

1 **Determination of host cell proteins constituting the molecular microenvironment of**  
2 **coronavirus replicase complexes by proximity-labeling**

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20 **Keywords:** RNA virus, Coronavirus, replication complex, virus-host interaction, replication  
21 compartments, proximity-labeling, viral replicase molecular microenvironment.

## 22 **Abstract**

23 Positive-sense RNA viruses hijack intracellular membranes that provide niches for viral RNA  
24 synthesis and a platform for interactions with host proteins. However, little is known about host  
25 factors at the interface between replicase complexes and the host cytoplasm. We engineered a  
26 biotin ligase into a coronaviral replication/transcription complex (RTC) and identified >500  
27 host proteins constituting the RTC microenvironment. siRNA-silencing of each RTC-proximal  
28 host factor demonstrated importance of vesicular trafficking pathways, ubiquitin-dependent  
29 and autophagy-related processes, and translation initiation factors. Notably, detection of  
30 translation initiation factors at the RTC was instrumental to visualize and demonstrate active  
31 translation proximal to replication complexes of several coronaviruses.

32 Collectively, we establish a spatial link between viral RNA synthesis and diverse host factors  
33 of unprecedented breadth. Our data may serve as a paradigm for other positive-strand RNA  
34 viruses and provide a starting point for a comprehensive analysis of critical virus-host  
35 interactions that represent targets for therapeutic intervention.

## 36 **Introduction**

37 Positive-strand RNA viruses replicate at membranous structures that accommodate the viral  
38 replication complex and facilitate RNA synthesis in the cytosol of infected host cells (1-5).  
39 Rewiring host endomembranes is hypothesized to provide a privileged microenvironment  
40 physically separated from the cytosol, thereby ensuring adequate concentrations of  
41 macromolecules for viral RNA synthesis, preventing recognition of replication intermediates  
42 such as double-stranded RNA (dsRNA) by cytosolic innate immune receptors (6, 7), and  
43 providing a platform that facilitates molecular interactions with host cell proteins.

44 Ultrastructural studies have reported the origin, nature, and extent of membrane modifications  
45 induced by coronaviruses (order *Nidovirales*, family *Coronaviridae*), which materialize as an  
46 ER-derived network of interconnected double-membrane vesicles (DMVs) and convoluted  
47 membranes (CM) in perinuclear regions of infected cells to which the viral  
48 replication/transcription complex (RTC) is anchored (4, 8, 9). The RTC is generated by  
49 translation of the genomic RNA into two large polyproteins that are extensively auto-  
50 proteolytically processed by viral proteases to give rise to 16 processing end-products, termed  
51 non-structural proteins (nsps) 1-16. Nsp1 is rapidly cleaved from the polyproteins and not  
52 considered an integral component of the coronaviral RTC, but interferes with host cell  
53 translation by inducing degradation of cellular mRNAs (10-12). Although it has not yet been  
54 formally demonstrated, the remaining nsps (2-16) are thought to comprise the RTC and harbor  
55 multiple enzymes and functions, such as de-ubiquitination, proteases, helicase, polymerase,  
56 exo- and endonuclease, and N7- and 2'O-methyltransferases (13-18). Many of these functions  
57 have been studied using reverse genetic approaches, which revealed their importance in virus-  
58 host interactions (19-23). In most cases phenotypes were described via loss-of-function  
59 mutagenesis, however, in the context of virus infection, the specific interactions of RTC  
60 components with host cell factors remain largely unknown.

61 A number of individual host cell proteins have been shown to impact coronavirus replication  
62 by using various screening methods, such as genome-wide siRNA, kinome, and yeast-two-  
63 hybrid screens (24-28). Likewise, genome-wide CRISPR-based screens have been applied to  
64 other positive-stranded RNA viruses, such as flaviviruses, and identified critical host proteins  
65 required for replication (29, 30). Some of these proteins were described in the context of  
66 distinct ER processes, such as N-linked glycosylation, ER-associated protein degradation  
67 (ERAD), and signal peptide insertion and processing. Although individual proteins identified  
68 by these screens may interact with viral replication complexes, they likely constitute only a  
69 small fraction of the global replicase microenvironment.

70 To capture the full breadth of host cell proteins and cellular pathways that are spatially  
71 associated with viral RTCs, we employed a proximity-based labeling approach involving a  
72 promiscuous *E. coli*-derived biotin ligase (BirA<sub>R118G</sub>). BirA<sub>R118G</sub> biotinylates proximal (<10  
73 nm) proteins in live cells without disrupting intracellular membranes or protein complexes, and  
74 hence, does not rely on high affinity protein-protein interactions, but is able to permanently tag  
75 transient interactions (31). Covalent protein biotinylation allows stringent lysis and washing  
76 conditions during affinity purification and subsequent mass spectrometric identification of  
77 captured factors. By engineering a recombinant MHV harboring BirA<sub>R118G</sub> as an integral  
78 component of the RTC we identified >500 host proteins reflecting the molecular  
79 microenvironment of MHV replication structures. siRNA-mediated silencing of each of these  
80 factors highlighted, amongst others, the functional importance of vesicular ER-Golgi apparatus  
81 trafficking pathways, ubiquitin-dependent and autophagy-related catabolic processes, and  
82 translation initiation factors. Importantly, the detection of active translation in close proximity  
83 to the viral RTC highlighted the critical involvement of translation initiation factors during  
84 coronavirus replication. Collectively, the determination of the coronavirus RTC-associated  
85 microenvironment provides a functional and spatial link between conserved host cell processes

86 and viral RNA synthesis, and highlights potential targets for the development of novel antiviral  
87 agents.

88

## 89 **Results**

### 90 **Engineering the BirA<sub>R118G</sub>-biotin ligase into the MHV replicase transcriptase complex**

91 To insert the promiscuous biotin ligase BirA<sub>R118G</sub> as an integral subunit of the MHV RTC, we  
92 used a vaccinia virus-based reverse genetic system (32, 33) to generate a recombinant MHV  
93 harboring a myc-tagged BirA<sub>R118G</sub> fused to nsp2. This strategy was recently employed by  
94 Freeman *et al.* for a fusion of green fluorescent protein (GFP) with nsp2 (34). MHV-BirA<sub>R118G</sub>-  
95 nsp2 retained the cleavage site between nsp1 and BirA<sub>R118G</sub>, while a deleted cleavage site  
96 between BirA<sub>R118G</sub> and nsp2 ensured the expression of a BirA<sub>R118G</sub>-nsp2 fusion protein (Fig.  
97 1a).

98 MHV-BirA<sub>R118G</sub>-nsp2 replicated to comparable peak titers and replication kinetics as the  
99 parental wild-type MHV-A59 (Fig. 1b). MHV-EGFP-nsp2, which was constructed in parallel  
100 and contained the coding sequence of EGFP (34) instead of BirA<sub>R118G</sub>, was used as a control  
101 and also reached wild-type virus peak titers, with slightly reduced viral titers at 9 hours post-  
102 infection (h.p.i.) compared to MHV-A59 and MHV-BirA<sub>R118G</sub>-nsp2 (Fig. 1b).

103 To confirm the accommodation of BirA<sub>R118G</sub> within the viral RTC, MHV-A59-, MHV-  
104 BirA<sub>R118G</sub>-nsp2-, and mock-infected L929 fibroblasts were visualized using indirect  
105 immunofluorescence microscopy. BirA<sub>R118G</sub>-nsp2 remained strongly associated with the MHV  
106 RTC, as indicated by the co-localization of BirA<sub>R118G</sub>-nsp2 with established markers of the  
107 MHV replicase, such as nsp2/3 and nsp8 (Fig. 1c; Supplemental Fig. S1). This observation  
108 corroborates previous studies demonstrating that nsp2, although not required for viral RNA  
109 synthesis, co-localizes with other nsps of the coronaviral RTC (35-37). Importantly, by

110 supplementing the culture medium with biotin, we could readily detect biotinylated proteins  
111 with fluorophore-coupled streptavidin that appeared close to the MHV RTC in MHV-  
112 BirA<sub>R118G</sub>-nsp2-infected cells, demonstrating efficient proximity-dependent biotinylation of  
113 RTC-proximal host factors (Fig. 1c; Supplemental Fig. S1). Furthermore, to define the  
114 localization of the nsp2 fusion protein at the ultrastructural level, we replaced the BirA<sub>R118G</sub>  
115 biotin ligase with the APEX2 ascorbate peroxidase to generate recombinant MHV-APEX2-  
116 nsp2. APEX2 mediates the catalysis of 3,3'-diaminobenzidine (DAB) into an insoluble  
117 polymer that can be readily observed by electron microscopy (38). As shown in figure 1d,  
118 APEX2-catalyzed DAB polymer deposition was readily detectable at characteristic coronavirus  
119 replication compartments, such as DMVs and CM, categorically demonstrating that the nsp2  
120 fusion proteins localize to known sites of coronavirus replication (4, 8).

121 Importantly, our collective results establish that the recombinant MHV-BirA<sub>R118G</sub>-nsp2  
122 replicates with comparable kinetics to wild-type MHV-A59, expresses a functional BirA<sub>R118G</sub>  
123 biotin ligase that is tightly associated with the MHV RTC, and that biotinylated, RTC-proximal  
124 proteins can be readily detected in MHV-BirA<sub>R118G</sub>-nsp2 infected cells.

125

## 126 **Determination of the coronavirus RTC-proximal proteome**

127 To further demonstrate the efficiency and specificity of BirA<sub>R118G</sub>-mediated biotinylation we  
128 assessed, by western blot analysis, fractions of biotinylated proteins derived from MHV-A59-  
129 , MHV-BirA<sub>R118G</sub>-nsp2-, or non-infected cells that were grown with or without the addition of  
130 biotin (Fig. 2a,b). A characteristic pattern of endogenously biotinylated proteins was observed  
131 under all conditions where no exogenous biotin was added to the culture medium (Fig. 2b).  
132 The same pattern was detectable in non-infected and wild-type MHV-A59-infected cells when  
133 the culture medium was supplemented with biotin, suggesting that the addition of biotin in the

134 absence of the BirA<sub>R118G</sub> biotin ligase does not recognizably change the fraction of  
135 endogenously biotinylated proteins. In contrast, we observed a greatly increased fraction of  
136 biotinylated proteins in lysates derived from MHV-BirA<sub>R118G</sub>-nsp2-infected cells treated with  
137 biotin. This result demonstrates that virus-mediated expression of the BirA<sub>R118G</sub> biotin ligase  
138 results in efficient biotinylation when biotin is added to the culture medium. Moreover, we  
139 could readily affinity purify, enrich, and recover the fraction of biotinylated proteins under  
140 stringent denaturing lysis and washing conditions by using streptavidin-coupled magnetic  
141 beads (Fig. 2b).

142 Affinity purified proteins derived from biotin-treated MHV-A59- and MHV-BirA<sub>R118G</sub>-nsp2-  
143 infected cells were subjected to mass spectrometric analysis (n=3). Liquid chromatography  
144 tandem-mass spectrometry (LC-MS/MS) was performed from in-gel digested samples and log-  
145 transformed label free quantification (LFQ) levels were used to compare protein enrichment  
146 between samples (Fig. 2c). Overall, 1381 host proteins were identified, of which 513 were  
147 statistically significantly enriched in MHV-BirA<sub>R118G</sub>-nsp2-infected samples over MHV-A59-  
148 infected samples. These host proteins represent a comprehensive repertoire of RTC-proximal  
149 factors throughout MHV infection (Fig. 2c, table S1). Importantly, viral replicase gene  
150 products nsp2-10 and nsp12-16, and the nucleocapsid protein were significantly enriched in  
151 fractions derived from MHV-BirA<sub>R118G</sub>-nsp2-infected cells (Fig. 2c, d). This is in agreement  
152 with studies demonstrating co-localization and interactions amongst individual nsps, and with  
153 studies showing association of the nucleocapsid protein with the coronavirus RTC (8, 39-41).  
154 It also highlights the specificity and effectiveness of the labeling approach in live cells and is  
155 the first experimental evidence showing that collectively these viral nsps and the nucleocapsid  
156 (N) protein are subunits of the coronavirus RTC. Furthermore, these results corroborate  
157 previous reports that nsp1 is likely not an integral component of the coronavirus RTC (10-12,  
158 42). Amongst the “not detected” or “not enriched” viral proteins are (i) nsp11, which is a short

159 peptide of only 14 amino acids at the carboxyterminus of polyprotein 1a with a yet unassigned  
160 role or function in coronavirus replication, (ii) the structural proteins spike (S) protein,  
161 envelope (E) protein, and membrane (M) protein, which mainly localize to sites of viral  
162 assembly before being incorporated into newly-formed viral particles, and (iii) all accessory  
163 proteins (NS2a, HE, ORF4, ORF5a). Altogether, these results validate the proximity-  
164 dependent biotinylation approach and demonstrate the specific and exclusive labeling of MHV-  
165 RTC-associated proteins (Fig. 2d).

166 The BirA<sub>R118G</sub> biotin ligase biotinylates proteins in its close proximity that must not necessarily  
167 have tight, prolonged, or direct interaction (31). Therefore, the identified RTC-proximal host  
168 proteins, recorded over the entire duration of the MHV replication cycle, likely include proteins  
169 that display a prolonged co-localization with the MHV RTC, proteins that may locate only  
170 transiently in close proximity to the RTC, and proteins of which only a minor fraction of the  
171 cellular pool may associate with the RTC. To this end, we assessed the localization of a limited  
172 number of host proteins from our candidate list in MHV-infected cells. Accordingly, we  
173 identified RTC-proximal host proteins displaying a pronounced co-localization with the MHV  
174 RTC, such as the ER protein reticulon 4 (rtn4; Fig. 2e), and host proteins where co-localization  
175 by indirect immunofluorescence microscopy was not readily detectable, such as the eukaryotic  
176 translation initiation factor 3E (eIF3E; Fig. 2e). However, in the latter case, a more sensitive  
177 detection technique, such as a proximity ligation assay that relies on proximity-dependent  
178 antibody-coupled DNA probe amplification (43), demonstrated proximity of eIF3E and  
179 dsRNA in MHV-infected cells (Fig. 2f).

180 Collectively, our results show that the approach of integrating a promiscuous biotin ligase as  
181 an integral subunit into a coronavirus RTC revealed a comprehensive list of host cell proteins  
182 that comprises the RTC microenvironment. The efficacy and specificity of our approach is best  
183 illustrated by the fact that we were able to identify all expected viral components of the MHV



184 RTC, while other viral proteins, such as nsp1, structural proteins S, E, and M, and accessory  
185 proteins, were not amongst the significantly enriched proteins. Our data further suggest that  
186 the RTC microenvironment may be highly dynamic and likely also contains proteins that are  
187 only transiently present in the microenvironment or only comprise a sub-fraction of the cellular  
188 pool in close proximity to the MHV RTC.

189

### 190 **Functional classification of RTC-proximal host factors**

191 To categorize functionally-related proteins from the list of RTC-proximal host proteins and  
192 identify enriched biological themes in the dataset, we performed a functional classification of  
193 RTC-proximal factors using Gene Ontology (GO) enrichment analysis. 86 GO biological  
194 process (BP) terms were significantly enriched in the dataset (p-value <0.05), of which 32  
195 terms were highly significant (p-value <0.005) (Fig. 3a, Table S2). Additional analysis using  
196 AmiGO revealed that 25 of these 32 highly significant GO BP terms fell into 5 broad functional  
197 categories, namely cell adhesion, transport, cell organization, translation, and catabolic  
198 processes. To examine these categories further, identify important cellular pathways within  
199 them, and extract known functional associations among RTC-proximal host proteins, we  
200 performed STRING network analysis on the RTC-proximal proteins in each category (Fig. 3b,  
201 c, Fig S2).

202 Despite “cell-cell adhesion” scoring high, it likely represents a typical limitation of gene  
203 annotation databases, where many genes play multiple roles in numerous pathways and  
204 processes. Accordingly, most genes assigned to the GO BP term cell-cell adhesion are also  
205 found in the other categories described below.

206 The category “transport” included protein trafficking and vesicular-mediated transport  
207 pathways and comprised the majority of RTC-proximal factors (Fig. 3a, b). Protein interaction

208 network analysis, using STRING, revealed at least 4 distinct clusters of interacting factors  
209 within this category (Fig. 3b). Cluster I, protein transport, comprised nuclear transport  
210 receptors at nuclear pore complexes, such as importins and transportins. Interestingly, this  
211 cluster also contained Sec63, which is part of the Sec61 translocon (44) and has been implicated  
212 in protein translocation across ER membranes. The list of RTC-proximal factors also included  
213 signal recognition particles SRP54a and SRP68 (Table S2) proteins that promote the transfer  
214 of newly synthesized integral membrane proteins or secreted proteins across translocon  
215 complexes. Furthermore, the list contained Naca and BTF3, which prevent the translocation of  
216 non-secretory proteins towards the ER lumen (45, 46). Interestingly, genome-wide CRISPR  
217 screens have identified proteins involved in biosynthesis of membrane and secretory proteins  
218 as required for flavivirus replication (29, 30), suggesting similarities between flaviruses and  
219 coronaviruses concerning the requirement of ER-associated protein sorting complexes for viral  
220 replication.

221 Cluster II included vesicle components, tethers and SNARE (Soluble N-ethylmaleimide-  
222 sensitive-factor Attachment protein Receptor) proteins characteristic of the COPII-mediated  
223 ER-to-Golgi apparatus anterograde vesicular transport pathway whereas, cluster III contained  
224 components of the COPI-related retrograde Golgi-to-ER transport machinery. Moreover,  
225 Cluster IV was comprised of proteins that mediate clathrin-coated vesicle (endosomal)  
226 transport between the plasma membrane and the trans-Golgi network (TGN), which is also  
227 closely associated with the actin cytoskeleton. Together with sorting nexins, cluster IV  
228 components can be regarded as regulating late-Golgi trafficking events and interacting with the  
229 endosomal system.

230 Many of the cellular processes and host proteins assigned to “transport” (specifically in clusters  
231 II-IV) are also listed in the category “cell organization” (Fig. 3a, S2a). However, this category  
232 actually extends the importance of vesicular transport as it also contains factors involved in the

233 architecture, organization, and homeostasis of the ER and Golgi apparatus, and the  
234 cytoskeleton-supporting these organelles. The prominent appearance of biological processes  
235 linked to protein and vesicular transport between the ER and both the cis- and trans-Golgi  
236 network, is in agreement with previous findings that have reported the relevance of the early  
237 secretory pathway for a number of RNA viruses, including coronaviruses (24, 26, 47-50).

238 Notably, a number of MHV RTC-proximal factors were part of the host translation machinery  
239 and assigned to category “translation” (Fig. 3a, c). We found enrichment of factors involved in  
240 the initiation of translation, particularly multiple subunits of eIF3 and eIF4 complexes, as well  
241 as eIF2, eIF5, the Ddx3y helicase, and the Elongation factor-like GTPase 1, which are required  
242 for the formation of 43S pre-initiation complexes, 48S initiation complexes, and the assembly  
243 of elongation-competent 80S ribosomes (51). The high degree of interaction between these  
244 subunits is suggestive of the presence of the entire translation initiation apparatus in close  
245 proximity to the viral RTC. The 60S ribosomal protein L13a (Rp113a), ribosome biogenesis  
246 protein RLP24 (Rsl24d1), ribosome-binding protein 1 (Rbp1), release factor Gspt1, and  
247 regulatory elements, such as Igf2bp1, Gcn111, Larp, Fam129a and Nck1, are further indicative  
248 of the host cell translation machinery near sites of viral RNA synthesis. Notably, our results  
249 are in line with a recent genome-wide siRNA screen where translation factors were suggested  
250 to play a role in the replication of avian infectious bronchitis coronavirus (IBV) (27). The  
251 implication of this finding has, to our knowledge, not been further investigated.

252 Lastly, the category “catabolic processes” (Fig. 3a, S2b) includes a subset of autophagy-related  
253 factors and numerous ubiquitin-dependent ERAD components, including the E3 ubiquitin-  
254 protein ligase complex and 26S proteasome regulatory subunits (Psmc2, Psmd4). Interestingly,  
255 the importance of the ERAD pathway has also been reported in the genome-wide CRISPR  
256 screen on flaviviruses, and the ubiquitin-proteasome pathway has been noted to be important  
257 for IBV and MHV replication (27, 52).

258 Collectively, the catalogue of coronavirus RTC-proximal proteins greatly expands the  
259 repertoire of candidate proteins implicated in the coronavirus replication cycle, and contains  
260 several factors that have previously been reported to impact the replication of other positive-  
261 sense RNA viruses. Importantly, since our screening approach was tailored to detect host  
262 factors associated with the coronavirus RTC, it provides a spatial link of these factors to the  
263 site of viral RNA synthesis.

264

### 265 **Identification of proviral factors within the coronavirus RTC microenvironment**

266 In order to assess the potential functional relevance of RTC-proximal factors identified in our  
267 MHV- BirA<sub>R118G</sub>-nsp2-mediated proximity-dependent screen, we designed a custom siRNA  
268 library individually targeting the expression of each of the 513 identified RTC-proximal host  
269 proteins. siRNA-treated L929 cells were infected (MOI=0.05, n=4) with a recombinant MHV  
270 expressing a *Gaussia* luciferase reporter protein (MHV-Gluc) (53) and replication was assessed  
271 by virus-mediated *Gaussia* luciferase expression (Fig. 4a). Cell viability after siRNA  
272 knockdown was also assessed and genes resulting in cytotoxicity following silencing were  
273 discarded from further analysis. Importantly, we included internal controls of known relevance  
274 for MHV entry (MHV receptor Ceacam1a) and replication (Gbf1, Arf1) on each plate and  
275 found in each case that siRNA silencing of these factors significantly reduced MHV  
276 replication, which underscores the robustness and effectiveness of our approach (Fig. S3a) (24).  
277 We found that siRNA-mediated silencing of 53 RTC-proximal host factors significantly  
278 reduced MHV replication compared to non-targeting siRNA controls. These factors can  
279 therefore be considered proviral and required for efficient replication (Fig. 3b; table S3).  
280 Notably, siRNA targets that had the strongest impact on MHV replication were in majority  
281 contained within the functional categories highlighted in Fig. 3a (Fig. 4b). Indeed, in line with

282 the hypothesis that MHV subverts key components mediating both anterograde and retrograde  
283 vesicular transport between the ER, Golgi apparatus and endosomal compartments for the  
284 establishment of replication organelles, several factors contained within these pathways  
285 impaired MHV replication as exemplified by the siRNA-mediated silencing of Kif11, Snx9,  
286 Dnm11, Scfd1, Ykt6, Stx5a, Clint1, Aak11, or Vapa (Fig. 4b). Consistently, ER-associated  
287 protein sorting complexes associated with the ribosome and newly synthesized proteins (Naca,  
288 BTF3, SRP54a, SRP68) that were revealed in the GO enrichment analysis (Fig. 3a, table S2),  
289 also appear to be required for efficient MHV replication (Fig. 4b).

290 Furthermore, we also observed significantly reduced MHV replication upon silencing of core  
291 elements of the 26S and 20S proteasome complex (Psm1 and Psmc2, and Psmb3,  
292 respectively), suggesting a crucial role of the ubiquitin-proteasome pathway for efficient CoV  
293 replication (27, 52). Indeed, this finding may provide a link to the described coronavirus RTC-  
294 encoded de-ubiquitination activity residing in nsp3 that has been implicated in innate immune  
295 evasion (16, 17, 54).

296 Most interestingly, this custom siRNA screen identified a crucial role of the host protein  
297 synthesis apparatus that was associated with the MHV RTC as indicated by the proximity-  
298 dependent proteomic screen (Fig. 3a, c). Silencing of ribosomal proteins Rpl13a and Rls24d1  
299 and several subunits of the eIF3 complex resulted in greatly reduced MHV replication and  
300 scored with highest significance in the siRNA screen, suggesting that proximity of the host cell  
301 translation machinery to the viral RTC likely has functional importance for coronavirus  
302 replication (Fig. 4b).

303

304 **Active translation near sites of viral mRNA synthesis**

305 Due to the striking dependence of MHV replication on a subset of RTC-proximal translation  
306 initiation factors, we extended these results in independent assays. For this, we selected all host  
307 factors assigned to the category “translation” (Fig. 3a) and assessed virus replication following  
308 siRNA-mediated silencing of each factor. Measurement of luciferase activity after MHV-Gluc  
309 infection confirmed initial findings obtained by screening the entire siRNA library of MHV  
310 RTC-proximal factors (Fig. 4c). Specifically for Rpl13a, and eIFs 3i, 3f, and 3e viral replication  
311 was reduced to levels comparable to our controls Ceacam1a (MHV receptor) and Gbfl (24).  
312 Consistently, cell-associated viral mRNA levels (Fig. 4d) and viral titers (Fig. 4e) were reduced  
313 upon siRNA silencing of these factors. Although the silencing of a subset of host translation  
314 factors severely restricted MHV replication, effective knockdown of these factors (Fig. S3c)  
315 did not affect cell viability (Fig. S3b, d) and only moderately affected host cell translation  
316 levels (Fig. 4f, S3e). This data demonstrates that the reduced viral replication observed after  
317 siRNA knockdown is not due to a general impairment of host translation.

318 Subsequently, we aimed to visualize the localization of active translation during virus infection  
319 by puromycin incorporation into nascent polypeptides on immobilized ribosomes  
320 (ribopuromycylation) followed by fluorescence imaging using antibodies directed against  
321 puromycin (55). In non-infected L929 cells, ribopuromycylation resulted in an expected  
322 diffuse, mainly cytosolic, staining pattern interspersed with punctate structures indicative of  
323 translation localized to dedicated subcellular cytosolic locations (Fig. 5). In striking contrast,  
324 MHV-infected L929 cells displayed a pronounced enrichment of actively translating ribosomes  
325 near the viral RTC as indicated by the strong overlap between the viral replicase and the  
326 ribopuromycylation stain. Interestingly, active translation in vicinity of the RTC was strongest  
327 during the early phase of infection at 6 h.p.i., and was observed until 8 h.p.i., before gradually  
328 decreasing as the infection advanced along with the appearance of typical syncytia formation  
329 indicative of cytopathic effect (CPE).

330 Remarkably, we observed a similar phenotype in Huh7 cells infected with human  
331 coronaviruses, such as HCoV-229E or the highly pathogenic MERS-CoV (Fig. 6). The HCoV-  
332 229E RTC, which was detected with an antiserum directed against nsp8, appeared as small and  
333 dispersed perinuclear puncta during early infection and eventually converged into larger  
334 perinuclear structures later in infection. Consistent with findings obtained for MHV, we  
335 observed a striking co-localization of the HCoV-229E RTC with sites of active translation  
336 during the early phase of the infection (Fig. 6, S4). The co-localization gradually decreased as  
337 the infection reached the late phase with upcoming signs of CPE. Finally, we further  
338 demonstrated that active translation is localized to the site of MERS-CoV RNA synthesis as  
339 dsRNA puncta highly overlapped with the ribopuromycylation stain in MERS-CoV-infected  
340 Huh7 cells (Fig. 6). Collectively, these results not only confirm the spatial link between  
341 individual components of the host cell translation machinery and coronavirus replication  
342 compartments as identified by proximity-dependent biotinylation using MHV-BirA<sub>R118G</sub>-nsp2,  
343 but they also demonstrate that active translation is taking place in close proximity to the viral  
344 RTC.

345

## 346 **Discussion**

347 In this study, we made use of a recently developed system based on proximity-dependent  
348 biotinylation of host factors in living cells (31). By engineering a promiscuous biotin ligase  
349 (BirA<sub>R118G</sub>) as an integral component of the coronavirus replication complex, we provide a  
350 novel approach to define the molecular microenvironment of viral replication complexes that  
351 is applicable to many other RNA and DNA viruses.

352 We show that nsp2 fusion proteins encoded by recombinant MHV-APEX2-nsp2 and MHV-  
353 BirA<sub>R118G</sub>-nsp2, are indeed part of the RTC and localize to characteristic coronavirus

354 replicative structures. On the ultrastructural level, APEX2-catalyzed DAB polymer  
355 depositions were detected at DMVs and CMs, and we observed co-localization of BirA<sub>R118G</sub>  
356 with established coronavirus RTC markers, such as nsp2/3 and nsp8, by indirect  
357 immunofluorescence microscopy. Notably, in MHV-BirA<sub>R118G</sub>-nsp2-infected cells the  
358 detection of biotinylated coronavirus replicase gene products nsp2-10, nsp12-16, and the  
359 nucleocapsid protein by mass spectrometry demonstrates that these proteins are in close  
360 proximity during infection. This extends previous immunofluorescence and electron  
361 microscopic studies that were limited by the availability of nsp-specific antibodies and could  
362 only show localization of individual nsps to coronavirus replicative structures (4, 8, 35-37).  
363 Moreover, the close proximity of BirA<sub>R118G</sub>-nsp2 to MHV replicative enzymes, such as the  
364 RNA-dependent RNA polymerase (nsp12), the NTPase/helicase (nsp13), the 5'-cap  
365 methyltransferases (nsp14, nsp16), the proof-reading exonuclease (nsp14), in MHV-BirA<sub>R118G</sub>-  
366 nsp2-infected cells further suggest close proximity of nsp2 to the site of viral RNA synthesis.  
367 We thus propose that nsp2-16 and the nucleocapsid protein collectively constitute a functional  
368 coronavirus replication and transcription complex in infected cells.

369 The analysis of the host proteome enriched at MHV replication sites revealed a comprehensive  
370 list of host proteins that constitute the coronavirus RTC microenvironment. This included  
371 several individual factors and host cell pathways, especially transport mechanisms involving  
372 vesicle-mediated trafficking, which have previously been shown to assist coronavirus  
373 replication. (24, 26, 27, 49, 52). Moreover, numerous coronavirus RTC-proximal host proteins  
374 and pathways also have documented roles in the life cycle of other, more intensively studied,  
375 positive-stranded RNA viruses, suggesting considerable commonalities and conserved virus-  
376 host interactions at the replication complexes of a broad range of RNA viruses (29, 30, 56).  
377 Importantly, our list of RTC-proximal proteins by far exceeds the number of host cell proteins  
378 currently known to interact with viral replication complexes and the vast majority of MHV



379 RTC-proximal proteins have not been described before. These likely include proteins with  
380 defined temporal roles during particular phases of the viral life cycle and proteins that did not  
381 yet attract our attention in previous screens because of functional redundancies. We therefore  
382 expect that this approach will find wide application in the field of virus-host interaction, target  
383 identification for virus inhibition, and provides a starting point to reveal similarities and  
384 differences between replication strategies of a broad range of viruses.

385 One novel finding that arose immediately from our RTC-proximity screen is the demonstration  
386 of a close spatial association of host cell translation with the coronavirus RTC. Indeed, the  
387 biotin ligase-based proteomic screen identified a number of translation initiation factors, most  
388 prominently several eIF3 subunits that were found to have functional importance for viral  
389 replication, and numerous ribosome- and translation-associated proteins within the coronavirus  
390 RTC microenvironment (Fig. 3, 4). In addition, the presence of subunits of the signal  
391 recognition particle in proximity to the coronavirus RTC and their functional relevance for  
392 viral replication is indicative of an importance for the translation of membrane proteins.  
393 Notably, the coronavirus RTC is translated as two polyproteins that contain nsp3, 4 and 6 with  
394 multiple trans-membrane domains that are believed to anchor the RTC at ER-derived  
395 membranes (4, 47). It is thus tempting to speculate that the coronavirus RTC is either attracting,  
396 or deliberately forming in proximity to, the ER-localized host translation machinery in order  
397 to facilitate replicase translation and insertion into ER membranes. This idea is also applicable  
398 to many other positive-stranded RNA viruses that express viral polyproteins with embedded  
399 trans-membrane domains to anchor the viral replication complex in host endomembranes.  
400 Recent experimental evidence for Dengue virus supports this hypothesis. By using cell  
401 fractionation and ribosomal profiling it has been shown that translation of the Dengue virus  
402 (family *Flaviviridae*) genome is associated with the ER-associated translation machinery  
403 accompanied by ER-compartment-specific remodeling of translation (57). Moreover, several

404 recent genome-wide CRISPR screens demonstrated the functional importance of proteins  
405 involved in biosynthesis of membrane and secretory proteins, further supporting a pivotal role  
406 of the ER-associated translation machinery for virus replication (30).

407 Compartmentalization of cellular translation to sites of viral RNA synthesis has been described  
408 for dsRNA viruses of the orthoreovirus family, which replicate and assemble in distinct  
409 cytosolic inclusions known as viral factories to which the host translation machinery is  
410 recruited (58). The data presented here indicate that coronaviruses have evolved a similar  
411 strategy by compartmentalizing and directing viral RNA synthesis to sites of ER-associated  
412 translation. Likewise, this strategy has a number of advantages. Coronaviruses would not  
413 require sophisticated transport mechanisms that direct viral mRNA to distantly located  
414 ribosomes. A close spatial association of viral RNA synthesis and translation during early post-  
415 entry events would rather allow for remodeling the ER-associated translation machinery to  
416 ensure translation of viral mRNA in a protected microenvironment. Viruses have evolved  
417 diverse mechanisms to facilitate translation of their mRNAs including highly diverse internal  
418 ribosomal entry sites, recruitment of translation-associated host factors to viral RNAs, and even  
419 transcript-specific translation (59, 60). Accordingly, by remodeling defined sites for viral  
420 mRNA translation, the repertoire and concentration of translation factors can be restricted to  
421 factors needed for translation of these viral mRNAs. A microenvironment that is tailored  
422 towards the translational needs of viral mRNAs in proximity to the viral replicase complex  
423 would also make virus replication tolerant to host- or virus-induced shut down of translation at  
424 distal sites within the cytosol. Finally, proximity of viral mRNA synthesis and translation can  
425 also be considered a mechanism to evade cytosolic mRNA decay mechanisms and innate  
426 immune sensors of viral RNA.

427 The novel finding of a close association of the host translation machinery with sites of viral  
428 RNA synthesis during coronavirus infection exemplifies the power of the MHV-BirA<sub>R118G</sub>-

429 nsp2 –mediated labelling approach to identify RTC-proximal cellular processes that  
430 significantly contribute to viral replication. Indeed, the ability of BirAR<sub>118G</sub> to label viral and  
431 host factors independently of high affinity and prolonged molecular interactions enables the  
432 establishment of a comprehensive repertoire reflecting the history of protein association with  
433 the viral RTC, recorded during the entire course of infection. In future studies it will be  
434 important to provide an “RTC-association map” with temporal resolution. Like we have seen  
435 for translation initiation factors in this study, association of host cell proteins with the viral  
436 RTC might not persist throughout the entire replication cycle but might be of importance only  
437 transiently or during specific phases of the replication cycle. Given its short labelling time,  
438 APEX2 indeed offers this possibility to dissect protein recruitment to the viral RTC in a time-  
439 resolved manner, i.e. to detect RTC-associated host proteins at specific time points post  
440 infection. This will ultimately result in a dynamic, high resolution molecular landscape of  
441 virus-host interactions at the RTC and provide an additional impetus to elucidate critical virus-  
442 host interactions that take place at the site of viral RNA synthesis. These interactions should  
443 be exploited in the development of novel strategies to combat virus infection, based on  
444 conserved mechanisms of interactions at replication complexes of a broad range of positive-  
445 stranded RNA viruses.

446

#### 447 **Acknowledgements**

448 We thank Mark Denison, Susan Baker, and John Ziebuhr for sharing virus sequence  
449 information and antisera. We thank Sandra Huber and Kerry Woods for helpful discussions.

450 This work was supported by the Swiss National Science Foundation (SNF; grants #  
451 310030\_173085, and # CRSII3\_160780 to V.T.). S.P. was supported by the European

452 Commission's Horizon 2020 research and innovation program under the Marie Skłodowska-  
453 Curie grant agreement no. 748627.

454

455 **Author Contributions**

456 Conceptualization, P.V., V.T.; Investigation, P.V., M.G., S.P., N.E., S.B.L., J.P., H.P., V.G.,  
457 R.D.; Formal Analysis, J.K., S.B.L., C.S., M.H.; Writing – Original Draft, P.V., V.T.;  
458 Supervision, V.T., R.D., M.H., R.B., M.S.; Funding Acquisition, V.T., R.D., M.H., R.B., M.S.,  
459 S.P.

460

461 **Declaration of Interests**

462 The authors declare no competing interests

463 **Methods**

464 **Cells**

465 Murine L929 fibroblasts (Sigma) and murine 17C11 fibroblasts (gift from S.G. Sawicki) were  
466 cultured in MEM supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100  
467 µg/ml streptomycin and 100 IU/ml penicillin (MEM+/+). Huh-7 hepatocarcinoma cells (gift  
468 from V. Lohnmann) and Vero B4 cells (kindly provided by M. Müller) were propagated in  
469 Dulbecco's Modified Eagle Medium-GlutaMAX supplemented with, 1 mM sodium pyruvate,  
470 10% (v/v) heat-inactivated fetal bovine serum, 100 µg/ml streptomycin, 100 IU/ml penicillin  
471 and 1% (w/v) non-essential amino acids.

472

473 **Viruses**

474 Recombinant MHV strain A59 (WT), MHV-Gluc (61), which expresses a *Gaussia* luciferase  
475 reporter replacing accessory gene 4 of MHV strain A59, and HCoV-229E were generated as  
476 previously described(32, 33, 62). Viruses were propagated on 17C11 cells (MHV) and Huh-7  
477 cells (hCoV-229E) and their sequence was confirmed by RT-PCR sequencing. MERS-CoV  
478 (63, 64) was propagated and titrated on Vero cells.

479

480 **Generation of recombinant MHV viruses**

481 Recombinant MHV viruses were generated using a vaccinia virus-based system as described  
482 before (33). In short, a pGPT-1 plasmid encoding an *Escherichia coli* guanine  
483 phosphoribosyltransferase (GPT) flanked by MHV-A59 nt 447-950 and 1315-1774 was used  
484 for targeted homologous recombination with a vaccinia virus (VV) containing a full-length  
485 cDNA copy of the MHV-A59 genome (32). The resulting GPT-positive VV was further used  
486 for recombination with a plasmid containing the EGFP coding sequence flanked by MHV-  
487 A59 nt 477-956 and 951-1774 for the generation of MHV-GFP-nsp2, based on the strategy

488 employed by Freeman et al.(34). Alternatively, a plasmid containing the BirA<sub>R118G</sub> coding  
489 sequence (31) or the APEX2 coding sequence (65), with a N-terminal myc-tag or V5-tag,  
490 respectively, and a C-terminal (SGG)<sub>3</sub> flexible linker flanked by MHV-A59 nt 477-956 and  
491 951-1774 was used for the generation of MHV- BirA<sub>R118G</sub>-nsp2 and MHV-APEX2-nsp2. The  
492 resulting VV were used to generate full-length cDNA genomic fragments by restriction  
493 digestion of the VV backbone. Rescue of MHV-GFP-nsp2, MHV-BirA<sub>R118G</sub>-nsp2 and MHV-  
494 APEX2-nsp2 was performed by electroporation of capped *in vitro* transcribed recombinant  
495 genomes into a BHK-21-derived cell line stably expressing the nucleocapsid (N) protein  
496 layered on permissive 17C11 mouse fibroblasts. Recombinant MHV viruses were plaque-  
497 purified three times and purified viruses were passaged three times for stock preparations. All  
498 plasmid sequences, VV sequences and recombinant MHV sequences were confirmed by PCR  
499 or RT-PCR sequencing. Viruses were propagated on 17C11 cells and virus stocks were titrated  
500 by plaque assay on L929 cells.

501

### 502 **Viral replication assay**

503 L929 cells were infected with MHV-A59, MHV-GFP-nsp2, MHV-BirA<sub>R118G</sub>-nsp2 or MHV-  
504 APEX2-nsp2 in quadruplicate at an MOI=1. Virus inoculum was removed 2 h.p.i., cells were  
505 washed with PBS and fresh medium was added. Viral supernatants were collected at the  
506 indicated time point and titrated by plaque assay on L929 cells. Titers reported are the averages  
507 of three independent experiments  $\pm$  standard error of the mean (SEM).

508

### 509 **Immunofluorescence imaging**

510 Biotinylation assays were carried out as described before with minor modifications(66).  $10^6$   
511 L929 cells grown on glass coverslips were infected with MHV-A59, MHV-BirA<sub>R118G</sub>-nsp2 or  
512 MHV-APEX2-nsp2 at an MOI=1, or non-infected in medium supplemented with 67  $\mu$ M biotin

513 (Sigma B4501). Cells were washed three times with PBS at the indicated time points and fixed  
514 with 4% (v/v) neutral buffered formalin before being washed three additional times. Cells were  
515 permeabilized in PBS supplemented with 50 mM NH<sub>4</sub>Cl, 0.1% (w/v) Saponin and 2% (w/v)  
516 BSA (CB) for 60 min and incubated 60 min with the indicated primary antibodies diluted in  
517 CB (polyclonal anti-MHV-nsp2/3 or nsp8 (gift from S. Baker), 1:200 (35, 67); anti-myc,  
518 1:8000 Cell Signalling 2276). Cells were washed three times with CB and incubated for 60 min  
519 with donkey-derived, AlexaFluor488-conjugated anti-mouse IgG (H+L) and donkey-derived,  
520 AlexaFluor647-conjugated anti-rabbit IgG (H+L) (Jackson ImmunoResearch). Cells were  
521 additionally labelled with streptavidin conjugated to AlexaFluor 594 (Molecular Probes) to  
522 detect biotinylated proteins. Coverslips were mounted on slides using ProLong Diamond  
523 Antifade mountant containing 4',6-diamidino-2-phenylindole (DAPI) (Thermo Fisher  
524 Scientific).

525 For indirect immunofluorescence detection of viral and host proteins, L929 cells were grown  
526 on glass coverslips in 24-well plates and infected with MHV-A59 or MHV-BirA<sub>R118G</sub>-nsp2  
527 (MOI=1). At the indicated time point, cells were fixed with 4% (v/v) formalin and processed  
528 using primary monoclonal antibodies directed against dsRNA (J2 Mab, English Scientific and  
529 Consulting) or myc-tab (Cell signalling 2276) and polyclonal antibodies recognizing eIF3E  
530 (Sigma, HPA023973) or RTN4 (Nogo A+B, Abcam 47085) as well as secondary donkey-  
531 derived, AlexaFluor488-conjugated anti-mouse and AlexaFluor647-conjugated anti-rabbit IgG  
532 (H+L), as described above.

533 For proximity ligation assays, L929 cells were seeded in 24-well plates on glass coverslips and  
534 infection with MHV-A59 or MHV-BirA<sub>R118G</sub>-nsp2 (MOI=1). At the indicated time point, cells  
535 were washed with PBS, fixed with 4% (v/v) formalin and permeabilized with 0.1% (v/v) Triton  
536 X-100. Proximity ligation was performed as recommended by the manufacturer (Duolink In  
537 Situ detection reagents Red, Sigma) using monoclonal antibodies directed against dsRNA (J2,

538 English & Scientific Consulting) or myc-tag (Cell Signaling 2276) and polyclonal antibodies  
539 recognizing eIF3E (Sigma, HPA023973) or RTN4 (Nogo A+B, Abcam 47085). Coverslips  
540 were mounted using Duolink® In Situ Mounting Media with DAPI (Sigma).

541 All samples were imaged by acquiring 0.2 µm stacks over 10 µm using a DeltaVision Elite  
542 High-Resolution imaging system (GE Healthcare Life Sciences) equipped with a 60x or 100x  
543 oil immersion objective (1.4 NA). Images were deconvolved using the integrated softWoRx  
544 software and processed using Fiji (ImageJ).

545

#### 546 **Biotinylation assay – western blot – mass spectrometry**

547 L929 cells were infected with MHV-A59 or MHV-BirA<sub>R118G</sub>-nsp2, and for comparison  
548 MHV<sub>H277A</sub> and MHV<sub>H277A</sub>-BirA<sub>R118G</sub>-nsp2, at an MOI=1 in medium supplemented with 67 µM  
549 biotin (Sigma B4501). At 15 h.p.i., cells were washed three times with PBS and lysed in ice-  
550 cold buffer containing 50 mM TRIS-Cl pH 7.4, 500 mM NaCl, 0.2% (w/v) SDS, 1 mM DTT  
551 and 1x protease inhibitor (cOmplete Mini, Roche). Cells were scraped off the flask and  
552 transferred to tubes. Cells were kept on ice until the end of the procedure. Triton X-100 was  
553 added to each sample to a final concentration of 2%. Samples were sonicated for two rounds  
554 of 20 pulses with a Branson Sonifier 250 (30% constant, 30% power). Equal volumes of 50  
555 mM TRIS-Cl were added to each sample and samples were centrifuged at 4 °C for 10 min at  
556 18000 x g. Supernatants were incubated with magnetic beads on a rotator at 4 °C overnight  
557 (800 µl Dynabeads per sample, MyOne Streptavidin C1, Life Technologies) that were  
558 previously washed with lysis buffer diluted 1:1 with 50 mM TRIS-Cl. Beads were washed  
559 twice with buffer 1 (2% (w/v) SDS), once with buffer 2 (0.1% (w/v) deoxycholic acid, 1%  
560 (v/v) Triton X-100, 1 mM EDTA, 500 mM NaCl, 50 mM HEPES pH 7.5), once with buffer 3  
561 (0.5% w/v deoxycholic acid, 0.5% NP40, 1 mM EDTA, 250 mM LiCl, 10 mM TRIS-Cl pH



562 7.4) and once with 50 mM TRIS-Cl pH 7.4. Proteins were eluted from beads by the addition  
563 of 0.5 mM biotin and Laemmli SDS-sample buffer and heating at 95 °C for 10 min.  
564 For SDS-PAGE and western blot analysis, cells were cultured in 6-well plates and lysates were  
565 prepared and affinity purified as described above. Proteins were separated on 10% (w/v) SDS-  
566 polyacrylamide gels (Bio-Rad), and proteins were electroblotted on nitrocellulose membranes  
567 (Amersham Biosciences, GE Healthcare) in a Mini Trans-Blot cell (Bio-Rad). Membranes  
568 were incubated in a protein-free blocking buffer (Advansta) and biotinylated proteins were  
569 probed by incubation with horseradish peroxidase-conjugated Streptavidin (Dako). Proteins  
570 were visualized using WesternBright enhanced chemiluminescence horseradish peroxidase  
571 substrate (Advansta) according to the manufacturer's protocol.  
572 For mass spectrometry analysis, lysates and affinity purification were performed as described  
573 above from  $4 \times 10^7$  cells cultured in 150 cm<sup>2</sup> tissue culture flasks. Proteins were separated 1 cm  
574 into a 10% (w/v) SDS-polyacrylamide gel. A Coomassie stain was performed and 4x 2 mm  
575 bands were cut with a scalpel. Proteins on gel samples were reduced, alkylated and digested  
576 with Trypsin(68). Digests were loaded onto a pre-column (C18 PepMap 100, 5  $\mu$ m, 100 A, 300  
577  $\mu$ m i.d. x 5 mm length) at a flow rate of 20  $\mu$ L/min with solvent C (0.05% TFA in  
578 water/acetonitrile 98:2). After loading, peptides were eluted in back flush mode onto the  
579 analytical Nano-column (C18, 3  $\mu$ m, 100 Å, 75  $\mu$ m x 150 mm, Nikkyo Technos C. Ltd., Japan)  
580 using an acetonitrile gradient of 5% to 40% solvent B (0.1% (v/v) formic acid in  
581 water/acetonitrile 4,9:95) in 40 min at a flow rate of 400 nL/min. The column effluent was  
582 directly coupled to a Fusion LUMOS mass spectrometer (Thermo Fischer, Bremen; Germany)  
583 via a nano-spray ESI source. Data acquisition was made in data dependent mode with precursor  
584 ion scans recorded in the orbitrap with resolution of 120'000 (at m/z=250) parallel to top speed  
585 fragment spectra of the most intense precursor ions in the Linear trap for a cycle time of 3  
586 seconds maximum. Spectra interpretation was performed with Easyprot on a local, server run

587 under Ubuntu against a forward + reverse *Mus musculus* (UniprotKB version 2016\_04) and  
588 MHV (UniprotKB version 2016\_07) database, using fixed modifications of  
589 carboamidomethylated on Cysteine, and variable modification of oxidation on Methionine,  
590 biotinylation on Lysine and on protein N-term, and deamidation of Glutamine and Asparagine.  
591 Parent and fragment mass tolerances were set to 10 ppm and 0.4 Da, respectively. Matches on  
592 the reversed sequence database were used to set a Z-score threshold, where 1% false  
593 discoveries (FDR) on the peptide spectrum match level had to be expected. Protein  
594 identifications were only accepted, when two unique peptides fulfilling the 1% FDR criterion  
595 were identified. MS identification of biotinylated proteins was performed in three independent  
596 biological replicates. For label-free protein quantification, LC-MS/MS data was interpreted  
597 with MaxQuant (version 1.5.4.1) using the same protein sequence databases and search  
598 parameters as for EasyProt. Match between runs was activated, however samples from different  
599 treatments were given non-consecutive fraction numbers in order to avoid over-interpretation  
600 of data. The summed and median normalized top3 peptide intensities extracted from the  
601 evidence table as a surrogate of protein abundance (69) and LFQ values were used for statistical  
602 testing. The protein groups were first cleared from all identifications, which did not have at  
603 least two valid LFQ values. Protein LFQ levels derived from MaxQuant were log-transformed.  
604 Missing values were imputed by assuming a normal distribution between sample replicates. A  
605 two-tailed t-test was used to determine significant differences in protein expression levels  
606 between sample groups and p-values were adjusted for multiple testing using the Benjamini-  
607 Hochberg (FDR) test.

608

### 609 **Computational analysis**

610 Database for Annotation, Visualization, and Integrated Discovery (DAVID) was used to  
611 perform GO enrichment analysis on the RTC-proximal cellular factors identified via mass

612 spectrometry(70-73). GO BP terms with a p-value <0.05 were considered to be terms that were  
613 significantly enriched in the dataset. Additional analysis of significant GO terms was conducted  
614 using AmiGO and revealed that the top 32 GO BP terms (p-value <0.005) were predominantly  
615 associated with five broad functional categories (cell-cell adhesion, transport, cell organization,  
616 translation, and catabolic processes)(74). Alternatively, enrichment analysis was performed  
617 using SetRank (data not shown), a recently described algorithm that circumvents pitfalls of  
618 commonly used approaches and thereby reduces the amount of false-positive hits (75) and the  
619 following databases were searched for significant gene sets: BIOCYC (76), GO (72), ITPF  
620 (77), KEGG (78), PhosphoSitePlus (79), REACTOME (80), and WikiPathways (81). Both  
621 independent approaches lead to highly similar results and consistently complement results  
622 obtained upon GO Cellular Components analysis.

623 STRING functional protein association networks were generated using RTC-proximal host  
624 proteins found within each of the five broad functional categories. Default settings were used  
625 for active interaction sources and a high confidence interaction score (0.700) was used to  
626 maximize the strength of data support. The MCL clustering algorithm was applied to each  
627 STRING network using an inflation parameter of 3 (82, 83).

628

## 629 **siRNA screen**

630 A custom siRNA library targeting each individual RTC-proximal factor (On Target Plus,  
631 SMART pool, 96-well plate format, Dharmacon, GE Healthcare) was ordered. 10 nM siRNA  
632 were reverse transfected into L929 cells ( $8 \times 10^3$  cells per well) using Viromer Green  
633 (Lipocalyx) according to the manufacturer's protocol. Cells were incubated 48 hours at 37 °C  
634 5% CO<sub>2</sub> and cell viability was assessed using the CytoTox 96<sup>®</sup> Non-Radioactive Cytotoxicity  
635 Assay (Promega). Cells were infected with MHV-Gluc (MOI=0.05, 1000 plaque forming  
636 units/well), washed with PBS 3 h.p.i. and incubated in MEM+/+ for additional 12 hours.

637 Gaussia luciferase was measured from the supernatant using Pierce™ Gaussia Luciferase Glow  
638 Assay Kit (ThermoFisher Scientific). Experiments were carried out in 4 independent replicates  
639 and both cytotoxicity values and luciferase counts were normalized to the corresponding non-  
640 targeting scrambled control of each plate. A one-way ANOVA (Kruskal-Wallis test,  
641 uncorrected Dunn's test) was used to test the statistical significance of reduced viral replication  
642 (mean < 95% as compared to scramble control, n=216). The R package ggplot2 was used to  
643 create the bubble plot (Fig 4B).

644

#### 645 **siRNA screen validation**

646 L929 cells were transfected with 10 nM siRNA as described above. 48 h post-transfection, cell  
647 viability was assessed using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega)  
648 and visually inspected by automated phase-contrast microscopy using an EVOS FL Auto 2  
649 Imaging System equipped with a 4x air objective. Cells were infected with MHV-Gluc  
650 (MOI=0.05), washed with PBS 3 h.p.i. and incubated for 9 additional hours. *Gaussia* luciferase  
651 activity, viral titers and cell viability were measured from the supernatant as described above.  
652 One-way ANOVAs (ordinary one-way ANOVA, uncorrected Fisher's LSD test) were used to  
653 test the statistical significance.

654 Total cellular RNA was isolated from cells using the NucleoMag® RNA Kit (Machery Nagel,  
655 Switzerland) on a KingFisher™ Flex Purification System (Thermo Fisher Scientific,  
656 Switzerland) according to the manufacture's instructions. The QuantiTect Probe RT-PCR Kit  
657 (Qiagen, Switzerland) was used according to the manufactures instructions for measuring the  
658 cell associated viral RNA levels with primers and probe specific to the MHV genome fragment  
659 coding the nucleocapsid gene (Table S4). Primers and Probe for mouse Glyceraldehyde 3-  
660 phosphate dehydrogenase (GAPDH) where obtained from ThermoFisher Scientific  
661 (Mm03302249\_g1, Catalog Number: 4331182). The MHV levels were normalized to GAPDH

662 and shown as  $\Delta\Delta Ct$  over mock ( $\Delta Ct$  values calculated as  $Ct$  reference -  $Ct$  target). The  
663 QuantiTect SYBR® Green RT-PCR Kit (Qiagen, Switzerland) was used according to the  
664 manufactures instructions for measuring the expression levels of Rpl13a, eIF3E, eIF3I, eIF3F,  
665 eIF4G1, eIF4G2, eIF2ak3, Rsl24d1 and Tbp. All primer pairs where placed over an exon intron  
666 junction (Table S4). All expression levels are displayed as  $\Delta\Delta Ct$  over non-targeting siRNA  
667 ( $\Delta Ct$  values calculated as  $Ct$  target -  $Ct$  Tbp) (84). One-way ANOVA (ordinary one-way  
668 ANOVA, uncorrected Fisher's LSD test) was used to test the statistical significance.

669

### 670 **Total cellular translation**

671 siRNA-based silencing was performed as described above. 48 h post-transfection, control cells  
672 were incubated with 355  $\mu M$  cycloheximide (Sigma) and 208  $\mu M$  Emetin (Sigma) for 30 min  
673 to block protein synthesis. Cells were treated with 3  $\mu M$  puromycin for 60 min followed by  
674 three PBS washes(85). Total cell lysates were prepared using M-PER mammalian protein  
675 extraction reagent (Thermo Scientific) supplemented with protease inhibitors (cOmplete Mini,  
676 Roche). Lysates were separated on a 10% (w/v) SDS-PAGE and electroblotted as described  
677 above. Western blots were probed using a monoclonal AlexaFluor647-conjugated anti-  
678 puromycin antibody (clone 12D10, Merk Millipore) and a donkey-derived HRP-conjugated  
679 anti-mouse (Jackson immunoresearch 715-035-151). Actin was detected using a monoclonal  
680 HRP-conjugated anti-actin antibody (Sigma A3854) and used to normalize input.

681

### 682 **Ribopuromylation assay**

683 Ribopuromylation of actively translating ribosomes was performed as described before (55).  
684 L929, Huh-7 cells were seeded on glass coverslips and infected with MHV-A59 (L929),  
685 HCoV-229E (Huh-7), MERS-CoV (Huh-7) and at MOI=1. One hour after inoculation, cells  
686 were washed with PBS and incubated further for the indicated time. Cells were treated with

687 355  $\mu$ M cycloheximide and 208  $\mu$ M Emetin (Sigma) for 15 min at 37°C. Cells were further  
688 incubated in medium containing 355  $\mu$ M cycloheximide, 208  $\mu$ M Emetin and 182  $\mu$ M  
689 puromycin (Sigma) for additional 5 min. Cells were washed twice in ice-cold PBS and fix on  
690 ice for 20 min in buffer containing 50 mM TRIS HCl, 5 mM MgCl<sub>2</sub>, 25 mM KCl, 355  $\mu$ M  
691 cycloheximide, 200 mM NaCl, 0.1% (v/v) TritonX-100, 3% formalin and protease inhibitors  
692 (cOmplete Mini, Roche). Cells were blocked for 30 min in CB, and immunostained as  
693 described above using polyclonal anti-MHV-nsp2/3 (gift from S. Baker), polyclonal anti-  
694 HCoV-229E-nsp8 (gift from J. Ziebuhr), or monoclonal anti-dsRNA (J2 MAB, English and  
695 Scientific Consulting) as primary antibodies to detect MHV, HCoV-229E and ZIKV  
696 replication complexes, respectively. Donkey-derived, AlexaFluor488-conjugated anti-mouse  
697 or anti-rabbit IgG (H+L) were used as secondary antibodies. Additionally, ribosome-bound  
698 puromycin was detected using a monoclonal AlexaFluor647-conjugated anti-puromycin  
699 antibody (clone 12D10, Merk Millipore). Slides were mounted, imaged and processed as  
700 described above.

701

#### 702 **DAB staining and transmission electron microscopy**

703 L929 fibroblasts were seeded in 24-well plates and infected with MHV-APEX2-nsp2, MHV-  
704 A59, or non-infected for 10 h. 3,3-diaminobenzidine (DAB) stains were performed as described  
705 previously (38). Briefly, cells were fixed at 10 h.p.i. using warm 2% (v/v) glutaraldehyde in  
706 100 mM sodium cacodylate, pH 7.4, supplemented with 2 mM calcium chloride (cacodylate  
707 buffer) and placed on ice for 60 min. The following incubations were performed on ice in ice-  
708 cold buffers unless stated otherwise. Cells were washed 3x with sodium cacodylate buffer,  
709 quenched with 20 mM glycine in cacodylate buffer for 5 min. before 3 additional washes with  
710 cacodylate buffer. Cells were stained in cacodylate buffer containing 0.5 mg/ml DAB and 10  
711 mM H<sub>2</sub>O<sub>2</sub> for 20 min until DAB precipitates were visible by light microscopy. Cells were

712 washed 3x with cacodylate buffer to stop the staining reaction. Processing of samples for  
713 transmission electron microscopy (TEM) was performed as described previously (86). Briefly,  
714 cells were washed once with PBS prewarmed to 37 °C and subsequently fixed with 2.5% (v/v)  
715 glutaraldehyde (Merck, Darmstadt, Germany) in 0.1 M cacodylate buffer (Merck, Hohenbrunn,  
716 Germany) pH 7.4 for 30 min at room temperature or overnight at 4 °C. After three washes in  
717 cacodylate buffer for 10 min each, cells were post-fixed with 1% OsO<sub>4</sub> (Chemie Brunschwig,  
718 Basel, Switzerland) in 0.1 M cacodylate buffer for 1 h at 4 °C and again washed three times  
719 with cacodylate buffer. Thereafter, cells were dehydrated in an ascending ethanol series (70%,  
720 80%, 90%, 94%, 100% (v/v) for 20 min each) and embedded in Epon resin, a mixture of Epoxy  
721 embedding medium, dodecenyl succinic anhydride (DDSA) and methyl nadic anhydride  
722 (MNA) (Sigma Aldrich, Buchs, Switzerland). Ultrathin sections of 90 nm were then obtained  
723 with diamond knives (Diatome, Biel, Switzerland) on a Reichert-Jung Ultracut E (Leica,  
724 Heerbrugg, Switzerland) and collected on collodion-coated 200-mesh copper grids (Electron  
725 Microscopy Sciences, Hatfield, PA, USA). Sections were double-stained with 0.5% (w/v)  
726 uranyl acetate for 30 min at 40 °C (Sigma Aldrich, Steinheim, Germany) and 3% (w/v) lead  
727 citrate for 10 min at 20 °C (Laurylab, Saint Fons, France) in an Ultrastain® (Leica, Vienna,  
728 Austria) and examined with a Philips CM12 transmission electron microscope (FEI,  
729 Eindhoven, The Netherlands) at an acceleration voltage of 80 kV. Micrographs were captured  
730 with a Mega View III camera using the iTEM software (version 5.2; Olympus Soft Imaging  
731 Solutions GmbH, Münster, Germany).

732

733 **Figure legends**

734 **Figure 1. Characterization of the recombinant MHV-BirA<sub>R118G</sub>-nsp2.** (a) Genome  
735 organization of recombinant MHV-BirA<sub>R118G</sub>-nsp2. The positive-sense RNA genome of MHV  
736 contains a 5' cap and a 3' poly(A) tail. ORF1a and ORF1b encode the viral replication and  
737 transcription complex (nsp1-16). myc-BirA<sub>R118G</sub> was inserted as an N-terminal fusion with  
738 nsp2 within ORF1a. The cleavage site between nsp1 and myc-BirA<sub>R118G</sub> was retained (black  
739 arrow) while a deleted cleavage site between BirA<sub>R118G</sub> and nsp2 ensured the release of a  
740 BirA<sub>R118G</sub>-nsp2 fusion protein from the pp1a polyprotein. The cleavage site between nsp2 and  
741 nsp3 was also retained. (b) Viral replication kinetics of recombinant MHV-BirA<sub>R118G</sub>-nsp2  
742 were compared to wild-type MHV-A59 and recombinant MHV-GFP-nsp2. Murine L929  
743 fibroblasts were infected at a multiplicity of infection (MOI) of 1 plaque forming unit (pfu) per  
744 cell. Viral supernatants were collected at the indicated time points, titrated by plaque assay and  
745 expressed in pfu per ml. Data points represent the mean and SEM of three independent  
746 experiments, each performed in quadruplicate. (c) Immunofluorescence analysis of MHV-  
747 BirA<sub>R118G</sub>-nsp2-mediated biotinylation of RTC-proximal factors. L929 cells were infected with  
748 MHV-BirA<sub>R118G</sub>-nsp2 (MOI=1) in medium supplemented with 67µM biotin. Cells were fixed  
749 15 hours post infection (h.p.i.) and processed for immunofluorescence analysis with antibodies  
750 directed against the BirA<sub>R118G</sub> (anti-myc), the viral replicase (anti-nsp2/3) and biotinylated  
751 factors (streptavidin). Nuclei are counterstained with DAPI. Z-projection of deconvolved z-  
752 stacks acquired with a DeltaVision Elite High-Resolution imaging system are shown. Scale  
753 bars: 20 µm. (d) Ultrastructural analysis of MHV-APEX2-nsp2 infection. L929 cells were  
754 infected with MHV-APEX2-nsp2 and MHV-A59 (MOI=2), or mock infected. At 10 h.p.i.,  
755 cells were fixed, stained with DAB and processed for electron microscopy investigations.  
756 Representative low and high magnifications are displayed.

757



758 **Figure 2. Determination of the coronavirus RTC-proximal proteome** (a) Schematic  
759 overview of the BirA<sub>R118G</sub>-mediated proximity biotinylation assay using MHV-BirA<sub>R118G</sub>-  
760 nsp2. (b) Western blot analysis of MHV-BirA<sub>R118G</sub>-nsp2-infected L929 cells. L929 cells were  
761 infected with MHV-BirA<sub>R118G</sub>-nsp2, MHV-A59 (parental wild-type strain) or non-infected in  
762 medium with and without supplementation of 67  $\mu$ M biotin. Cells were lysed 15 h.p.i. and  
763 biotinylated factors were subjected to affinity purification using streptavidin-coupled magnetic  
764 beads. Total cell lysates and affinity-purified fractions were separated by SDS-PAGE and  
765 analysed by western blot probed with horse radish peroxidase (HRP)-coupled Streptavidin. (c)  
766 Host and viral factors identified by LC-MS/MS. 4\*10<sup>7</sup> L929 cells were infected with MHV-  
767 BirA<sub>R118G</sub>-nsp2 or MHV-A59 in medium supplemented with 67 $\mu$ M biotin. 15 h.p.i., lysates  
768 were affinity purified and LC-MS/MS was performed from in-gel digested samples. MS  
769 identification of biotinylated proteins was performed in three independent biological replicates.  
770 Spectral interpretation was performed against a *Mus musculus* and MHV database and log<sub>2</sub>-  
771 transformed LFQ levels (x-axis) were used to determine significant differences in protein  
772 enrichment between sample groups (Student's T-test, y-axis). Identified cellular proteins are  
773 displayed as black dots, MHV proteins are highlighted in red (nsp: non-structural protein, N:  
774 nucleocapsid, S: spike, M: membrane, 2a: accessory protein 2a). (d) Summary of viral proteins  
775 identified by LC-MS/MS. nsp2-10, nsp12-16, and nucleocapsid were significantly enriched in  
776 fractions derived from MHV-BirA<sub>R118G</sub>-nsp2-infected cells whereas nsp1, nsp11, structural  
777 proteins spike (S), envelope (E) and membrane proteins (M) as well as all accessory proteins  
778 (NS2a, HE, ORF4, ORF5a) were either not significantly enriched or not detected. (e,f)  
779 Immunofluorescence analysis of RTC-proximal cellular factors. L929 cells were seeded on  
780 coverslips, infected with MHV-BirA<sub>R118G</sub>-nsp2 (e) or MHV-A59 (f), fixed at 9 h.p.i. and  
781 processed for immunofluorescence using anti-myc, anti-RTN4 and anti-eIF3E antibodies (e)  
782 or anti-dsRNA, anti-RTN4 and anti-eIF3E antibodies (f). Secondary fluorophore-coupled

783 antibodies were used to detect the viral replicase and endogenous levels of RTN4 and eIF3E  
784 (e). Proximity ligations were performed using Duolink In Situ detection reagents (f). Nuclei  
785 are counterstained with DAPI. Z-projection of deconvolved z-stacks acquired with a  
786 DeltaVision Elite High-Resolution imaging system are shown. Intensity profiles highlighted in  
787 the magnified regions are shown. Scale bars: 20  $\mu$ m.

788

789 **Figure 3. Functional classification of RTC-proximal host factors** (a) Gene Ontology  
790 enrichment analysis of RTC-proximal cellular factors. 32 terms were highly significant (p-  
791 value <0.005) and were assigned to 5 broad functional categories: cell-cell adhesion, transport,  
792 cell organization, translation, catabolic processes. (b-c) STRING protein interaction network  
793 analysis of the categories “transport” (b) and “translation” (c). The nodes represent RTC-  
794 proximal host proteins and the edges represent the interactions, either direct (physical) or  
795 indirect (functional), between two proteins in the network. Cellular proteins assigned to the  
796 “transport” category separated into 4 distinct interaction clusters. I: protein transport, II: COPII  
797 anterograde transport, III: COPI retrograde transport, IV: clathrin-mediated transport.

798

799 **Figure 4. Identification of proviral factors within the coronavirus RTC**  
800 **microenvironment** (a) Impact of siRNA-silencing of RTC-proximal cellular proteins on viral  
801 replication. L929 fibroblasts were reverse-transfected with siRNAs (10 nM) for 48 h before  
802 being infected with MHV-Gluc (MOI=0.05, n=4). Replication was assessed by virus-mediated  
803 Gaussia luciferase expression at 15 h.p.i. and was normalized to levels of viral replication in  
804 cells targeted by scrambled siRNA controls. Target proteins to the left of the dashed line  
805 represent RTC-proximal factors whose silencing decreased viral replication. (b) Bubble plot  
806 illustrating host proteins that significantly impact MHV replication. Bubble size is proportional

807 to the level of viral replication impairment. Colors correspond to the functional categories  
808 highlighted in Figure 3. Light grey bubbles (below the dashed line) represent host proteins that  
809 did not significantly impact MHV replication ( $p$ -value  $> 0.05$ ). (c, d, e, f) Silencing of RTC-  
810 proximal components of the cellular translation machinery. Upon 48h siRNA silencing of  
811 factors assigned to the category “translation” (Figure 3), L929 fibroblasts were infected with  
812 MHV-Gluc (MOI=0.05,  $n=3$ ). Luciferase activity (c), cell-associated viral RNA levels (d) and  
813 viral titers (e) were assessed at 12 h.p.i.. (f) Western blot quantification of total cellular  
814 translation following silencing of a subset of the host translation apparatus. Upon 48h siRNA-  
815 silencing, L929 fibroblasts were pulsed with 3  $\mu$ M puromycin for 60 min. Control cells were  
816 treated, prior to puromycin incubation, with 355  $\mu$ M cycloheximide and 208  $\mu$ M Emetin for  
817 30 min to block protein synthesis. Cell lysates were separated by SDS-PAGE and Western  
818 blots were probed using anti-puromycin antibodies to assess puromycin incorporation into  
819 polypeptides and normalized to actin levels. Error bars represent the mean  $\pm$  standard deviation,  
820 where \* is  $p \leq 0.05$ , \*\* is  $p \leq 0.005$ , \*\*\* is  $p \leq 0.0005$  and \*\*\*\* is  $p < 0.0001$ .

821

822 **Figure 5. Active translation near sites of MHV mRNA synthesis.** Visualization of active  
823 translation in MHV-infected L929 fibroblasts. Cells infected with MHV-A59 (MOI=1) or non-  
824 infected cells were cultured for 6, 8, 10 and 12 hours and pulsed with cycloheximide, emetine  
825 and puromycin for 5 min to label translating ribosomes. All cells, including non-treated control  
826 infections, were subjected to a coextraction/fixation procedure to remove free puromycin. Cells  
827 were labelled using anti-nsp2/3 antiserum and anti-puromycin antibodies. Nuclei are  
828 counterstained with DAPI. Z-projection of deconvolved z-stacks acquired with a DeltaVision  
829 Elite High-Resolution imaging system are shown. Note the gradual decrease of overlap  
830 between the viral replication and actively translating ribosomes highlighted in the intensity  
831 profiles. Scale bar: 20  $\mu$ m.

832

833 **Figure 6. Active translation near sites of HCoV-229E and MERS-CoV mRNA synthesis.**

834 Visualization of active translation during hCoV-229E and MERS-CoV infections. Huh7 cells  
835 were infected with HCoV-229E and MERS-CoV (MOI=1) for 12 h and 6 h, respectively. Cells  
836 were pulsed with cycloheximide, emetine and puromycin for 5min to label translating  
837 ribosomes and subjected to a coextraction/fixation procedure to remove free puromycin. Non-  
838 infected and/or non-pulsed cells were used as control. Cells were labelled using anti-nsp8  
839 (HCoV-229E) or dsRNA (MERS-CoV) and anti-puromycin antibodies. Nuclei are  
840 counterstained with DAPI. Z-projection of deconvolved z-stacks acquired with a DeltaVision  
841 Elite High-Resolution imaging system are shown. Intensity profiles of magnified regions are  
842 shown. Scale bar: 20  $\mu$ m.

843

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- 1043



1044 **Supplemental Figure 1.** Immunofluorescence analysis of MHV-BirA<sub>R118G</sub>-nsp2-mediated  
1045 biotinylation. MHV-BirA<sub>R118G</sub>-nsp2, MHV-A59- or non-infected L929 fibroblasts were  
1046 cultured in medium supplemented with 67 $\mu$ M biotin. Cells were fixed 12 hours post infection  
1047 (h.p.i.) and processed for immunofluorescence analysis with antibodies directed against the  
1048 BirA<sub>R118G</sub> (anti-myc), the viral replicase (anti-nsp2/3 or nsp8) and biotinylated factors  
1049 (streptavidin). Nuclei are counterstained with DAPI. Z-projection of deconvolved z-stacks  
1050 acquired with a DeltaVision Elite High-Resolution imaging system are shown. Scale bars: 20  
1051  $\mu$ m.

1052

1053 **Supplemental Figure 2.** STRING protein interaction network analysis of the categories “cell  
1054 organization” (a) and “catabolic processes” (b). The nodes represent RTC-proximal host  
1055 proteins and the edges represent the interactions, either direct (physical) or indirect  
1056 (functional), between two proteins in the network.

1057

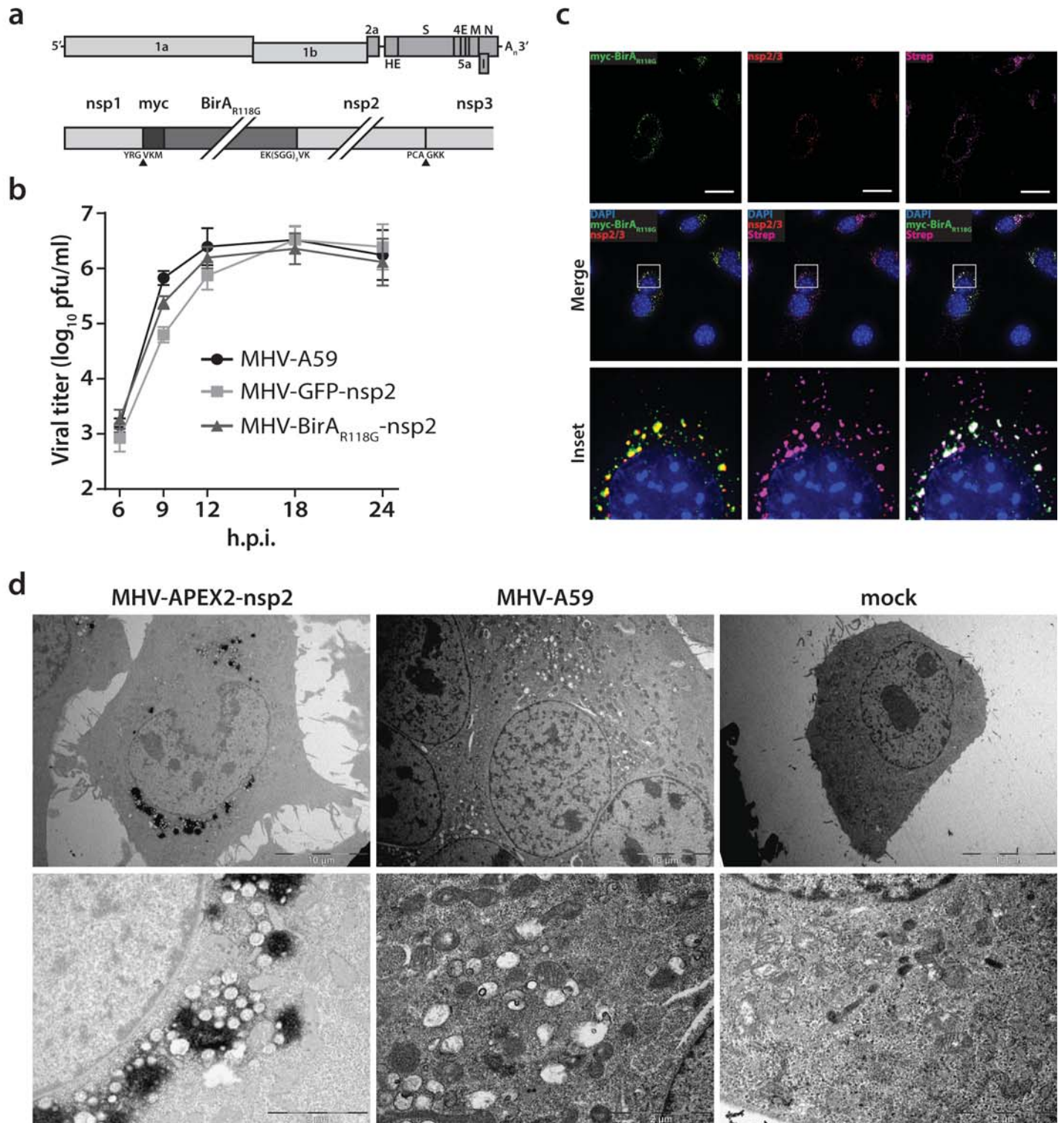
1058 **Supplemental Figure 3.** (a) siRNA controls contained in each 96-well plate during siRNA-  
1059 silencing of the RTC-proximal library. Controls included the established factors such as MHV  
1060 entry receptor (Ceacam1a), Gbfl1, Arf1. Arfgap2 was found to moderately affect MHV  
1061 replication during pilot experiments and was included to cover the entire inhibitory range. (b)  
1062 Cell viability following 48h siRNA-silencing of components of the cellular translation  
1063 machinery. (c) Expression levels of Rpl13a, eIF3E, eIF3I, eIF3F, eIF4G1, eIF4G2, eIF2ak3,  
1064 Rsl24d1 following siRNA knockdown compared to expression levels in cells treated with non-  
1065 targetting siRNA. (d) Visual inspection of L929 treated with siRNA targetting eIF3E, eIF3I,  
1066 eIF3F, Rrbp1, Rpl13a, non-targetting siRNA (scramble). Note that RNA silencing (b) and  
1067 translation activity (c) in Rpl13a-silenced cells could not be assessed, likely due to cytotoxicity

1068 observed by visual inspection of cells. (e) Western blot and western blot analysis of total  
1069 cellular translation. Upon 48h siRNA-silencing, L929 fibroblasts were pulsed with 3  $\mu$ M  
1070 puromycin for 60 min. Control cells were treated, prior to puromycin incubation, with 355  $\mu$ M  
1071 cycloheximide and 208  $\mu$ M Emetin for 30 min to block protein synthesis. Western blots were  
1072 probed using anti-puromycin antibodies to assess puromycin incorporation into polypeptides  
1073 and normalized to actin levels. Error bars represent the mean  $\pm$  standard deviation of three  
1074 independent experiments, where \* is \*\* is  $p \leq 0.005$ .

1075

1076 **Supplemental Figure 4.** Visualization of active translation during HCoV-229E infections.  
1077 Huh7 cells were infected with HCoV-229E (MOI=1) for 12, 15, 18, 24 h. Cells were pulsed  
1078 with cycloheximide, emetine and puromycin for 5min to label translating ribosomes and  
1079 subjected to a coextraction/fixation procedure to remove free puromycin. Non-infected and/or  
1080 non-pulsed cells were used as control. Cells were labelled using anti-nsp8 (HCoV-229E) and  
1081 anti-puromycin antibodies. Nuclei are counterstained with DAPI. Z-projection of deconvolved  
1082 z-stacks acquired with a DeltaVision Elite High-Resolution imaging system are shown.  
1083 Intensity profiles of magnified regions are shown. Scale bar: 20  $\mu$ m.

## Figure 1





## Figure 3

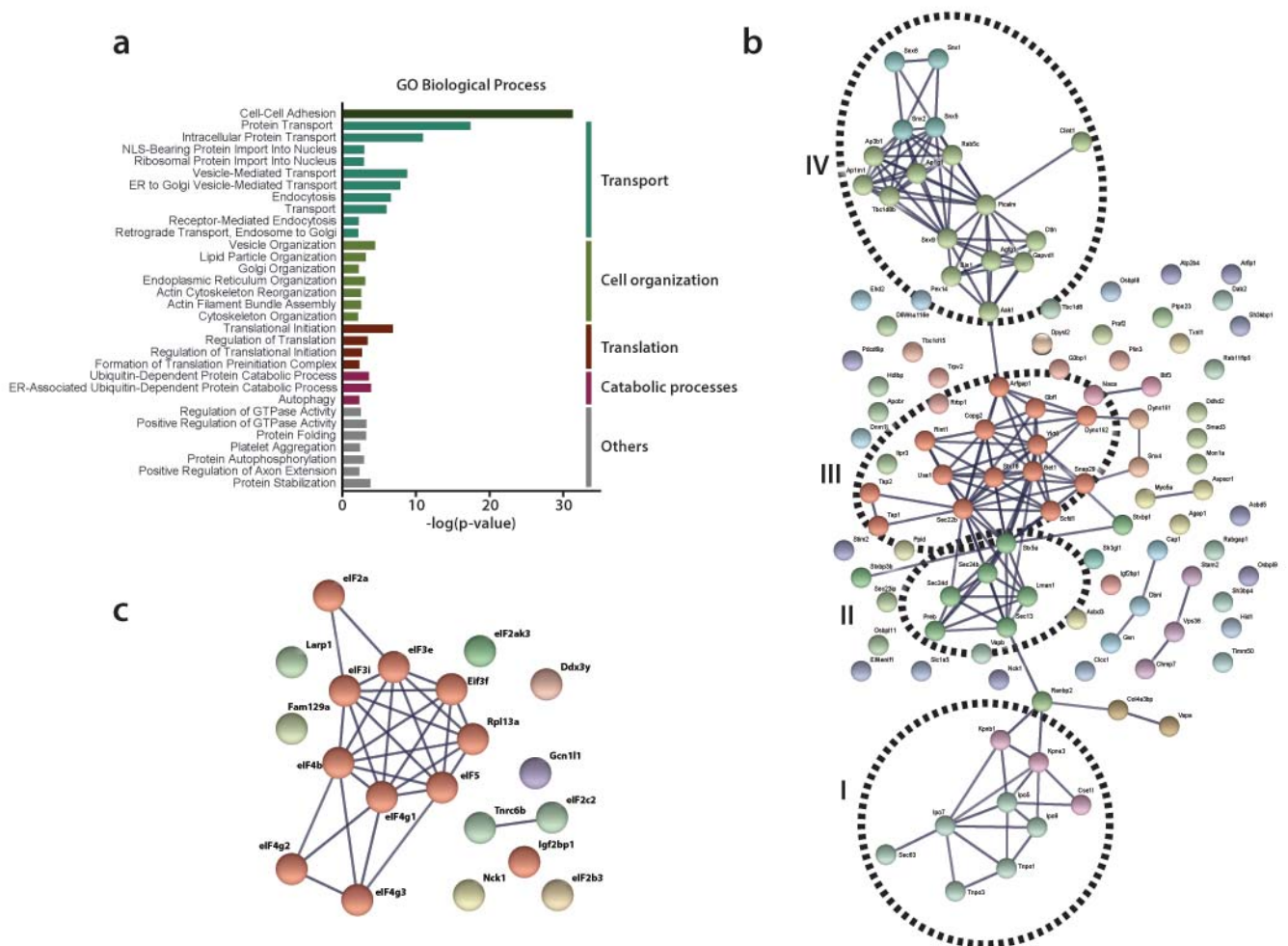


Figure 4

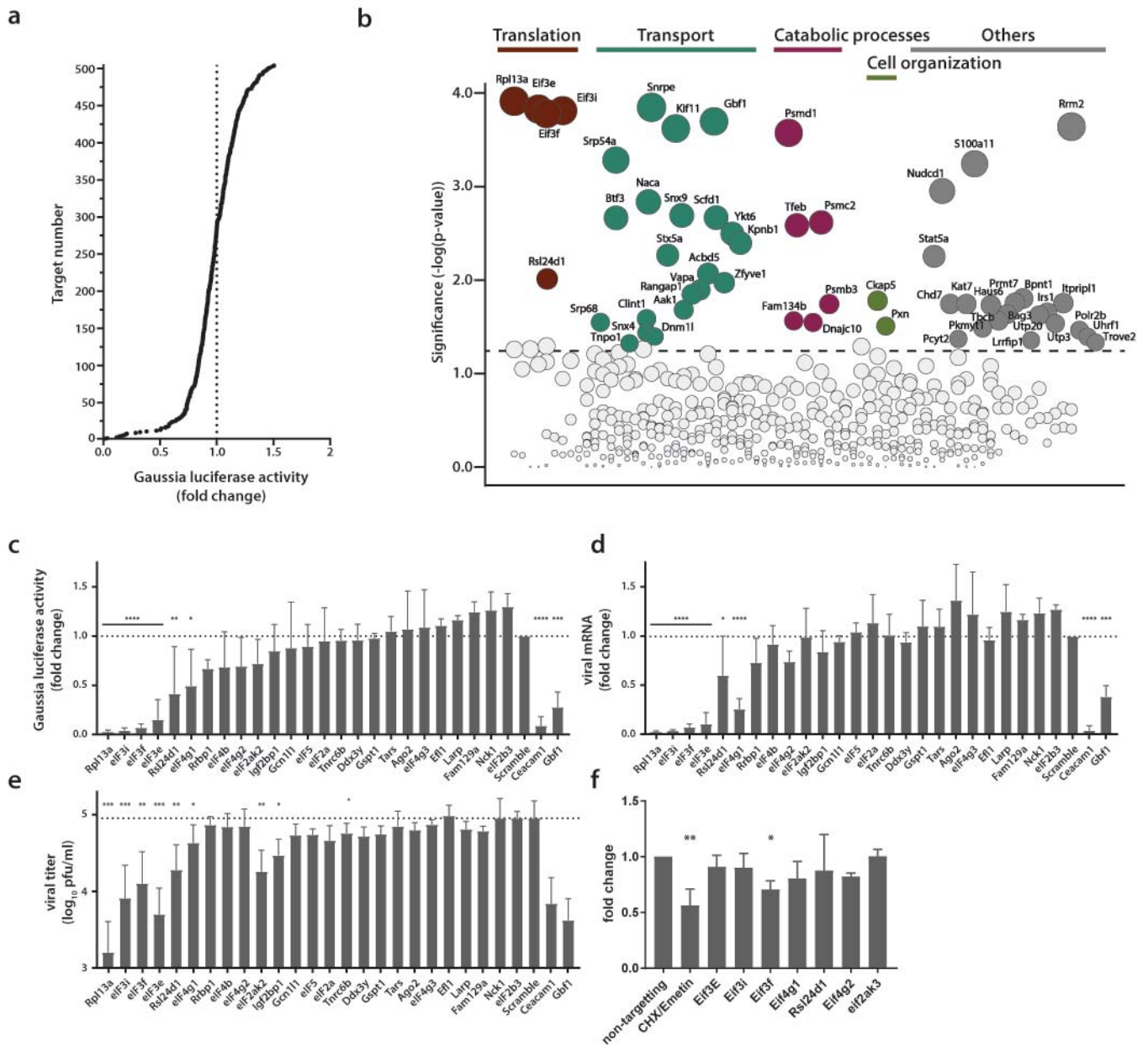
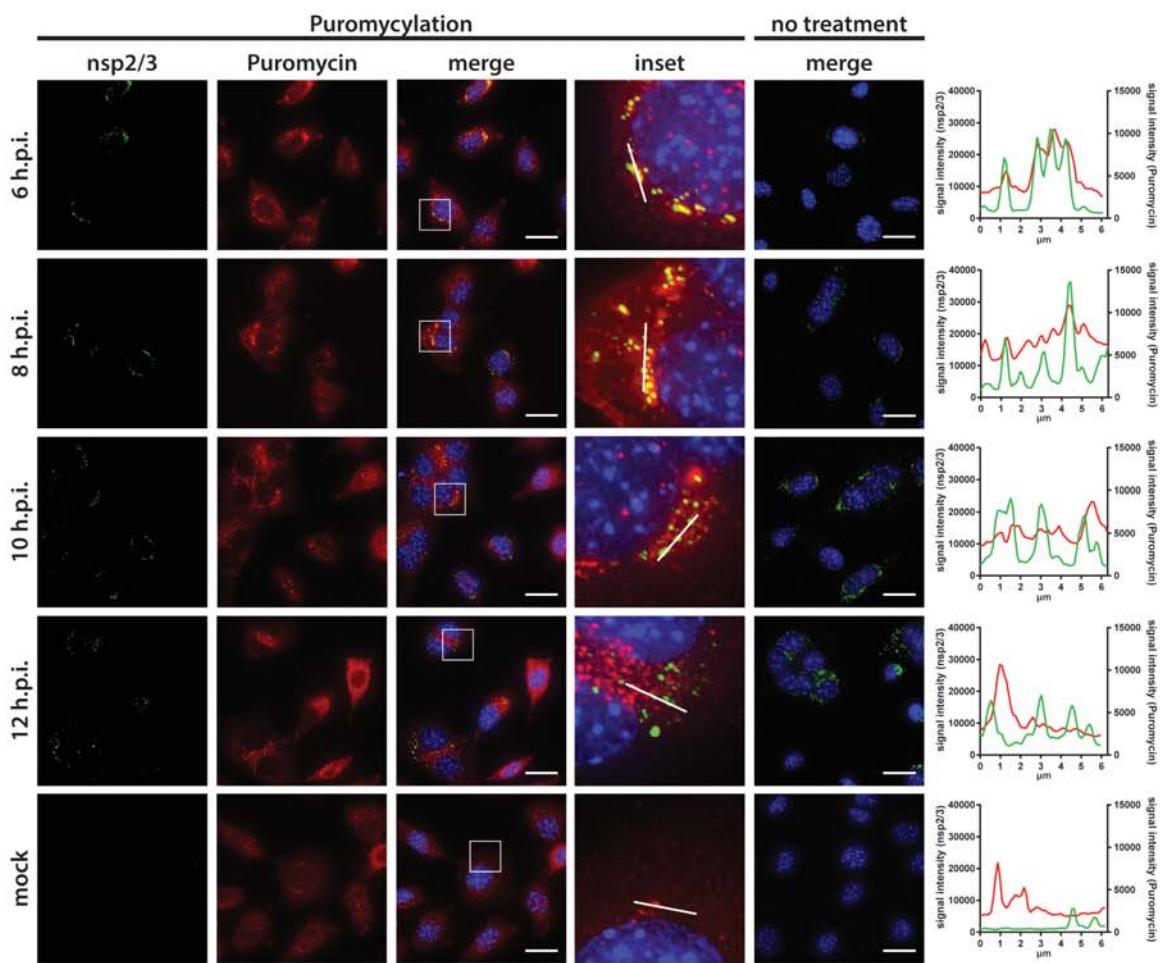
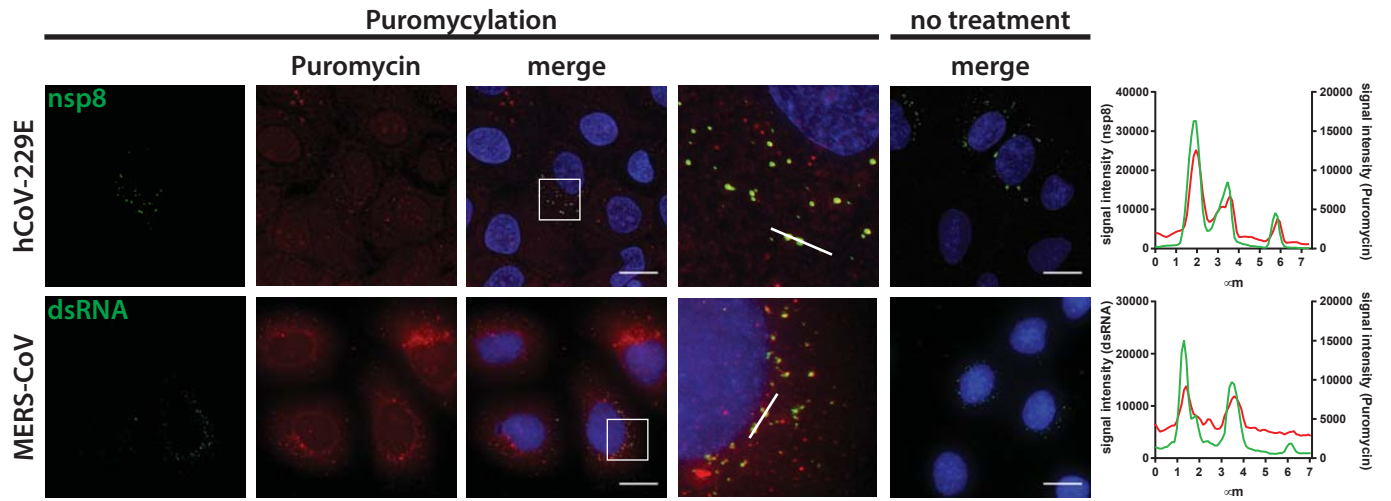


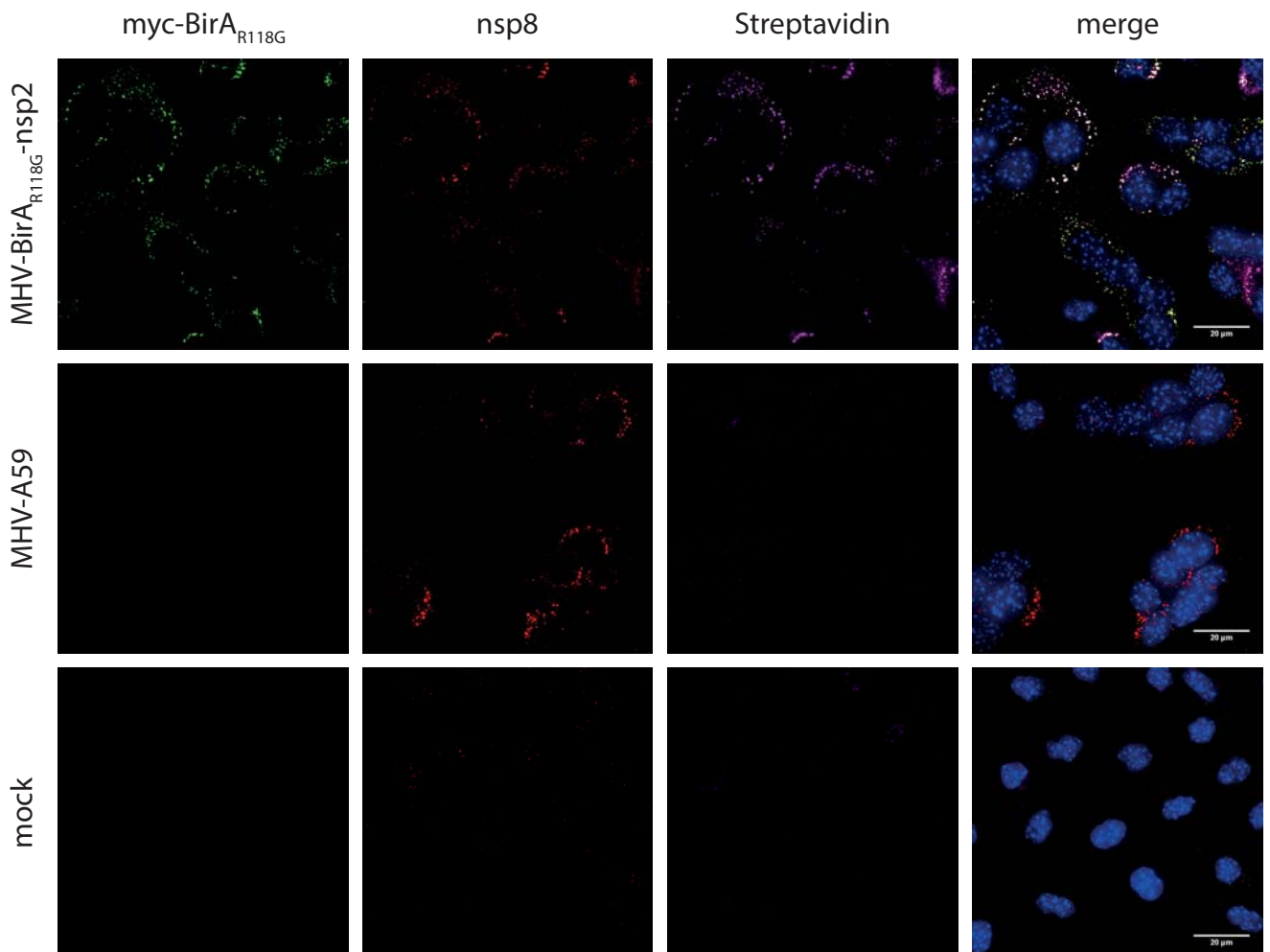
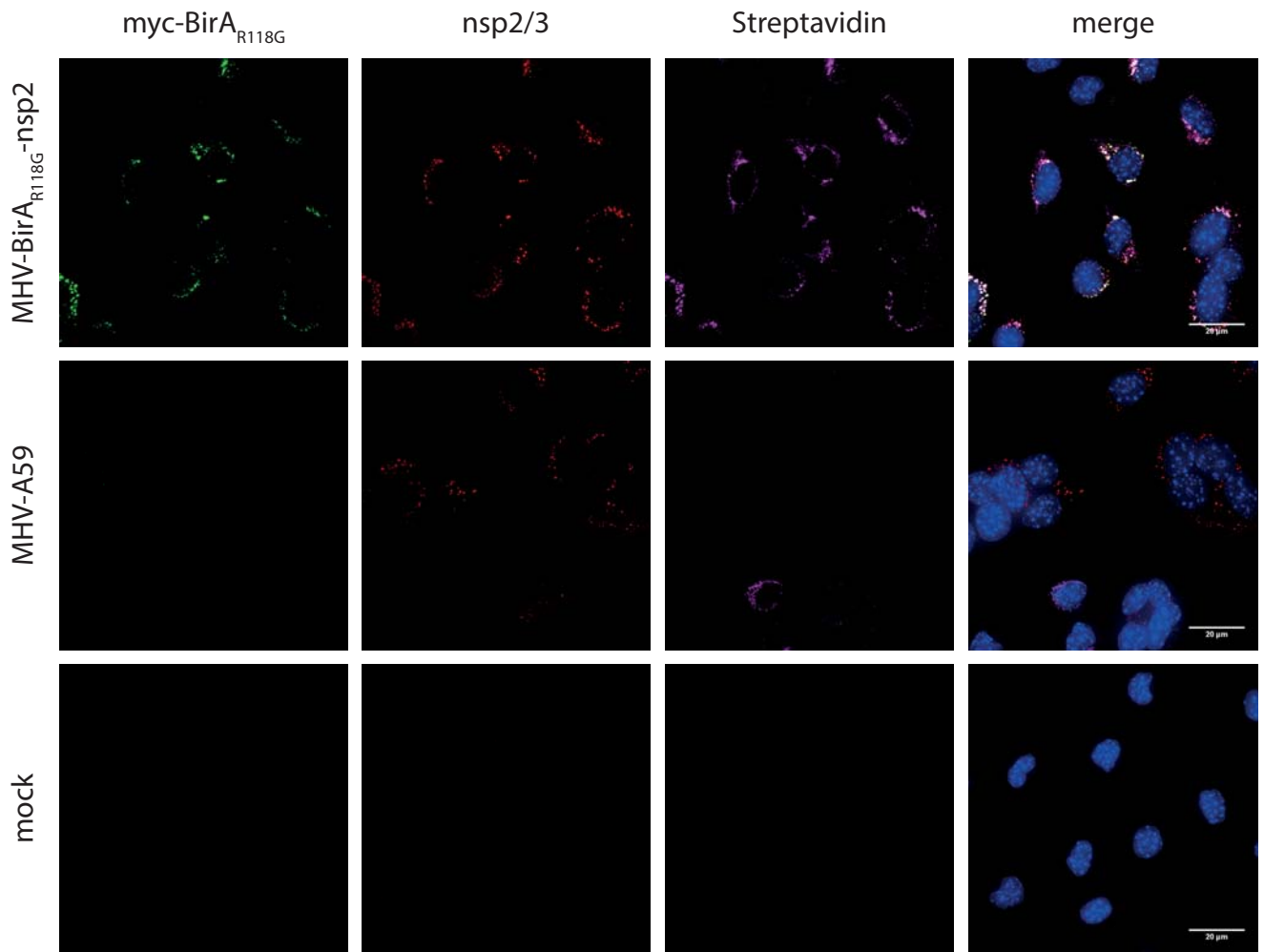
Figure 5



# Figure 6

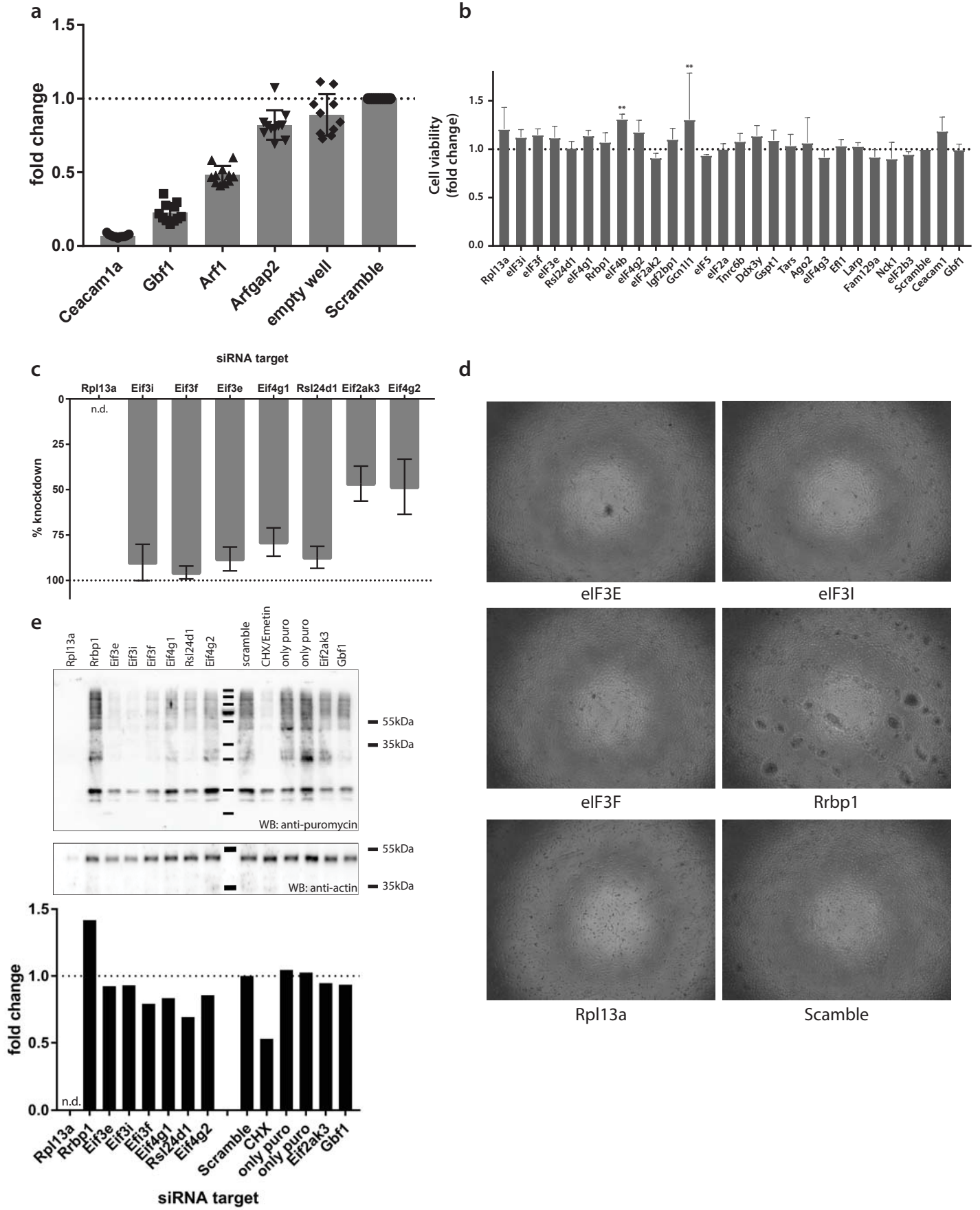








# Figure S3



# Figure S4

