the assay performed to obtain data in (d) was run in parallel with another described in fig 8e and so share control groups.

**Figure 5. Analysis of mutations using the intragenomic replicon and assessing their**

**dependency on NS3-5B for rescue.** Selection of mutations was on the basis that they were refractile to rescue with ORF1 NS3-5A when present in ORF2, but tolerate when ORF1 instead expressed NS3-5B. These mutations were introduced into intragenomic replicons in various combinations and replication assays performed. Schematics above each graph (a, c, d, e, f) provide details of the constructs used and positioning of mutations within them. Also shown are Northern blot data (b) using total cellular RNA taken from a replication assay (a). Data represent the mean  $\pm$  s.d. from two separate experiments.

**Figure 6. Rescue of lethal mutations in NS3 and NS4A that disrupt processes other than NS3 helicase activity.** Genetic complementation of genetic defects was assessed a variety of ways including use of intragenomic replicons transfected into Huh7.5 cells (a, c), or using bicistronic replicon expressing firefly luciferase from ORF1 transfected into either a helper replicon cell line (d) or into naive Huh7.5 cells transduced with either baculovirus expressing NS3-5A or  $\beta$ -galactosidase (mock control) (e). Intragenomic replicons were also used to assess whether V1665G, which appeared refractile to rescue with NS3-5B, was dominant negative (c). Schematics above each graph provide specific details of the constructs used and positioning of mutations within them. Data represent the mean  $\pm$  s.d. from two separate experiments.

**Figure 7. The effect of blocking NS4B5A boundary cleavage on intragenomic replication.**

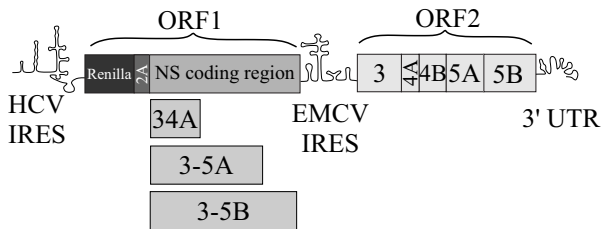
Schematics provide details of the constructs used and positioning of mutations within them. Cleavage of the NS4B5A boundary was blocked by the S1977P mutation. The impact this had on intragenomic replicons, both when it was introduced into ORF2 (a) and ORF1 (b) is shown, with cell lysates taken from the latter experiment 72 hours post-transfection also analysed by Western blot (c). Arrows indicate the position of NS5A and the uncleaved NS4B5A precursor. An asterisk indicates the position of a cross-reacting cellular protein detected by the anti-FLAG antibody. A replication assay comparing the ability of different mutated NS3-5B ORF1s, including that of S1977P, to suppress replication of intragenomic replicons carrying functional ORF2 (d) is also provided. Data represent the mean  $\pm$  s.d. from two separate experiments. Note: the assay performed to obtain data in (b) was run in parallel with that described in fig 8b and so share control groups.

**Figure 8. Impact of blocking NS4B5A cleavage on NS3-5A function.** In (a) intragenomic replicons with a functional NS3-5B encoded in ORF2 and expressing either NS5A, NS3-5A or a S1977P mutated NS3-5A from ORF1 were transfected into Huh7.5 cells. Confocal microscopy visualized the extent of ORF1 V5-tagged NS5A and ORF2 FLAG-tagged NS5A co-localization 72 hours post transfection. In (b) and (e) intragenomic replicon replication assays detail the ability of an S1977P mutated polyprotein expressed from ORF1 to complement either the NS5A S2208I (b) or NS5A G2323A (e) defect in ORF2, the schematics above the graphs indicating further details of the constructs used. In (c) *trans*-complementation of a firefly-expressing bicistronic replicon carrying the S2208I mutation is assessed using Huh7.5 cells transduced with baculovirus expressing either  $\beta$ -galactosidase (lacZ) or GFP-tagged versions of NS5A, NS3-5A and NS3-5A carrying the S1977P mutation. Western blot analysis of cell lysates taken 72 hours post transfection show levels of baculovirus-transduced expression (d). Graphical data represent the mean  $\pm$  s.d. from two separate experiments.

**Figure 9. Putative model of NS3-5B dependent complex assembly.** In (a) the NS3 helicase domain within a recently translated polyprotein precursors binds in a *cis*-dependent manner to the viral genome (for illustrative purposes the interaction is shown to occur within the 3'UTR X-region). Subsequent recruitment of additional copies of NS3-5B precursor is not a *cis*-acting event but does dependent on them possessing both NS5B and helicase-dependent RNA binding

907 activity. Our data showing blocking NS4B5A cleavage creates a dominant negative polyprotein  
908 precursor suggests that while complex activity requires completion of polyprotein proteolytic  
909 processing, complex assembly does not. In (b – f) the outcome of NS3-5B dependent complex  
910 formation is shown using the same criteria described above. Replication requires that: (1)  
911 complex assembly occurs; (2) any mutated NS protein present is rescued by a functional  
912 counterpart; (3) a polyprotein carrying a dominant negative mutation is not recruited.

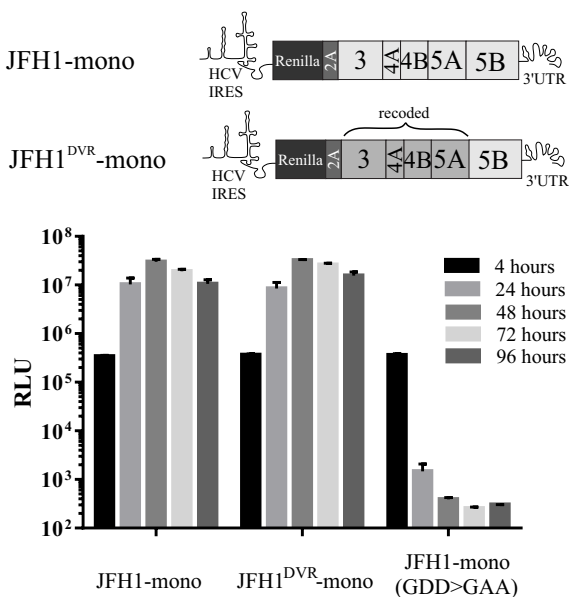
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**Fig 1**

Coding Region	Amino Acid Identity	Nucleotide Identity	ORF1 vs ORF2 CpG Frequency	ORF1 vs ORF2 UpA Frequency
NS3	100%	73%	5.92% vs 5.97%	2.96% vs 3.49%
NS4A	100%	78%	6.83% vs 8.70%	1.86% vs 2.48%
NS4B	100%	73%	4.60% vs 5.24%	2.05% vs 2.94%
NS5A	100%	72%	4.21% vs 5.87%	3.45% vs 2.43%
NS5B	100%	73%	5.36% vs 4.60%	2.81% vs 3.83%

Fig2

a



b

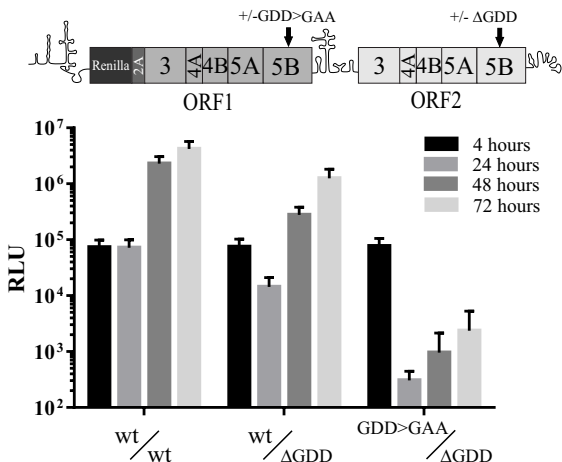
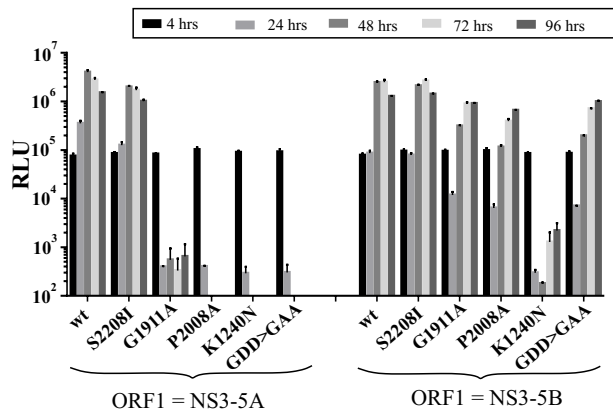
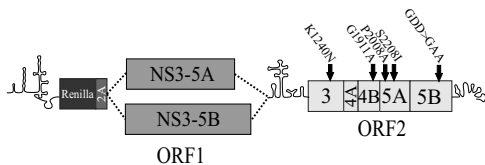
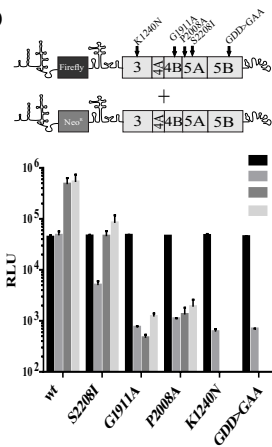


Fig 3

a



b



c

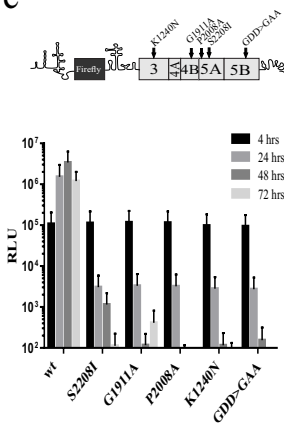
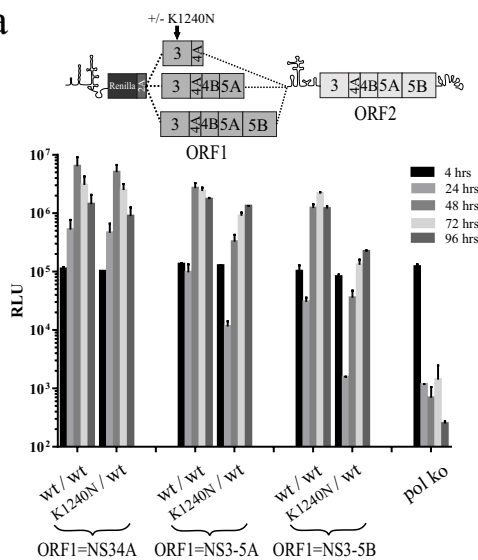
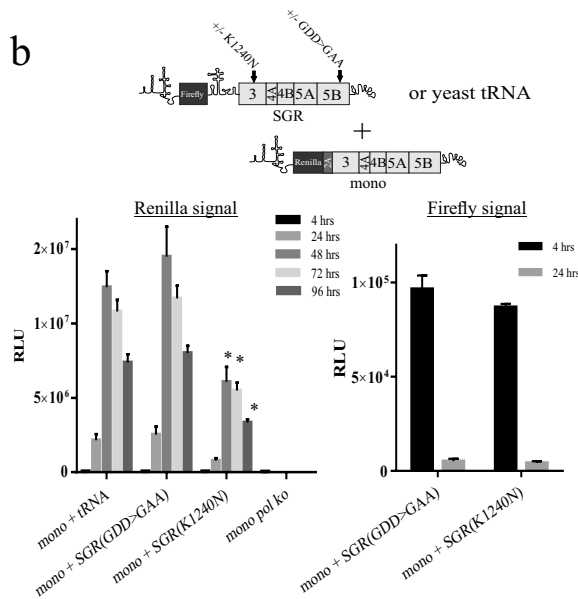


Fig 4

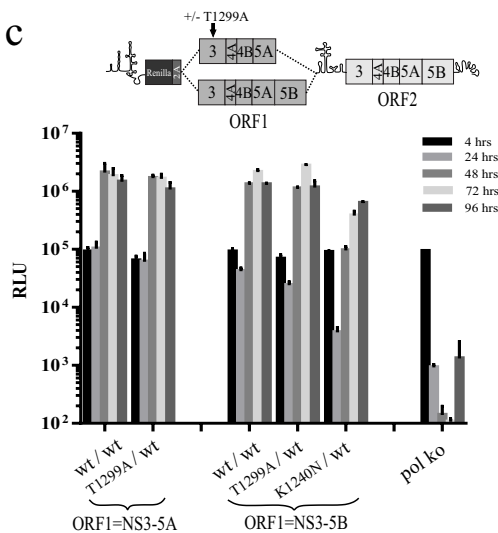
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b



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d

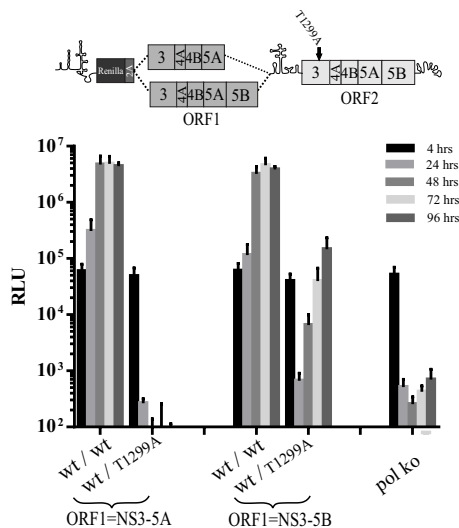
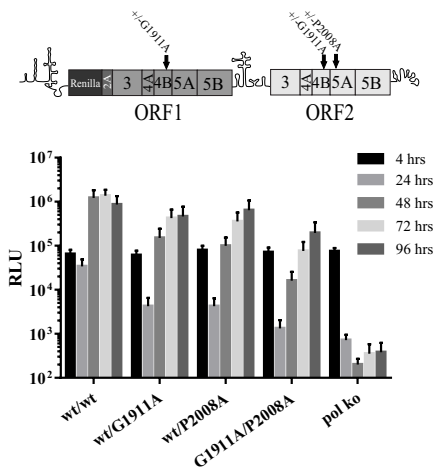


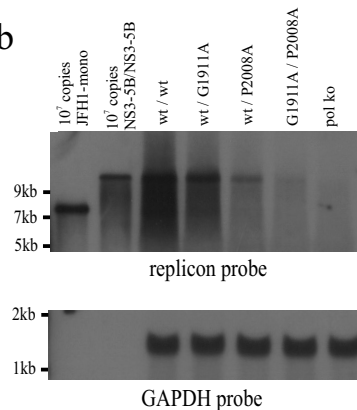


Fig 5

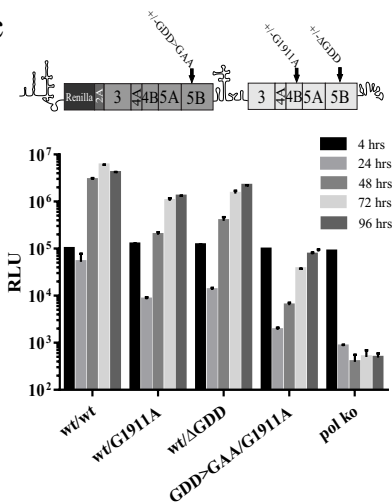
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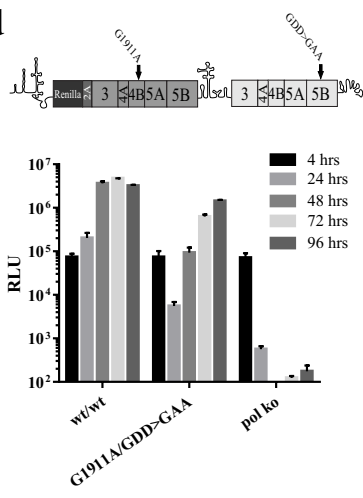
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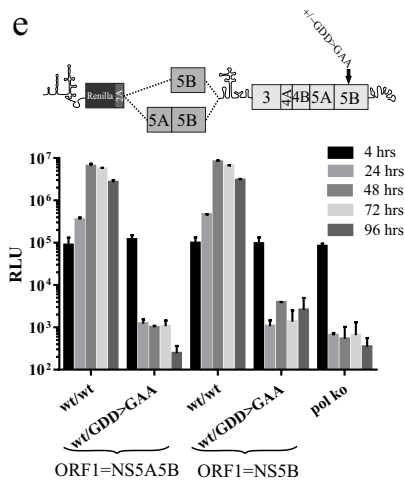
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d



e



f

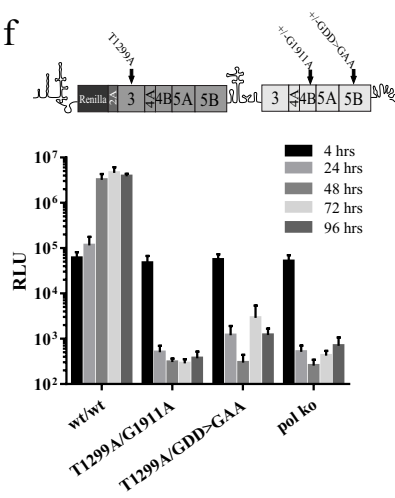
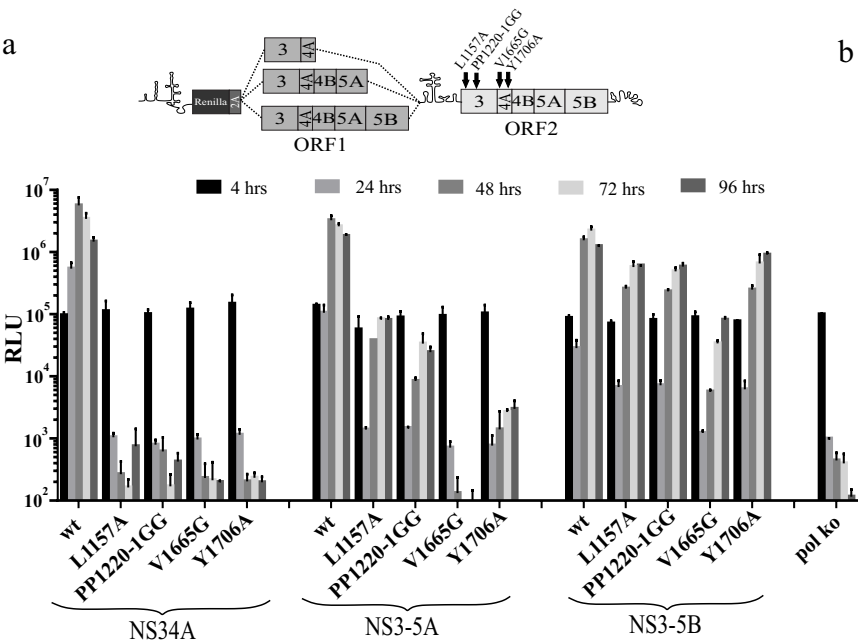
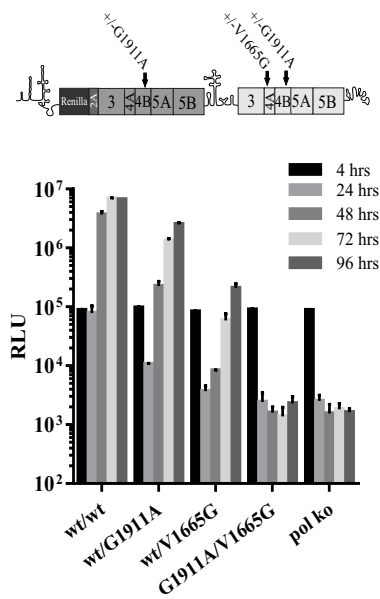


Fig 6

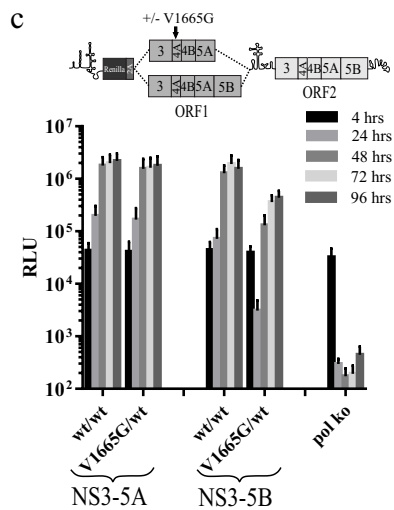
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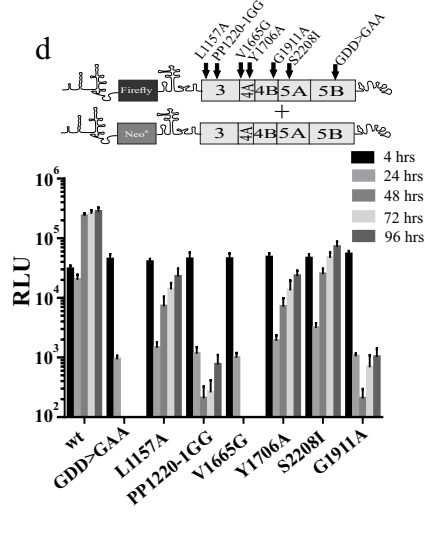
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d



e

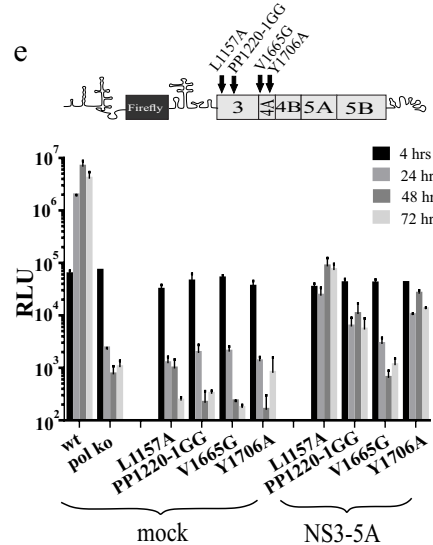
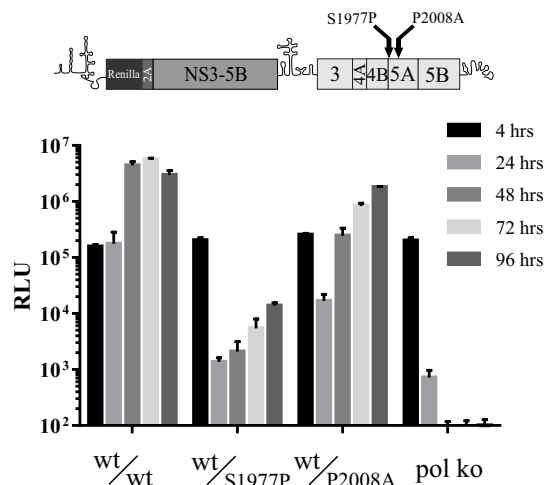
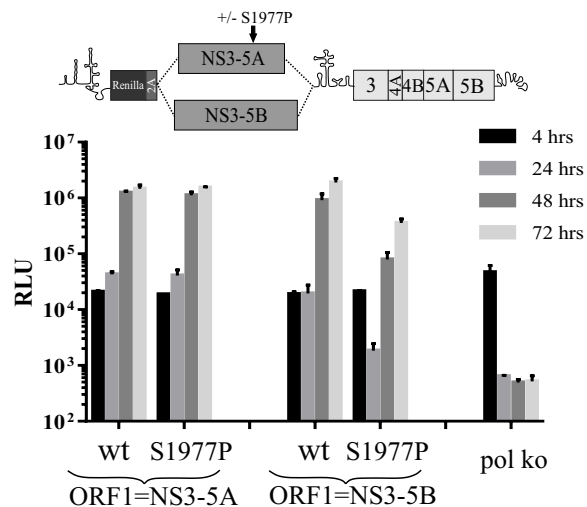


Fig 7

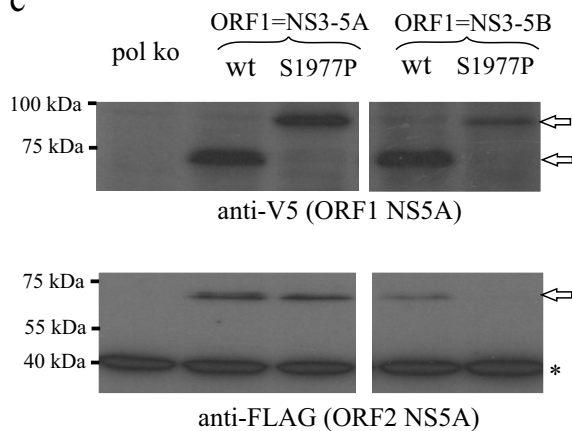
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b



c



d

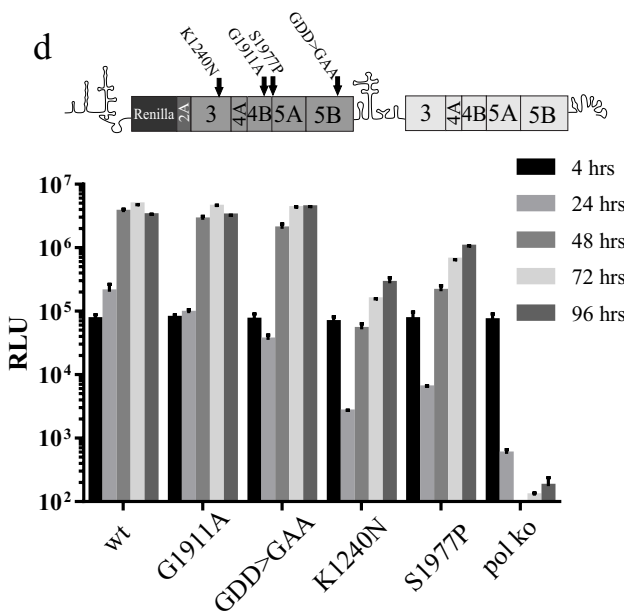
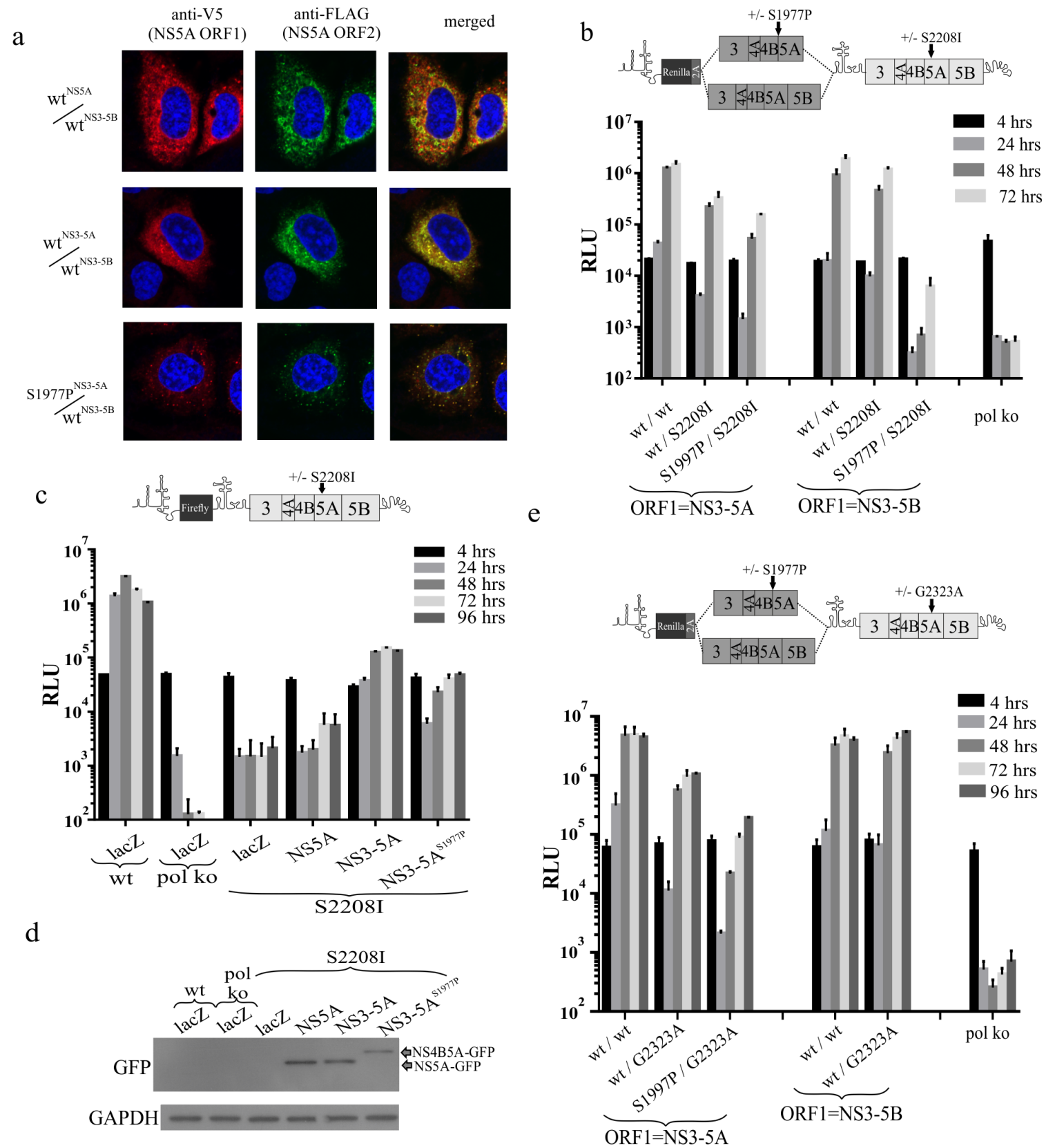
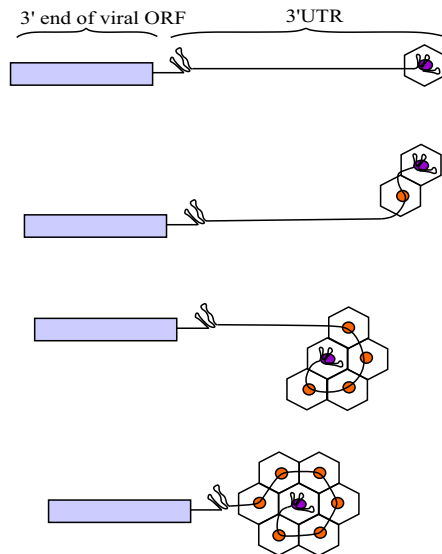


Fig 8



a



key



NS3-5B precursor

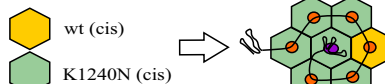


helicase cis-acting interaction site



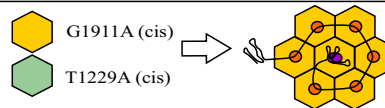
helicase trans-acting interaction site

b



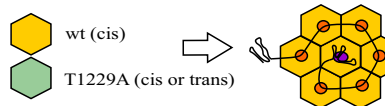
Binding of RNA by NS3 helicase enhanced by K1240N. K1240N-mutated NS3-5B over represented in complex and 'padlocks' genome, blocking replication.

c



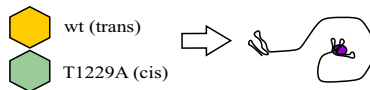
Binding of RNA by NS3 helicase blocked by T1299A. T1299A-mutated NS3-5B unable to make both cis-acting and trans-acting helicase interactions with RNA. Complex formed exclusively by G12911A-mutated NS3-5B, blocking replication.

d



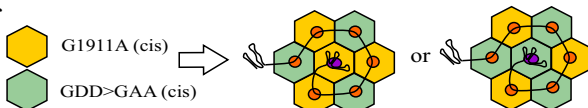
Binding of RNA by NS3 helicase blocked by T1299A. T1299A-mutated NS3-5B unable to make both cis-acting and trans-acting helicase interactions with RNA. Complex formed exclusively by wt NS3-5B, allowing replication.

e



Binding of RNA by NS3 helicase blocked by T1299A. T1299A-mutated NS3-5B unable to make initial cis-acting interactions with RNA. Complex fails to form, blocking replication.

f



Both mutated NS3-5B polyproteins able to undertake cis- and trans-interactions with viral genome. Composite NS3-5B complex formed enabling replication.