SARS-CoV-2 diverges from other betacoronaviruses in only partially activating the IRE1α/XBP1 ER stress pathway in human lung-derived cells

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Running title

Coronavirus activation of the IRE1α ER stress pathway

SUMMARY

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has killed over 6 million individuals worldwide and continues to spread in countries where vaccines are not yet widely available, or its citizens are hesitant to become vaccinated. Therefore, it is critical to unravel the molecular mechanisms that allow SARS-CoV-2 and other coronaviruses to infect and overtake the host machinery of human cells. Coronavirus replication triggers endoplasmic reticulum (ER) stress and activation of the unfolded protein response (UPR), a key host cell pathway widely believed essential for viral replication. We examined the master UPR sensor IRE1α kinase/RNase and its downstream transcription factor effector XBP1s, which is processed through an IRE1α-mediated mRNA splicing event, in human lung-derived cells infected with betacoronaviruses. We found human respiratory coronavirus OC43 (HCoV-OC43), Middle East respiratory syndrome coronavirus (MERS-CoV), and murine coronavirus (MHV) all induce ER stress and strongly trigger the kinase and RNase activities of IRE1α as well as XBP1 splicing. In contrast, SARS-CoV-2 only partially activates IRE1α through autophosphorylation, but its RNase activity fails to splice XBP1. Moreover, while IRE1α was dispensable for replication in human cells for all coronaviruses tested, it was required for maximal expression of genes associated with several key cellular functions, including the interferon signaling pathway, during SARS-CoV-2 infection. Our data suggest that SARS-CoV-2 actively inhibits the RNase of autophosphorylated IRE1α, perhaps as a strategy to eliminate detection by the host immune system.

IMPORTANCE

SARS-CoV-2 is the third lethal respiratory coronavirus after MERS-CoV and SARS-CoV to emerge this century, causing millions of deaths world-wide. Other common coronaviruses such as HCoV-OC43 cause less severe respiratory disease. Thus, it is imperative to understand the similarities and differences among these viruses in how each interacts with host cells. We focused here on the inositol-requiring enzyme 1α (IRE1α) pathway, part of the host unfolded protein response to virus-induced stress. We found that while MERS-CoV and HCoV-OC43 fully activate the IRE1α kinase and RNase activities, SARS-CoV-2 only partially activates IRE1α, promoting its kinase activity but not RNase activity. Based on IRE1α-dependent gene expression changes during infection, we propose that SARS-CoV-2 prevents IRE1α RNase activation as a strategy to limit detection by the host immune system.

INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerged in China in late 2019. It was the third lethal zoonotic coronavirus to emerge into humans after SARS-CoV (2002) and Middle East respiratory syndrome coronavirus (MERS-CoV) (2012), each of which has been associated with acute lung injury and hypoxemic respiratory failure. While coronaviruses are divided into four genera (alpha, beta, gamma, and delta)(1, 2), all three of the lethal human coronaviruses are betacoronaviruses, albeit from different lineages (Figure 1). SARS-CoV and SARS-CoV-2 are sarbecoviruses, while MERS-CoV is a merbecovirus. Other human CoVs, including HCoV-OC43 (OC43) and HCoV-HKU1 (HKU-1), are embecoviruses as is the model murine coronavirus mouse hepatitis virus (MHV). All CoVs have similar genome structures, replication cycles, and the human CoVs as well as some MHV strains exhibit tropism for the epithelia of the respiratory tract, the portal of entry. They replicate their RNAs and produce subgenomic mRNAs by conserved mechanisms and encode homologous structural as well as replicase proteins. Despite the similarities among all coronaviruses, each lineage expresses distinct accessory proteins that may confer differences in host-virus interactions. Indeed, we have previously found that SARS-CoV-2, MERS-CoV and MHV all induce somewhat different levels of activation and/or antagonism of interferon (IFN) signaling and other dsRNA induced antiviral innate responses (3-5).

One key pathway involved in the virus-induced host response is the endoplasmic reticulum (ER) stress response that regulates protein homeostasis (referred to as proteostasis) in this organelle. One third of all eukaryotic proteins, including most that are inserted into membranes or secreted, are synthesized through co-translational translocation into the ER lumen. Likewise, viral membrane associated proteins are translated and processed in association with the ER (6, 7). Once in the ER, these polypeptides undergo stringent quality control monitoring to ensure that they are properly processed and folded. If the capacity to fold proteins is unable to keep up with

demand, misfolded proteins will accumulate in the ER lumen—a condition referred to as "ER stress." The presence of misfolded proteins in the ER is sensed by three transmembrane sentinel proteins - activating transcription factor 6 (ATF6), PKR-like ER kinase (PERK), and inositol-requiring enzyme (IRE)1α - which trigger an intracellular signaling pathway called the unfolded protein response (UPR). In an effort to restore proteostasis, activation of these sensors induces transcription factors that turn on genes encoding chaperones, oxidoreductases, and ER-associated decay (ERAD) components(8). The UPR also inhibits cap-dependent translation, thus decreasing the load on the ER and giving it extra time to fold proteins already in production (9, 10). If successful, these adaptive UPR programs restore ER homeostasis.

The most ancient UPR pathway is controlled by IRE1α — an ER transmembrane bifunctional kinase/endoribonuclease (RNase) that employs auto-phosphorylation to control its catalytic RNase function (11, 12). In response to ER stress, IRE1α undergoes auto-phosphorylation and dimerization to allosterically activate its RNase domain to excise a 26nt non-conventional intron in *XBP1* mRNA; re-ligation of spliced *XBP1* shifts the open reading frame, and its translation produces the homeostatic transcription factor XBP1s (s=spliced) (13, 14). Once synthesized, XBP1s upregulates genes that expand the ER and its protein folding machinery (15). IRE1α can additionally lead to apoptosis and inflammation via JUN N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) signaling (16). Prolonged ER stress can induce regulated IRE1-dependent decay (RIDD), promoting the cleavage of additional targets beyond XBP1 mRNA, such as secretory protein and ER-localized mRNAs (17). In the short term, RIDD may promote adaptation through further reducing translation and protein burden on the ER. However, prolonged RIDD leads to the depletion of vital ER resident enzymes and structural components to exacerbate ER stress and hasten cell death (11, 18).

116 There is a large body of evidence that viral replication of mammalian cells can trigger ER stress
117 and UPR activation in infected cells (19), and numerous studies report that the UPR is activated
118 upon infection of host cells by coronavirus family members (6, 7, 20-25) Coronaviruses induce
119 stress in the ER in several ways. First, conserved replicase encoded, nonstructural proteins nsp3,
120 nsp4 and nps6 are embedded into the ER membrane, and along with unknown host factors,
121 promote membrane curvature to form double membrane vesicles (DMVs), the site of viral
122 replication/transcription centers (RTC) (26). In addition to remodeling the ER, coronaviruses
123 further condition infected cells by shifting translation away from host mRNAs and instead to viral
124 mRNAs. Translation of viral mRNAs causes the ER to be flooded with heavily glycosylated viral
125 structural proteins [e.g., spike (S), membrane (M) and envelope (E)], challenging the organelle's
126 folding capacity and overall integrity. Indeed, overexpression of CoV spike proteins (27) as well
127 as several sarbecovirus accessory poteins (22, 28) has been reported to induce ER stress.
128 Finally, cell membranes are depleted as enveloped virus particles are assembled into new virions
129 in the ER-Golgi intermediate compartment before budding from the infected cell (1). Thus,
130 coronaviruses as well as other enveloped viruses promote a massive ER expansion and
131 modification necessary to replicate their genomes, transcribe mRNAs, and finally to process and
132 package their protein products into viral particles.
133
134 We have compared the activation status and requirement of the IRE1α/XBP1 arm of the UPR in
135 well-characterized human lung epithelial cell lines and in induced pluripotent stem cell (iPSC)-
136 derived type II alveolar (iAT2) cells, following infection with four betacoronaviruses representing
137 three distinct lineages. We find that infection with MERS-CoV, OC43 and MHV leads to
138 phosphorylation of IRE1α and the consequent production of spliced XBP1 transcription factor.
139 Surprisingly, while we observed phosphorylation of IRE1α in SARS-CoV-2 infected cells, there
140 was notable absence of XBP1s, suggesting SARS-CoV-2 inhibits downstream signaling of the

141 IRE1α/XBP1 arm of the UPR. In addition, we report reduced SARS-CoV-2 induced interferon
142 signaling gene expression in the absence of IRE1α.
143
144 **RESULTS**
145
146 **Induction of IRE1α phosphorylation following coronavirus infection.**
147 To determine whether betacoronaviruses activate IRE1α, we first examined the level of
148 phosphorylated IRE1α after viral infection of the A549 human lung carcinoma cell line. We used
149 A549 cells stably expressing the following receptors to facilitate optimal entry for each of the
150 viruses: carcinoembryonic antigen cell adhesion molecule (CEACAM)1a or MHVR (MHV),
151 dipeptidyl peptidase DPP4 (MERS-CoV), or angiotensin converting enzyme (ACE)2 (SARS-CoV-
152 2). HCoV-OC43 can infect parental A549 or cells expressing ACE2 (3). Consistent with previous
153 reports that embeco lineage coronaviruses MHV (20, 29) and OC43 (24) induce ER stress, we
154 observed a significant increase in phospho-IRE1α (p-IRE1α) during infection by either OC43 (24
155 or 48hpi) or MHV (24hpi) (Figure 2A-C). To confirm the specificity of the p-IRE1α band, we
156 pretreated cells prior to infection with KIRA8, a highly selective kinase inhibitor of IRE1α known
157 to inhibit both autophosphorylation and consequently RNase activity. As expected, KIRA8
158 significantly inhibited the induction of p-IRE1α by OC43 and MHV (Figure 2A&C). Thapsigargin
159 (Tg) and tunicamycin (Tm), both inducers of ER stress, were used as further controls (Figure
160 2B,D&E). Robust induction of p-IRE1α was observed with 1 hour of Tg (1μM) treatment, while no
161 activation of p-IRE1α was observed after 8 hours of treatment with Tm (1μg/ mL), consistent with
162 the negative feedback regulation observed with extended Tm treatment (30). We also observed
163 robust phosphorylation of IRE1α in A549-DDP4 cells and A549-ACE2 cells infected by MERS-
164 CoV and SARS-CoV-2, respectively at 24 and 48 hpi (Figures 2D-F and S1A&B). As with OC43
165 and MHV, IRE1α phosphorylation during SARS-CoV-2 infection was inhibited by KIRA8 (Figure

166 2F). These results are not limited to a single cell type as we observed similar induction of p-IRE1α
167 in Calu-3 cells, another lung epithelial derived cells line, which can be productively infected with
168 both MERS-CoV or SARS-CoV-2 (Figure 2G). These results demonstrate that MERS-CoV,
169 SARS-CoV-2, HCoV-OC43 and MHV activate the host IRE1α kinase after infection.

170

171 **MHV, OC43, MERS-CoV but not SARS-CoV-2 induce splicing of XBP1 mRNA.**

172 We next examined the effect of coronavirus infection on the RNase activity of IRE1α as assessed
173 by XBP1 splicing. Using specific primers to quantify spliced XBP1 mRNA (XBP1s), we observed
174 a marked increase in the percentage of spliced XBP1 mRNA (% XBP1s) as well as an increase
175 in the relative amount of spliced XBP1 mRNA (XBP1s) compared to mock control after infection
176 by OC43, MERS-CoV or MHV in receptor-expressing A549 cells (Figures 3A&B and S2A&B).
177 This induction of XBP1s by OC43 and by MERS-CoV infection was confirmed by assessing XBP1
178 splicing by agarose gel electrophoresis (Figure 3E&F). DNAJB9, a canonical target of XBP1s,
179 was also markedly upregulated with OC43, MERS-CoV, and MHV infection at both 24 and 48
180 hours post-infection (Figures 3A&B and S2B). This induction of IRE1α RNase activity is coincident
181 with the observed autophosphorylation of p-IRE1α upon OC43, MHV or MERS-CoV infection.

182

183 Surprisingly, despite the observed IRE1α autophosphorylation following SARS-CoV-2 infection,
184 there was no significant upregulation of XBP1s mRNA in A549-ACE2 cells up to 52 hours post-
185 infection (Figure 3C&G). Similarly, DNAJB9 expression levels were unchanged at all time points
186 observed with SARS-CoV-2 (Figure 3C). To confirm this effect is not limited to A549 cells, we
187 measured XBP1 mRNA splicing in MERS-CoV and SARS-CoV-2-infected Calu-3 cells. Again,
188 infection with MERS-CoV, but not SARS-CoV-2, significantly induced XBP1s and its downstream
189 effector DNAJB9 (Figure 3D&H). In agreement with these results, OC43, but not SARS-CoV-2,
190 infection induced XBP1s protein levels (Figure 3I&J).

191

192

193 **Upon infection, MHV, OC43, MERS-CoV induce IRE1α and related genes to a greater extent**
194 **than SARS-CoV-2 .**

195 To determine how different coronaviruses impact the UPR at the transcriptional level, we
196 performed RNA-sequencing of A549-DPP4 cells infected with MERS-CoV for 24 and 36 hours.
197 We compared the results to published RNA-seq data sets (29, 31) of MHV infection of murine
198 bone marrow derived macrophages (BMDM) or SARS-CoV-2 infection of A549-ACE2, normal
199 human bronchial epithelial (NHBE) cells, and Calu-3 cell lines. In agreement with our IRE1α
200 activation results, Ingenuity Pathway Analysis (IPA) predicted activation of the UPR and ER stress
201 pathways by MERS-CoV and MHV (Figure 4A). In contrast, SARS-CoV-2 consistently showed
202 little to no activation of the UPR and ER stress pathway across different MOI conditions and cell
203 lines.

204

205 To support the results of the gel electrophoresis splicing assays for XBP1 mRNA that
206 distinguished SARS-CoV-2 infection from that of the other betacoronaviruses (Figure 3), we
207 further utilized the RNA sequencing results to quantitatively measure XBP1 mRNA splicing by
208 these coronaviruses. Through RNA-seq, we visualized both the unspliced and spliced XBP1
209 mRNA reads based on whether they contain the 26 nucleotide non-conventional intron that is
210 removed as a result of RNase activity of IRE1α as previously described (32) (Figure 4B&C).
211 MERS-CoV infection resulted in significant XBP1 mRNA splicing, in contrast with no difference
212 detected in SARS-CoV-2 infected versus mock-infected cells (Figure 4B&C). We further
213 quantified total XBP1 spliced vs unspliced reads, which consistently showed a substantial
214 increase in the percent expression of the XBP1s reads when normalized to total XBP1 reads for
215 MERS-CoV at both 24 and 36 hours post-infection but not for SARS-CoV-2 infected cells (Figure

216 4D&E). This was consistent with significant upregulation of DNAJB9 and total XBP1 during
217 infection with MERS-CoV but not SARS-CoV-2 (Figure 4F-I).
218
219 **MERS-CoV but not SARS-CoV-2 induces XBP1 splicing during infection of biologically**
220 **relevant iPSC-derived alveolar type II cells**
221 To confirm our results in a more physiologically relevant cell, we infected iPSC-derived type II
222 alveolar (iAT2) cells. We employed the SPC2 line, which expresses tdTomato from the surfactant
223 protein-C (SFTPC) locus as an AT2 marker, which we have previously used to characterize innate
224 immune responses to SARS-CoV-2 infection (3). Type II alveolar cells are a major target during
225 both MERS-CoV and SARS-CoV-2 infection in humans, and their destruction may be a
226 contributing factor to lung pathogenesis in severe cases (33, 34).
227
228 Both MERS-CoV and SARS-CoV-2 replicate in these cells and release infectious virus as
229 quantified by plaque assay (Figure 5A). Notably, MERS-CoV replicated to higher titers than
230 SARS-CoV-2 in these lung-derived cells. This complements our previous findings that SARS-
231 CoV-2 replicates more efficiently than MERS-CoV in upper respiratory derived primary nasal cells
232 (3), and may suggest that MERS-CoV is better adapted to replicate within the lower respiratory
233 tract while SARS-CoV-2 replicates more efficiently in the upper airway. Despite this difference in
234 replication, both viruses were observed to induce p-IRE1α over the course of infection (Figure
235 5B). In agreement with our results in A549 and Calu-3 cells, SARS-CoV-2 failed to induce XBP1
236 splicing in iAT2 cells, as measured by RT-qPCR (Figure 5C). By contrast, MERS-CoV induced
237 XBP1 splicing, albeit to a lower extent than in immortalized cell lines. Lastly, we visualized XBP1
238 splicing using RT-PCR and agarose gel electrophoresis (Figure 5D). Again, our data indicate that
239 SARS-CoV-2 fails to induce XBP1 splicing at either 24 or 48hpi in iAT2 cells, despite inducing p-
240 IRE1α. MERS-CoV, however, induced increasing XBP1 splicing over the course of infection,
241 matching the results in A549 and Calu-3 cells (Figures 2 and 3). Overall, these results indicate

242 that both SARS-CoV-2 and MERS-CoV induce ER stress as evidenced by IRE1α phosphorylation
243 during infection of primary iAT2 cells, but only MERS-CoV induces the downstream effects of
244 active IRE1α RNase.
245

246 **SARS-CoV-2 inhibits XBP1 splicing**

247 We then tested whether SARS-CoV-2 actively inhibits splicing of XBP1 induced by the N-linked
248 glycosylation inhibitor tunicamycin (TM), a common agent used to chemically induce ER stress.
249 To do so, A549-ACE2 cells were either mock infected or infected with SARS-CoV-2 or OC43 for
250 24 hours and then treated with TM for 6 hours prior to analysis. Interestingly, while SARS-CoV-2
251 infection did not completely prevent XBP1 splicing induced by TM, it led to significantly lower
252 XBP1 splicing levels compared with mock infected cells (Figure 6A). In contrast, OC43 increased
253 XBP1 splicing at all tested concentrations of TM (Figure 6B). This result suggests that SARS-
254 CoV-2 actively inhibits activation IRE1α RNase.
255

256 **Betacoronaviruses do not require IRE1α for replication**

257 Given the presumed importance of IRE1α/XBP1s to expand the ER and maintain protein folding
258 during viral replication, and the interesting differences we observed between SARS-CoV-2 and
259 the other betacoronaviruses, we next explored the consequences for its inhibition on the
260 replication of each virus. To determine whether IRE1α activity is required for replication and
261 propagation of MHV, OC43, MERS-CoV or SARS-CoV-2, we utilized CRISPR/Cas9 gene editing
262 to knock out IRE1α in A549 cell lines expressing receptors for each coronavirus (Figure S3 A-F).
263 Surprisingly, we did not observe any significant differences in the capability of all tested
264 coronaviruses to replicate in cells lacking IRE1α (Figure 6C-F). These results suggest IRE1α is
265 neither essential nor inhibitory for coronavirus replication in these cells. Since SARS-CoV-2 does
266 not lead to IRE1α-mediated XBP1 splicing, we also tested replication of SARS-CoV-2 and OC43

267 does in XBP1s KO cells (Figures 6C&D and S3G). Consistently, there was no detectable effect
268 of XBP1s KO on SARS-CoV-2 or HCoV-OC43 replication in A549-ACE2. Together, these results
269 demonstrate that none of the coronaviruses tested require the activation IRE1α/XBP1 pathway
270 for optimal replication.
271
272 **Loss of IRE1α expression causes robust alterations in gene expression, including reduced**
273 **interferon signaling, following SARS-CoV-2 infection**. To gain insight into the role of IRE1α in
274 regulating betacoronaviruses, we conducted RNA sequencing analysis of wildtype or IRE1α
275 knockout A549-ACE2 cells infected with either SARS-CoV-2 or OC43, compared to mock infected
276 cells. Infections of A549-ACE2 cells were carried out at 33C to enable direct comparison of the
277 two viruses [OC43 replication is significantly more robust at 33C compared to 37C, while SARS-
278 CoV-2 replicates to a similar extent at both temperatures (Figure S4A)]. Principal component
279 analysis showed a modest change in cellular gene expression upon OC43 infection of wildtype
280 cells relative to SARS-CoV-2, which caused a robust alteration in gene expression (Figure 7A).
281 In contrast to uninfected or OC43-infected cells, loss of IRE1α significantly impacted host gene
282 expression in SARS-CoV-2-infected A549 cells (Figure 7A,B). Clustering analysis of RNA-seq
283 data revealed 6 distinct clusters altered upon loss of IRE1α related to key cellular functions,
284 including chromatin organization (Cluster 1), mRNA metabolism and processing (Cluster 2) and
285 protein translation (Cluster 3) (Figure 7B: S5A). Detailed analysis of the IRE1α-mediated UPR
286 pathway confirms activation by OC43 infection that is inhibited upon loss of IRE1α (Figure S4C-
287 E). In contrast, minimal change in this pathway was observed in SARS-CoV-2-infected cells,
288 consistent with previous results in this study. Loss of IRE1α also appears to alter other elements
289 of the UPR in SARS-CoV-2-infected cells, including some genes in the PERK and ATF6 pathways
290 (Figure S6), which may reflect compensatory effects on the UPR in an attempt to control
291 proteostasis in the absence of IREα (35-37). Strikingly, we observed significantly lower induction

292 of some interferon stimulated genes (ISGs) during SARS-CoV-2 infection of IRE1α KO cells
293 (Figure 7D, S4F, S5B). We have previously reported that SARS-CoV-2 induces type I and type
294 III IFN signaling and ISGs in multiple cell types (3). Interestingly, OC43 infection did not induce
295 notable IFN or ISG responses with or without IRE1α expression, so we were unable to make the
296 same observations with this virus (Figure 7D). To confirm these results, we performed RT-qPCR
297 on representative IFN and ISG genes that we have previously reported to be upregulated during
298 SARS-CoV-2 infection (3) . Consistent with our RNA-seq data, we observed significantly lower
299 induction of ISGs such as OAS2, MX1, and IFIT1 during SARS-CoV-2 infection of cells lacking
300 IRE1α expression at both 37 C (Figure 7E) and 33 C (Figure S4F). These data suggests that
301 IRE1α may play a role in augmenting IFN signaling, while not being necessary for ISG induction,
302 in SARS-CoV-2 infected cells. Our data taken together lead us to propose the model shown in
303 Figure 8.
304

305 **DISCUSSION**

306 Human respiratory betacoronavirusese initiate infection in the upper respiratory tract and have
307 the potential to cause life-threatening pneumonia as a result of infection and inflammation of the
308 lower respiratory tract. The host response to severe infection with CoV is associated with marked
309 dysfunction in the distal lung (alveolar) epithelium, which includes disruption of barrier function,
310 dysregulated immune responses, transcriptomic reprogramming to a transitional cell state, and
311 senescence (38, 39).
312

313 To better understand the host epithelial response to CoV, we systematically compared the
314 activation of the IRE1α/XBP1 pathway of the UPR during infection with betacoronaviruses in lung-
315 derived A549 and Calu-3 cells lines and iPSC-derived AT2 cells. We employed three human
316 viruses, each from a different betacoronavirus lineage: OC43 (embeco), SARS-CoV-2 (sarbeco)
317 and MERS-CoV (merbeco), and included the model murine coronavirus MHV, an embecovirus.

318 We found a striking difference between the host response to SARS-CoV-2 and the other three
319 viruses. OC43, MHV and MERS-CoV all activated the canonical IRE1α/XBP1 pathway in both
320 A549 and Calu-3 cell lines as evidenced by phosphorylation of IRE1α (Figure 2), XBP1 mRNA
321 splicing (Figures 3&4) and induction of DNAJB9 (Figure 3), a target of XBP1s. Additionally,
322 MERS-CoV was observed to induce IRE1α/XBP1 activation in iAT2 cells (Figure 5). In contrast,
323 while SARS-CoV-2 also promoted autophosphorylation of IRE1α, there was no evidence of
324 XBP1s, indicating that the pathway was only partially activated and suggesting that the IRE1α
325 kinase was active while the XBP1 splicing RNase activity was not. The differential splicing of
326 XBP1 mRNA during SARS-CoV-2 and MERS-CoV infection was also observed in iPSC-derived
327 AT2 cells, confirming the results in a more physiologically relevant system (Figure 5). The
328 difference among these viruses is surprising as all of them encode highly conserved replicase
329 and structural proteins that promote ER membrane rearrangements and challenge the ER folding
330 capacity, respectively (26). We had originally hypothesized that these conserved genes would
331 induce similar stress on the ER and lead to UPR activation. Instead, our data suggest that that
332 SARS-CoV-2 actively prevents XBP1 splicing (Figure 6A&B). Consistent with this idea, a
333 recombinant SARS-CoV lacking the E protein (rSARS-CoV-ΔE) was reported to induce more
334 XBP1 splicing as well as induction of UPR genes compared to parental wild type virus (40).
335
336 To investigate the importance of IRE1α for coronavirus replication, we evaluated replication of
337 each of the betacoronaviruses in IRE1α KO A549 cells compared to parental wild type cells. In
338 contrast to influenza (41), all of the betacoronaviruses examined were able to replicate efficiently
339 in the absence of IRE1α signaling, consistent with a previous report of the gammacoronvirus IBV
340 (25). This raises interesting possibilities for the role of IRE1α during coronavirus infection. As
341 previously stated, IRE1α can produce both cytoprotective (through XBP1s) and destructive
342 responses (via RIDD and JNK/p38 signaling) depending on the extent of the encountered stress.

343 It seems likely that coronavirus infection would induce extensive and prolonged ER stress, which
344 may push IRE1α beyond the initial pro-recovery responses and towards a pro-apoptotic response.
345 Indeed, our data reveal that, at least with MERS-CoV and SARS-CoV-2 infection, IRE1α
346 phosphorylation is readily detectable by 24hpi and remains steady throughout the course of
347 infection (Figure S1A&B). Additionally, unlike what has been observed with chemically induced
348 ER stress (30, 42), IRE1α phosphorylation does not appear to attenuate at any point during
349 coronavirus infection, again suggesting a hyperactive and destructive outcome. As stated above,
350 destruction of cells, in particular AT2 cells in the lung, may contribute to pathogenesis during
351 coronavirus infection. However, SARS-CoV-2 appears to limit the downstream consequences of
352 IRE1α activation, most notably XBP1 splicing via its RNase activity, and thus may be protected
353 from this destructive phenotype. MERS-CoV may induce apoptosis redundantly in the UPR, as it
354 has been reported that MERS-CoV induces and benefits from apoptosis mediated by the PERK
355 arm of the UPR (21, 43).
356
357 To further probe the impact of IRE1α signaling on host gene expression following coronavirus
358 infection, we performed RNA sequencing analysis of wildtype or IRE1α knockout A549-ACE2
359 cells infected with either SARS-CoV-2 or HCoV-OC43. IRE1α deletion significantly reduced the
360 expression of genes downstream of XBP1s during OC43 infection, as expected, with otherwise
361 only modest changes in overall gene expression. In contrast, genetic ablation of IRE1α
362 significantly impacted host gene expression in SARS-CoV-2-infected A549 cells. The two most
363 dramatic effects that appear to be specific to SARS-CoV-2 relate to chromatin organization and
364 protein folding and transport. Effects on mRNA metabolism and processing are also observed
365 for SARS-CoV-2 and, more modestly, for OC43. Finally, protein translation is down-regulated in
366 both OC43 and SARS-CoV-2-infected cells but, in the latter case, occurs primarily upon loss of
367 IRE1α. Taken together, these results suggest that IRE1α plays a key role in mediating changes
368 in host cell gene transcription and protein production caused by SARS-CoV-2.

369

370 We found here that deletion of IRE1α modestly blunted the induction of some but not all ISGs by
371 SARS-CoV-2 infection. In contrast, OC43 was not observed to induce significant levels of IFN or
372 ISG mRNAs in either WT or IRE1α KO cells. The mechanism by which loss of IRE1α activity
373 during SARS-CoV-2 infection dampens the induction of interferon signaling remains to be
374 determined. It has been reported that the UPR can precede and prime innate immune signaling
375 in flavivirus-infected cells (44). XBP1s has been found upstream of IFNα and IFNβ transcription
376 and may work through binding upstream cis-acting enhancer elements (45, 46). Moreover, XBP1s
377 can directly bind and transcriptionally activate IL-6, TNFα and other inflammatory cytokines (47).
378 It is possible that a low level of background XBP1 splicing may occur during SARS-CoV-2
379 infection, which could contribute to these responses. Independent of its RNase activity, the
380 autophosphorylated cytoplasmic domain of IRE1α can oligomerize and serve as a scaffold that
381 recruits TRAF2, JNK, ASK, Nck, and other molecules that can lead to varied signaling outputs
382 (48, 49). Therefore, the ability of SARS-CoV-2 to prevent full IRE1α activation might dampen
383 inflammatory signaling and prevent detection and elimination by the immune system in an intact
384 organism. However, it is important to note that the diminution of ISG expression in the absence
385 of IRE1α is small for most ISGs, and SARS-CoV-2 still induces IFN and IFN signaling to a greater
386 extent than OC43 in IRE1α KO cells. Thus, the significance of IRE1α dependent IFN signaling is
387 not clear and will be a subject of future investigation.

388

389

390 Overall, despite the lack of apparent virus replication defects with IRE1α deficiency, further
391 characterization of the repertoire of betacoronavirus induced IRE1α signaling is warranted,
392 including contributions to cytokine production, apoptosis, and pro-inflammatory responses. While
393 we initially investigated this pathway from the perspective of the impact on virus replication, future

394 studies should examine effects of IRE1α activation on the host, including inflammation and cell
395 death through the JNK and p38 MAPK signaling scaffolded by IRE1α (16) and/or RIDD, as a
396 consequence of prolonged IRE1α activation (11, 50). These responses could be particularly
397 important in AT2 cells, which must rely on the UPR to maintain proteostasis in the face of the
398 challenge from the biosynthesis and secretion of surfactant proteins (51). Dysregulation of these
399 responses by coronavirus infection could promote AT2 cell reprogramming, epithelial apoptosis,
400 alteration of surfactant components in alveoli, and the rampant inflammation associated with
401 severe coronavirus infection (52-54). Finally, the UPR response is complex and made up of the
402 PERK and ATF6 pathways in addition to IRE1α, and signals from all three of these pathways
403 almost certainly integrate into the final outcome of an infected cell.
404
405 We recently reported that SARS-CoV-2 and MERS-CoV also diverge in their activation and
406 antagonism of the double-stranded RNA induced host cell innate immune responses, another
407 early innate response to viruses (3). While MERS-CoV actively antagonizes type I and type III
408 interferon production and signaling, the oligoadenylate ribonuclease L (OAS/RNase L) system
409 and the protein kinase R (PKR) pathway, SARS-CoV-2 activates OAS/RNase L, PKR and
410 induces a low level of IFN and ISG expression (3, 4). Here, we observed that OC43 infection did
411 not lead to the induction of IFN or ISGs (Figure 7D), and we have shown previously that OC43
412 encoded accessory proteins NS2, antagonizes of activation of the OAS/RNase L pathway (55).
413 Activation of these pathways during MERS-CoV mutant infection significantly reduces virus
414 replication (56), while SARS-CoV-2 can tolerate the innate responses activated during infection
415 (3).
416
417 Considering the differences we have observed between betacoronaviruses with innate immune
418 responses and now IRE1α activation and signaling, it is striking that MERS-CoV and SARS-CoV-

419 2 are reciprocal in what they activate and antagonize. To optimize replication, coronaviruses must
420 likely strike a balance in the cellular responses they antagonize, tolerate, or benefit from.
421 Supporting this, our data suggest that IRE1α influences ISG induction during infection. It is
422 intriguing to consider if MERS-CoV tolerates this by antagonizing IFN and ISG induction, while
423 SARS-CoV-2 instead limits IRE1α activity. Future studies should examine the synergy between
424 innate immune responses and the UPR during coronavirus infection, and how perturbations on
425 one side may change viral replicative capacity, tropism, and spread. Understanding how signals
426 from each one of these pathways are integrated into viral replication and cell fate decisions during
427 coronavirus infection may illuminate new therapeutic strategies for combating emerging
428 betacoronaviruses.

429
430
431

432 **MATERIALS AND METHODS**

433

434 **Cell lines**

435 Human A549 cells (ATCC CCL-185) and its derivatives were cultured in RPMI 1640 (Gibco
436 catalog no. 11875) supplemented with 10% FBS, 100 U/ml of penicillin, and 100 µg/ml
437 streptomycin (Gibco catalog no. 15140). African green monkey kidney Vero cells (E6) (ATCC
438 CRL-1586) and VeroCCL81 cells (ATCC CCL-81) were cultured in Dulbecco's modified Eagle's
439 medium (DMEM; Gibco catalog no. 11965), supplemented with 10% fetal bovine serum (FBS),
440 100 U/ml of penicillin, 100 µg/ml streptomycin, 50 µg/ml gentamicin (Gibco catalog no. 15750),
441 1mM sodium pyruvate (Gibco catalog no. 11360), and 10mM HEPES (Gibco catalog no. 15630).
442 Human HEK 293T cells (ATCC) were cultured in DMEM supplemented with 10% FBS. Human
443 Calu-3 cells (ATCC HTB-55) were cultured in DMEM supplemented with 20% FBS without
444 antibiotics. Mouse L2 cells(57) were grown in DMEM supplemented with 10% FBS, 100U/mL of

445 penicillin, 100µg/mL streptomycin, 10nM HEPES, 2mM L-glutamine (Gibco catalog no.
446 25030081), and 2.5µg/mL Amphotericin B (Gibco catalog no. 15290).
447
448 A549-DPP4 (4), A549-ACE2 (3) and A549-MHVR (4) cells were generated as described
449 previously. A549-ACE2 cells, used in Figure 3I&J, Figure 4, Figure 6, and Figure S3 were a kind
450 gift of Benjamin TenOever, Mt Sinai Icahn School of Medicine. CRISPR-Cas9 knockout cell lines
451 were generated using lentiviruses. Lentivirus stocks were generated by using lentiCRISPR v2
452 (Addgene) with single guide RNA (sgRNA) targeting IRE1α sequences: Version1
453 (V1):CGGTCACTCACCCCGAGGCC, version (V2): TTCAGGAAGCGTCACTGTGC, version
454 (V3): CGGTCACTCACCCCGAGGCC; or XBP1 sequence: TCGAGCCTTCTTTCGATCTC. The
455 infected A549-ACE2 cells were polyclonally selected and maintained by culture in media
456 supplemented with 4 µg/mL puromycin for 1 week.
457
458 iPSC- (SPC2 iPSC line, clone SPC2-ST-B2, Boston University) derived alveolar epithelial type 2
459 cells (iAT2) were grown and infected as previously described (3). In brief, cells were differentiated
460 and maintained as alveolospheres embedded in 3D Matrigel in CK+DCI media, as previously
461 described (58). For generation of 2D alveolar cells for viral infection, alveolospheres were
462 dispersed into single cells, then plated on pre-coated 1/30 Matrigel plates at a cell density of
463 125,000 cells/cm2 using CK+DCI media with ROCK inhibitor for the first 48h and then the medium
464 was changed to CK+DCI media at day 3 and infected with either mock infected or infected with
465 MERS-CoV or SARS-CoV-2 at a MOI of 5.
466
467
468 **Viruses**
469 SARS-CoV-2 (USA-WA1/2020) was obtained from BEI Resources, NIAID, NIH or provided by
470 Natalia Thornburg, World Reference Center for Emerging Viruses and Arboviruses (Galveston,

471 Texas), and propagated in VeroE6-TMPRSS2 cells. The genome RNA was sequenced and found
472 to be identical to GenBank: MN985325.1. Recombinant MERS-CoV was described previously (1)
473 and propagated in VeroCCL81 cells. SARS-CoV-2 and MERS-CoV infections were performed at
474 the University of Pennsylvania or at the Howard Taylor Ricketts Laboratory (HTRL) at Argonne
475 National Laboratory (Lemont, IL), in biosafety level 3 laboratories under BSL-3 conditions, using
476 appropriate and approved personal protective equipment and protocols. OC43 was obtained from
477 ATCC (VR-1558) grown and titrated on VeroE6 cells at 33C or on A549-mRuby cells as described
478 (59). MHV-A59 (5, 60) was propagated on A549-MHVR cells or on murine 17CL-1 cells.

479

480 **Viral growth kinetics and titration**

481 SARS-CoV-2 and MERS-CoV infections and plaque assays were performed as previously
482 described (1, 5). In brief, A549 cells were seeded at $3x10^5$ cells per well in a 12-well plate for
483 infections. Calu-3 cells were seeded similarly onto rat tail collagen type I coated plates (Corning
484 #356500). Cells were washed once with PBS before infecting with virus diluted in serum free
485 media – RPMI for A549 cells or DMEM for Calu-3 cells. Virus was absorbed for 1 hour (A549
486 cells) or 2 hours (Calu-3 cells) at 37 degrees Celsius before the cells were washed 3 times with
487 PBS and the media replaced with 2% FBS RPMI (A549 cells) or 4% FBS MEM (Calu-3 cells). At
488 the indicated timepoints, 200µL of media was collected to quantify released virus by plaque assay
489 and stored at -80 degrees Celsius. Infections for MHV growth curves were performed similarly in
490 BSL-2 conditions. For OC43 infections, similar infection conditions and media were used,
491 however virus was absorbed, and the infections incubated at 33C rather than 37C.

492

493 Plaque assays were performed using VeroE6 cells for SARS-CoV-2 and OC43; VeroCCL81 cells
494 for MERS-CoV; and L2 cells for MHV. SARS-CoV-2 and MERS-CoV plaque assays were
495 performed in 12-well plates at 37C. OC43 and MHV plaque assays were performed in 6-well
496 plates at 33C and 37C, respectively. In all cases, virus was absorbed onto cells for one hour at

497 the indicated temperatures before overlay was added. For SARS-CoV-2, MERS-CoV, and OC43
498 plaque assays, a liquid overlay was used (DMEM with 2% FBS, 1x sodium pyruvate, and 0.1%
499 agarose). A solid overlay was used for MHV plaque assays (DMEM plus 2% FBS, 1x HEPES, 1x
500 glutamine, 1x Fungizone, and 0.7% agarose). Cell monolayers were fixed with 4%
501 paraformaldehyde and stained with 1% crystal violet after the following incubation times: SARS-
502 CoV-2 and MERS-CoV, 3 days; OC43, 5 days; MHV, 2 days. All plaque assays were performed
503 in biological triplicate and technical duplicate.
504

505 **Pharmacologic agents**

506 KIRA8 was purchased at >98% purity from Chemveda Life Sciences India Pvt. Ltd. For use in
507 tissue culture, KIRA8 stock solution was prepared by dissolving in DMSO. Tunicamycin (cat.
508 #T7765) and thapsigargin (cat. #T9033) were purchased at >98% purity from Sigma. For use in
509 tissue culture, tunicamycin and thapsigargin stock solutions were prepared by dissolving in
510 DMSO.
511

512 **Immunoblotting**

513 Cells were washed once with ice-cold PBS and lysates harvested at the indicated times post
514 infection with lysis buffer (1% NP-40, 2mM EDTA, 10% glycerol, 150mM NaCl, 50mM Tris HCl,
515 pH 8.0) supplemented with protease inhibitors (Roche complete mini EDTA-free protease
516 inhibitor) and phosphatase inhibitors (Roche PhosStop easy pack). After 5 minutes, lysates were
517 incubated on ice for 20 minutes, centrifuged for 20 minutes at 4°C and supernatants mixed 3:1
518 with 4x Laemmli sample buffer (Bio-rad 1610747). Samples were heated at 95°C for 5 minutes,
519 then separated on SDS-PAGE, and transferred to PVDF membranes. Blots were blocked with
520 5% nonfat milk or 5% BSA and probed with antibodies (table below) diluted in the same block
521 buffer. Primary antibodies were incubated overnight at 4°C or for 1 hour at room temperature. All
522 secondary antibody incubation steps were done for 1 hour at room temperature. Blots were

523 visualized using Thermo Scientific SuperSignal chemiluminescent substrates (Cat #: 34095 or
524 34080).
525

Primary Antibody	Antibody species	Blocking buffer	Dilution	Catalog number
Phospho-IRE1α	rabbit	5% BSA	1:1000	Abcam (EPR5253)
IRE1α (14C10)	rabbit	5% milk/TBST	1:1000	Cell Signaling Technology 3294S
XBP1	mouse	5% milk/TBST	1:1000	Biolegend 9D11A43
GAPDH (14C10)	rabbit	5% milk/TBST	1:2000	Cell Signaling Technology 2118S
SARS-CoV-2 N	rabbit	5% milk/TBST	1:2000	GTX135357 (Gentex)
MERS-CoV N	mouse	5% milk/TBST	1:2000	40068-MM10 (Sino Biological)
OC43 N	rabbit	5% milk/TBST	1:2000	40643-T62 (Sino Biological)

526
527
528 **RNA sequencing**
529 A549 cells expressing the MERS-CoV receptor DPP4 (4) were cultured in 10% FBS RPMI media.
530 At 70% cell confluence, cells were washed once with PBS before being mock infected or infected
531 with MERS-CoV (EMC/2012) at MOI = 1. Virus was absorbed for 1 hour at 37 degrees Celsius in
532 serum-free RPMI media. After one hour, virus was removed, cells washed three times with PBS,

533 and 2% FBS RPMI was added. The cells were incubated for another 24 hours or 36 hours, then
534 washed once with PBS and lysed using RLT Plus lysis buffer before genomic DNA removal and
535 total RNA extraction using the Qiagen RNeasy Plus Mini Kit (Qiagen 74134). Three independent
536 biological replicates were performed per experimental condition. RNA sample quality check,
537 library construction, and sequencing were performed by GeneWiz following standard protocols.
538 All samples were sequenced by an Illumina HiSeq sequencer to generate paired-end 150bp
539 reads. Read quality was assessed using FastQC v0.11.2 as described by Andrews, S. (2010)
540 "FastQC: A Quality Control Tool for High Throughput Sequence Data"
541 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Raw sequencing reads from each
542 sample were quality and adapter trimmed using BBDuk 38.73 as described by Bushnell, B at
543 "BBTools software package" (http://sourceforge.net/projects/bbmap). The reads were mapped to
544 the human genome (hg38 with Ensembl V98 annotation) using RNA STAR 2.7.1a(61). The
545 resulting BAM files were counted by featureCounts 1.6.4 to count the number of reads for each
546 gene(62). Differential expression between mock, 24hpi, and 36hpi experimental conditions were
547 analyzed using the raw gene counts files by DESeq2 1.22.1(63). A PCA plot of RNA-seq samples
548 and a normalized gene expression matrix were also generated by DESeq2.
549
550 For SARS-CoV-2 and OC43 infections, ACE2-A549 control or IRE1 KO cells were cultured in
551 10% FBS RPMI to 70% confluence. Cells were washed once with PBS before being mock infected
552 or infected with each virus at MOI = 1 for one hour in serum-free RPMI at 33C. Cells were then
553 washed three times with PBS before 2% FBS RPMI was added. At 48 hours post infection, cells
554 were lysed with RLT Plus lysis buffer before genomic DNA removal and total RNA extraction using
555 the Qiagen RNeasy Plus Mini Kit (Qiagen 74134). Three independent biological replicates were
556 performed per experimental condition. RNA sample quality check, library construction, and
557 sequencing were performed by the University of Chicago Genomics Facility following standard
558 protocols. All samples were sequenced in two runs by a NovaSeq 6000 sequencer to generate

559 paired-end 100bp reads. For each sample, the reads from two flow cells were combined before
560 downstream processing. Quality and adapter trimming were performed on the raw sequencing
561 reads using Trim Galore! 0.6.3 (https://github.com/FelixKrueger/TrimGalore). The reads were
562 mapped to the human genome (UCSC hg19 with GENCODE annotation) and the downstream
563 analyses performed using the same methods as above.

564

565 **Host pathway activity analysis of viruses**

566

567 RNA-seq data from GSE147507(31), GSE168797 (32), GSE144882 (29) and above were used
568 to compare effects of different viruses on host ER stress response. Specifically, Ingenuity
569 Pathway Analysis (IPA) (https://www.qiagenbioinformatics.com/products/ingenuitypathway-
570 analysis) was used to predict activities of related canonical pathways based on host gene
571 expression changes following viral infection. Activation z-scores for every virus and canonical
572 pathway combination were plotted as a heatmap using Morpheus
573 (https://software.broadinstitute.org/morpheus). IPA used the following q-value cutoffs for each
574 dataset to perform the canonical pathway cross comparison: Calu-3 SARS-CoV-2 MOI 2 24hr q
575 < 0.05, NHBE SARS-CoV-2 MOI 2 24hr q < 0.1, A549-ACE2 SARS-CoV-2 MOI 0.2 24hr q < 0.1,
576 A549-ACE2 SARS-CoV-2 MOI 2 24hr q < 0.05, A549-ACE2 SARS-CoV-2 MOI 3 24hr q < 0.01,
577 A549-ACE2 SARS-CoV-2 MOI 1 48hr 33°C q < 0.05, A549-ACE2 OC43 MOI 1 48hr 33°C q <
578 0.001, A549-DPP4 MERS-CoV MOI 1 24hr q < 0.1, A549-DPP4 MERS-CoV MOI 1 36hr q < 0.01,
579 BMDM MHV-A59 MOI 1 12hr q < 0.1 and over 1-fold up or down-regulated. These cutoffs were
580 implemented due to the limitations set by the IPA software. IPA was also used to overlay gene
581 expression data ($\log_2$ fold-change) onto the interferon signaling pathway map (Figure S5B).

582

583 **Gene expression heatmaps**

584 Expression levels for genes involved in various pathways from RNA-seq data were drawn using
585 Morpheus. For each gene, the normalized expression values of all samples were transformed by
586 subtracting the mean and dividing by the standard deviation. The transformed gene expression
587 values were used to generate the heatmap. For the clustering analysis of RNA-seq experiments
588 for OC43 and SARS-CoV-2-infected A549-ACE2 cells with or without IRE1α, the top 5,000 most
589 variable genes were selected. The normalized gene expression data were analyzed using
590 Morpheus. K-means clustering with 6 clusters was applied to the gene expression data.
591

592 **Gene set enrichment analyses**

593

594 To identify themes across the 6 clusters, functional gene set enrichment analyses for the genes
595 in each cluster were performed using Metascape (64) . The following categories were selected
596 for the enrichment analyses: GO Molecular Functions, GO Biological Processes, and KEGG
597 Pathway. Metascape analysis was performed with a minimum *P* value significance threshold of
598 0.05, a minimum overlap of 10 genes, and a minimum enrichment score of 5. Notable pathways
599 enriched by Metascape from each cluster were summarized in a heatmap using Morpheus. GSEA
600 v4.1.0 (65)was used to perform specific gene set enrichment analyses on Gene Ontology terms
601 : IRE1 mediated unfolded protein response (66, 67); response to type I interferon (68); and
602 response to interferon alpha (69) using the normalized expression data from the RNA-seq
603 experiment for OC43 and SARS-CoV-2-infected A549-ACE2 cells with or without IRE1α.
604

605 **Statistical analysis**

606

607 All statistical analyses and plotting of data were performed using GraphPad Prism software. RT-
608 qPCR data were analyzed by Student's *t*-test. Plaque assay data were analyzed by two-way

609 ANOVA with multiple comparisons correction. Displayed significance is determined by p-value
610 (P), where * = P < 0.05; ** = P < 0.01; *** = P < 0.001; **** = P < 0.0001; ns = not significant.
611
612 **Quantification of XBP1 alternative splicing using RNA-seq data**
613
614 BAM files produced by RNA STAR were analyzed in Integrative Genomics Viewer 2.9.4 to count
615 the number of XBP1 reads containing the alternative splicing (70). The total number of XBP1
616 reads were counted by featureCounts. The percentage of XBP1 alternative splicing for each
617 sample was determined by dividing the number of alternatively spliced reads by the number of
618 total XBP1 reads (spliced plus unspliced).
619
620 **Quantitative PCR (RT-qPCR)**
621
622 Cells were lysed with RLT Plus buffer and total RNA was extracted using the RNeasy Plus Mini
623 Kit (Qiagen). RNA was reverse transcribed into cDNA with a High Capacity cDNA Reverse
624 Transcriptase Kit (Applied Biosystems 4387406). cDNA samples were diluted in molecular biology
625 grade water and amplified using specific RT-qPCR primers (see Table below). RT-qPCR
626 experiments were performed on a Roche LightCycler 96 Instrument. SYBR Green Supermix was
627 from Bio-Rad. Host gene expression displayed as fold change over mock-infected samples was
628 generated by first normalizing cycle threshold (C_T) values to 18S rRNA to generate ΔC_T values
629 (ΔC_T = C_T gene of interest - C_T 18S rRNA). Next, Δ (ΔC_T) values were determined by subtracting
630 the mock infected ΔC_T values from the virus infected samples. Technical triplicates were averaged
631 and means displayed using the equation $2^{-\Delta(\Delta CT)}$.
632
633 Primer sequences list:

	Forward primer (5' to 3')	Reverse primer (5' to 3')
XBP1s	GCTGAGTCCGCAGCAGGT	CTGGGTCCAAGTTGTCCAGAAT
XBP1 total	TGAAAACAGAGTAGCAGCTCAGA	CCCAAGCGCTGTCTTAACTC
RPL13A	CTCAAGGTGTTTGACGGCATCC	TACTTCCAGCCAACCTCGTGAG
18S rRNA	TTCGATGGTAGTCGCTGTGC	CTGCTGCCTTCCTTGAATGTGGTA
SARS-CoV-2 genome (nsp12/RdRp)	GGTAACTGGTATGATTTCG	CTGGTCAAGGTTAATATAGG
MERS-CoV genome (nsp7)	GCACATCTGTGGTTCTCCTCTCT	AAGCCCAGGCCCTACTATTAGC
DNAJB9	AGTCGGAGGGTGCAGGATATT	TTGATTTGGCGCTCTGATGC

634

635 **XBP1 splicing assay by RT-qPCR**

636 RT-qPCR was used to quantify the relative expression of the spliced version of XBP1 (XBP1s) by
637 using specific pairs of primers for human alternatively spliced XBP1 and total XBP1 (primer
638 sequences are described above) as previously described (71). The relative percentage of
639 alternative splicing of XBP1 (%XBP1s) was indicated by calculating the ratio of signals between
640 XBP1s and total XBP1.

641

642 **Data Availability**

Raw and processed RNA-seq data for MERS-CoV, OC43, and SARS-CoV-2 were deposited into the Gene Expression Omnibus database (GSE193169).

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Disclosures

S.R.W. is on the Scientific Advisory Boards of Immunome, Inc and Ocugen, Inc.
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FIGURE LEGENDS

Figure 1. Coronavirus family.

Phylogenetic tree of betacoronaviruses and their lineages. Viruses examined in this study are show in red font.

Figure 2. Induction of IRE1α phosphorylation following coronavirus infection.

A549 cells expressing the indicated viral receptors were mock infected or infected. Protein was harvested at 24 or 48hpi and analyzed by immunoblotting with antibodies, as indicated. (A,C,F) Cells infected with OC43 at MOI=4 (A) or MHV at MOI=0.1 (C) or SARS-CoV-2 at MOI=3 (F) were pre-treated 2 hours prior to infection with 1μM KIRA8. (B,D,E) Cells were infected with OC43 at MOI=1 (B), MERS-CoV at MOI=5 (D), or SARS-CoV-2 at MOI=5 (E) or treated with DMSO, thapsigargin (Tg, 1μM) for 1 hour or tunicamycin (TM, 1μg/ mL) for 8 hours. (G) Calu-3 cells were mock infected, or infected with MERS-CoV, or SARS-CoV-2 (MOI=5).. Data shown are from one representative of at least two independent experiments.

859 **Figure 3. IRE1α-mediated XBP1 splicing occurs following infection with OC43 or MERS-**
860 **CoV, but not SARS-CoV-2.**

861 A549 cells were mock infected or infected (in triplicate) with OC43 at MOI=1 (A, E), MERS-CoV
862 at MOI=5 (B, F), SARS-CoV-2 at MOI=5 (C, G) or treated with Tm (1μg/ mL) for 8 hours and total
863 RNA harvested at indicated time points. (A-C) Relative %XBP1s, XBP1s, total XBP1 and DNAJB9
864 mRNA expression were quantified by RT-qPCR. C_T values were normalized to 18S rRNA and
865 expressed as fold-change over mock displayed as $2^{-\Delta(\Delta Ct)}$. Technical replicates were averaged,
866 the means for each replicate displayed, ±SD (error bars). (D) Calu-3 cells were mock infected or
867 infected with MERS-CoV or SARS-CoV-2 (MOI=5) and total RNA harvested at indicated time
868 points. Relative %XBP1s, XBP1s, total XBP1 and DNAJB9 mRNA expression were quantified by
869 RT-qPCR, calculated, and displayed as described above. Values are means ± SD (error bars).
870 Statistical significance was determined using two-tailed, paired Student's *t*-test. Displayed
871 significance (infected relative to mock) is determined by p-value (P), where * = P < 0.05; ** = P <
872 0.01; *** = P < 0.001; **** = P < 0.0001; ns = not significant. (E-H) RNA was harvested from A549
873 cells mock infected or infected with OC43 at MOI=1 (E), MERS-CoV at MOI=5 (F), SARS-CoV-2
874 at MOI=5 (G), or Calu-3 cells infected with MERS-CoV and SARS-CoV-2 at MOI=5 (H) or treated
875 with tunicamycin (Tm, 1μg/ mL) for 8 hour, or thapsigargin (Tg, 1μM) for 1 hour or DMSO. RT-
876 PCR was performed using primers crossing the XBP1 splicing site. The product was resolved on
877 an agarose gel to visualize XBP1 splicing. (I-J) Lysates from A549-ACE2 cells mock infected, or
878 Tm (500 ng/mL) for 6 hours or infected with OC43 (MOI=4) or SARS-CoV-2 (MOI=3), treated with
879 or without KIRA8 (1μM), were harvested at indicated time points as in Figure 2A,C&F and
880 immunoblotted with antibody directed against XBP1s protein. Data shown are from one
881 representative experiment from at least three independent experiments.

882

883

884 **Figure 4. Unlike other coronaviruses, SARS-CoV-2 infection does not lead to robust UPR**
885 **activation.**
886 (A) Heatmap of predicted pathway status based on Ingenuity Pathway Analysis (IPA) of activation
887 z-scores for each pathway from RNA-sequencing data from indicated cells infected with OC43
888 (MOI =1), MERS-CoV (MOI = 1), MHV (MOI = 1) and SARS-CoV-2 under specified conditions.
889 Red: pathway predicted to be activated. Blue: pathway predicted to be inhibited. White: pathway
890 predicted to be unchanged. Gray: no prediction due to lack of significance. (B&C) Quantification
891 of XBP1 splicing by analyzing RNA-Seq data from A549-DPP4 and A549-ACE2 cells mock-
892 infected or infected with MERS-CoV or SARS-CoV-2, respectively, under indicated conditions.
893 Reads representing spliced or unspliced XBP1 mRNA were identified based on the presence or
894 absence of the 26 nucleotides intron and quantified. (D-I) Percentage of XBP1 spliced reads, or
895 relative expression of total XBP1 and DNAJB9 mRNA from the RNA-seq samples. Values are
896 means ± SD (error bars). Statistical significance was determined by Unpaired t-tests (* = P < 0.05;
897 ** = P < 0.01; ns = not significant).

898

899 **Figure 5. SARS-CoV-2 and MERS-CoV induce IRE1α phosphorylation in iAT2 cells but**
900 **diverge in induction of XBP1 splicing.**
901 iPSC-derived AT2 cells (iAT2 cells) were mock infected or infected (in triplicate) with MERS-CoV
902 or SARS-CoV-2 at a MOI of 5. (A) At the indicated timepoints, supernatants were collected, and
903 infectious virus quantified by plaque assay. Values are means ± SD (error bars). Statistical
904 significance was determined by two-way ANOVA (* = P < 0.05; ns = not significant). (B) Total
905 protein was harvested at the indicated timepoints and analyzed by immunoblotting using the
906 indicated antibodies. Thapsigargin treatment for 1 hour (Tg; 1μM) was used as a positive control
907 for IRE1α activation while DMSO served as a vehicle control. (C) Total RNA was harvested at the
908 indicated timepoints and relative %XBP1s, XBP1s, and total XBP1 mRNA expression were

909 quantified by RT-qPCR, calculated, and displayed as described above. Values are means ± SD
910 (error bars). Statistical significance (infected compared to mock) was determined using two-tailed,
911 paired Student's *t*-test. Displayed significance is determined by p-value (P), where * = P < 0.05;
912 ** = P < 0.01; *** = P < 0.001; **** = P < 0.0001; ns = not significant. (D) RT-PCR was performed
913 using extracted RNA and primers crossing the XBP1 splicing site. The product was run out on an
914 agarose gel to visualize XBP1 splicing. Tunicamycin treatment (1μg/mL for 6 hours) was used as
915 a positive control for RT-(q)PCR, while DMSO treatment served as a vehicle control. Data shown
916 are from one representative experiment from at least two independent experiments.

917

918

919 **Figure 6. SARS-CoV-2 inhibits IRE1α-mediated XBP1 splicing under ER stress and does**
920 **not require IRE1α for replication.**

921 (A&B) A549-ACE2 cells were mock infected or infected (in triplicate) with SARS-CoV-2 (MOI=3)
922 (A) or OC43 (MOI=1) (B) for 24 hours prior to treatment with low doses of tunicamycin (100-175
923 ng/mL) for 6 hours. Total RNA was harvested and used to quantify the relative %XBP1s and
924 XBP1s expression by RT-qPCR. C_T values were normalized to 18S rRNA and expressed as fold-
925 change over mock displayed as $2^{-\Delta(\Delta Ct)}$. Technical replicates were averaged, the means for each
926 replicate are displayed as ±SD (error bars). Statistical significance (infected compared to mock)
927 was determined by one-tailed, paired t-tests (* = P < 0.05; ** = P < 0.01; *** = P < 0.001; ns = not
928 significant). (C-F) Infection of CRISPR/Cas9-edited IRE1α KO A549 cells with different
929 coronaviruses. Experiments were performed using sgControl or IRE1α KO or XBP1s KO (where
930 indicated) A549 cells stably expressing viral receptors: A549-ACE2 (OC43 or SARS-CoV-2),
931 A549-DDP4 (MERS-CoV) and A549-MHVR (MHV). Cells were infected (in triplicate) with SARS-
932 CoV-2, MERS-CoV, OC43, or MHV at a MOI of 1. At the indicated times, supernatants were
933 collected and infectious virus quantified by plaque assay. Values are means ± SD (error bars).

934 Statistical significance was determined by two-way ANOVA (* = P < 0.05; ** = P < 0.01; ns = not
935 significant). Data shown are from one representative of at least two independent experiments.
936
937 **Figure 7. IRE1α promotes the induction of interferon stimulated genes upon SARS-CoV-2**
938 **infection.**
939 (A-E) A549-ACE2 CRISPR/Cas9-edited IRE1α KO or control cells were mock infected or infected
940 (in triplicate) with SARS-CoV-2 or OC43 (MOI=1) for 48 hours. All infections were performed in
941 the same culture conditions at 33C. Total RNA was harvested and RNA sequencing was
942 performed as described in Materials and Methods. (A) Principal component analysis (PCA) of
943 RNA-seq data from samples in triplicate. The first and second principal components (PC1 and
944 PC2) of each sample are plotted. (B) Heatmap of normalized expression levels of the 5000 most
945 variable genes across all samples were plotted and K-means clustering was used to divided
946 genes into six clusters based on expression patterns among different treatment conditions. (C-D)
947 Heatmap of normalized expression levels from RNA-seq of ER stress IRE1α mediated genes (C)
948 or interferon stimulated genes (D) for all treatment conditions. (E) Total RNA was used to quantify
949 and validate expression of ISGs by RT-qPCR. C_T values were normalized to 18S rRNA and
950 expressed as fold-change over mock displayed as $2^{-\Delta(\Delta Ct)}$. Technical replicates were averaged,
951 the means for each replicate are displayed as ±SD (error bars). Statistical significance (infected
952 compared to mock) was determined by Ordinary one-way ANOVA (* = P < 0.05; ** = P < 0.01; ***
953 = P < 0.001; **** = P < 0.0001 ns = not significant).
954
955 **Figure 8**. **Model of betacoronavirus activation of the IRE1α/XBP1 pathway and downstream**
956 **effects on interferon signaling**. MHV, OC43 and MERS-CoV infection induces ER stress that
957 leads to IRE1α autophosphorylation and downstream IRE1α RNase mediated XBP1 splicing
958 producing XBP1s. In contrast, SARS-CoV-2 infection only partially activates IRE1α through

959 autophosphorylation but prevents the activation of the RNase activity. XBP1s maintains a low
960 basal level upon SARS-CoV-2 infection. MERS, OC43 and MHV efficiently antagonize dsRNA
961 induction of IFN signaling. In contrast, SARS-CoV-2 allows dsRNA induction of some IFN
962 signaling and basal XBP1s potentiates the induction of IFN signaling upon SARS-CoV-2 infection.
963

964 **Supplemental Figure 1. Kinetics of activation of IRE1α phosphorylation during infection**
965 **with MERS-CoV or SARS-CoV-2.**
966 (A-B) A549 cells expressing the indicated viral receptors were mock infected or infected with
967 MERS-CoV (A) or SARS-CoV-2 (B) at a MOI of 5. At the indicated timepoints, total protein was
968 harvested and analyzed by immunoblotting with indicated antibodies. Cells treated with
969 thapsigargin (Tg, 1μM) for 1 hour or tunicamycin (TM, 1μg/ mL) for 8 hours or were used as a
970 positive control for IRE1α phosphorylation and attenuation, respectively. Data shown are from
971 one representative experiment from at least two independent experiments.
972
973

974 **Supplemental Figure 2. XBP1 is spliced in MHV infected cells**
975 (A) Schematic of method and primer design used to quantify %XBP1. (B) A549-MHVR cells were
976 mock infected or infected with MHV (MOI=0.1). Total RNA was harvested at 48 hours post
977 infection. Relative %XBP1s, XBP1s, total XBP1 and DNAJB9 mRNA expression were quantified
978 by RT-qPCR. C_T values were normalized to 18S rRNA and expressed as fold-change over mock
979 displayed as $2^{-\Delta(\Delta Ct)}$. Technical replicates were averaged, the mean for each biological replicate
980 (n=2) is displayed, ±SD (error bars).
981

982 **Supplemental Figure 3. Validation of IRE1α and XBP1 knockout cell lines using**
983 **CRISPR/Cas9.** (A-C) A549 cells expressing the indicated viral receptors subjected to
984 CRISPR/Cas9 editing using different guide RNAs targeting IRE1α were immunoblotted for IRE1α

985 protein to assess knockout efficiency. (D) CRISPR/Cas-9 gene edited IRE1α KO A549-ACE2 cell
986 lines were treated with tunicamycin (500 ng/mL) or DMSO for 6 hours. Total RNA was harvested
987 and %XBP1 quantified by RT-qPCR. Technical replicates were averaged, the means for each
988 replicate displayed. Data shown are one representative experiment from at least three
989 independent experiments. (E) CRISPR/Cas9 gene edited IRE1α KO A549-ACE2 (guide 3) or
990 control A549-ACE2 were treated with tunicamycin (Tm, 1μg/mL) for 8 hours. Total RNA was
991 harvested, reverse transcribed, and amplied for XBP1. XBP1 cDNA product was assayed on an
992 agarose gel to visualize splicing. (F) Control or IRE1α KO A549-DDP4 cells were infected with
993 MERS-CoV (MOI=1). At the indicated time points, total RNA was collected. RT-PCR was
994 performed using primers crossing the XBP1 splicing site. The product was analyzed on an
995 agarose gel to visualize XBP1 splicing. (G) CRISPR/Cas9 gene edited control or XBP1 KO A549-
996 ACE2 were treated with DMSO or tunicamycin (Tm, 1μg/mL) for 6 hours. Lysates were then
997 immunblotted for XBP1s to confirm knockout efficiency.
998
999 **Supplemental Figure 4. IRE1α promotes the induction of interferon stimulated genes upon**
1000 **SARS-CoV-2 infection.** (A) Infection of CRISPR/Cas9-edited IRE1α KO A549-ACE2 cells with
1001 OC43 and SARS-CoV-2 (MOI=1) with same culture conditions at 33C. Experiments were
1002 performed in triplicate. At the indicated times, supernatants were collected and infectious virus
1003 quantified by plaque assay. Values are means ± SD (error bars). Statistical significance was
1004 determined by two-way ANOVA (ns = not significant). Data shown are from one representative of
1005 at least two independent experiments. (B) Quantification of XBP1 splicing by analyzing RNA-seq
1006 data (Figure 7). Reads representing spliced or unspliced XBP1 mRNA were identified based on
1007 the presence or absence of the 26-nucleotide intron and quantified. Percentage of XBP1 spliced
1008 reads were then plotted. Values are means ± SD (error bars). Statistical significance was
1009 determined by ordinary one-way ANOVA. * = P < 0.05; ** = P < 0.01; *** = P < 0.001; **** = P <

1010 0.0001 ns = not significant, adjusted after Tukey's multiple comparisons test). (C-D) Gene set
1011 enrichment analysis (GSEA) of IRE1α mediated unfolded protein response genes with normalized
1012 enrichment score (NES) and p-values compared between IRE1α KO and control cells infected
1013 with OC43 (C) or SARS-CoV-2 (D). (E) GSEA of genes that belong to GO terms response to type
1014 I interferon (left) or response to interferon alpha (right) compared between IRE1α KO and Control
1015 cells infected SARS-CoV-2. (F) Infection of IRE1α KO or control A549-ACE2 SARS-CoV-2
1016 (MOI=1) at 33 C. At the indicated times post-infection, total RNA was collected and gene
1017 expression quantified by RT-qPCR. C_T values were normalized to 18S rRNA and expressed as
1018 fold-change over mock displayed as $2^{-\Delta(\Delta Ct)}$. Technical replicates were averaged, the means for
1019 each replicate are displayed as ±SD (error bars). Statistical significance (infected compared to
1020 mock) was determined by Ordinary one-way ANOVA (* = P < 0.05).

1021

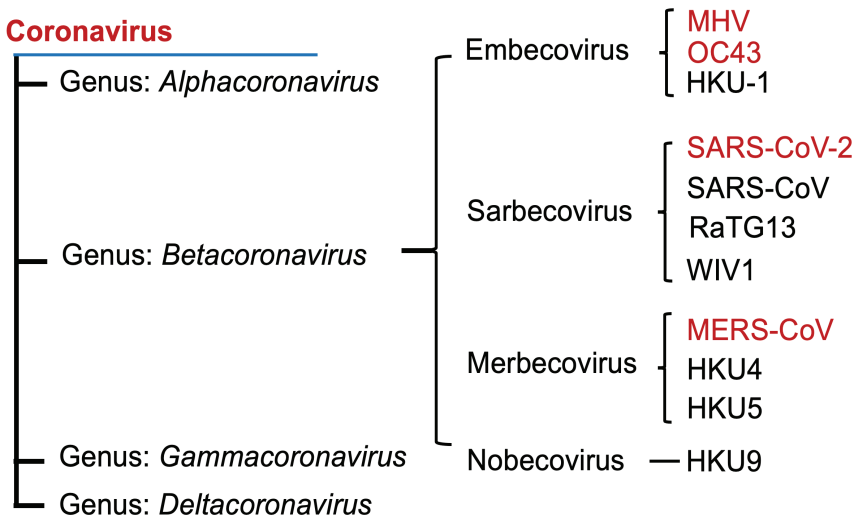
1022 **Supplemental Figure 5. Metascape analysis of SARS-CoV-2 and OC43 infections RNA-seq**
1023 **data** (A) Metascape analyses of genes from six clusters (Figure 7B). GO terms and KEGG
1024 pathways (hsa) are shown with -Log10 p-values. (B) Ingenuity-generated interferon signaling
1025 pathways analysis compared IRE1α KO over control cells upon SARS-CoV-2 infection from RNA-
1026 seq result (Figure 7). Up-regulated genes (red), down-regulated genes (green) or no significant
1027 differential expression genes (gray) are shown with color intensity corresponding to log2(fold-
1028 change) values from RNA-seq data.

1029

1030 **Supplemental Figure 6. Transcriptomic changes in the host canonical pathway of**
1031 **unfolded protein response upon SARS-CoV-2 and OC43 infection.** (A-C) Heatmap of
1032 normalized expression levels from RNA-seq (Figure 7) of genes from the canonical pathway of
1033 the UPR (A), PERK branch of UPR (B), or ATF6 branch of UPR (C).

1034

Figure 1


Coronavirus
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Genus: Betacoronavirus
Genus: Gammacoronavirus
Genus: Deltacoronavirus
Embecovirus
MHV
OC43
HKU-1
Sarbecovirus
SARS-CoV-2
SARS-CoV
RaTG13
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MERS-CoV
HKU4
HKU5
Nobecovirus
HKU9

Figure 2

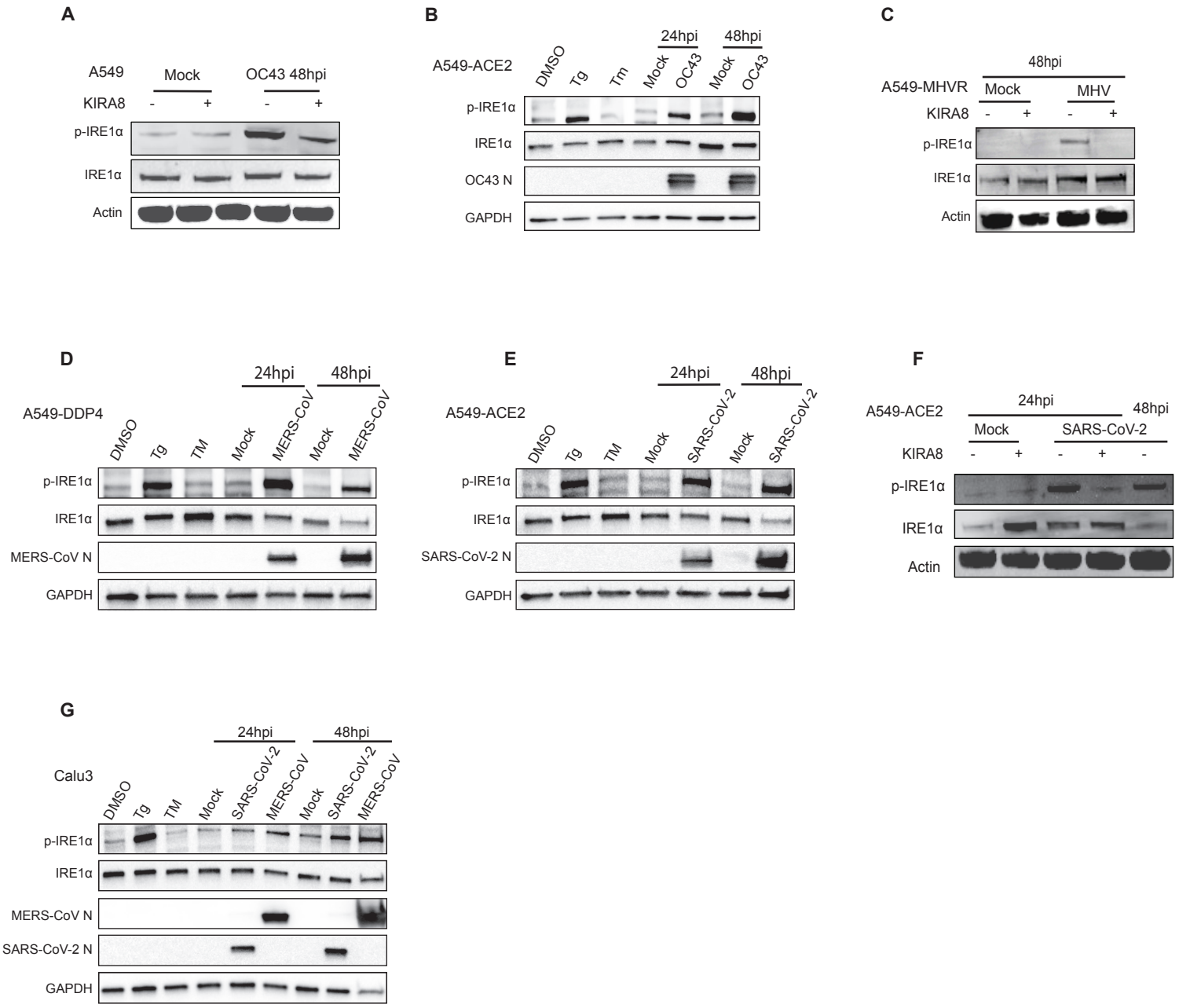
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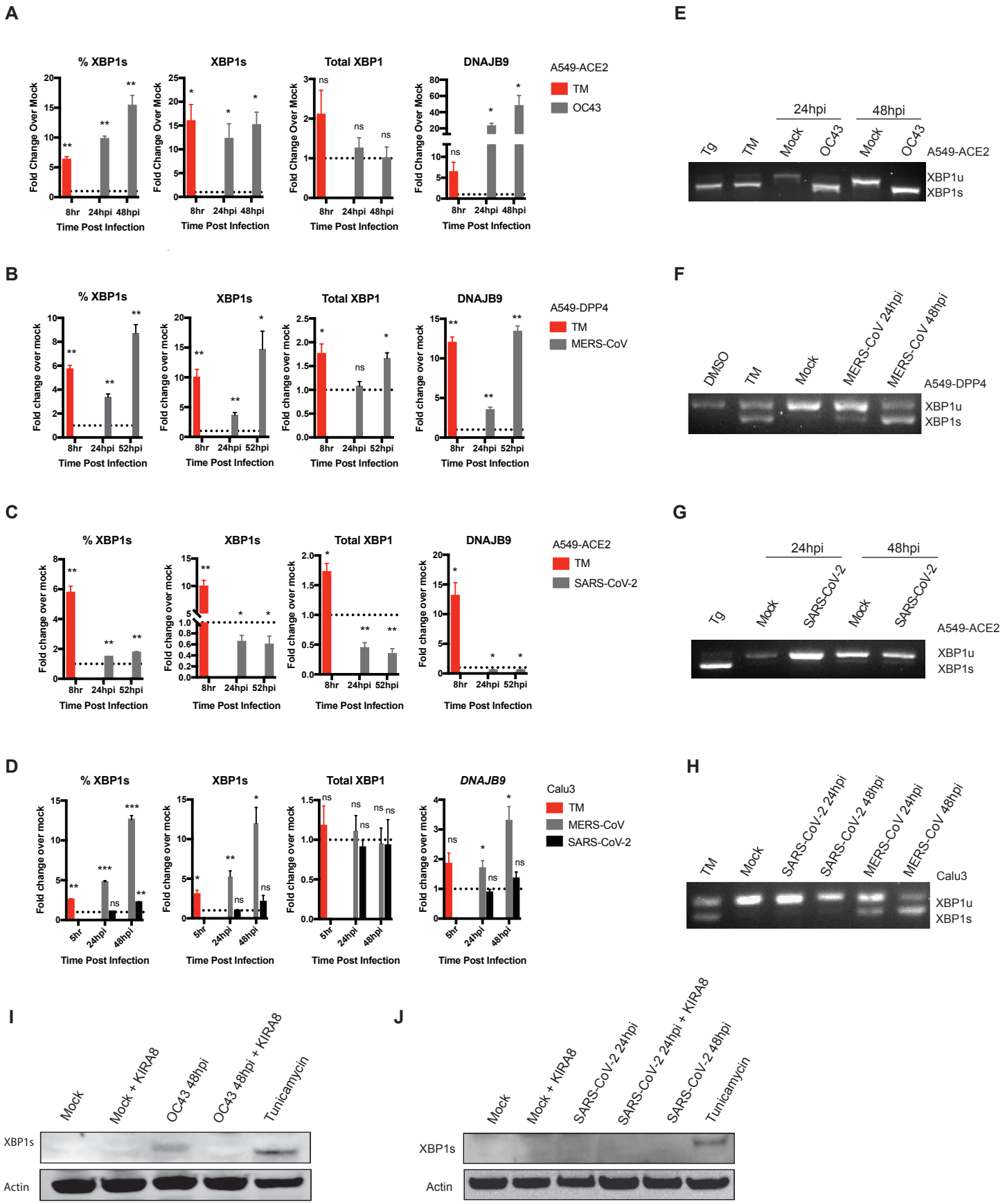
Figure 3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SARS-CoV-2
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A549-ACE2
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SARS-CoV-2 48hpi
MERS-CoV 24hpi
MERS-CoV 48hpi
Calu3
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Actin
J
Mock
Mock + KIRA8
SARS-CoV-2 24hpi
SARS-CoV-2 24hpi + KIRA8
SARS-CoV-2 48hpi
Tunicamycin
XBP1s
Actin

Figure 4

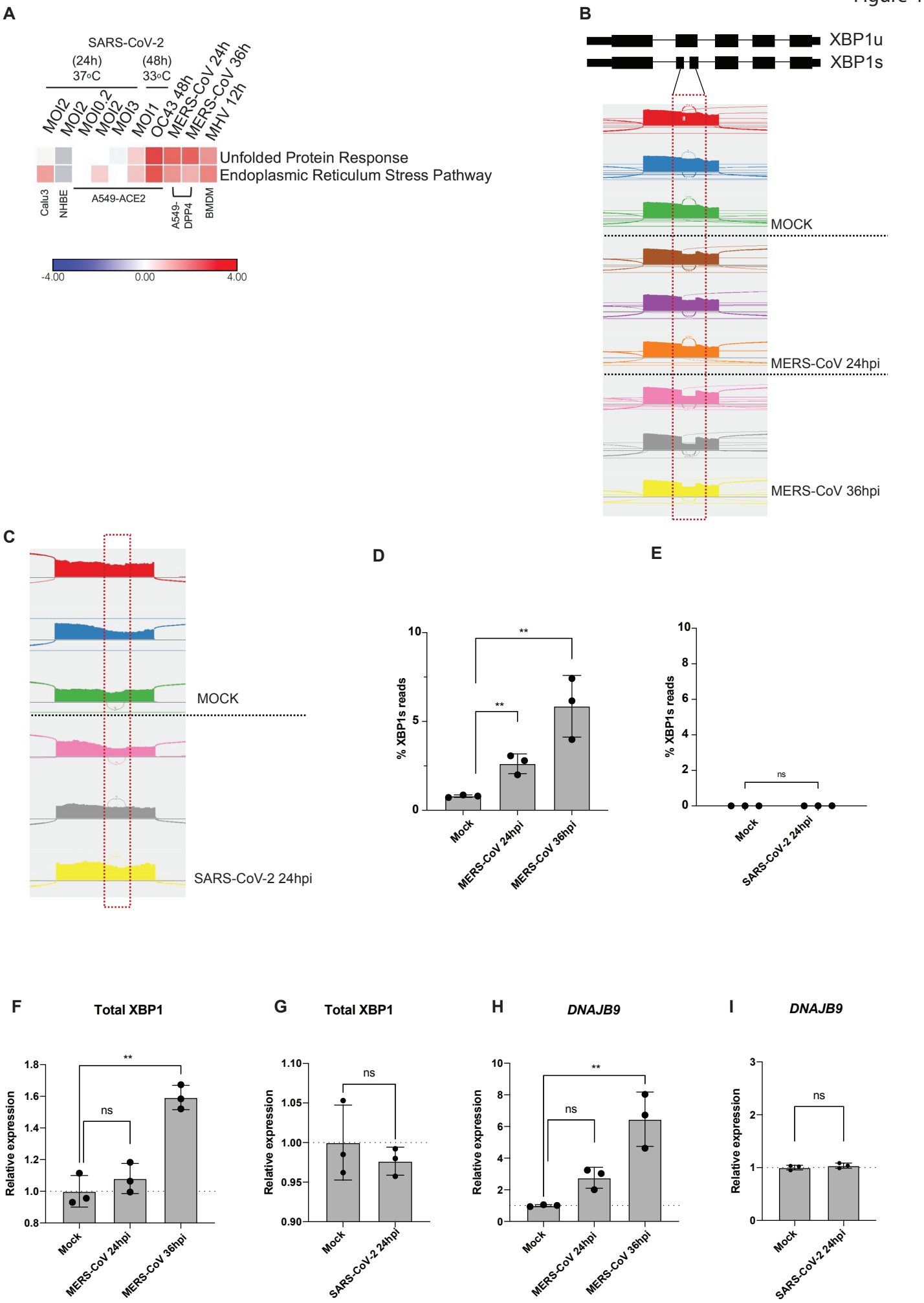
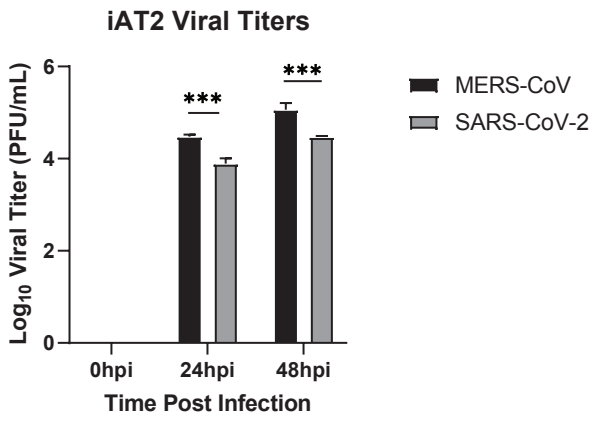
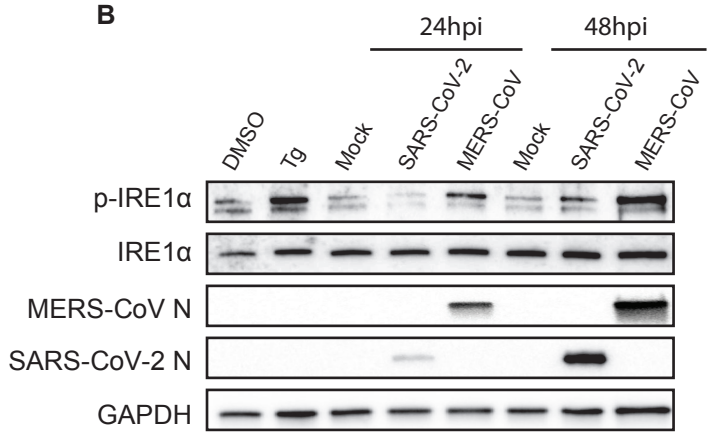

A
SARS-CoV-2
(24h)
37°C
(48h)
33°C
MOI2
MOI2
MOI0.2
MOI2
MOI3
MOI1
OC43 48h
MERS-CoV 24h
MERS-CoV 36h
MHV 12h
Unfolded Protein Response
Endoplasmic Reticulum Stress Pathway
Calu3
NHBE
A549-ACE2
A549-DPP4
BMDM
-4.00
0.00
4.00
B
XBP1u
XBP1s
MOCK
MERS-CoV 24hpi
MERS-CoV 36hpi
C
MOCK
SARS-CoV-2 24hpi
D
% XBP1s reads
Mock
MERS-CoV 24hpi
MERS-CoV 36hpi
E
% XBP1s reads
Mock
SARS-CoV-2 24hpi
ns
F
Total XBP1
Relative expression
Mock
MERS-CoV 24hpi
MERS-CoV 36hpi
G
Total XBP1
Relative expression
Mock
SARS-CoV-2 24hpi
H
DNAJB9
Relative expression
Mock
MERS-CoV 24hpi
MERS-CoV 36hpi
I
DNAJB9
Relative expression
Mock
SARS-CoV-2 24hpi

Figure 5

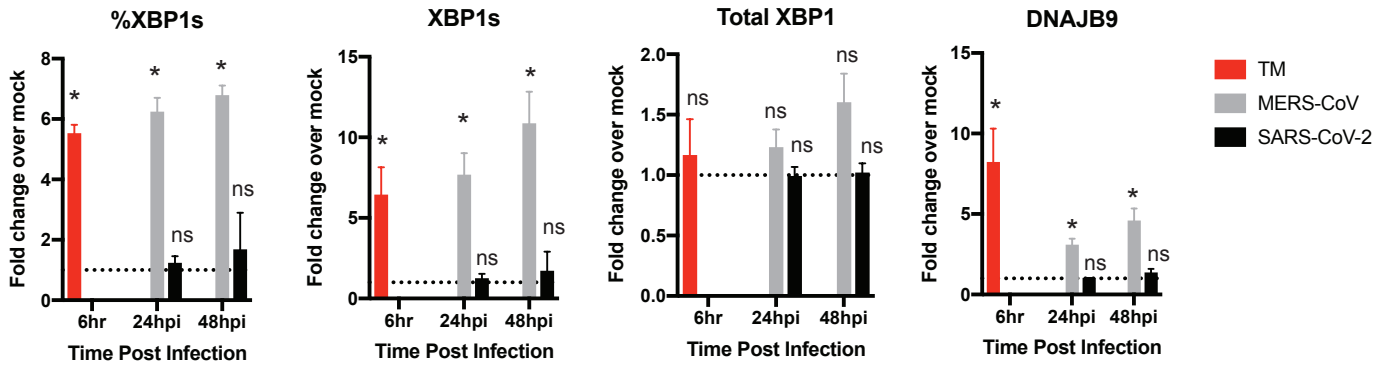
A



B



C



D

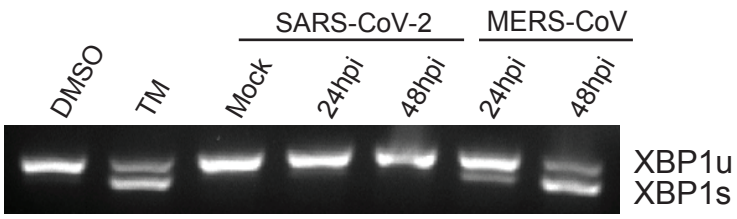
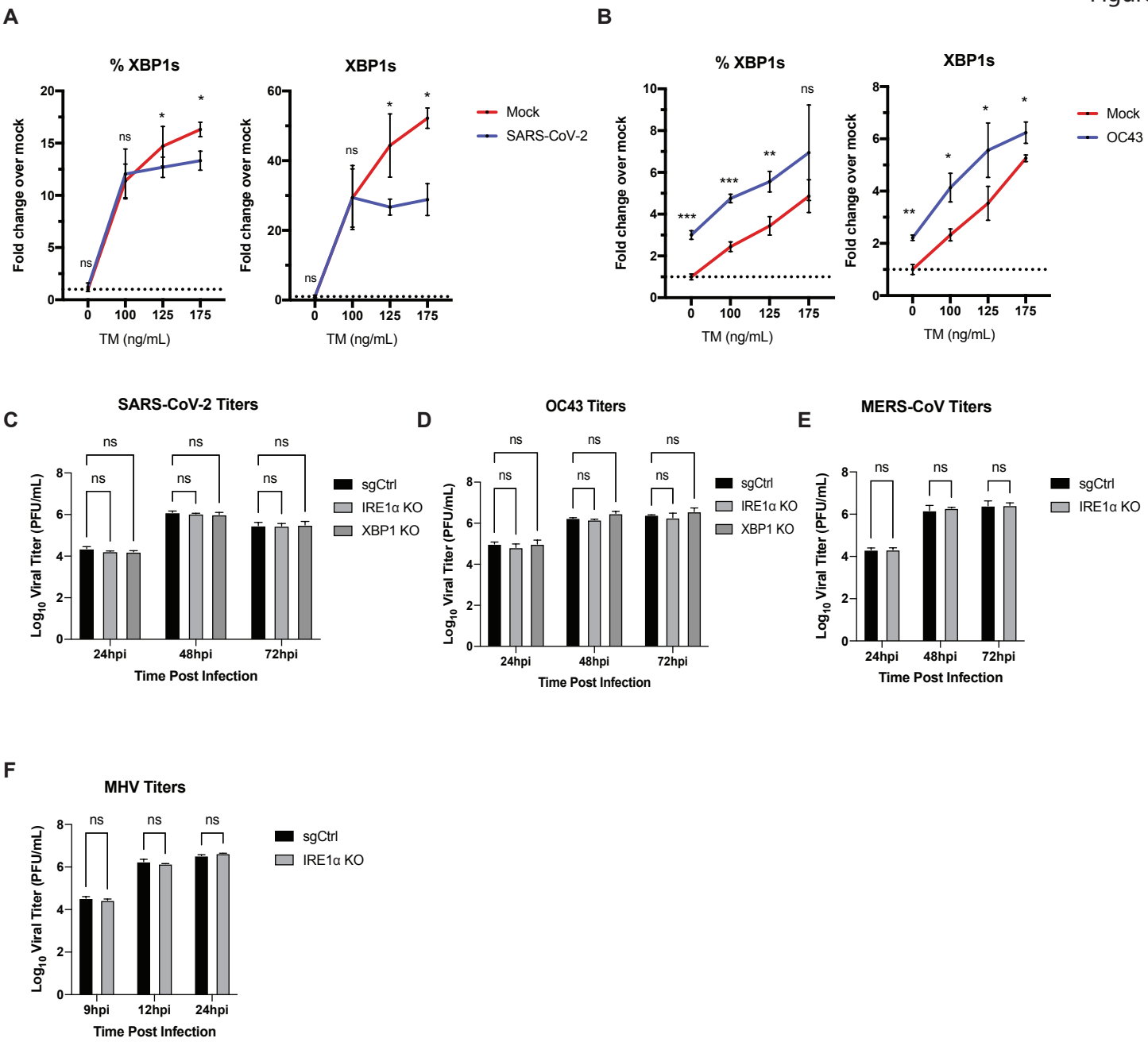
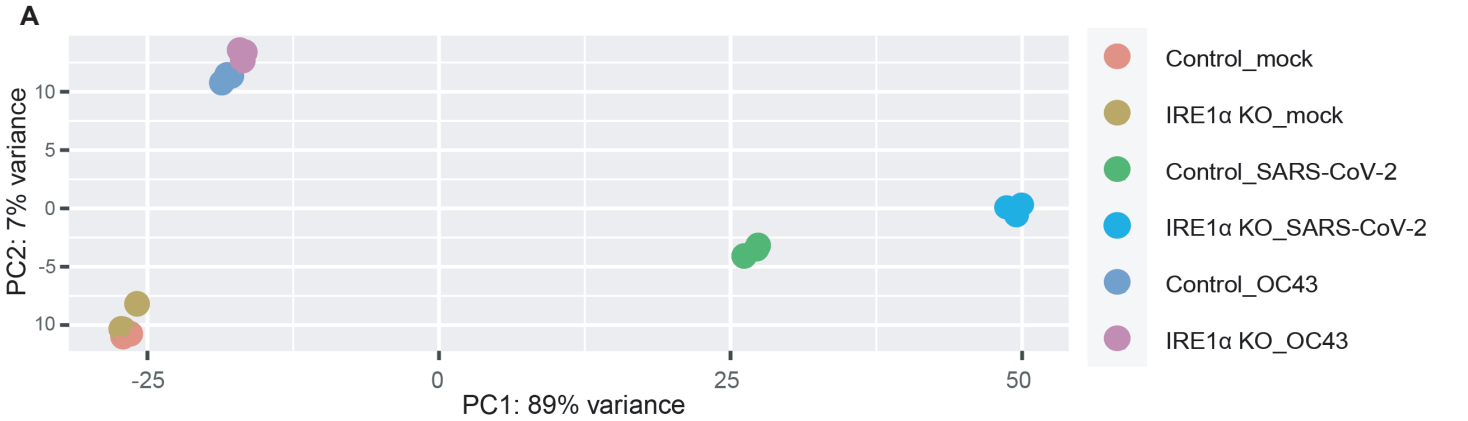


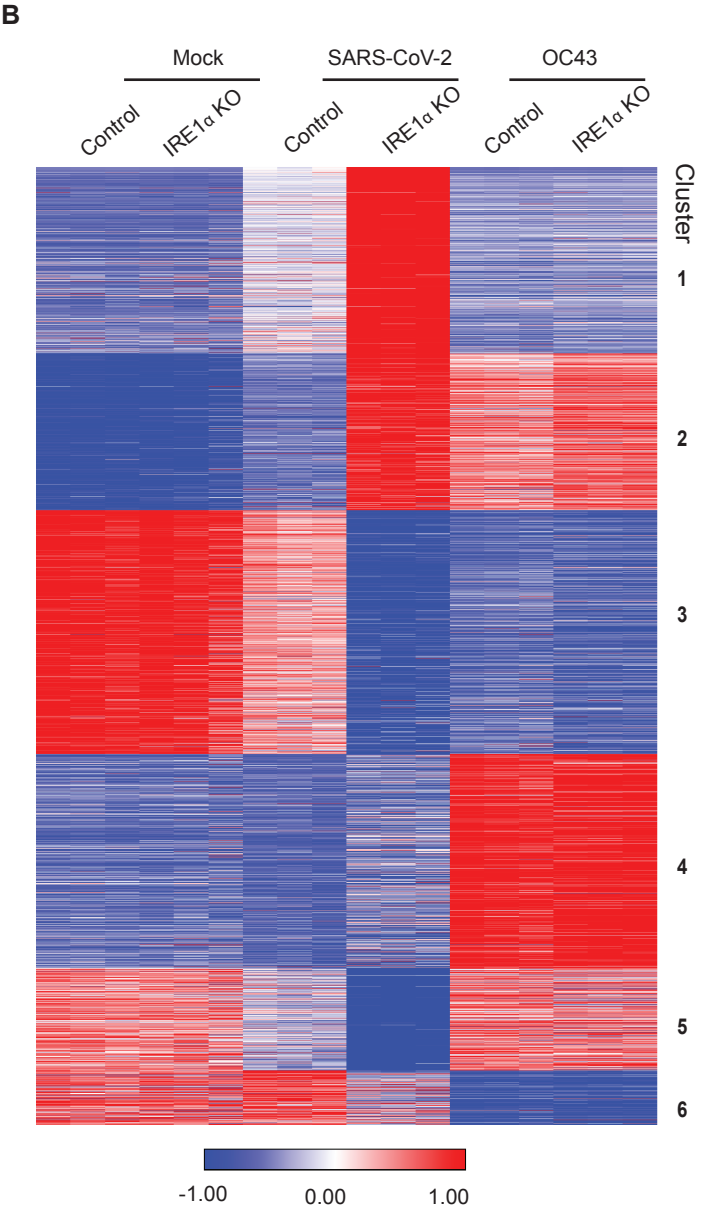
Figure 6

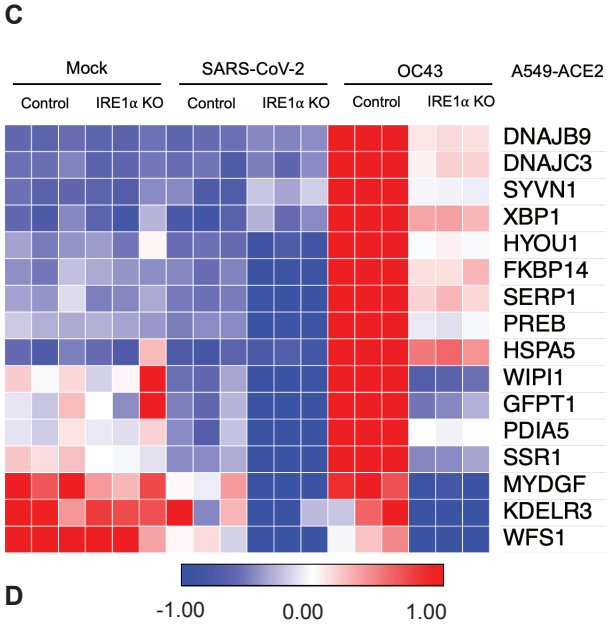

A
% XBP1s
XBP1s
Fold change over mock
TM (ng/mL)
Mock
SARS-CoV-2
B
% XBP1s
XBP1s
Fold change over mock
TM (ng/mL)
Mock
OC43
C
SARS-CoV-2 Titers
D
OC43 Titers
E
MERS-CoV Titers
Log10 Viral Titer (PFU/mL)
24hpi
48hpi
72hpi
Time Post Infection
sgCtrl
IRE1α KO
XBP1 KO
ns

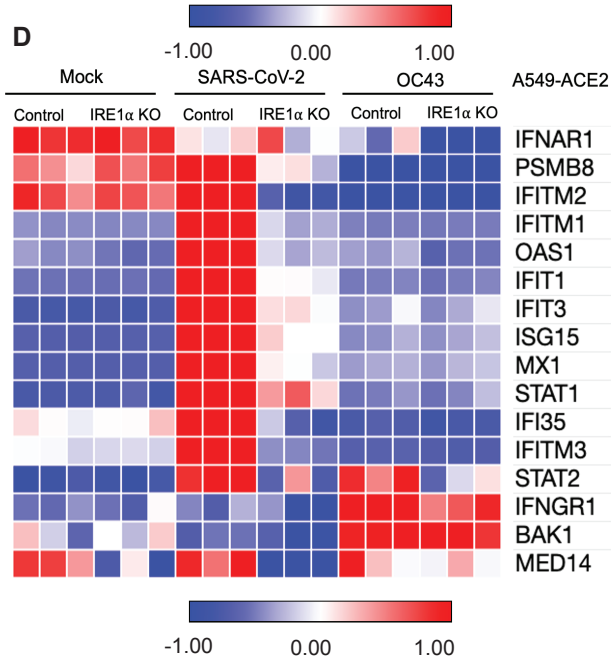
F
MHV Titers
Log10 Viral Titer (PFU/mL)
9hpi
12hpi
24hpi
Time Post Infection
sgCtrl
IRE1α KO
ns

Figure 7


A
PC2: 7% variance
PC1: 89% variance
Control_mock
IRE1α KO_mock
Control_SARS-CoV-2
IRE1α KO_SARS-CoV-2
Control_OC43
IRE1α KO_OC43


B
Mock
SARS-CoV-2
OC43
Control
IRE1α KO
Cluster
1
2
3
4
5
6
-1.00
0.00
1.00


C
Mock
SARS-CoV-2
OC43
A549-ACE2
Control
IRE1α KO
DNAJB9
DNAJC3
SYVN1
XBP1
HYOU1
FKBP14
SERP1
PREB
HSPA5
WIPI1
GFPT1
PDIA5
SSR1
MYDGF
KDELR3
WFS1
-1.00
0.00
1.00


D
Mock
SARS-CoV-2
OC43
A549-ACE2
Control
IRE1α KO
IFNAR1
PSMB8
IFITM2
IFITM1
OAS1
IFIT1
IFIT3
ISG15
MX1
STAT1
IFI35
IFITM3
STAT2
IFNGR1
BAK1
MED14
-1.00
0.00
1.00

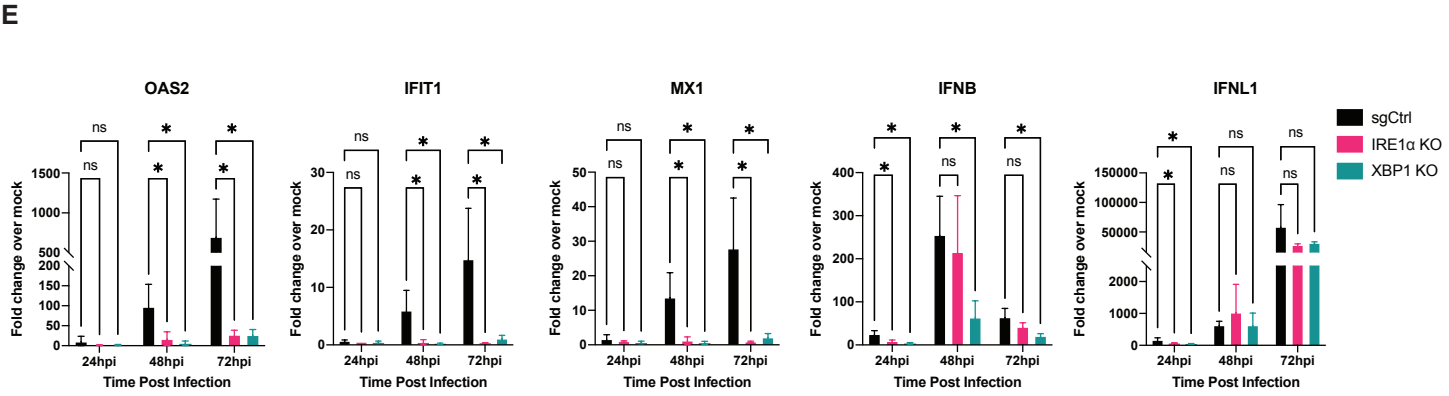
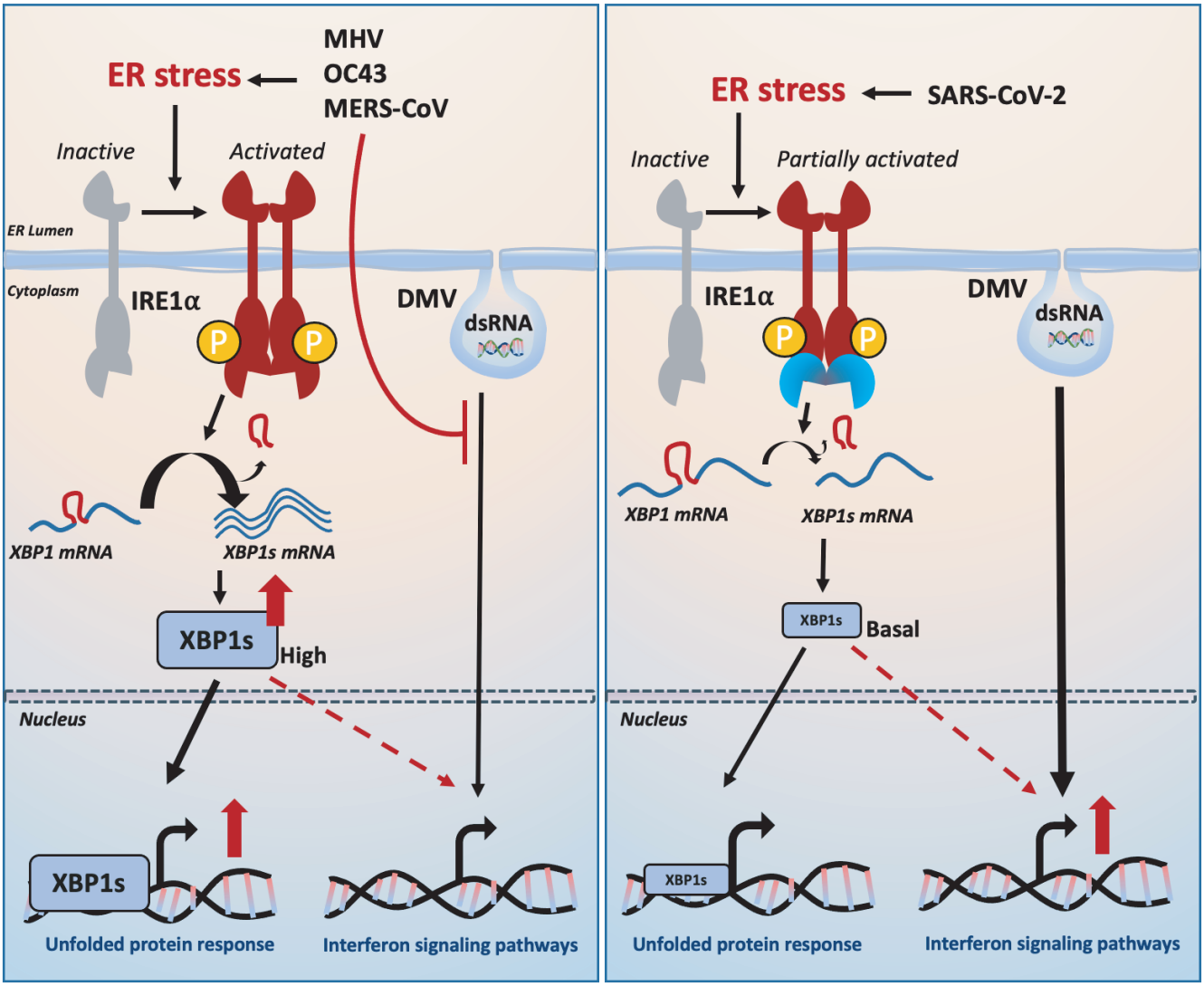
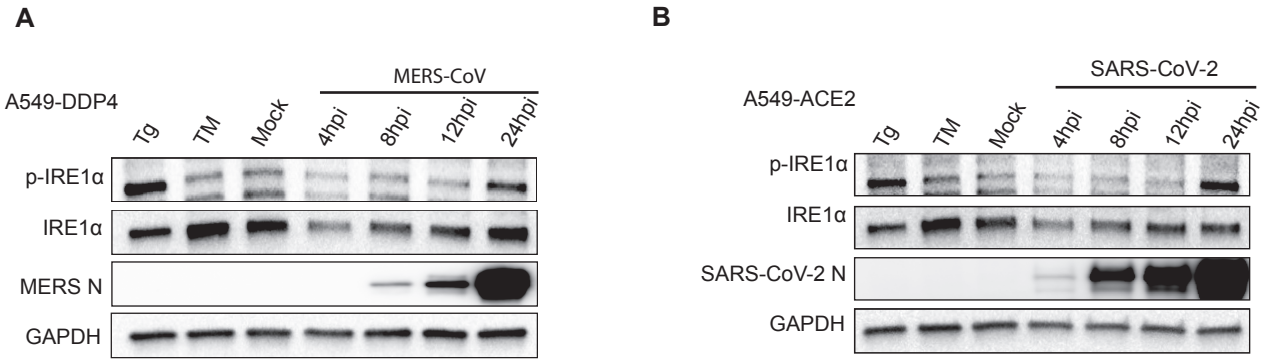

E
OAS2
IFIT1
MX1
IFNB
IFNL1
sgCtrl
IRE1α KO
XBP1 KO
Fold change over mock
Time Post Infection
24hpi
48hpi
72hpi
ns
*

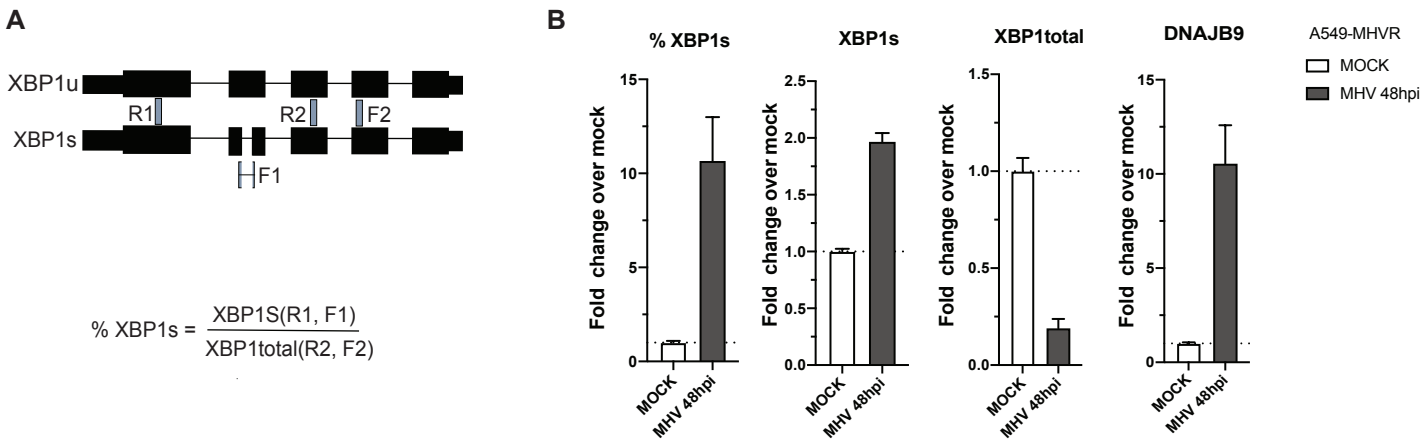
Figure 8


ER stress
MHV
OC43
MERS-CoV
Inactive
Activated
ER Lumen
Cytoplasm
IRE1α
P
P
DMV
dsRNA
XBP1 mRNA
XBP1s mRNA
XBP1s
High
Nucleus
XBP1s
Unfolded protein response
Interferon signaling pathways
ER stress
SARS-CoV-2
Inactive
Partially activated
IRE1α
P
P
DMV
dsRNA
XBP1 mRNA
XBP1s mRNA
XBP1s
Basal
Nucleus
XBP1s
Unfolded protein response
Interferon signaling pathways

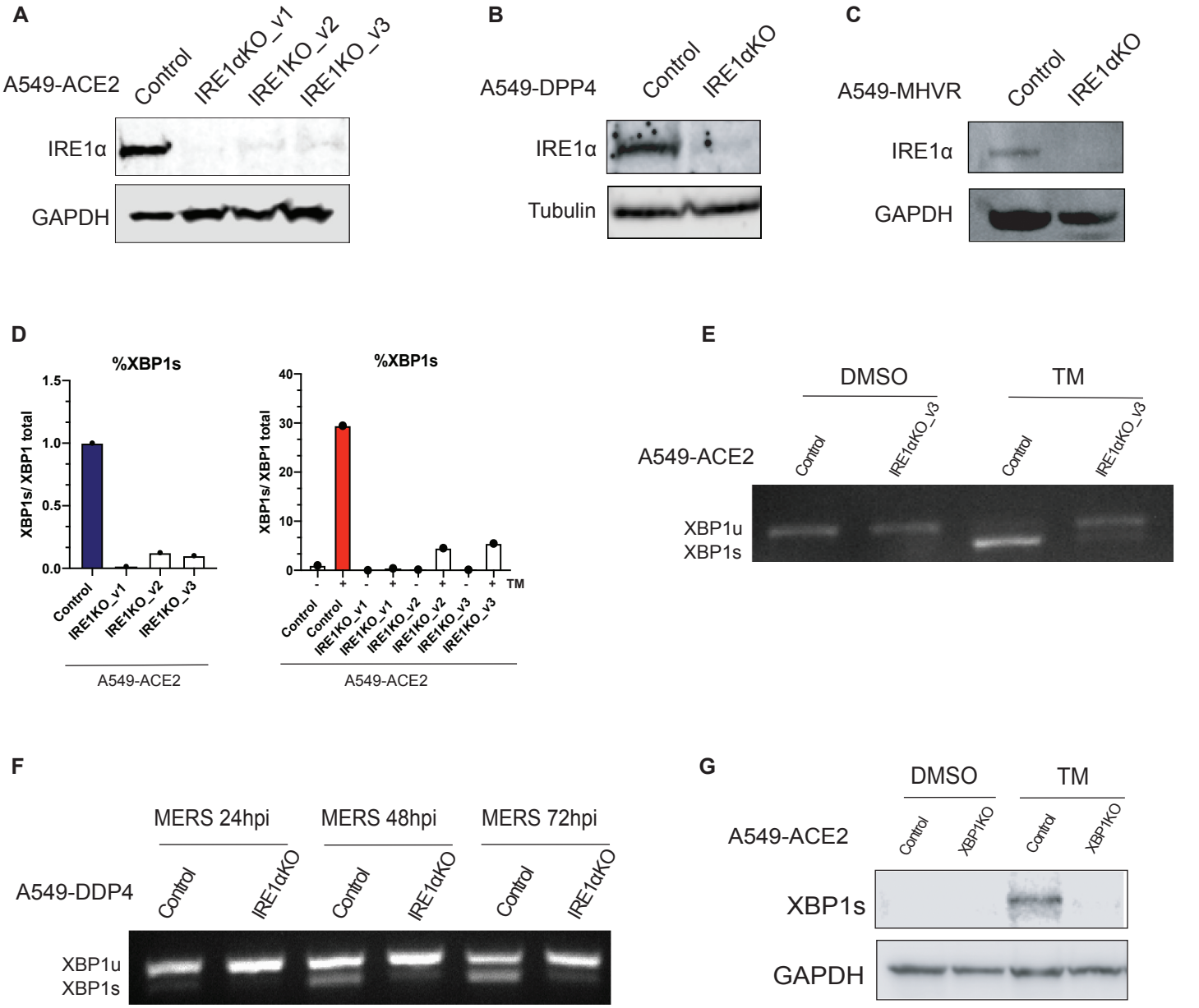
Supplement Figure 1



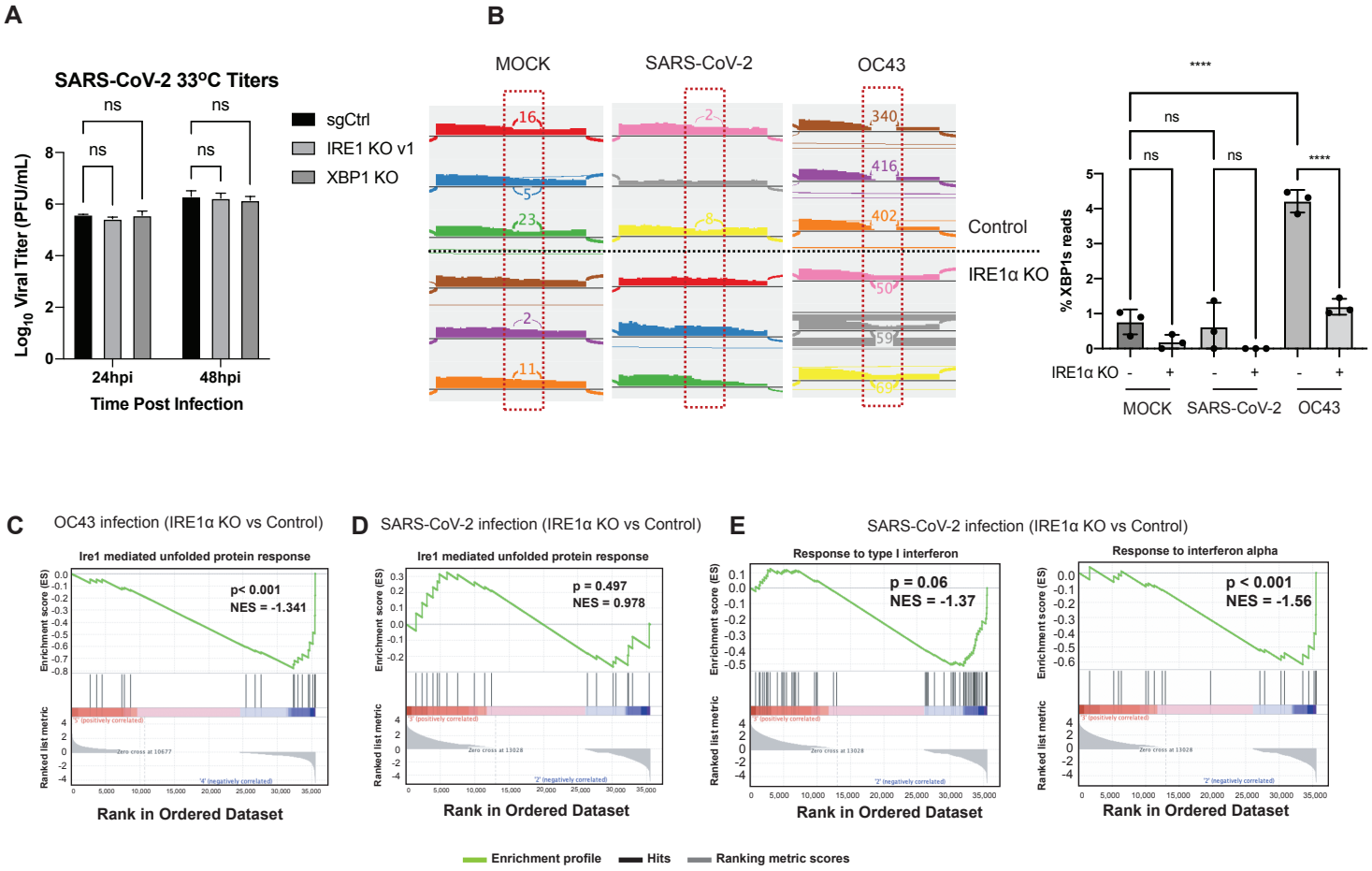
Supplement Figure 2

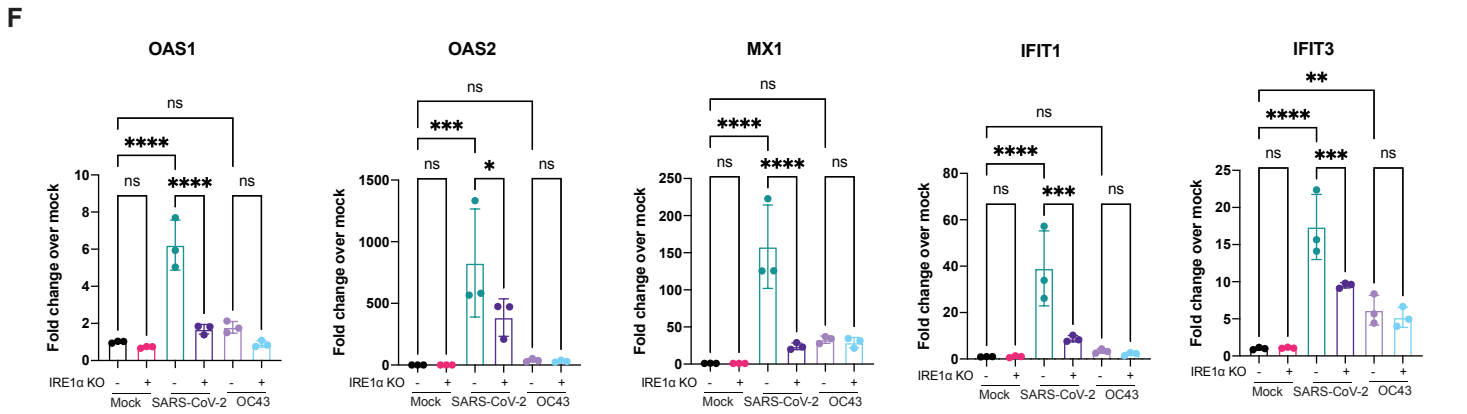


Supplement Figure 3

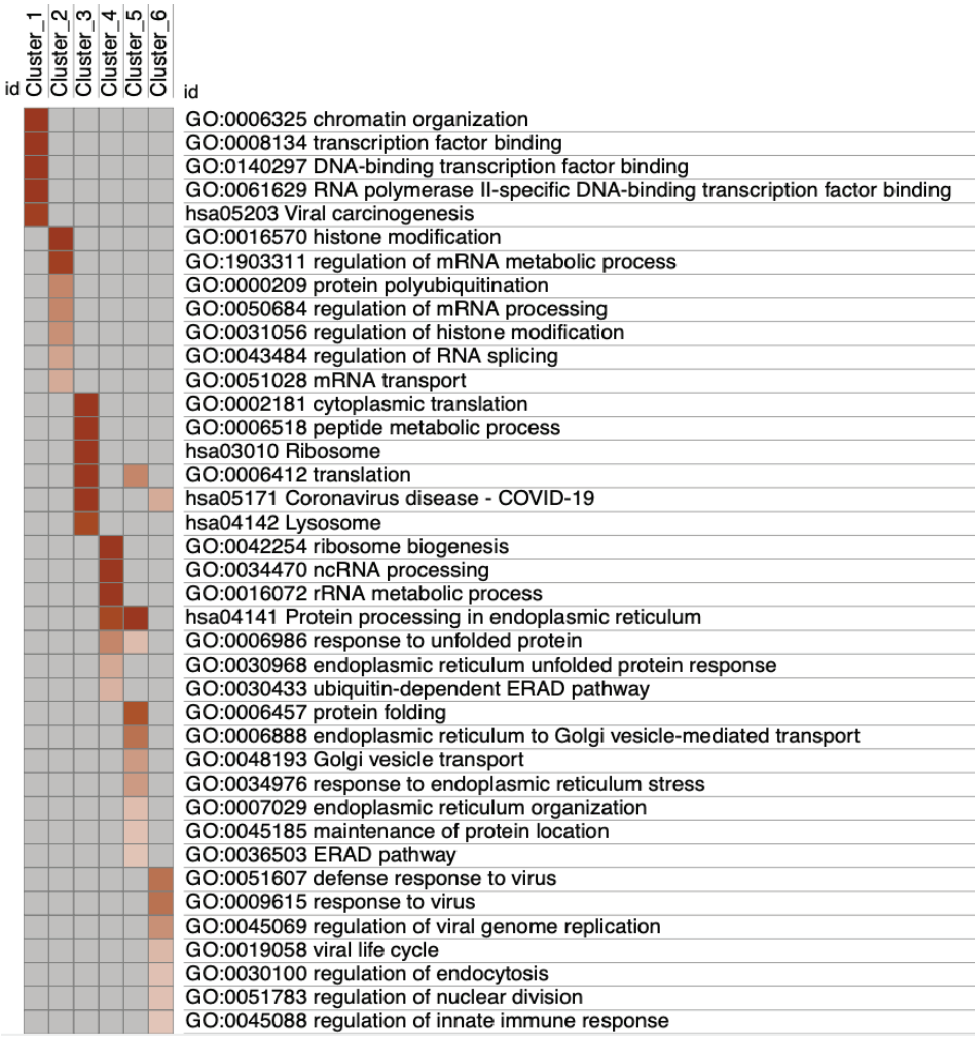


Supplement Figure 4

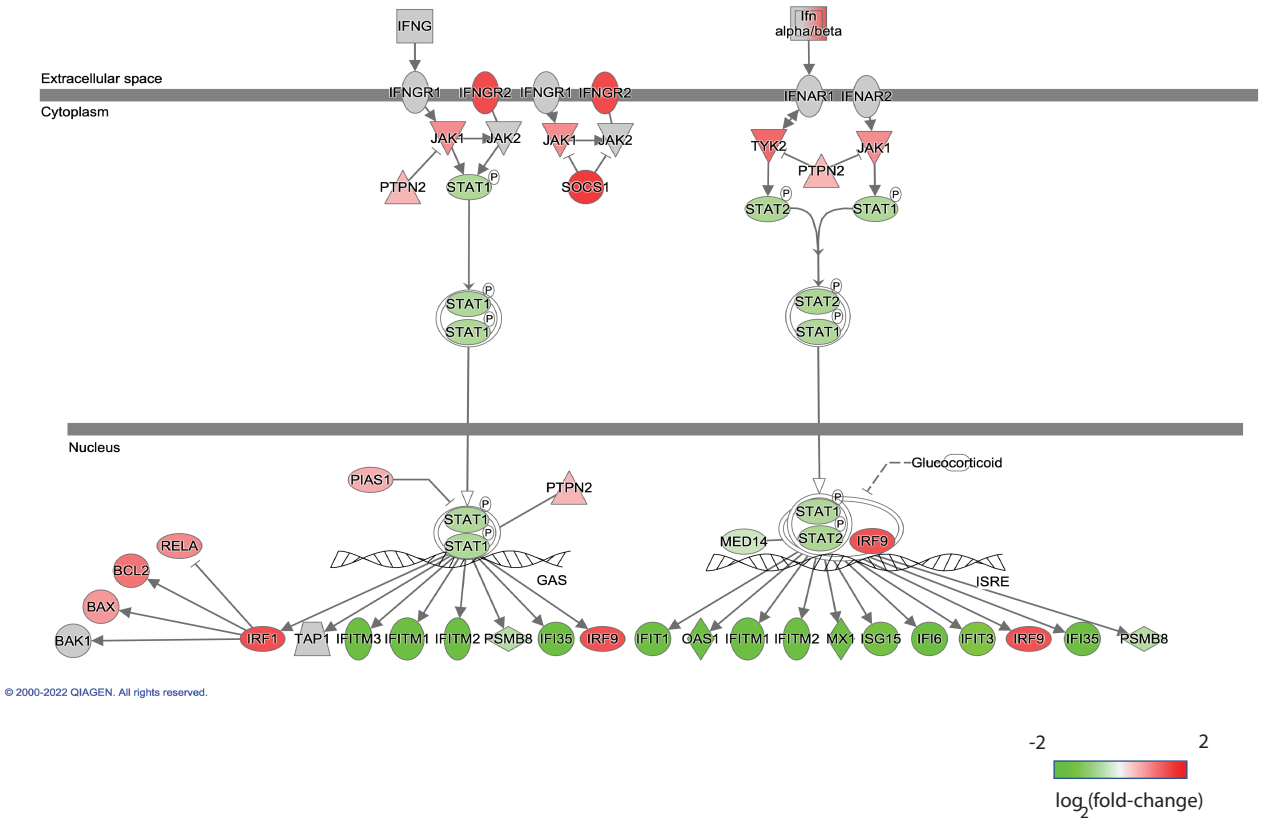




A



B



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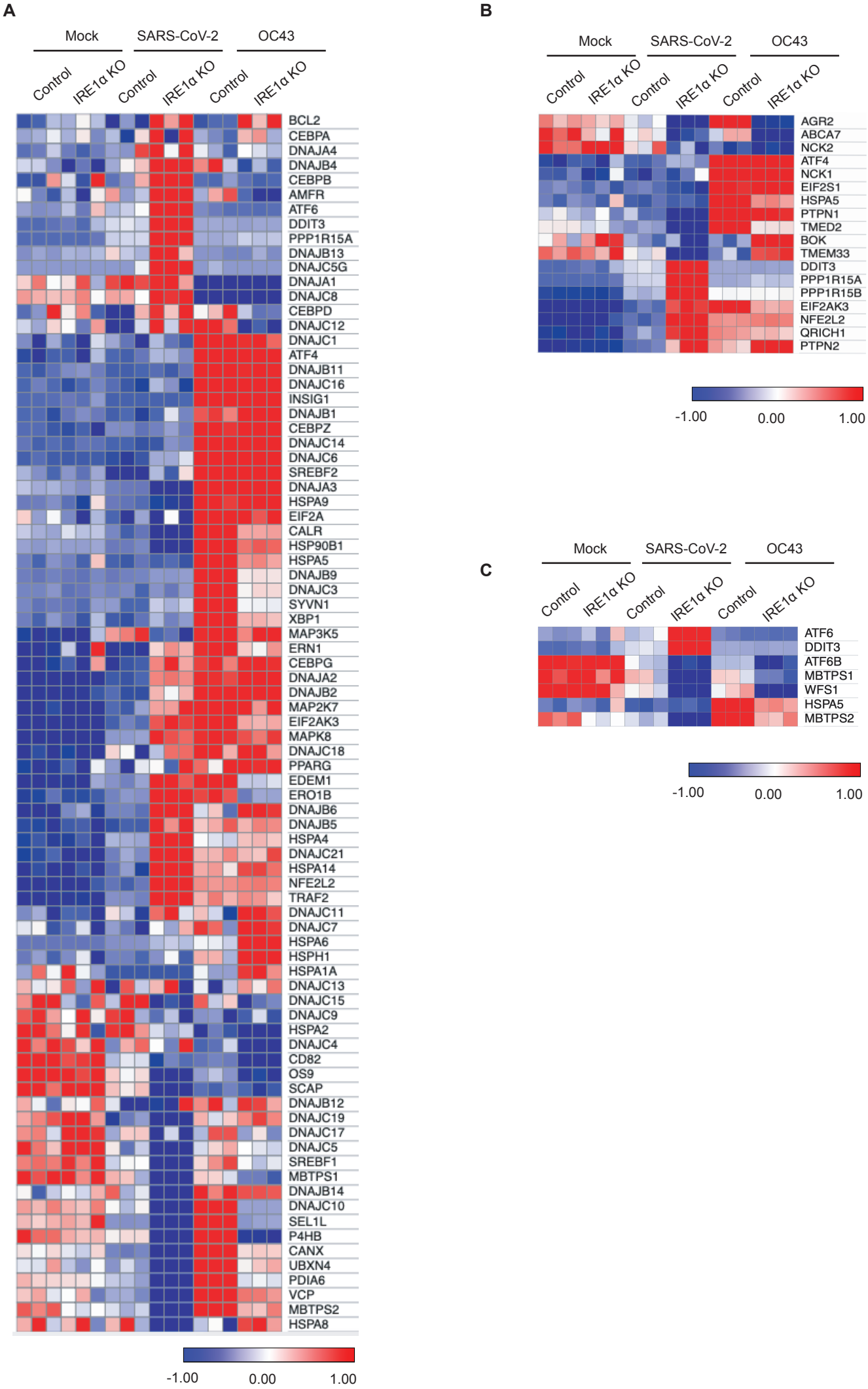
Supplement Figure 6