Supplementary Material

In-depth longitudinal study on hepatitis E virus evolution in chronic infection with ribavirin treatment failure

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Table S1. Details of nested PCR fragments amplified from HEV cDNA and used for sequencing.

Fragment	Length [bp]	Nucleotide position (47832c)	Nucleotide position (1a-Burma)
F1.2	2200	34–2233	36–2238
F2.2	1514	2569–4082	2219–3868
F3.2	2212	3716–5927	3502–5716
F4.2	1684	5688–7371	5477–7159
F5.2	7220	30–7249	32–7257
HVR2	472	2133–2604	2135–2390

Table S2. Details on HEV-specific primers and cycling conditions for amplification of first PCR fragments (F1.1-F4.1) and nested fragments (F1.2-F4.2), and HVR fragments (HVR1 and HVR2). Sequences of forward (F) and reverse (R) primers indicated. For a first round of PCR (Fx.1/HVR1), 2.5 µl cDNA was used in a 25 µl reaction. For nested PCR (Fx.2/HVR2), 0.5 µl of the first was used in a 25 µl reaction. For culture samples, F2.1/F2.2 required primer re-design due to the presence of a dominant 3' mismatch in the primer binding region, and subsequent re-amplification and re-sequencing. Similar re-design was required for F3.1, albeit only for culture A Ctrl samples from w34, w54, and w60.

Fragment	**Primers**	**Cycling conditions**
F1.1	Fw1: 5'- CCACCAGTTCATTAAGGCTCCT Rv1: 5'- CCTCTTGGGGTTATGCTCAAC	30" 98°C; [10" 98°C; 30" 68°C; 90" 72°C] 35x; 120" 72°C
F1.2	Fw2: 5'- CCACCAGTTCATTAAGGCTCCT Rv2: 5'- AGGAGGTTTAGGGTGGCRTA	30" 98°C; [10" 98°C; 30" 68°C; 75" 72°C] 30x; 120" 72°C
F2.1	Fw1: 5'- CACCCGCCGCCTTCTTTA Fw1a: 5'- CACCCGCCGCCTTCTT Rv1: 5'- GGGATGAATCTAGCCAGGGAC	30" 98°C; [10" 98°C; 30" 68°C; 60" 72°C] 35x; 120" 72°C
F2.2	Fw2: 5'- CACCCGCCGCCTTCTTTA Fw2a: 5'- CACCCGCCGCCTTCTT Rv2: 5'- CAGACACCGTAAGCTCCTGAG Rv2a: 5'- ACTATGTCCGTGAGTTCAAAGACT	30" 98°C; [10" 98°C; 30" 68°C; 50" 72°C] 30x; 120" 72°C
F3.1	Fw1: 5'- GCHAGGGGGCTYATYCAATC Rv1: 5'- CGGGAYACACGAGTGTTRGTG Rv1a: 5'- CGGGAYACACGAGTGTTGGTR	30" 98°C; [10" 98°C; 30" 69°C; 90" 72°C] 35x; 120" 72°C
F3.2	Fw2: 5'- GCHAGGGGGCTYATYCAATC Rv2: 5'- AGCRCGYACAACCCGATACTG	30" 98°C; [10" 98°C; 30" 69°C; 90" 72°C] 30x; 120" 72°C
F4.1	Fw1: 5'- CAGTGGTTTCTGGGGTGACA Rv1: 5'- AAAGCTATGAAGGGGGCACAA	30" 98°C; [10" 98°C; 30" 68°C; 60" 72°C] 35x; 120" 72°C
F4.2	Fw2: 5'- CAGTGGTTTCTGGGGTGACA Rv2: 5'- ACTGCCGTTTCACCTGCT	30" 98°C; [10" 98°C; 30" 68°C; 60" 72°C] 30x; 120" 72°C
F5.1	Fw1: 5'- AGGCCCAYCAGTTYATTAAGGCTCCTGGCATYACT Rv1: 5'- CACACCCCTGCAAACCAAGRGCGCGRCACTCCGG	5' 95°C; [20" 98°C; 8' 74°C] 35x; 5' 74°C
F5.2	Fw1: 5'- AGGCCCAYCAGTTYATTAAGGCTCCTGGCATYACT Rv2: 5'- CGGCACTCAGGGCAGAAATCATCRAAAGTRTGGG	5' 95°C; [20" 98°C; 8' 74°C] 35x; 5' 74°C
HVR1	Fw1: 5'- CGCATAGCCTCTCCTACGA Rv1: 5'- CCTCTTGGGGTTATGCTCAAC	30" 98°C; [10" 98°C; 30" 65°C; 40" 72°C] 35x; 120" 72°C
HVR2	Fw2: 5'- GGACATGGTCAACATCTGGT Rv2: 5'- GCCCCATCCGGATAGGTG	30" 98°C; [10" 98°C; 30" 66°C; 15" 72°C] 30x; 120" 72°C

Table S3. Absolute numbers of synonymous and non-synonymous variants detected at ≥5%, ≥20%, and ≥50% frequencies in patient and *in vitro* (adapted; naïve) timepoints. *In vitro* data is background-corrected against w1 variant counts.

		# synonymous			# non-synonymous		
Model	**Timepoint**	**≥5%**	**≥20%**	**≥50%**	**≥5%**	**≥20%**	**≥50%**
Patient	m76.5	42	269	202	14	69	57
	m95.2	177	17	274	51	6	71
Adapted	Ctrl_w18	22	8	0	7	4	0
	Ctrl_w31	46	14	0	4	5	0
	RBV_w31	69	27	1	23	6	0
	Ctrl_w34	29	13	2	9	2	1
	RBV_w34	88	13	15	39	2	6
	Ctrl_w54	43	2	10	7	0	3
	RBV_w54	77	24	27	29	5	7
	Ctrl_w60	23	1	11	5	0	3
	RBV_w60	102	14	40	39	7	7
Naïve	Ctrl_w5	17	0	0	4	2	0
	Ctrl_w20	26	7	0	14	2	0
	RBV_w20	113	12	0	35	7	0
	Ctrl_w23	21	10	0	12	5	8
	RBV_w23	94	6	4	36	6	4
	Ctrl_w40	42	13	1	13	6	2
	RBV_w40	126	20	24	44	5	14
	Ctrl_w43	43	13	1	14	2	6
	RBV_w43	98	25	19	40	6	14

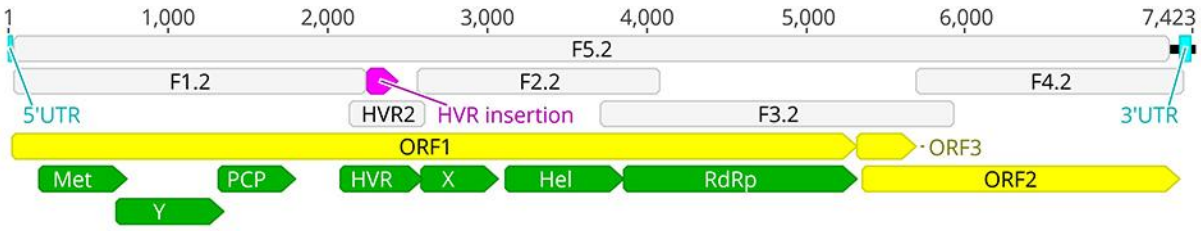

Figure S4. Position of nested PCR fragments within the HEV 47832c genomes (nucleotide positions on top). 5' and 3'UTRs, ORFs with functional domains, as well as the ORF1 insertion within the HVR are annotated.

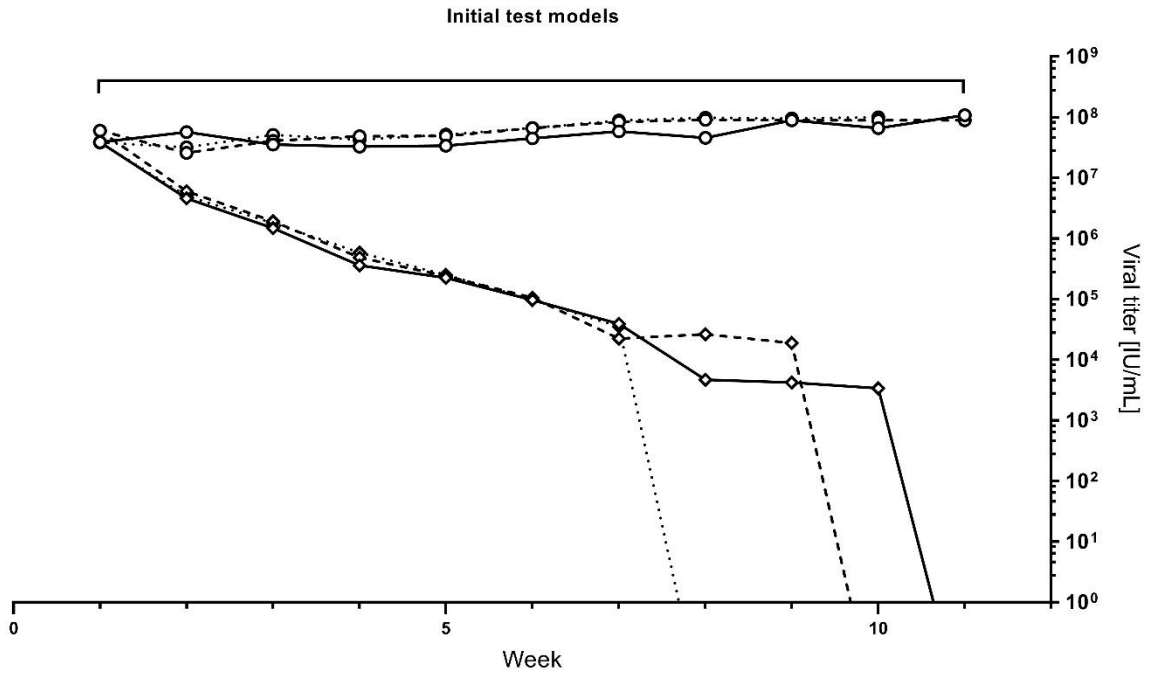

Figure S5. HEV titer (in IU/ml) progression in *in vitro* pre-testing of untreated (circles) or continuously treated (diamonds) with RBV 100 μM. Replicates are denoted by solid, broken, and dotted lines. Period of RBV treatment denoted by black bracket.

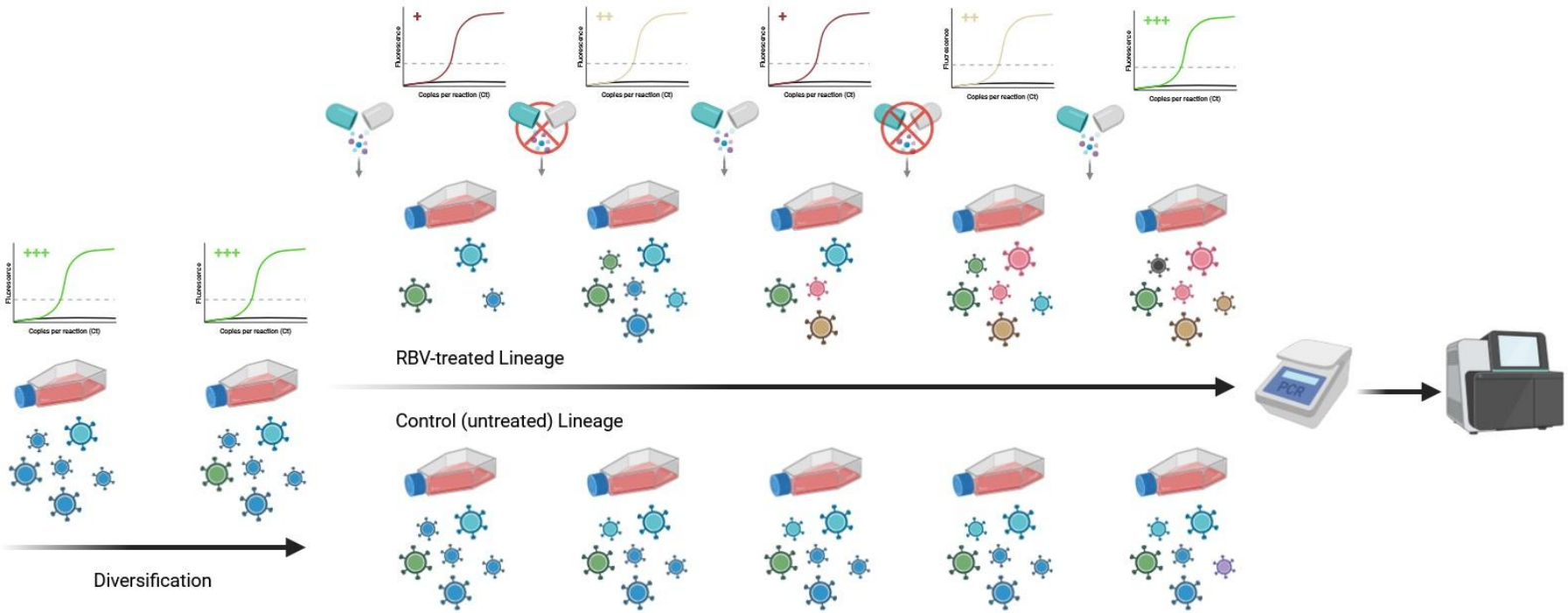
+
++
+
++
+++
Fluorescence
Copies per reaction (Ct)
+++
+++
RBV-treated Lineage
Control (untreated) Lineage
Diversification
PCR

Figure S6. Experimental outline of *in vitro* evolution of HEV-3c strain 47832c in cell culture. Created with BioRender.com.

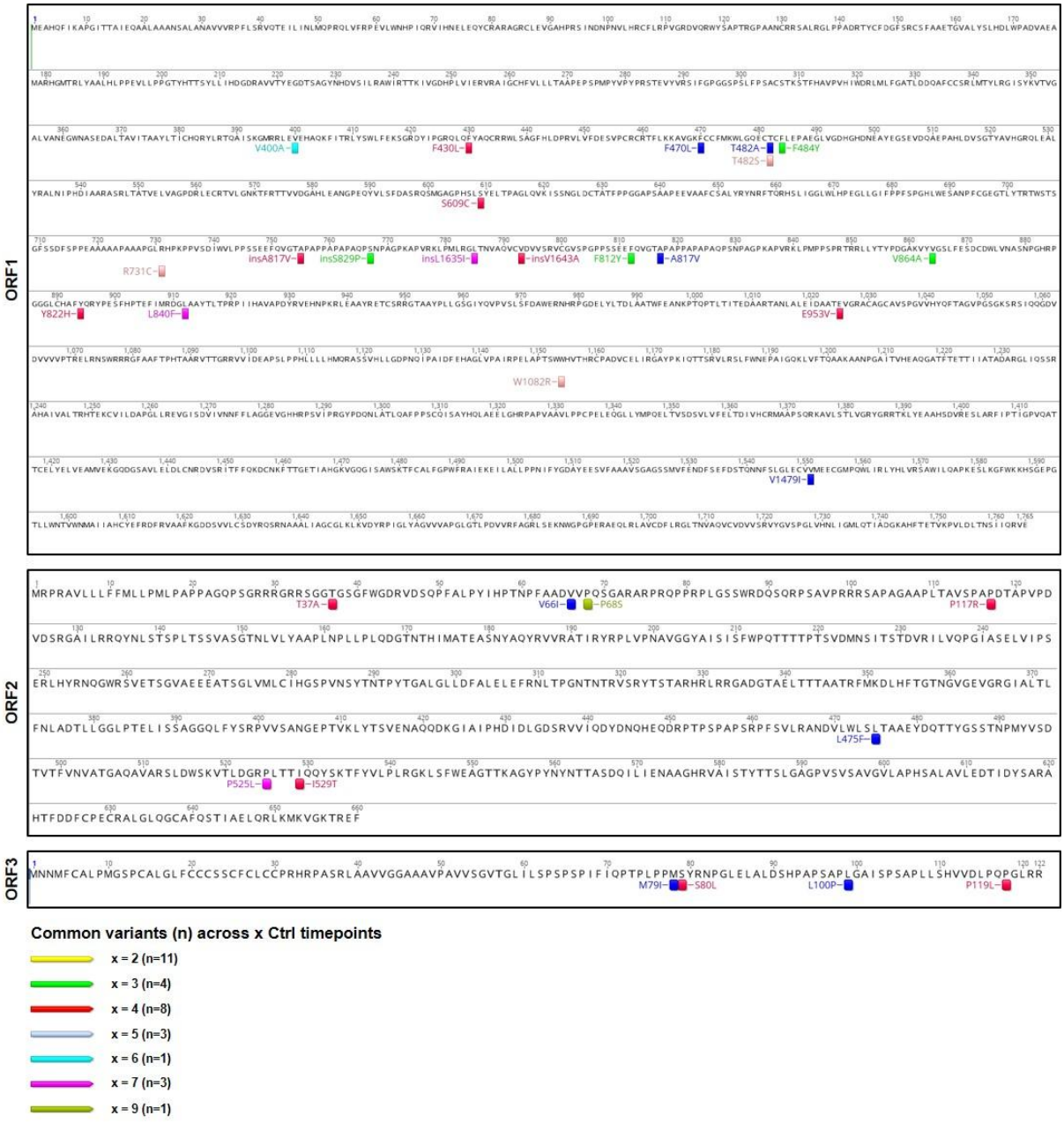

Figure S7. Distribution of non-synonymous variants (n) commonly detected in in vitro Ctrl timepoints of both cultures. Color code according to number of timepoints (x) in which variant is detected ≥5% frequency.

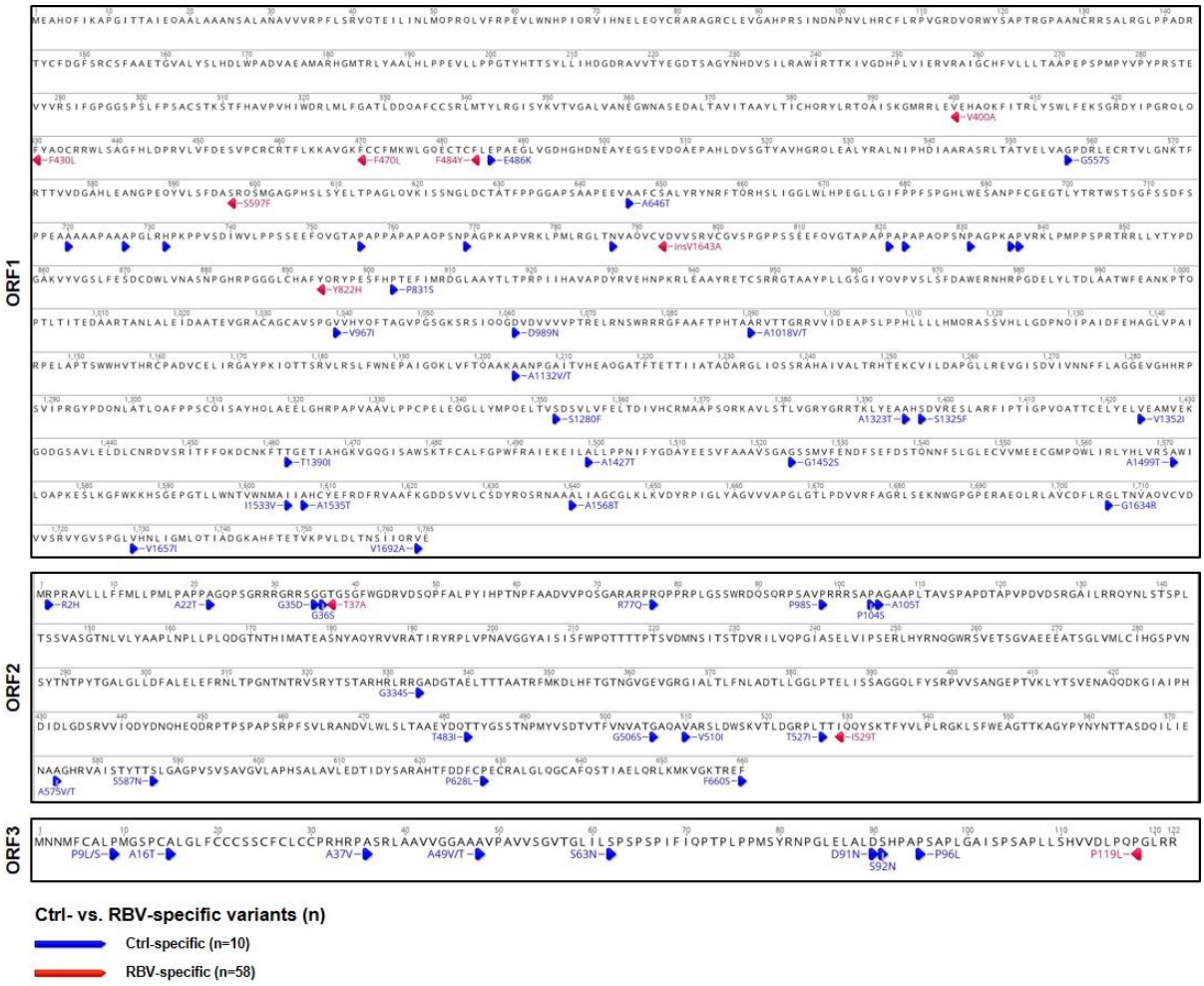

Figure S8. Distribution of Ctrl-specific (blue) and RBV-specific (red) non- synonymous variants (n) commonly detected ≥5% frequency in in vitro timepoints of both cultures.