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Enhancement of the replication of HCV replicons of genotypes 1-4 by manipulation of CpG and UpA dinucleotide frequencies and use of cell lines expressing SECL14L2 - application for antiviral resistance testing

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2 Enhancement of the replication of HCV replicons of genotypes 1-4

3 by manipulation of CpG and UpA dinucleotide frequencies and use of cell lines

4 expressing SECL14L2 – application for antiviral resistance testing

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21 ABSTRACT

22

23 Treatment for hepatitis C virus (HCV) has improved greatly through the use of direct acting antivirals
24 (DAAs). However, their effectiveness and potential for drug resistance development in non-genotype
25 1 variants of HCV remains relatively unexplored as *in vitro* assays to assess drug susceptibility are
26 poorly developed and unsuited for a transient transfection format. In the current study, we have
27 evaluated effects of dinucleotide frequency changes in the replicon and the use of a SEC14L2-
28 expressing cell line on the replication of HCV of different genotypes and evaluated the resulting
29 assay formats for susceptibility measurements to the DAA, Sofosbuvir. Removal of CpG and UpA
30 dinucleotides from the luciferase gene used in HCV replicons of genotype 1b (Con1) and 2a (JFH-1)
31 achieved between 10-100-fold enhancement of replication over wild type post transfection.
32 Removal of CpG/UpA-high neomycin genes in replicons of genotype 3a (S52) and 4a (ED43)
33 enhanced replication but phenotypic effects on altering luciferase gene composition were minimal.
34 Further ten-fold replication enhancement of replicons from all four genotypes was achieved using a
35 transgenic Huh7.5 cell line expressing SECL14L2, whose expression showed a dose-dependent effect
36 of HCV replication that was reversible by siRNA knockdown of gene expression. Combining these
37 strategies, the 100 to 1000-fold enhancement of replication allowed susceptibility to the RNA
38 polymerase inhibitor, Sofosbuvir, in a transient transfection assay format to be robustly determined
39 for all four genotypes. These methods of replication enhancement provide new tools for the
40 monitoring of susceptibility and resistance of a wide range of HCV genotypes to DAAs.

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45 INTRODUCTION

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47 Hepatitis C virus (HCV) is a positive-sense RNA virus of the *Flaviviridae* family first described in 1989
48 (1) and subsequently identified as the principal cause of non-A, non-B hepatitis in blood recipients
49 and haemophiliacs, as well as widely infecting injecting drug users (2,3). Based on meta-analysis of
50 serological data it is estimated that about 185 million people worldwide are infected with HCV (4).
51 Currently there are 7 genotypes of HCV recognised of which genotype 1 accounts for 46.2% of all
52 infections, followed by genotype 3 with 30.1% and genotypes 2, 4 and 6 for 22.8% (5). It is estimated
53 that 70-80% of all new infections progress to chronicity that can ultimately lead to end-stage liver
54 disease (6,7).

55 Until recently, HCV was treated by combination-therapy of pegylated interferon-α and the
56 nucleoside inhibitor Ribavirin which is effective in genotypes 2 and 3 infections (approximately 80%
57 sustained virological response), but less so in genotypes 1 and 4, where 50% or less achieve virus
58 clearance (8-10). The phenotypic diversity of HCV genotypes, as manifested by these major
59 differences in treatment response therefore requires the effectiveness of and resistance
60 development to novel antiviral treatments to be evaluated separately for different HCV genotypes
61 and subtypes. Such testing is generally performed using subgenomic replicons in which the
62 structural genes are replaced by a luciferase or other reporter gene to allow replication to be rapidly
63 quantified (11,12). However, most wild-type replicons replicate poorly in cell culture and generally
64 restricted to the Huh7.5 cell line in which a number of cellular defence pathways are non-functional,
65 including the cytosolic RNA receptor, RIG-I (13). However, under antibiotic selection for replicon-
66 containing cells, stable cell lines can be selected in which replicons rapidly acquire cell culture
67 adaptive mutations that enhance replication. For example, in the genotype 1b Con 1 replicon,
68 adaptive mutations occur in the NS3, NS4B and NS5A genes (12,14,15) that may enhance HCV
69 protein-protein interactions and viral morphogenesis, although the mechanisms remains poorly

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70 understood (16). Such mutants, however, show increases in viral RNA in transient replication assays
71 without antibiotic selection. Although there now exist a wide range of stably transfected Huh7.5 cell
72 lines containing replicons of different HCV genotypes, the existence of functionally poorly defined
73 adaptive mutations has the potential to influence their susceptibility to directly acting antivirals
74 (DAAs) and effects of DAA-associated mutations on antiviral susceptibility. While transient
75 replication assays are clearly preferable for such testing, many of the currently available replicons do
76 not have the required level of replication needed in this assay format to accurately estimate changes
77 in replication levels in drugs inhibition studies

78 In the current study, we have investigated the effectiveness of two novel approaches to enhance
79 HCV replication in cell culture. The first approach is to reduce frequencies of CpG and UpA
80 dinucleotides in the reporter gene of HCV replicons to enhance replication. The rationale is based on
81 previous studies that demonstrate that lowering CpG and UpA frequencies in coding regions of
82 echovirus 7 or the luciferase gene in a derived replicon substantially enhanced their replication over
83 wild type virus (17,18). A second, separate approach is based upon the recent finding that
84 expression of the SEC14L2 gene is a limiting factor in HCV cell culture replication and enhancement
85 of HCV replication was achieved through the use of cell lines that overexpressed the SEC14L2 gene,
86 increasing the number of colony forming units in antibiotic-selected non-transient replicon studies
87 (19).

88

89

90 MATERIALS AND METHODS

91

92 **Replicon construction, RNA *in vitro* transcription and translation.** The Con1 subgenomic replicon
93 was provided by R. Bartenschlager (11), SGR-JFH1 and SGR-JFH1/GND were provided by J.
94 McLauchlan (20), S52/SG-Feo (AII) and ED43/SG-Feo (VYG) were obtained from C. Rice (21). A
95 synthetic DNA sequence used to replace the wild type (WT) luciferase sequence was specified using

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96 the program Sequence Mutate in the SSE package (22) and was cloned in the various replicons using
97 unique restriction sites at the 5' end (*Asc*I) and 3' end (*Pme*I) of the luciferase coding sequence (Fig.
98 1).

99 Replicon plasmids were linearized with *Xba*I, Mung-bean nuclease treated, purified, quantified and
100 used as template for *in vitro* RNA transcription (MEGAscript, Invitrogen). RNA was precipitated using
101 LiCl, washed, resuspended in water, aliquoted and stored at -80°C.

102 Transcript RNAs (125 ng) were used in nuclease-treated rabbit reticulocyte lysate translation assays
103 (Promega) to compare the translation speed of the different luciferase constructs according to the
104 manufacturer's instructions. Samples were taken after 30 minutes incubation and the luciferase
105 expression quantified as described below.

106 **Cell lines, electroporation and luciferase quantification.** Huh7.5 cells were maintained in Dulbecco's
107 modified Eagle's medium (DMEM; Invitrogen) supplemented with 4,500 mg/l glucose, 2 mM L-
108 glutamine, 10% heat-inactivated foetal calf serum (FCS; Harlan Sera-Lab), nonessential amino acids,
109 20 mM HEPES, 100 U/ml penicillin, and 100 µg/ml streptomycin and incubated at 37°C, 5% CO_2, and
110 100% relative humidity.

111 For electroporation, cells were washed 3x in cold PBS, counted and diluted to 10^7 cells/ml. Using
112 4mm electroporation cuvettes, 400µl of the cell suspension was mixed with 1 µg of replicon RNA and
113 electroporated (at 270V, 950µF) using the exponential setting in an electroporator (Bio-rad,
114 Genepulser XCell). Cells were immediately resuspended in warm, complete DMEM and transferred
115 to the appropriate sized cell culture dishes. At the desired time points, medium was removed, the
116 cells washed with PBS and lysed in passive lysis buffer (Promega) and the luciferase expression
117 measured using the 'Steady-glo' assay system (Promega) and a luminometer (Promega, Glomax
118 Multi detection system).

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119 To obtain stable cell lines expressing SEC14L2, a lentiviral vector expressing this gene (Applied
120 Biological Materials) was used to generate lentiviruses according to the manufacturer's instructions.
121 Huh7.5 cells were transduced with the lentivirus particles and selected with 5 µg/ml of puromycin.
122 Single colonies were isolated and grown for further evaluation.

123 **Protein isolation and Western blotting.** Cell monolayers were washed with PBS before being lysed
124 in RIPA buffer (50mM Tris, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate and 1% Triton-X100),
125 mixed with sample buffer (Sigma, Laemmli 2x concentrate) and boiled for 10 minutes. Protein
126 samples were loaded on 10% gels (Bio-rad, mini-protean TGX), transferred by semi-dry blotting to
127 Immobilon paper (Millipore) and blocked in 5% milk-powder in PBS. After washing in PBS-T (PBS with
128 0.05% Tween), blots were probed with anti-SEC14L2 (Santa Cruz Biotechnology) or anti β-tubulin
129 (Abcam) as a loading control for one hour at room temperature, washed and probed with anti-
130 mouse HRP or anti-rabbit HRP respectively, for one hour at room temperature, washed and
131 developed using ECL (ECL prime, Amersham).

132 **siRNA transfection.** Knockdown of SEC14L2 was performed using the commercially available and
133 validated esiRNA, EHU146781 (Sigma-Aldrich). The irrelevant siRNA sequences used as a control was
134 obtained from the same manufacturer (EHUEGFP). To knock-down SEC14L2 expression in the stable
135 cell line, a titration of SEC14L2-specfic siRNA was performed by transfecting 0, 100, 200 and 400 ng
136 of siRNA/ 24-well using Lipofectamine RNAiMAX transfection reagent (Invitrogen) using the
137 manufacturer's instructions. Mock treated cells received 400 ng of validated non-targeted control
138 siRNA. After 48 hours, SEC14L2 expression levels were quantified by Western-blot and ImageJ
139 software.

140 **Sofosbuvir susceptibility testing.** For the titration of Sofosbuvir (PSI-7977, Cayman chemical), cells
141 were electroporated with replicon RNA and seeded at the required density. After 4 hours the
142 medium was replaced with DMEM containing the desired concentration of Sofosbuvir dissolved in

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143 ethanol or mock treatment control (ethanol) and refreshed every 24 hours to ensure a constant
144 Sofosbuvir presence in the medium.

145 **qRT-PCR.** SEC14L2 mRNA expression levels were quantified by qRT-PCR using the forward primer: 5'-
146 TGCAGTGATCCTGGCATCTATG-3' and reverse: 5'-TGAGGCTTTGTCTGGAAGCAG-3'. RNA was extracted
147 using the RNeasy kit (Qiagen), DNAse-treated and reverse transcribed using GoScript reverse
148 transcriptase (Promega). The qRT-PCR was performed in a Rotorgene real-time PCR cycler (Qiagen)
149 using the SensiFAST sybr kit (Bioline) and GAPDH for normalization (Forward 5'-
150 GAAATCCCATCACCATCTTCCAGG-3'; Reverse 5'-GAGCCCCAGCCTTCTCCATG-3'). RNA stability of the
151 different replicons was determined by measuring luciferase-RNA levels at four hours post
152 electroporation by qPCR (Forward 5'- CCCTGGTTCCTGGAACAATTGC-3' and Reverse 5'-
153 AAGAATTGAAGAGAGTTTTCACTGC-3').

154

155

156 RESULTS

157

158 **Effect of CpG/UpA dinucleotide composition on the replication of HCV replicons.** In its unmodified
159 state, the genotype 2a replicon (JFH1) already shows a robust level of replication in a number of cell
160 lines, without any need for cell-culture adaptive mutations (20, 23, 24). We used the JFH1 replicon
161 expressing the firefly luciferase gene driven by the HCV 5' UTR (20). The WT luciferase sequence
162 shows a striking elevation of the observed to expected frequency of CpG dinucleotides (1.21; Table
163 1) compared to that in HCV (0.71 – 0.74 in genotypes 1-6) and in human mRNA sequences (mean
164 0.43). High frequencies of this dinucleotide and UpA substantially restricted the replication of
165 echovirus 7 (17) and we reasoned this may also influence the replication capability of the HCV
166 replicons in mammalian cell culture. We therefore replaced the wild type luciferase gene (L) in JFH1
167 with the CpG/UpA-low mutant (l) in both the replication-competent (GDD) and replication

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168 incompetent background (GND). The modified luciferase sequence was mutated to remove all 100
169 of the CpG dinucleotides present in the native sequence and 64 of the 86 UpA dinucleotides, the
170 maximum possible while retaining identical amino acid coding to the WT sequence (Table 1; Fig. 1A).
171 The modified sequence, however, contained a similar G+C content, codon adaptation index (CAI)
172 and codon pair bias (CPB) to the WT sequence.

173 Genotype 2a replicons with WT and CpG/UpA-low luciferase reporter genes (2a/l-GDD and 2a/L-GDD
174 respectively) were transfected into Huh7.5 cells and luciferase expression quantified at different
175 time points post-electroporation (Fig. 2A). To ensure equal amounts of RNA were delivered in the
176 cells, an aliquot of cells were taken 15 minutes after electroporation, RNAse-treated to remove
177 extracellular RNA and the internalized luciferase-RNA quantified by qRT-PCR. No significant
178 differences in amounts of RNA were found between the different mutants used was observed (data
179 not shown). Despite this equal delivery of RNA, the replication kinetics of the 2a/L-GDD (original)
180 and 2a/l-GDD (CpG/UpA-minimised) were distinct with an approximate hundred-fold (2-log)
181 difference in luciferase expression at 48 hours and 96 hours. This difference was greater than the 5-
182 fold-difference in luciferase gene expression 4 hours post-transfection and at earlier time points
183 before significant replication of HCV had taken place (Fig. S1; Supplementary Data).

184 The same changes were also made in the genotype 1b background, using the Con1 subgenomic
185 replicon (1b/L-GDD, 1b/l-GDD and replication defective counterparts, 1b/L-GND, 1b/l-GND; Fig. 2B).
186 Unlike the 2a replicons, the WT (1b/L-GDD) replicon showed very poor replication levels in Huh7.5
187 cells (Fig. 2B) in the first 24 hours. RT-qPCR analysis confirmed that comparable amounts of RNA
188 were electroporated in the cells at one hour (data not shown). Similarly, from 24 hours, their
189 replication kinetics were distinct, where the low-CpG/UpA luciferase replicon showed a greater than
190 2-log higher luciferase levels at 96 hours post electroporation compared to 1b/L-GDD.

191 To investigate effects of dinucleotide composition changes for other genotypes, a range of replicons
192 were constructed using genotype 3a (S52/SG-Feo(AII)) and 4a (ED43/SG-Feo (VYG)) backbones

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193 (Saeed et al. (21)). The original replicons expressed luciferase as a fusion with neomycin (N) which
194 also possesses high frequencies of CpG and UpA dinucleotides (Fig. 1B). Mutants were constructed in
195 which luciferase was replaced with the CpG/UpA-low sequence (3a/lN-GDD and 4a/lN-GDD), those
196 with deletion of the neomycin gene (3a/L-GDD and 3a/L-GND) and a combination of CpG/UpA-low
197 luciferase sequences and neomycin deletion (3a/l-GDD and 4a/l-GDD).

198 Both of the original replicons, 3a/LN-GDD and 4a/LN-GDD, of genotypes 3 and 4 replicated poorly in
199 transient replication assays compared to genotype 2a (Figs. 1C, 1D). In contrast to previous
200 experiments using 1b and 2a-based replicons, replacing the luciferase sequence in genotype 3
201 (3a/lN-GDD) did not enhance replication. However, deletion of the neomycin sequence in the 3a/L-
202 GDD increased replication compared to 3a/LN-GDD, although this effect was not reproduced in the
203 low luciferase version of the neomycin-deleted construct (3a/l-GDD). For genotype 4, deletion of the
204 neomycin gene (4a/L-GDD) and introduction of low-CpG/UpA luciferase (4a/lN-GDD) both enhanced
205 replication over the original replicon (4a/LN-GDD), effects that were synergistic at early time points
206 (4a/l-GDD). For all genotype 3 and 4 replicon mutants, quantitiation of transfected RNA at one hour
207 demonstrated that comparable amounts of RNA were electroporated into the cells (data not
208 shown).

209 **Effect of SEC14L2 expression on HCV replication.** To investigate whether the reported enhancement
210 of replication by expression of SEC14L2 (19) could also be achieved in a transient transfection
211 replication assay, a number of clonal cell-lines stably expressing SEC14L2 were made. Based on
212 SEC14L2 expression levels measured by RT-qPCR, several lines were selected and tested for SEC14L2
213 proteins expression levels by Western blot (Fig. 3A). All five cell lines selected constitutively
214 expressed detectable but variable levels of SEC14L2 protein while it was undetectable in the
215 parental Huh7.5 cell line (labelled "P").

216 Three out of five cell lines supported enhanced replication of 1b/L-GDD replicon (Fig. 3B), with cell
217 line #23 showing the highest increase in replication compared to the parental cell line

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218 (approximately x25-fold). To verify this replication enhancement arose directly from increased
219 expression of SEC14L2 and was not an artefact introduced the transduction and consequent single
220 cell selection, siRNA was transfected in #23 cells to knock-down SEC14L2 protein expression (Fig.
221 3C). The effect of SEC14L2 knockdown on HCV replication was investigated in cells transfected with a
222 siRNA concentration that achieved a 75% knock-down of expression (Fig. 3D). These cells were
223 electroporated with 1b/l-GDD, and luciferase expression measured compared to mock-treated #23
224 cells (Fig. 3D). Knock down SEC14L2 decreased replicon replication by approximately 4-fold at 72
225 hours, confirming the involvement of SEC14L2 in the control of replicon replication.

226 **Replication of HCV genotypes 1-4 in SEC14L2-expressing cells.** Replicons with all four genotype
227 backbones and corresponding CpG/UpA-low luciferase mutants were electroporated into the
228 SEC14L2 expressing cell line #23 and replication compared to that of the parental Huh7.5 cells (Fig.
229 4). Replication of the 2a/L-GDD replicon was increased approximately 17-fold in the SEC14L2
230 expressing cell line at the 72 h.p.e. time point; a slightly lower enhancement was observed in the
231 2a/l-GDD (CpG/UpA-low) construct (Fig. 4A). Replication enhancement was synergistic, with a 340-
232 fold increase in luciferase expression in 2a/l-GDD in #23 cells compared to 2a/L-GDD in Huh7.5 cells.
233 Consistent with a role for SEC14L2 expression in enhancing replication, luciferase expression from
234 both 2a/L-GND and 2a/l-GND defective replicons was comparable between cell lines (Fig. 4A).

235 A comparable 10-fold enhancement of the replication of the Con1 1b/L-GDD and 1b/l-GDD replicons
236 was observed in #23 cells, with similarly no effect on their replication defective counterparts (Fig.
237 4B). SEC14L2 expression and lowering CpG/UpA frequencies had a synergistic effect, leading to an
238 overall 1200-fold replication enhancement compared to the original replicon in Huh7.5 cells.
239 Replicons based on genotype 3a and 4a backbones (3a/L-GDD and 4a/L-GDD) showed approximately
240 1-log increased luciferase expression. A much larger cell line-dependent increase was observed in
241 the Neomycin-deleted 3a replicon, 3a/l-GDD (Figs. 3C, 3D).

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242 **Mechanism of replication enhancement.** The increased replication of replicons with alterations to
243 dinucleotide composition may have originated from differences in the efficiency of translation of the
244 luciferase gene through associated alterations in codon usage or codon pair bias (25-27).
245 Alternatively, as demonstrated for E7, changes in CpG and UpA frequency may influence the cellular
246 response to infection and induce greater restriction of replication (17). To investigate effects of CpG
247 and UpA dinucleotide frequency changes on translation, replicons from all four genotypes containing
248 the original (L) or modified (l) luciferase genes were assayed for translation efficiency in an *in vitro*
249 translation assay (Fig. 5). Despite the large differences in codon usage and codon pair bias between
250 the insect-derived luciferase gene and the CpG/UpA-minimised mutant sequence (Table 1),
251 expression of the original and mutant forms of the luciferase gene in all four replicons of genotypes
252 1-4 was similar. The two forms of luciferase gene showed at most 2-fold differences in translation
253 efficiency but with no evidence for any consistent greater expression of the CpG/UpA-low luciferase
254 sequences over wild type (Fig. 5). To ensure the assay was not saturated with RNA that narrowed
255 differences in expression, the assay was repeated using different RNA transcript amounts.
256 Transfecting 4 times more and four times less RNA confirmed that the read-outs for the assay
257 concentrations used were in the linear range (Fig. S2; Supplementary Data).

258 Replicons containing modified luciferase gene sequences showed comparable stability post-
259 transfection. In the absence of replication, RNA levels of both genotype 1 and 2a replicons showed a
260 comparable at 4 hours post-transfection (Fig. S3; Supplementary Data).

261 As the restriction in replication engendered by increased frequencies of CpG and UpA dinucleotides
262 composition was not mediated though differences in translation efficiency or greater RNA instability,
263 we next investigated whether the inhibition of replication in replicons expressing native (high
264 CpG/UpA) luciferase genes was mediated on the replicon containing the gene sequence (*in cis*) or
265 induced a global change in the dell in permissivity to HCV replication (*in trans*). 1b/L-GDD and 1b/l-
266 GDD (containing WT- and CpG/UpA-low luciferase gene sequences respectively) were co-

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267 electroporated into Huh7.5 cells (Fig. 6A). The presence of 1b/L-GDD RNA minimally reduced the
268 replication of 1b/l-GDD (approximately 50%) compared to expression levels of 1b/l-GDD
269 electroporated alone. The experiment was repeated in the SEC14L2 expressing cell line, where no
270 effect on the expression of luciferase compared to electroporation on 1b/l-GDD alone (Fig. 6B).
271 These findings provide evidence that effects of dinucleotide composition are mediated locally (in *cis*)
272 on the RNA molecule possessing the altered dinucleotide composition rather than such sequence
273 inducing a whole cellular restriction on replication (*eg.* mediated through induction of interferon-β).

274 **Use of enhanced transient transfection assays to measure susceptibility to DAAs.** For testing the
275 inhibitory capacity of HCV antiviral agents in transient replication assays, achieving sufficient
276 replication levels to quantify degrees of inhibition is essential. We therefore investigated whether
277 the enhanced replication achieved by replacing the wild-type luciferase or culture in SEC14L2-
278 expressing cell line facilitated the evaluation the NS5B inhibitor Sofosbuvir in genotypes 1b, 3a and
279 4a. Cells were electroporated with each replicons in the presence of a range of Sofosbuvir
280 concentrations spanning the previously established IC_{50} concentration (28,29). Luciferase expression
281 levels were measured at time points 48, 72 and 96 hours post electroporation. Non-replicating
282 replicons were included as baseline levels of luciferase expression. The 1b/L-GDD and 4a/L-GDD
283 replicons replicated at such low levels in the Huh7.5 cell lines that replication inhibition by
284 Sofosbuvir could not be detected (Fig. 7, left two panels). However, performing the assay in SEC14L2
285 expressing cells improved the replication and enabled a concentration-dependent inhibition of
286 replication by sofosbuvir to be detected (Fig. 7A/7C, panels 7-9). A major enhancement was
287 observed using the 1b/l-GDD replicon in Huh7.5 cells which improved even more when using
288 SEC14L2 expressing cells. This same pattern, but to a lesser extent was observed in genotype 4a.
289 Although the wild type genotype 3a replicon already shows usable titration data in Huh7.5 cells,
290 removal of Neomycin and especially the use of SEC14L2 expressing cells improved overall replication
291 and enabled a robust estimation of IC_{50} values for sofosbuvir for this genotype (Table 2).

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292 DISCUSSION

293

294 Despite only reproducing the intracellular replication steps of the HCV lifecycle, replicons are a
295 valuable tool in HCV research, especially in drug discovery programs and have been instrumental in
296 the discovery of the first direct-acting antivirals (DAAs) (30), including Sofosbuvir (31), Simeprevir
297 (32,33) and Boceprevir (34). In addition to susceptibility testing, *in vitro* systems play an essential
298 role in monitoring the phenotypic effects of resistance-associated mutations that arise during DAA
299 treatment on drug susceptibility (35,36). Resistance mutations are frequently genotype-specific and
300 for their effects to be quantified, inhibition assays require that these mutations should be tested in
301 the same genotypic background *in vitro.* DAA susceptibility and resistance testing should also be
302 performed without the confounding effect of unpredictable cell-culture adaptive mutations that are
303 likely to arise with antibiotic selection.

304 To date however, most information on susceptibility and resistance testing has been based on *in*
305 *vitro* assays using cell lines stably expressing HCV replicon-RNA, typically H77 (genotype 1a) and
306 Con1 (genotype 1b) (36). Selection of stable cell lines prior to testing is also normally used in the
307 analysis or selection of resistance mutations (38-40). However, construction of stably transformed
308 cell lines is a time-consuming procedure and which is also likely to introduce additional cell culture
309 adaptive mutations that may also influence DAA susceptibility, complicating the comparison with the
310 wild-type replicons. Therefore the use of a transient expression assay with a range of genotypes
311 would be highly advantageous in terms of speed, simplicity and avoidance of cell culture-induced
312 artefacts. Although some of these have been used in the analysis of, for example, several resistance-
313 associated mutations to Sofosbuvir (29), the low level of replication generally achieved means that
314 such studies are problematic to extend for a wider range of HCV strains and genotypes.

315 To improve the replication of replicons in transient expression assays we first replaced the wild-type
316 luciferase gene with a mutated version in which all CpG dinucleotides were removed and as many

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317 UpA dinucleotides as possible while retaining the same coding sequence and similar codon usage. In
318 both genotypes 1b (Con1) and 2a (JFH1), this replacement resulted in 2-log improved replication
319 rates compared to replicons containing the original wild type luciferase sequence (Figs. 1A and 1B).
320 This enhancement of replication was comparable to that exhibited in the echovirus replicon with
321 similar replacement of the luciferase gene (17). How this enhancement of both initial gene
322 expression and subsequent increased replication is mediated remains uncertain, although
323 observation in the current study can rule out some mechanisms. Firstly, the absence of any
324 consistent difference in translation efficiency of original and modified luciferase genes in different
325 replicon constructs clearly demonstrates that the enhancement of replication of CpG/UpA-low
326 replicons was not mediated though a translational mechanism. These findings are consistent with
327 previous studies demonstrating comparable translation efficiency of mutants of E7 with regions of
328 genome with altered dinucleotide frequencies and codon pair bias (18), and of poliovirus in which
329 relatively small differences in translation rates between PV-Min, WT and PV-Max mutants differing
330 in codon pair usage / dinucleotide frequencies were not predictive of their replicative ability (26). A
331 detailed investigation of a range of compositional variables on the replication of poliovirus reported
332 that CpG and UpA dinucleotide frequencies primarily influenced the replication of poliovirus, and
333 was unaffected by variation in codon usage, codon pair bias and other metrics of predictive of
334 translational optimisation, such as CAI (27). Modification of codon pair bias and CpG/UpA
335 dinucleotide frequencies in echovirus 7 similarly indicated the primary influence of dinucleotide
336 frequencies on virus replication (18). In a broader context, differences in expression of the luciferase
337 gene mediated purely through translational effects cannot contribute to the replication fitness of
338 HCV replicons as its purpose is simply to act as reporter gene. The enhancement of replication of
339 replicons containing CpG/UpA-low luciferase coding sequences must therefore be mediated though
340 alternative mechanisms.

341 The minimal or absent interference on replication of the 1b/l-GDD by the wild type 1b/L-GDD
342 replicon similarly argues against a global restriction in permissivity for viral replication that would be

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343 expected in interferon-primed cells. The lack of interference between replicons is however
344 compatible with the hypothesis advanced previously to explain the restriction of CpG/UpA-high
345 mutants of E7. In this account, possession of high CpG and UpA dinucleotides may induce a localised
346 stress response in the cell that influences the ability of viruses to establish replication complexes
347 (17). The HCV replicon system established in the current study provides a valuable tool for future
348 dissection of the restriction mechanisms associated with altered dinucleotide frequencies

349 The clear replication enhancement observed in the genotype 1b and 2a replicons containing
350 CpG/UpA-low luciferase sequence was not fully reproduced in genotypes 3a and 4a, but showed a
351 more subtle and complicated pattern. The original versions of both replicons expressed luciferase as
352 a fusion protein with neomycin (21). Removal of neomycin in genotype 3a increased replication
353 although in contrast to other replicons, replacement of the luciferase component of the fusion
354 protein with the CpG/UpA-low sequence negatively impacted replication of the genotype 3a replicon
355 (Fig. 2C) for reasons that remain undetermined. In the genotype 4a replicon, removal of the
356 neomycin gene similarly resulted in enhanced replication at late time points but in this case the
357 further introduction of the low CpG/UpA luciferase further increased replication to a similar degree
358 to that observed in genotype 1b and 2a. Understanding how the restrictions in replication mediated
359 though dinucleotide composition interact with the limited or complete inability of many genotype or
360 strains of HCV to replicate in cell culture will require a much better understanding of the cellular
361 pathways that mediate these replication phenotypes and their potential for interaction.

362 As a further manifestation of the complexity of the restriction of HCV replication *in vitro*, substantial
363 increases in the replication of replicons with all four genotype backgrounds were achieved in cells
364 over-expressing SEC14L2. SEC14L2 was originally reported to enhance the replication of non-cell
365 culture adapted isolates of HCV that are not resistant to lipid peroxidation (19). We investigated
366 whether same positive effect on replication could be achieved for replicons in a transient expression
367 assay format. Transduction of parental Huh7.5 cells and consequent selection yielded colonies with

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368 varying levels of SEC14L2 mRNA- and protein expression (Fig. 3A). Knock-down of SEC14L2 by siRNA
369 was used to confirm the involvement of this gene and not an artefact of transduction or selection
370 (Figs. 2C and 2D). Transient expression assays of HCV replicons showed a broadly consistent ten-fold
371 increase in replication in cells expressing high levels of SEC14L2 compared to the parental Huh7.5
372 cell line with some higher (genotype 3a, neomycin-low CpG/UpA) and lower (genotype 4a, low
373 CpG/UpA) exceptions (Figs. 4A-C). Expression of SEC14L2 had no effect on the expression of
374 luciferase in replication-incompetent (GND) replicons, suggesting that SEC14L2 directly influenced
375 replication rather than RNA stability and/or translation. However, in contrast to previously reported
376 results (19), we observed a consistent and substantial increase in the replication in the lipid-
377 peroxidation resistant genotype 2a JFH1 replicon, whose replication was not enhanced in stably-
378 transfected cell lines. Differences in assay systems may have contributed to this difference; firstly
379 our experiments were performed in a cell line with SEC14L2 expressed from a transgene that is not
380 under the same regulatory control as the native gene. Secondly, we electroporated the cells instead
381 of using transfection; this may deliver RNA at a different location and efficiency and influence the
382 efficiency of initial translation and gene expression and consequent replication efficiency.

383 Irrespective of the likely complex mechanisms underlying the restriction of HCV replication in cell
384 culture, this study achieved its original goal of improving the replication of replicons that enable
385 their use in transient expression assays. This enabled a pilot study of the susceptibility of different
386 genotypes to the NS5B inhibitor Sofosbuvir (Fig. 7). The enhancement of replication in SEC14L2-
387 expressing cells and changing to the low CpG/UpA luciferase reporter gene enabled robust
388 measurement of IC_{50}s for genotypes 1-4 (Table 2), generating values that were comparable to those
389 reported previously from other assay systems (28,29).

390 In summary, we have shown that reducing the number of CpG and UpA dinucleotides in HCV
391 subgenomic replicons can greatly enhance replication levels but with some variability between
392 genotypes. Combined with further increases in HCV replication in cell expressing SEC14L2, the 30-

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393 1000-fold increases in replication achieved across all four genotypes will greatly facilitate
394 susceptibility and resistance mutation testing in a convenient and rapid transient expression assay
395 format.

396

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400

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403 McLaughlan for providing us with HCV plasmids. One author (PS) has filed a patent describing the
404 use of dinucleotide frequency changes to enhance virus, replicon and vector replication.

405 Author Contributions: JW and MMG carried out the laboratory work described in the study. PS and
406 JW conceived the study and designed the vectors and experimental design and wrote the
407 manuscript.

408

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536 TABLE 1

537 Composition and coding parameters of WT and CpG / UpA-low luciferase sequences

538

Sequence	Symbol	Subs.[a]	C+G%	CpG	ΔCpG [b]	O/E [c]	UpA	ΔUpA [b]	O/E	CAI[d]	CPS [e]
WT	L	---	0.44	100	---	1.21	86	---	0.70	0.719	-.011
CpG/UpA-low	l	174	0.45	0	100	0	22	64	0.17	0.804	0.021

539

540 [a]Number of sequence changes from WT sequence

541 [b]Change in the numbers of CpG and UpA dinucleotides

542 [c]Observed to expected frequencies of CpG and UpA dinucleotides

543 [d]Calculated using the website http://genomes.urv.es/CAIcal/ (41)

544 [e]Calculated as previously described (26,42)

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550 TABLE 2

551 IC_{50} values for Sofosbuvir in genotypes 1b (Con1), 3a (S52) and 4a (ED43)

Cell	Replicon	48 h.p.e.	72 h.p.e.	96 h.p.e.
7.5	1b/L-GDD	-[a]	-	-
	1b/I-GDD	-	-	-
	3a/LN-GDD	48±4[b]	42.3±1.3	57±2
	3a/L-GDD	53±8	47.3±8.8	40±2
	4a/LN-GDD	-	-	-
	4a/I-GDD	-	-	49±27
#23	1b/L-GDD	-	-	-
	1b/I-GDD	95±8	42±4	55±6
	3a/LN-GDD	35±3	38±6	29±1
	3a/L-GDD	42±3	41±2	29±1
	4a/LN-GDD	-	-	28±10
	4a/I-GDD	-	45±15	38.5±0.5

552

553 [a] - : Not determined (insufficient replication)

554 [b]IC_{50} values are the concentration of Sofosbuvir that inhibits the replicon of the HCV replicon by 50%

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556 FIGURE LEGENDS

557

558 **Fig. 1**. Structure of sub-genomic replicons and mutants. Diagrammatic representations of the
559 replicons used in the current study, with changes to the luciferase gene (L, l), deletion of the
560 neomycin gene (N) and replacement of the GDD motif in NS5B with GND to make it replication
561 defective labelled.

562 **Fig. 2**. Replication of unmodified replicons and mutants with low CpG/UpA luciferase. Replication of
563 genotype (A) 2a, (B) 1b, (C) 3a and (D) 4a replicons after electroporation into Huh7.5 cells.
564 Luciferase activity of was measured at four time points (x-axis) and plotted as the absolute values (y-
565 axis). Error bars represent standard deviations.

566 **Fig. 3**. Expression of SEC14L2 in different cell lines and effect of HCV replication. (A) Quantification of
567 SEC14L2 expression by western blot of cell lysates from different cell lines. P: parental Huh7.5 cell
568 line. Samples were normalized to total amount of protein loaded; a conrol immunoblot using β-
569 tubulin is shown below (B) luciferase expression in different cell lines at different time points after
570 electroporation of 1b/l-GDD. The values are normalised to luciferase expression of the 1b/l-GDD
571 replicon in the parental Huh7.5 cell line at each time point. (C) Effect of transfection of SEC14L2-
572 specific siRNA in line #23 on SEC14L2 expression levels as determined by western blot (D) Replication
573 of 1b/l-GDD in siRNA-treated and irrelevant siRNA-treated #23 cells. Error bars depict standard
574 deviations.

575 **Fig. 4**. Comparison of the replication of replicons from genotypes 1-4 in Huh7.5 and #23 cell lines.
576 Replication of replicons from genotypes 1-4 generated in the study in Huh7.5 and #23 cell lines; right
577 hand panels show luciferase expression from the replication incompetent control. Error bars depict
578 standard deviations.

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579 **Fig. 5**. *In vitro* translation assay of wild type and low CpG/UpA luciferase replicons. Measurement of
580 luciferase activity of translation products of WT and CpG/UpA-low HCV replicons of genotypes 1-4
581 after a 30 minute incubation time. Bars show the mean of two replicates; error bars show standard
582 deviations.

583 **Fig. 6**. Co-electroporation of CpG/UpA-low and WT luciferase containing replicons. Luciferase
584 expression in (A) Huh7.5 cells and (B) #23 cells after electroporation of CpG/UpA-low, WT or both
585 replicons; y-axis scale is normalised to luciferase expression by 1b/l-GDD (100%). Error bars depict
586 standard deviations.

587 **Fig. 7**. Susceptibility testing to Sofosbuvir of original and modified replicons. Inhibition of replication
588 of CpG-low and WT luciferase replicons (A) 1b/l-GDD and 1b/L-GDD, (B) 3a/LN-GDD and 3a/L-GDD
589 and (C) 4a/LN-GDD and 4a/l-GDD to differing concentration of Sofosbuvir. The experiment was
590 performed in Huh7.5 and #23 cell lines. Error bars depict standard deviations.

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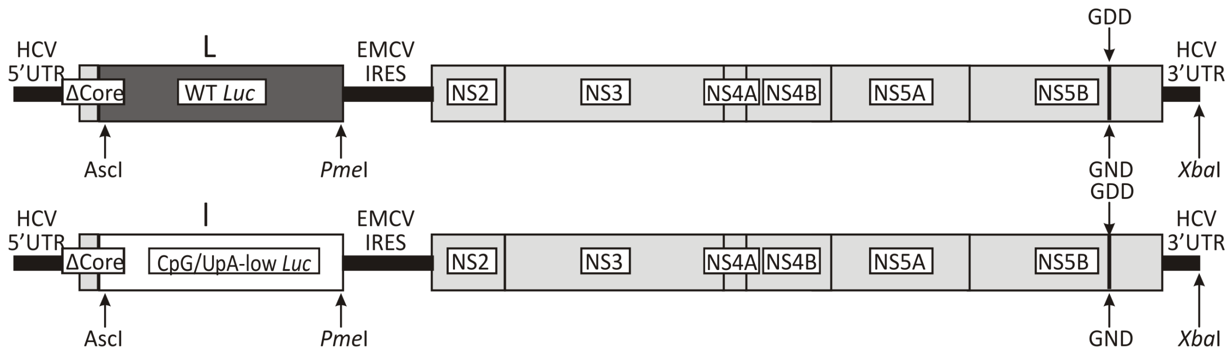
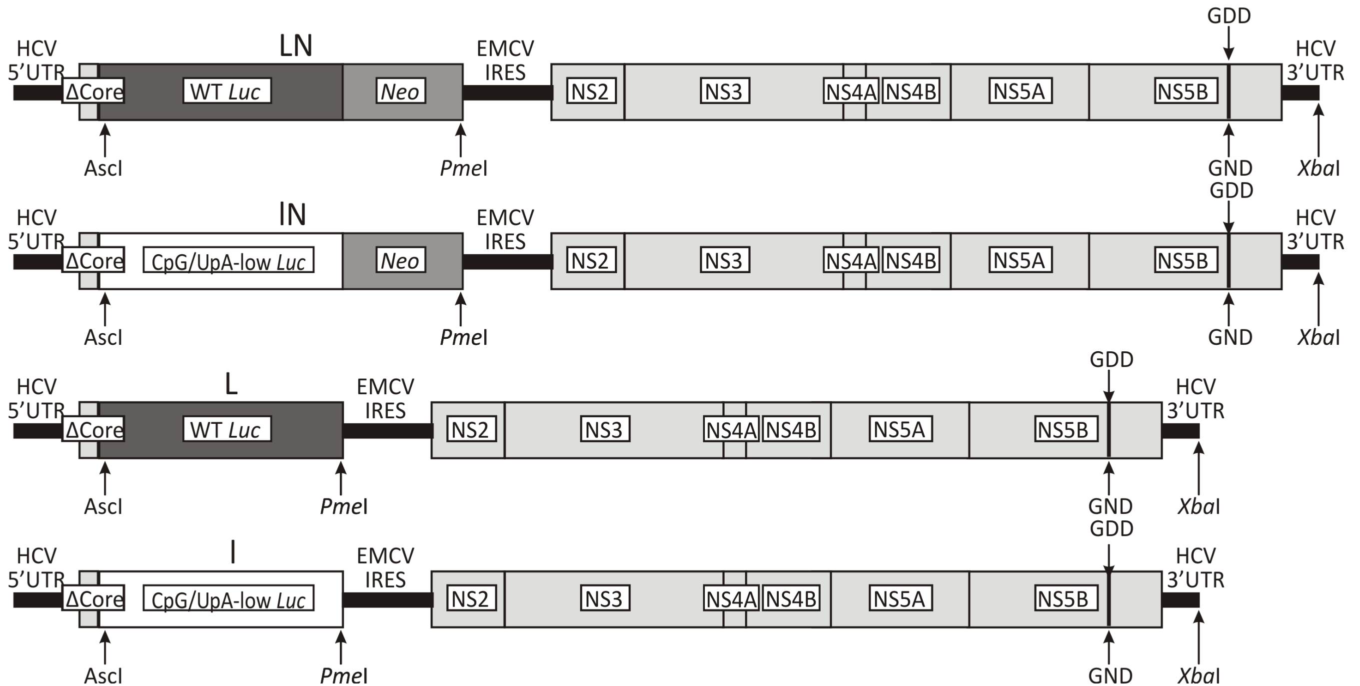
A) Genotype 2a (pSGR-JFH-1) and 1b (pSGR-Con1) replicons



B) Genotype 3a (S52/SG-Feo) and 4a (ED43/SG-Feo) replicons



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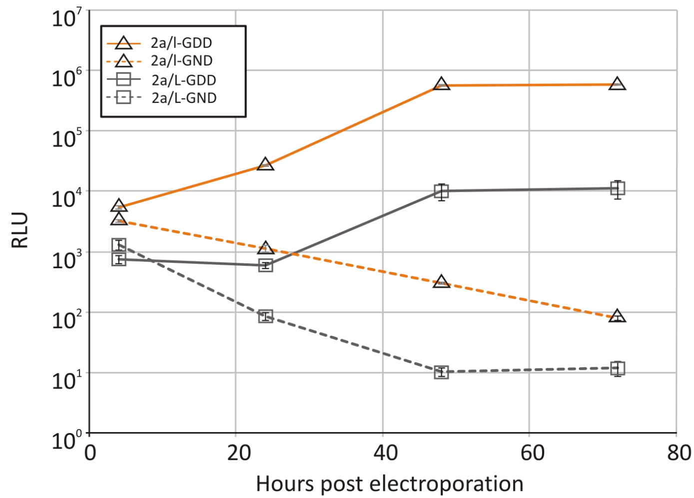

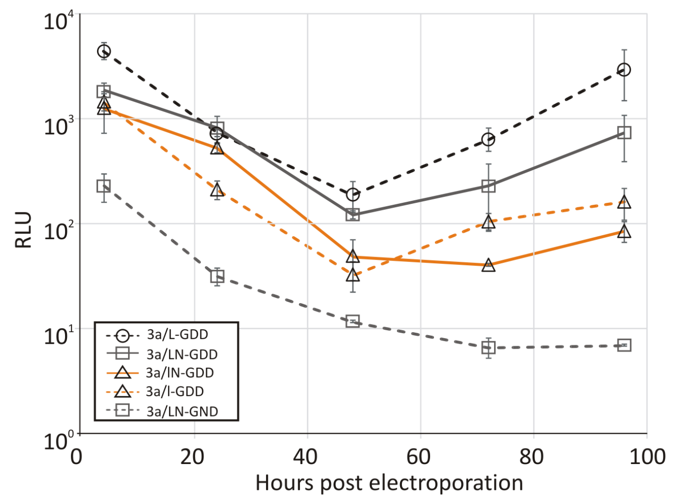

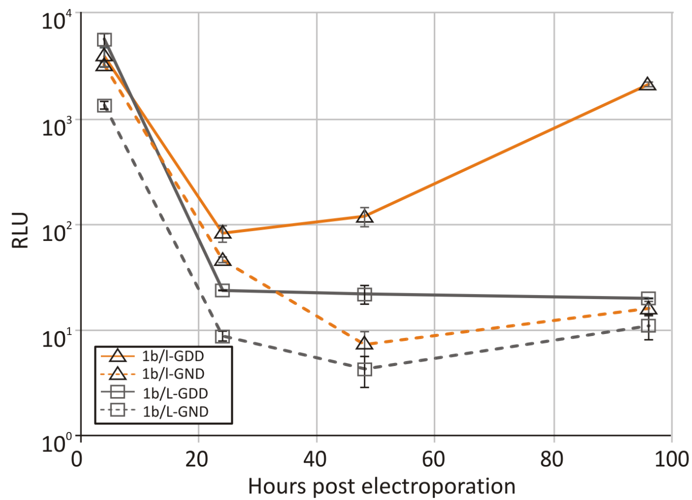

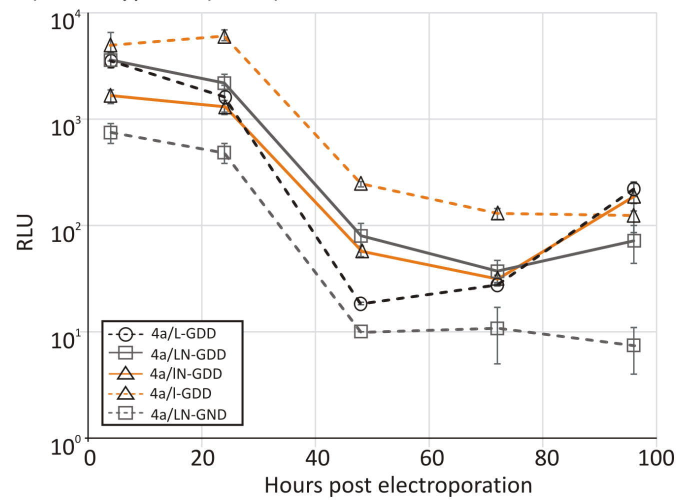

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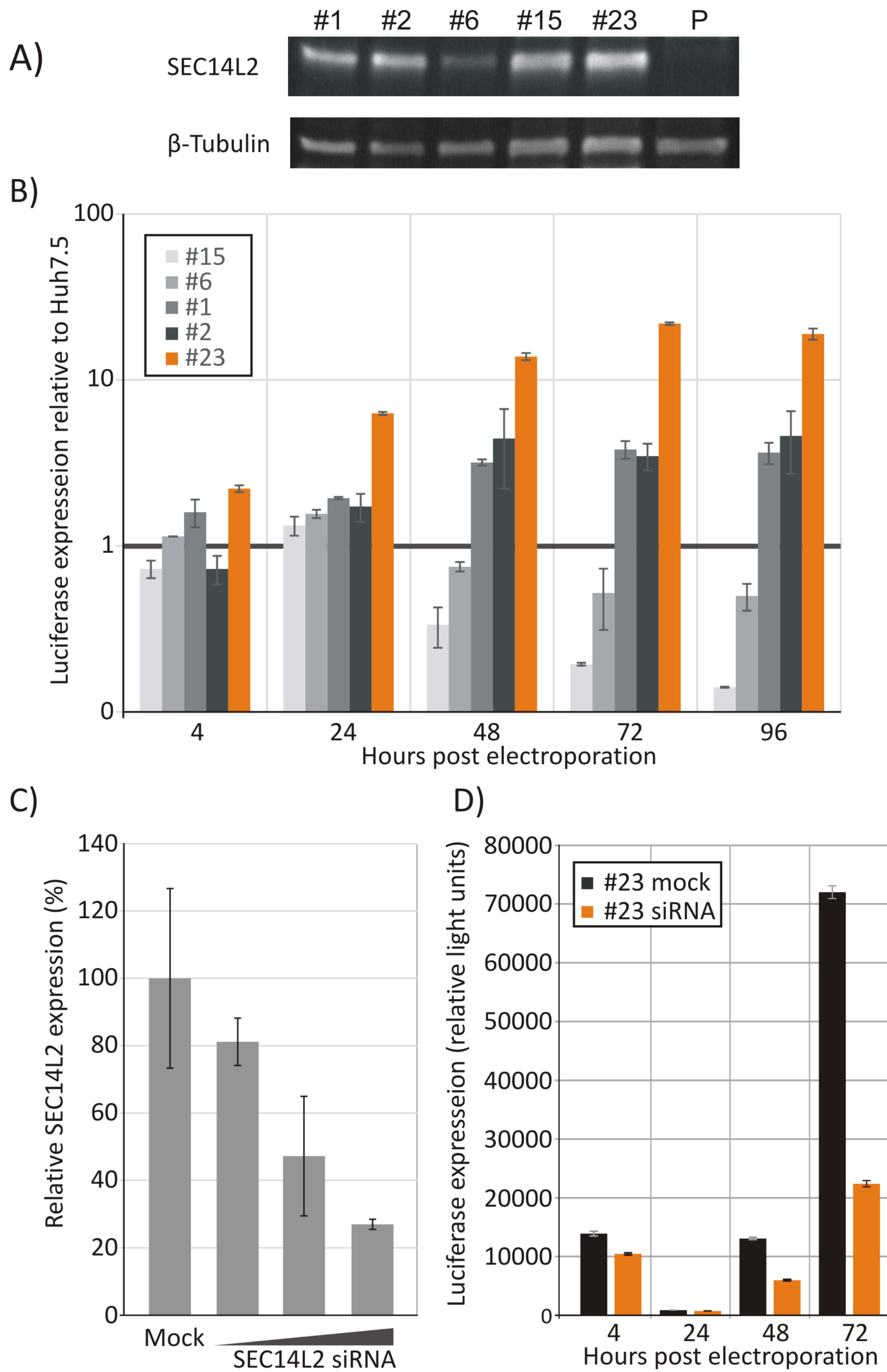

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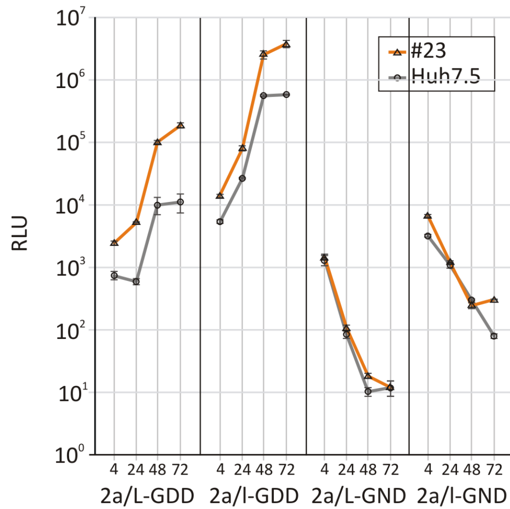
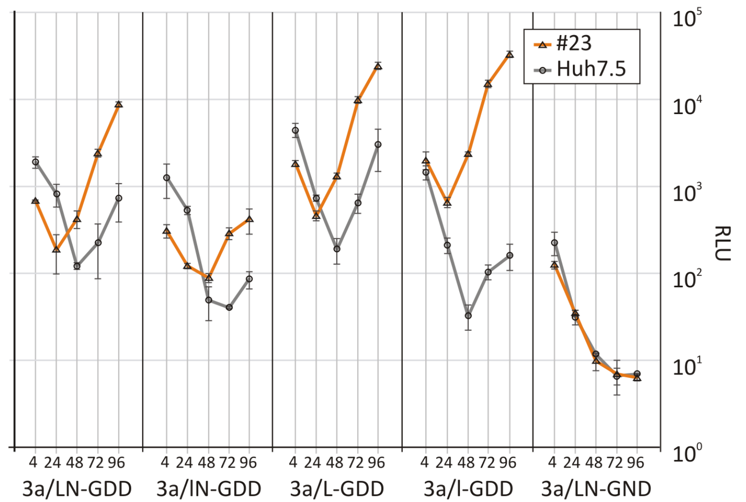
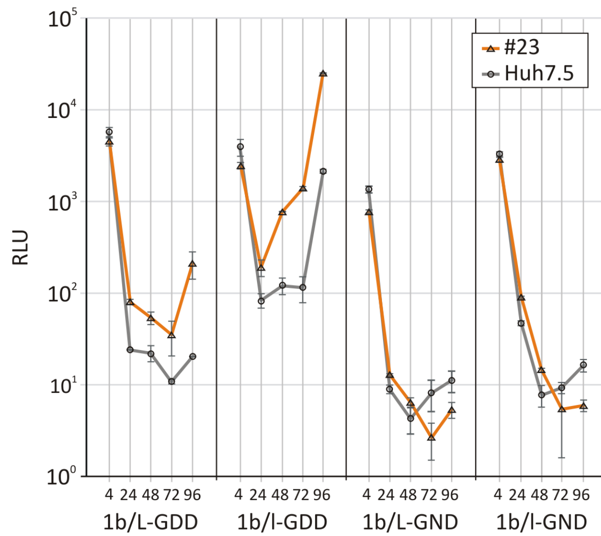
A) Genotype 2a (JFH)



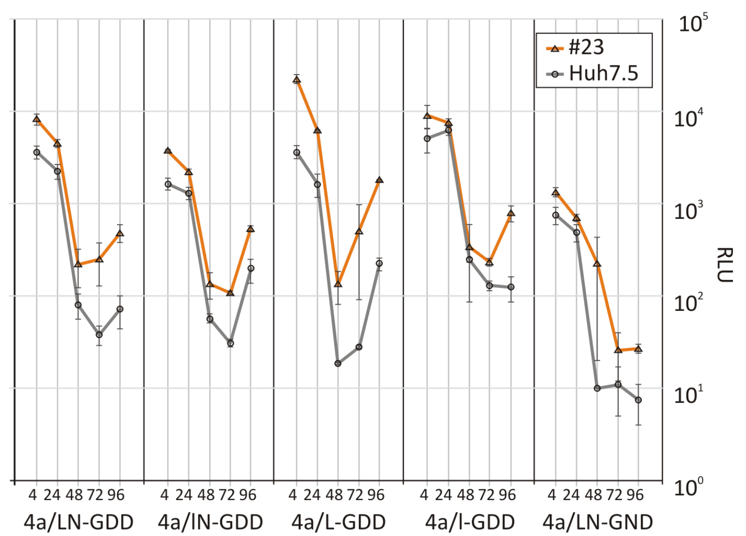
C) Genotype 3a (S52)



B) Genotype 1b (Con1)



D) Genotype 4a (ED43)



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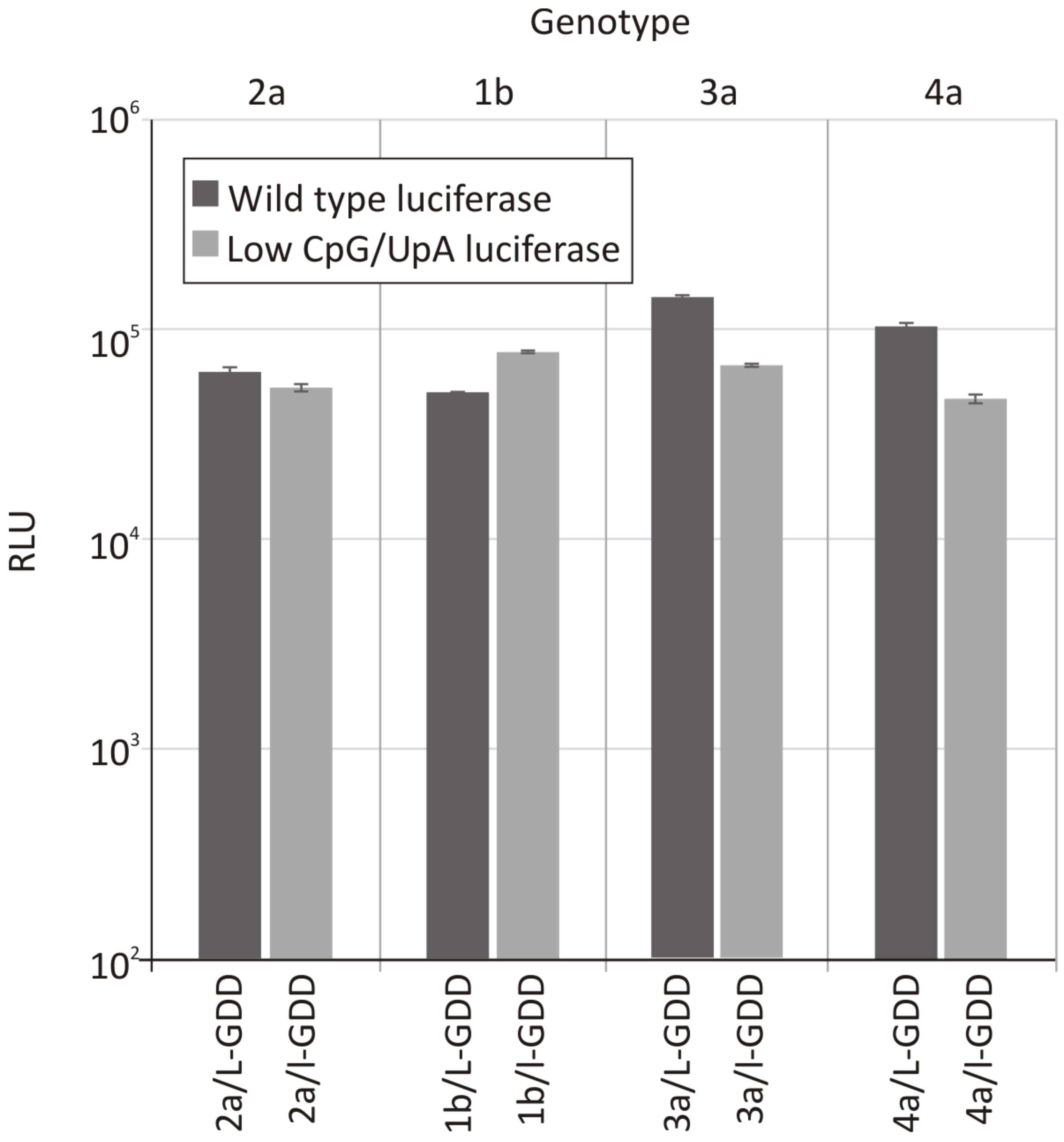

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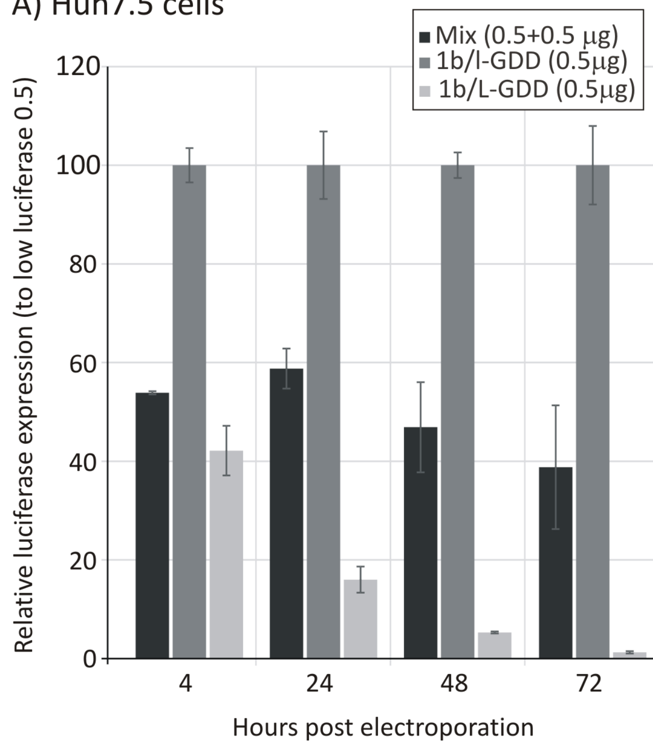

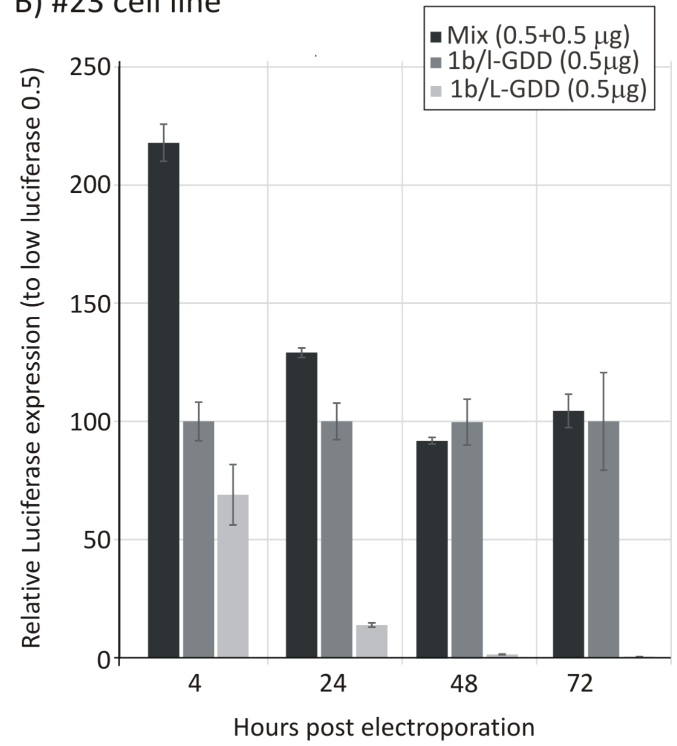

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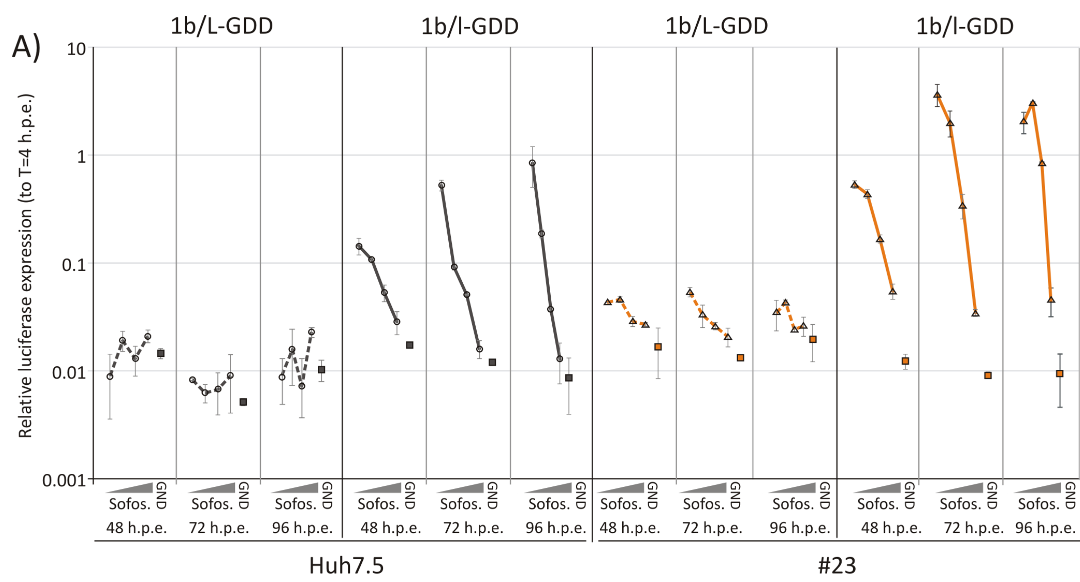

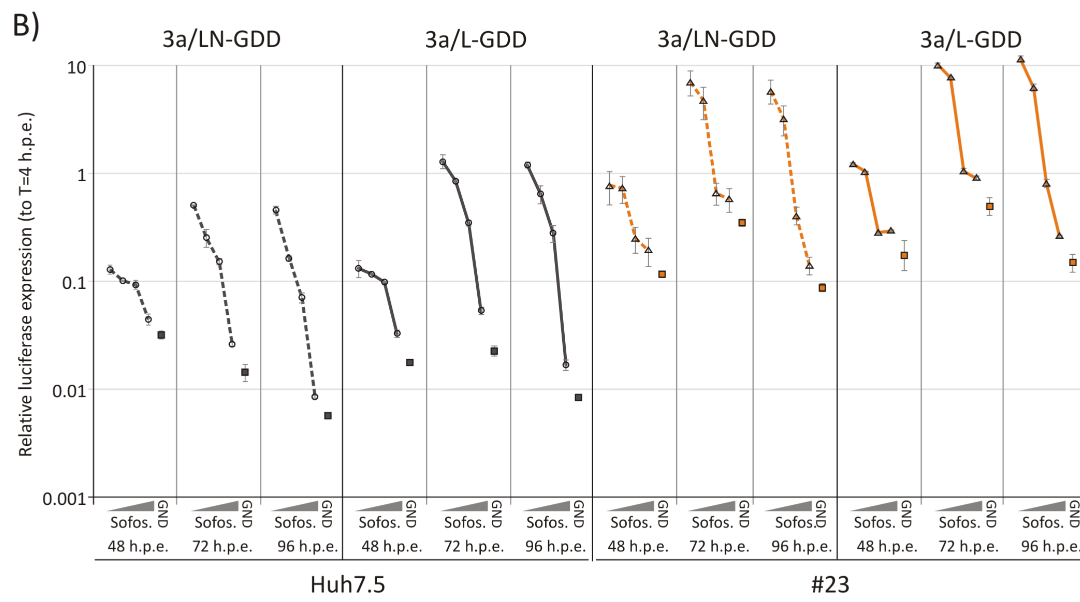

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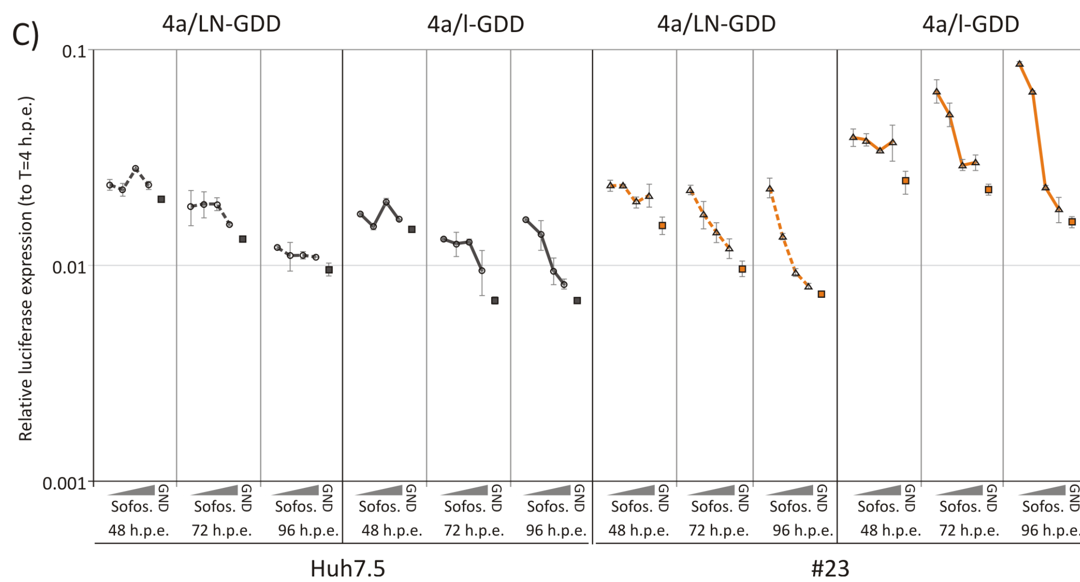

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