Identification of DAXX As A Restriction Factor Of SARS-CoV-2 Through A CRISPR/Cas9 Screen

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Abstract:

Interferon restricts SARS-CoV-2 replication in cell culture, but only a handful of Interferon Stimulated Genes with antiviral activity against SARS-CoV-2 have been identified. Here, we describe a functional CRISPR/Cas9 screen aiming at identifying SARS-CoV-2 restriction factors. We identified DAXX, a scaffold protein residing in PML nuclear bodies known to limit the replication of DNA viruses and retroviruses, as a potent inhibitor of SARS-CoV-2 and SARS-CoV replication in human cells. Basal expression of DAXX was sufficient to limit the replication of SARS-CoV-2, and DAXX over-expression further restricted infection. In contrast with most of its previously described antiviral activities, DAXX-mediated restriction of SARS-CoV-2 was independent of the SUMOylation pathway. SARS-CoV-2 infection triggered the re-localization of DAXX to cytoplasmic sites and promoted its degradation. Mechanistically, this process was mediated by the viral papain-like protease (PLpro) and the proteasome. Together, these results demonstrate that DAXX restricts SARS-CoV-2, which in turn has evolved a mechanism to counteract its action.

Introduction. Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) is the causative agent of COVID-19 and the third coronavirus to cause severe disease in humans after the emergence of SARS-CoV in 2002 and Middle East Respiratory Syndrome-related Coronavirus (MERS-CoV) in 2012. Since the beginning of the pandemic, SARS-CoV-2 has infected more than 200 million people and claimed more than 4 million lives. While the majority of infected individuals experience mild (or no) symptoms, severe forms of COVID-19 are associated with respiratory failure, shock and pneumonia. Innate immune responses play a key role in COVID-19 pathogenesis: immune exhaustion (1) and reduced levels of type-I and type-III interferons (IFN) have been observed in the plasma of severe COVID-19 patients (2,3). Imbalanced immune responses to SARS-CoV-2, with a low and delayed IFN response coupled to early and elevated levels of inflammation, have been proposed to be a major driver of COVID-19 (4,5). Neutralizing auto-antibodies against type-I IFN (6) and genetic alterations in several IFN pathway genes (7) have also been detected in critically ill COVID-19 patients. These studies highlight the crucial need to characterize the molecular mechanisms by which IFN effectors may succeed, or fail, to control SARS-CoV-2 infection.

Although SARS-CoV-2 has been described to antagonize the IFN pathway by different mechanisms involving the viral proteins ORF3b, ORF9b ORF6, and Nsp15 (8), detection of SARS-CoV-2 by the innate immune sensor MDA5 (9,10) leads to the synthesis of IFN and expression of IFN Stimulated Genes (ISGs) in human airway epithelial cells (4). IFN strongly inhibits SARS-CoV-2 replication when added in cell culture prior to infection (11,12) or when administered intranasally in hamsters (13), suggesting that some ISGs might have antiviral activity (14). Relatively few ISGs with antiviral activity against SARS-CoV-2, however, have been identified so far. For instance, spike-mediated viral entry and fusion is restricted by LY6E (15,16) and IFITMs (17,18). Mucins have also been suggested to restrict viral entry (19). ZAP, which targets CpG dinucleotides in RNA viruses, also restricts SARS-CoV-2, albeit moderately (20). OAS1 has been recently identified in an ISG overexpression screen to restrict SARS-CoV-2 replication, through the action of RNAseL, both in cell lines and in patients (21). Another overexpression screen identified 65 ISGs as potential inhibitors of SARS-CoV-2 (22), and found that BST-2/Tetherin is able to restrict viral budding, although this activity is counteracted by the viral protein ORF7a. We hypothesize that additional ISGs with antiviral activity against SARS-CoV-2 remain to be discovered. Other antiviral factors that are not induced by IFN may also inhibit SARS-CoV-2: for instance, the RNA helicase DDX42 restricts several RNA viruses, including SARS-CoV-2 (23). While several whole-genome CRISPR/Cas9 screens identified host factors required for SARS-CoV-2 replication (24–29), none focused on antiviral genes.

Here, we performed a CRISPR/Cas9 screen designed to identify restriction factors for SARS-CoV-2, assessing the ability of 1905 ISGs to modulate SARS-CoV-2 replication in human epithelial lung cells. We report that the Death domain-associated protein 6 (DAXX), a scaffold protein residing in PML nuclear bodies (30) and restricting DNA viruses (31) as well as retroviruses (32,33), is a potent inhibitor of SARS-CoV-2 replication. SARS-CoV-2 restriction by DAXX is largely independent of the action of IFN, and unlike most of its other known activities, of the SUMOylation pathway. Within hours

of infection, DAXX re-localizes to sites of viral replication in the cytoplasm, likely targeting viral transcription. We show that the SARS-CoV-2 papain-like protease (PLpro) induces the proteasomal degradation of DAXX, demonstrating that SARS-CoV-2 developed a mechanism to evade, at least partially, the restriction imposed by DAXX.

Results.

A restriction factor-focused CRISPR/Cas9 screen identifies genes potentially involved in SARS-CoV-2 inhibition. To identify restriction factors limiting SARS-CoV-2 replication, we generated a pool of A549-ACE2 cells knocked-out (KO) for 1905 potential ISGs, using the sgRNA library we previously developed to screen HIV-1 restriction factors (34). This library includes more ISGs than most published libraries, as the inclusion criteria was less stringent (fold-change in gene expression in THP1 cells, primary CD4+ T cells or PBMCs $\geq$ 2). Therefore, some genes present in the library may not be ISGs *per se* in A549 cells. Transduced cells were selected by puromycin treatment, treated with IFNα and infected with SARS-CoV-2. Infected cells were immuno-labelled with a spike (S)-specific antibody and analyzed by flow cytometry. As expected (11,12), IFNα inhibited infection by 7-fold (**Fig. S1**). Infected cells were sorted based on S expression (**Fig. 1a**), and DNA was extracted from infected and non-infected control cells. Integrated sgRNA sequences in each cell fraction were amplified by PCR and sequenced by Next Generation Sequencing (NGS). Statistical analyses using the MAGeCK package (35) led to the identification of sgRNAs significantly enriched or depleted in infected cells representing antiviral and proviral factors, respectively (**Fig. 1b**). Although our screen was not designed to explicitly study proviral factors, we did successfully identify the well-described SARS-CoV-2 co-factor cathepsin L (CTSL) (36), validating our approach. USP18, a negative regulator of the IFN signaling pathway (37), and ISG15, which favors Hepatitis C Virus replication (38), were also identified as proviral ISGs. Core IFN pathway genes such as the IFN receptor (IFNAR1), STAT1, and STAT2, were detected as antiviral factors, further validating our screening strategy. LY6E, a previously described inhibitor of SARS-CoV-2 entry (15,16), was also a significant hit. Moreover, our screen identified APOL6, IFI6, DAXX and HERC5, genes that are known to encode proteins with antiviral activity against other viruses (39–42), but had not previously been studied in the context of SARS-CoV-2 infection. For all these genes except APOL6, individual sgRNAs were consistently enriched (for antiviral factors) or depleted (for proviral factors) in the sorted population of infected cells, while non-targeting sgRNAs were not (**Fig. 1c**).

LY6E and DAXX display antiviral activity against SARS-CoV-2. To validate the ability of the identified hits to modulate SARS-CoV-2 replication in human cells, we generated pools of A549-ACE2 knocked-out (KO) cells for different genes of interest by electroporating a mix of 3 sgRNA/Cas9 ribonucleoprotein (RNP) complexes per gene target. Levels of gene editing were above 80% in all of the A549-ACE2 KO cell lines, as assessed by sequencing of the edited *loci* (**Table 1**). As controls, we used cells KO for IFNAR1, for the proviral factor CTSL or for the antiviral factor LY6E, as well as cells electroporated with non-targeting (NTC) sgRNAs/Cas9 RNPs. These different cell lines were then treated with IFNα and infected with SARS-CoV-2. Viral replication was assessed by measuring the levels of viral RNA in the supernatant of infected cells using RT-qPCR (**Fig. 2a**). In parallel, we titrated the levels of infectious viral particles released into the supernatant of infected cells (**Fig. 2b**). As expected, infection was significantly reduced in CTSL KO cells, confirming the proviral effect of this gene (36). Among the selected antiviral candidate genes, only 2 had a significant impact on SARS-CoV-2 replication: LY6E (as expected), and to an even greater degree, DAXX. Both genes restricted replication in absence of IFNα, an effect which was detectable at the level of viral RNA (8-fold and 42-fold reduction of infection, respectively, **Fig. 2a**) and of infectious virus (15-fold and 62-fold reduction, **Fig. 2b**). Based on available single-cell RNAseq datasets (43), DAXX is expected to be expressed in cell types physiologically relevant for SARS-CoV-2 infection such as lung epithelial cells and macrophages (**Fig. S2**).

In IFNα-treated cells, DAXX and LY6E KO led to a modest, but significant rescue of viral replication, which was particularly visible when measuring the levels of infectious virus by plaque

assay titration (**Fig. 2b**), while the antiviral effect of IFNα treatment was completely abrogated in IFNAR1 KO cells, as expected (**Fig. 2c**). However, IFNα still had robust antiviral effect on SARS-CoV-2 replication in both DAXX KO and LY6E KO cells (**Fig. 2c**). While DAXX and LY6E contribute to the IFN-mediated restriction, this suggests that there are likely other ISGs contributing to this effect. DAXX is sometimes referred to as an ISG, and was originally included in our ISG library, although its expression is only weakly induced by IFN in some human cell types (32,44). Consistent with this, we found little to no increase in DAXX expression in IFNα-treated A549-ACE2 cells (**Fig. S3**). In addition, we tested the antiviral effect of DAXX on several SARS-CoV-2 variants that have been suggested to be partially resistant to the antiviral effect of IFN in A549-ACE2 cells (45). Our results confirmed that Lineage B.1.1.7. (Alpha) and Lineage P1 (Gamma) SARS-CoV-2 variants were indeed less sensitive to IFN (**Fig. 2d**). DAXX, however, restricted all variants to a similar level than the original Lineage B strain of SARS-CoV-2 (**Fig. 2d**), suggesting that while some variants may have evolved towards IFN-resistance, they are still efficiently restricted by DAXX. To determine whether DAXX is specific to SARS-CoV-2 or also inhibits other RNA viruses, including coronaviruses, we infected A549-ACE2 WT and DAXX KO cells with SARS-CoV, MERS-CoV, and 2 RNA viruses belonging to unrelated viral families: Yellow Fever Virus (YFV) and Measles Virus (MeV), which are positive and negative strand RNA viruses, respectively. Our results show that DAXX restricts SARS-CoV, but has no effect on the replication of YFV, MeV or MERS-CoV (**Fig. 2e-f**). Thus, our data suggests that DAXX restriction may exhibit some level of specificity.

DAXX targets SARS-CoV-2 transcription. Next, we investigated whether DAXX targets early steps of the SARS-CoV-2 viral life cycle such as viral entry or transcription. The intracellular levels of two viral transcripts were assessed at different time post-infection in A549-ACE2 WT or DAXX KO cells (**Fig. 3**). At early time points (from 2h to 6h p.i.), the levels of viral RNA were similar in WT and DAXX KO cells, suggesting that comparable amounts of SARS-CoV-2 virions were entering cells. The levels of viral transcripts significantly increased starting at 8h p.i., representing the initiation of viral transcription. The levels of the 5' UTR viral transcript (**Fig. 3a)** were 6.4-fold higher at 8h; 4.1-fold higher at 10h; and 8-fold higher at 24h post-infection in DAXX KO cells compared to WT cells. The levels of RdRp transcripts were less affected by the absence of DAXX than 5'UTR transcripts (**Fig. 3b**) with levels of viral transcripts 1.7-fold and 3.5-fold higher in DAXX KO cells compared to WT cells at 10h and 24h pos-infection, respectively. These results suggest that DAXX acts early during the SARS-CoV-2 replication cycle, likely targeting the step of viral transcription.

DAXX restriction is SUMO-independent. DAXX is a small scaffold protein that acts by recruiting other SUMOylated proteins in nuclear bodies through its C-terminal SUMO-Interacting Motif (SIM) domain (46). The recruitment of these factors is required for the effect of DAXX on various cellular processes such as transcription and apoptosis, and on its antiviral activities (32,47–49). DAXX can also be SUMOylated itself (50), which may be important for some of its functions. To investigate the role of SUMOylation in DAXX-mediated SARS-CoV-2 restriction, we used overexpression assays to compare the antiviral activity of DAXX WT with two previously described DAXX mutants (51). First, we used a version of DAXX in which 15 lysine residues have been mutated to arginine (DAXX 15KR), which is unable to be SUMOylated; and second, a truncated version of DAXX that is missing its C-terminal SIM domain (DAXXΔSIM) (48) and is unable to interact with its SUMOylated partners. A549-ACE2 were refractory to SARS-CoV-2 infection upon transfection with any plasmid, precluding us from using this cell line. Instead, we transfected 293T-ACE2 cells, another SARS-CoV-2 permissive cell line (18).

We examined the effect of DAXX WT overexpression on the replication of SARS-CoV-2-mNeonGreen (52) by microscopy. DAXX overexpression starkly reduced the number of infected cells (**Fig. 4a-b**), revealing that DAXX-mediated restriction is not specific to A549-ACE2 cells. Using double staining for HA-tagged DAXX and SARS-CoV-2, we found that most of the DAXX-transfected cells were negative for infection, and conversely, that most of the infected cells did not express transfected DAXX (**Fig. 4c**), indicating that DAXX imposes a major block to SARS-CoV-2 infection.

159 In order to quantify the antiviral effect of overexpressed DAXX WT and mutants, we assessed the
160 number of cells positive for the S protein (among transfected cells) by flow cytometry and the
161 abundance of viral transcripts by qRT-PCR. Western blot (**Fig. S4a**) and flow cytometry (**Fig. S4b**)
162 analyses showed that DAXX WT and mutants were expressed at similar levels, with a transfection
163 efficiency of around 40 to 50% for all three constructs. DAXX WT, 15KR and ΔSIM all efficiently
164 restricted SARS-CoV-2 replication. Indeed, at 24 hours p.i., the proportion of infected cells (among
165 HA-positive cells) was reduced by 2 to 3-fold as compared to control transfected cells for all 3
166 constructs (**Fig. 4d**). This effect was less pronounced but still significant at 48 hours p.i. (**Fig. 4e**).
167 Moreover, DAXX overexpression led to a significant reduction of the levels of two different viral
168 transcripts (**Fig. S5**), in line with our earlier results showing that DAXX targets viral transcription (**Fig.**
169 **3a-b**). Together, these results show that DAXX overexpression restricts SARS-CoV-2 replication in a
170 SUMOylation-independent mechanism.
171
172 **SARS-CoV-2 infection triggers DAXX re-localization.** DAXX mostly localizes in nuclear bodies (30),
173 whereas SARS-CoV-2 replication occurs in the cytoplasm. We reasoned that DAXX localization may
174 be altered during the course of infection in order for the restriction factor to exert its antiviral effect. To
175 test this hypothesis, we infected 293T-ACE2 cells with SARS-CoV-2 and used high-resolution
176 confocal microscopy to study the localization of endogenous DAXX (**Fig. 5**). As expected (30), DAXX
177 mostly localizes in the nuclei of non-infected cells, forming discrete *foci*. At 6h post-infection, DAXX re-
178 localizes to the cytoplasm, although nuclear *foci* can still be detected. At 24h post-infection, DAXX is
179 completely depleted from nuclear bodies, and is found almost exclusively in the cytoplasm of infected
180 cells, in close association with in close association with dsRNAs, likely representing SARS-CoV-2 viral
181 dsRNAs. These results suggest that early events following SARS-CoV-2 infection trigger the re-
182 localization of DAXX from the nucleus to the cytoplasm.
183
184 **SARS-CoV-2 PLpro induces proteasomal degradation of DAXX.** Next, we asked whether this
185 relocalization of DAXX following infection destabilizes the protein. Western blot analysis revealed that
186 SARS-CoV-2 infection induces a marked decrease of total DAXX expression in infected cells (**Fig.**
187 **6a**). In contrast, SARS-CoV-2 infection had no effect on DAXX mRNA levels (**Fig. S6**). Importantly, the
188 decrease in DAXX protein levels is likely not attributed to a global host cell shut down, as the levels of
189 Lamin B, HSP90, Actin, GAPDH, Tubulin, TRIM22 and RIG-I were unchanged following infection (**Fig.**
190 **6a**). These results suggest that DAXX may be actively and specifically targeted by SARS-CoV-2 for
191 degradation during the course of infection. SARS-CoV-2 papain-like protease (PLpro) is a possible
192 candidate for this activity, as it cleaves other cellular proteins such as ISG15 (53,54), and ULK1 (55). It
193 was also shown that foot-and-mouth disease virus (FDMV) PLpro degrades DAXX (56). Thus, we
194 treated cells with different inhibitors: GRL0617, an inhibitor of SARS-CoV-2 PLpro (54); MG132, a
195 well-described proteasome inhibitor; or Masitinib, an inhibitor of SARS-CoV-2 3CL protease (57).
196 These inhibitors had minimal effects on cell viability at the selected concentrations (**Fig**. **S7**).
197 Strikingly, GRL0617 treatment partially restored DAXX expression (**Fig. 6b**), especially at the highest
198 concentration. Similarly, MG132 also prevented DAXX degradation in SARS-CoV-2 infected cells. In
199 contrast, Masitinib treatment had no effect on DAXX levels. These results suggest that PLpro, but not
200 3CL, targets DAXX for proteasomal degradation. Consistently, GRL0617 treatment also restored
201 DAXX subcellular localization to nuclear bodies (**Fig. 6c**). As expected, GRL0617 treatment also
202 inhibited the production of SARS-CoV-2 proteins such as spike (**Fig. 6b**), and may thus have an
203 indirect effect on DAXX by inhibiting SARS-CoV-2 replication itself. However, the fact that Masitinib
204 also inhibits spike production but does not restore DAXX expression suggested that DAXX
205 degradation is not an unspecific consequence of viral replication but rather a specific activity of PLpro.
206 To investigate the potential direct contribution of PLpro to DAXX degradation, we assessed the impact
207 of overexpressing individual SARS-CoV-2 proteins in 293T-ACE2 cells on DAXX levels. We included
208 in the analysis mCherry-tagged SARS-CoV-2 Non-structural proteins (Nsp) (58), which are not
209 expressed from a lentiviral vector that may be targeted by DAXX antiviral activity (33). This included
210 Nsp3 (which encodes PLro), Nsp4, Nsp6, Nsp7, Nsp10, Nsp13 and Nsp14. All proteins were
211 expressed at similar levels (**Fig. S8a**). Only the overexpression of Nsp3 led to DAXX degradation (**Fig.**

212 **6d**). This effect was dose-dependent (**Fig. 6e and Fig. S8b**), and was abrogated when cells were
213 treated with GRL0617 (**Fig. 6f**). Taken together, these results strongly indicate that PLpro directly
214 induces the proteasomal degradation of DAXX.
215
216 **Discussion.**
217
218 **Comparison with other screens.** The whole-genome CRISPR/Cas9 screens conducted to date on
219 SARS-CoV-2 infected cells mostly identified host factors necessary for viral replication (24–29) and
220 did not focus on antiviral genes, as did our screen. Three overexpression screens, however, identified
221 ISGs with antiviral activity against SARS-CoV-2 (16,22,21). In the first one, Pfaender *et al.* screened
222 386 ISGs for their antiviral activity against the endemic human coronavirus 229E, and identified LY6E
223 as a restriction factor inhibiting both 229E and SARS-CoV-2. Our screen also identified LY6E as a top
224 hit (**Fig.1**), further validating the findings of both studies. Four additional genes had significant p-
225 values in both Pfaender *et al.* and our work: IFI6, HERC5, OAS2 and SPSB1 (**Table S5-S6**). We
226 showed that knocking-out LY6E and DAXX only partially rescued SARS-CoV-2 replication in IFN-
227 treated cells (**Fig. 2)**, suggesting that other IFN effectors active against SARS-CoV-2 remain to be
228 identified. For instance, other ISGs, such as IFITMs, inhibit SARS-CoV-2 viral entry (17–19). In the
229 second screen, Martin Sancho *et al.* tested 399 ISGs against SARS-CoV-2. Among the 65 antiviral
230 ISGs identified, they focused on BST-2/Tetherin, which targets viral budding. BST-2/Tetherin was not
231 a significant hit in our screen (**Table S5-6**). This discrepancy is likely due to the fact that our screen
232 relies on the sorting of S-positive cells, and is therefore unable to detect factors restricting the late
233 stages of the viral replication cycle. The most recent overexpression screen assessed the contribution
234 of 539 human and 444 macaque ISGs in SARS-CoV-2 restriction, and further characterized the role of
235 OAS1 in sensing SARS-CoV-2 and restricting its replication through RNAseL. While we did not identify
236 OAS1 or RNAseL in our screen (**Table S5-6**), we did identify hits in common with this screen,
237 including IFI6 and OAS2 (that were also identified by Pfaender *et al.*). Of note, DAXX was absent from
238 the ISG libraries used by these overexpression screens, which explains why it was not previously
239 identified as an antiviral gene for SARS-CoV-2. Our sgRNA library, by including 1905 genes, targeted
240 a wider set of ISGs and "ISG-like" genes, including genes like DAXX that are not (or only weakly)
241 induced by IFN in some cell types (32,44). Interestingly, IFN has a stronger effect on DAXX
242 expression levels in cells from other mammals such as bats (59). Future studies may investigate
243 whether DAXX orthologs of different species are also able to restrict SARS-CoV-2 and whether DAXX
244 participates in IFN-mediated viral restriction in these species.
245
246 **DAXX is a restriction factor for SARS-CoV-2.** We identify DAXX as a potent antiviral factor
247 restricting the replication of SARS-CoV-2, acting independently of IFN and likely targeting an early
248 step of the viral life cycle such as transcription (**Fig. 3**). DAXX fulfills all of the criteria defining a *bona*
249 *fide* SARS-CoV-2 restriction factor: knocking-out endogenous DAXX leads to enhanced viral
250 replication (**Fig. 2**), while over-expression of DAXX restricts infection (**Fig. 4**). DAXX co-localizes with
251 viral dsRNAs (**Fig. 5**) and SARS-CoV-2 antagonizes DAXX to some extent, as evidenced by the
252 proteasomal degradation of DAXX induced by PLpro (**Fig. 6**). Although DAXX expression is not
253 upregulated by IFNα in A549 cells (**Fig. S3**), basal levels of expression are sufficient for its antiviral
254 activity, as has been shown for other potent restriction factors. Publicly available single-cell RNAseq
255 analyses (**Fig. S2**) indicated that DAXX is expressed in cell types targeted by the virus *in vivo*, such as
256 lung epithelial cells and macrophages. Interestingly, DAXX exhibited some degree of specificity in its
257 antiviral activity, as unrelated viruses such as YFV and MeV, as well as the closely related MERS-CoV
258 were not sensitive to its action, in contrast to SARS-CoV and SARS-CoV-2 (**Fig. 2**). Future work will
259 determine which viral determinants are responsible for this specific antiviral activity of DAXX.
260
261 **Mechanism of DAXX-mediated restriction.** DAXX is mostly known for its antiviral activity against
262 DNA viruses replicating in the nucleus, such as adenovirus 5 (AdV5) (60) and human papillomavirus
263 (HPV) (61). Most of these viruses antagonize PML and/or DAXX, which interacts with PML in nuclear
264 bodies (30). We show here that DAXX is also able to restrict SARS-CoV-2, a positive sense RNA virus

265 that replicates in the cytoplasm. Recent studies have shown that DAXX inhibits the reverse
266 transcription of HIV-1 in the cytoplasm (32,33). Within hours of infection, DAXX subcellular localization
267 was altered, with DAXX accumulating in the cytoplasm and colocalizing with incoming HIV-1 capsids
268 (33). Here, we observed a similar phenomenon, with a rapid re-localization of DAXX from the nucleus
269 to cytoplasmic viral replication sites (**Fig. 5**), where it likely exerts its antiviral effect. Early events in the
270 replication cycle of both HIV-1 and SARS-CoV-2, such as viral fusion or virus-induced stress, may
271 thus trigger DAXX re-localization to the cytoplasm. DAXX seems to inhibit SARS-CoV-2 by a distinct
272 mechanism: whereas the recruitment of interaction partners through the SIM-domain is required for
273 the effect of DAXX on HIV-1 reverse transcription (32), it was not the case in the context of SARS-
274 CoV-2 restriction. This result was unexpected, since DAXX has no enzymatic activity and rather acts
275 as a scaffold protein recruiting SUMOylated partners through its SIM domain (51). Some DAXX
276 functions, such as interaction with the chromatin remodeler ATRX (30) or its recently described role as
277 a chaperone protein (62) are, however, SIM-independent. Future work should determine which DAXX
278 domains and residues are required for its antiviral activity.
279
280 **Antagonism of DAXX by SARS-CoV-2.** Our results suggest that SARS-CoV-2 developed a
281 mechanism to antagonize DAXX restriction, with PLpro inducing its degradation to the proteasome
282 (**Fig. 6**). his antagonism, however, is only partial, since knocking-out DAXX still enhances SARS-CoV-
283 2 replication (**Fig. 2**). Another possibility is that DAXX, by acting early in the viral life cycle (i.e. as soon
284 as 8 hours p.i., **Fig. 3**) may exert its antiviral effect before PLpro is able to complete its degradation.
285 Proteins expressed by other viruses are also able to degrade DAXX: for instance, the AdV5 viral factor
286 E1B-55K targets DAXX for proteasomal degradation (60), and FDMV PLpro cleaves DAXX (56). We
287 showed in **Fig. 2** that SARS-CoV, but not MERS-CoV, is sensitive to DAXX. Thus, it will be interesting
288 to test whether PLpro from these different coronaviruses differ in their ability to degrade DAXX, and
289 whether this has an impact on their sensitivity to DAXX restriction. Future research may also establish
290 whether PLpro induces the degradation of DAXX through direct cleavage, or whether it acts in a more
291 indirect way, such as cleaving or recruiting cellular co-factors. Such investigations may be relevant for
292 the development of PLpro inhibitors (63): indeed, in addition to directly blocking SARS-CoV-2
293 replication, PLpro inhibitors may also sensitize the virus to existing antiviral mechanisms such as
294 DAXX restriction.
295
296 **Material & Methods.**
297
298 **Cells, viruses & plasmids.** HEK 293T (ATCC #CRL-11268) were cultured in MEM (Gibco #11095080)
299 complemented with 10% FBS (Gibco #A3160801) and 2 mM L-Glutamine (Gibco # 25030081). VeroE6 (ATCC
300 #CRL-1586), A549 (ATCC #CCL-185) and HEK 293T, both overexpressing the ACE2 receptor (A549-ACE2 and
301 HEK 293T-ACE2, respectively), were grown in DMEM (Gibco #31966021) supplemented with 10% FBS (Gibco
302 #A3160801), and penicillin/streptomycin (100 U/mL and 100 µg/mL, Gibco # 15140122). Blasticidin (10 µg/mL,
303 Sigma-Aldrich #SBR00022-10ML) was added for selection of A549-ACE2 and HEK 293T-ACE2. All cells were
304 maintained at 37°C in a 5% CO_2 atmosphere. Universal Type I Interferon Alpha (PBL Assay Science #11200-2)
305 was diluted in sterile-filtered PBS 1% BSA according to the activity reported by the manufacturer. The strains
306 BetaCoV/France/IDF0372/2020 (Lineage B); hCoV-19/France/IDF-IPP11324/2020 (Lineage B.1.1.7); and hCoV-
307 19/France/PDL-IPP01065/2021 (Lineage B.1.351) were supplied by the National Reference Centre for
308 Respiratory Viruses hosted by Institut Pasteur and headed by Pr. Sylvie van der Werf. The human samples from
309 which the lineage B, B.1.1.7 and B.1.351 strains were isolated were provided by Dr. X. Lescure and Pr. Y.
310 Yazdanpanah from the Bichat Hospital, Paris, France; Dr. Besson J., Bioliance Laboratory, saint-Herblain
311 France; Dr. Vincent Foissaud, HIA Percy, Clamart, France, respectively. These strains were supplied through
312 the European Virus Archive goes Global (Evag) platform, a project that has received funding from the European
313 Union's Horizon 2020 research and innovation programme under grant agreement #653316. The hCoV-
314 19/Japan/TY7-501/2021 strain (Lineage P1) was kindly provided by Jessica Vanhomwegen (Cellule
315 d'Intervention Biologique d'Urgence; Institut Pasteur). The mNeonGreen reporter SARS-CoV-2 was provided by
316 Pei-Yong Shi (52). SARS-CoV FFM-1 strain (64) was kindly provided by H.W. Doerr (Institute of Medical
317 Virology, Frankfurt University Medical School, Germany). The Middle East respiratory syndrome (MERS)
318 Coronavirus, strain IP/COV/MERS/Hu/France/FRA2 (Genbank reference KJ361503) isolated from one of the
319 French cases (65) was kindly provided by Jean-Claude Manuguerra (Cellule d'Intervention Biologique

320 d'Urgence; Institut Pasteur). SARS-CoV-2 viral stocks were generated by infecting VeroE6 cells (MOI 0.01,
321 harvesting at 3 dpi) using DMEM supplemented with 2% FBS and 1 µg/mL TPCK-trypsin (Sigma-Aldrich #1426-
322 100MG). SARS-CoV and MERS-CoV viral stocks were generated by infecting VeroE6 cells (MOI 0.0001) using
323 DMEM supplemented with 5% FCS and harvesting at 3 dpi or 6 dpi, respectively. The Yellow Fever Virus (YFV)
324 Asibi strain was provided by the Biological Resource Center of the Institut Pasteur. The Measles Schwarz strain
325 expressing GFP (MeV-GFP) was described previously (70). Both viral stocks were produced on Vero NK cells.
326 The Human Interferon-Stimulated Gene CRISPR Knockout Library was a gift from Michael Emerman and is
327 available on Addgene (Pooled Library #125753). The plentiCRISPRv.2 backbone was ordered through Addgene
328 (Plasmid #52961). pMD2.G and psPAX2 were gifts from Didier Trono (Addgene #12259; #12260). pcDNA3.1
329 was purchased from Invitrogen. Plasmids constructs expressing WT and mutant HA-tagged DAXX constructs
330 were kindly provided by Hsiu-Ming Shih (51). The plasmids encoding mCherry-tagged viral proteins were a gift
331 from Bruno Antonny and ordered through Addgene: Nsp3 -mCherry (#165131); Nsp4-mCherry (#165132); Nsp6-
332 mCherry (#165133); Nsp7-mCherry (#165134); Nsp10-mCherry (#165135); Nsp13-mCherry (#165136); Nsp14-
333 mCherry (#165137).
334
335 **Antibodies.** For Western Blot, we used mouse anti-DAXX (diluted 1:1000, Abnova #7A11), rat anti-HA clone
336 3F10 (diluted 1:3000, Sigma #2158167001), mouse anti-GAPDH clone 6C5 (diluted 1:3000, Millipore
337 #FCMAB252F), Goat anti-Lamin B clone M-20 (diluted 1:500, Santa Cruz sc-6217), mouse monoclonal
338 HSP90α/β clone F-8 (diluted 1 :500, Santa Cruz sc-13119), mouse monoclonal β-actin clone AC-15 (1:3000
339 Sigma #A1978), mouse monoclonal α-Tubulin clone DMA1 (diluted 1:1000, Sigma #T9026), rabbit anti-TRIM22
340 (diluted 1 :1000, Proteintech #13744-1-AP) and mouse Monoclonal RIG-I clone Alme-1 (diluted 1: 1000,
341 adipoGen #AG-20B-0009). To detect SARS-CoV-2 Spike protein, we used mouse anti-spike clone 1A9 (diluted
342 1:1000, GeneTex GTX632604). Secondary antibodies were goat anti-mouse and anti-rabbit HRP-conjugates
343 (diluted 1:5000, ThermoFisher #31430 and #31460) and horse anti-goat HRP (diluted 1: 1000, Vector # PI-
344 9500). For immunofluorescence, we used rabbit anti-DAXX (diluted 1:50, Proteintech #20489-1-AP) and mouse
345 anti-dsRNA J2 (diluted 1:50, Scicons #10010200). Secondary antibodies were goat anti-rabbit AF555 and anti-
346 mouse AF488 (diluted 1:1000, ThermoFisher #A-21428 and #A-28175). For flow sorting of infected cells, we
347 used the anti-S2 H2 162 antibody (diluted 1:150), a kind gift from Dr. Hugo Mouquet, (Institut Pasteur, Paris,
348 France). Secondary antibody was donkey anti-mouse AF647 (diluted 1:1000, Invitrogen #A31571). For FACS
349 analysis, we used rat anti-HA clone 3F10 (diluted 1:100, Sigma #2158167001) and mouse anti-dsRNA J2
350 (diluted 1:500, Scicons #10010200). Secondary antibodies were goat anti-rat AF647 and anti-mouse AF488
351 (diluted 1:1000, ThermoFisher #A-21247 #A-28175). The pan-flavivirus anti-Env 4G2 antibody was a kind gift
352 from Phillipe Desprès.
353
354 **Generation of CRISPR/Cas9 library cells.** HEK 293T cells were transfected with the sgRNA library plasmid
355 together with plasmids coding for Gag/Pol (R8.2) and for the VSVg envelope (pVSVg) using a ratio of 5:5:1 and
356 calcium phosphate transfection. Supernatants were harvested at 36h and 48h, concentrated 80-fold by
357 ultracentrifugation (22,000 g, 4°C for 1h) and pooled. To generate the ISG KO library cells, $36x10^6$ A549-ACE2
358 cells were seeded in 6 well plates (10^6 cells per well) 24h before transduction. For each well, 100 µL of
359 concentrated lentivector was diluted in 500 µL of serum-free DMEM, supplemented with 10 µg/mL of DEAE
360 dextran (Sigma #D9885). After 48h, transduced cells were selected by puromycin treatment for 20 days (1
361 µg/mL; Sigma #P8833).
362
363 **CRISPR/Cas9 screen.** $4x10^7$ A549-ACE2 cells were treated with IFNα (200U/mL). 16h later, cells were infected
364 at a MOI of 1 in serum-free media complemented with TPCK-trypsin and IFNα (200 U/mL). After 90 min, the viral
365 inoculum was removed, and cells were maintained in DMEM containing 5% FBS and IFNα (200 U/mL). After
366 24h, cells were harvested and fixed for 15 min in Formalin 1%. Fixed cells were washed in cold FACS buffer
367 containing PBS, 2% Bovine Serum Albumin (Sigma-Aldrich #A2153-100G), 2 mM EDTA (Invitrogen #15575-
368 038) and 0.1% Saponin (Sigma-Aldrich #S7900-100G). Cells were incubated for 30 min at 4°C under rotation
369 with primary antibody diluted in FACS buffer. Incubation with the secondary antibody was performed during 30
370 min at 4°C under rotation. Stained cells were resuspended in cold sorting buffer containing PBS, 2% FBS, 25
371 mM Hepes (Sigma-Aldrich #H0887-100ML) and 5 mM EDTA. Infected cells were sorted on a BD FACS Aria
372 Fusion. Sorted and control (non-infected, not IFN-treated) cells were centrifugated (20 min, 2,000g) and
373 resuspended in lysis buffer (NaCl 300 mM, SDS 0.1%, EDTA 10 mM, EGTA 20 mM, Tris 10 mM) supplemented
374 with 1% Proteinase K (Qiagen #19133) and 1% RNAse A/T1 (ThermoFisher #EN0551) and incubated overnight
375 at 65°C. Two consecutive phenol-chloroform (Sigma #P3803-100ML) extractions were performed and DNA was
376 recovered by ethanol precipitation. Nested PCR was performed using the Herculase II Fusion DNA Polymerase
377 (Agilent, #600679) and the DNA oligos indicated in **Table S1**. PCR1 products were purified using QIAquick PCR

Purification kit (Qiagen #28104). PCR2 products were purified using Agencourt AMPure XP Beads (Beckman Coulter Life Sciences #A63880). DNA concentration was determined using Qubit dsDNA HS Assay Kit (Thermo Fisher #Q32854) and adjusted to 2 nM prior to sequencing. NGS was performed using the NextSeq 500/550 High Output Kit v2.5 75 cycles (Illumina #20024906).

Screen analysis. Reads were demultiplexed using bcl2fastq Conversion Software v2.20 (Illumina) and fastx_toolkit v0.0.13. Sequencing adapters were removed using cutadapt v1.9.1 (66). The reference library was built using bowtie2 v2.2.9 (67). Read mapping was performed with bowtie2 allowing 1 seed mismatch in --local mode and samtools v1.9 (68). Mapping analysis and gene selection were performed using MAGeCK v0.5.6, normalizing the data with default parameters. sgRNA and gene enrichment analyses are available in **Table S5-S6**, respectively and full MAGeCK output at https://github.com/Simon-LoriereLab/crispr_isg_sarscov2.

Generation of multi-guide gene knockout cells. 3 sgRNAs per gene were designed (**Table S2**). 10 pmol of NLS-Sp.Cas9-NLS (SpCas9) nuclease (Aldevron #9212) was combined with 30 pmol total synthetic sgRNA (10 pmol for each sgRNA) (Synthego) to form RNPs in 20 µL total volume with SE Buffer (Lonza #V5SC-1002). The reaction was incubated at room temperature for 10 min. $2x10^5$ cells per condition were pelleted by centrifugation at 100*g* for 3 min, resuspended in SE buffer and diluted to $2x10^4$ cells/µL. 5 µL of cell solution was added to the pre-formed RNP solution and gently mixed. Nucleofections were performed on a Lonza HT 384-well nucleofector system (Lonza #AAU-1001) using program CM-120. Immediately following nucleofection, each reaction was transferred to a 96-well plate containing 200 µL of DMEM 10% FBS ($5x10^4$ cells per well). Two days post-nucleofection, DNA was extracted using DNA QuickExtract (Lucigen #QE09050). Cells were lysed in 50 µL of QuickExtract solution and incubated at 68°C for 15 min followed by 95°C for 10 min. Amplicons were generated by PCR amplification using NEBNext polymerase (NEB #M0541) or AmpliTaq Gold 360 polymerase (ThermoFisher #4398881) and the primers indicated in **Table S3**. PCR products were cleaned-up and analyzed by Sanger sequencing. Sanger data files and sgRNA target sequences were input into Inference of CRISPR Edits (ICE) analysis https://ice.synthego.com/#/ to determine editing efficiency and to quantify generated indels (69). Percentage of alleles edited is shown in **Table 1** (n=3).

SARS-CoV-2, SARS-CoV and MERS-CoV infection assays. A549-ACE2 cells were infected by incubating the virus for 1h with the cells maintained in DMEM supplemented with 1 µg/ml TPCK-trypsin (Sigma #4370285). The viral input was then removed and cells were kept in DMEM supplemented with 2% FBS. For 293T-ACE2 cells, infections were performed without TPCK-trypsin. MERS-CoV and SARS-CoV infections were performed in DMEM supplemented with 2% FBS and cells were incubated 1h at 37°C 5% CO2. Viral inoculum was then removed and replaced by fresh DMEM supplemented with 2% FBS. All experiments involving infectious material were performed in Biosafety Level 3 facilities in compliance with Institut Pasteur's guidelines and procedures.

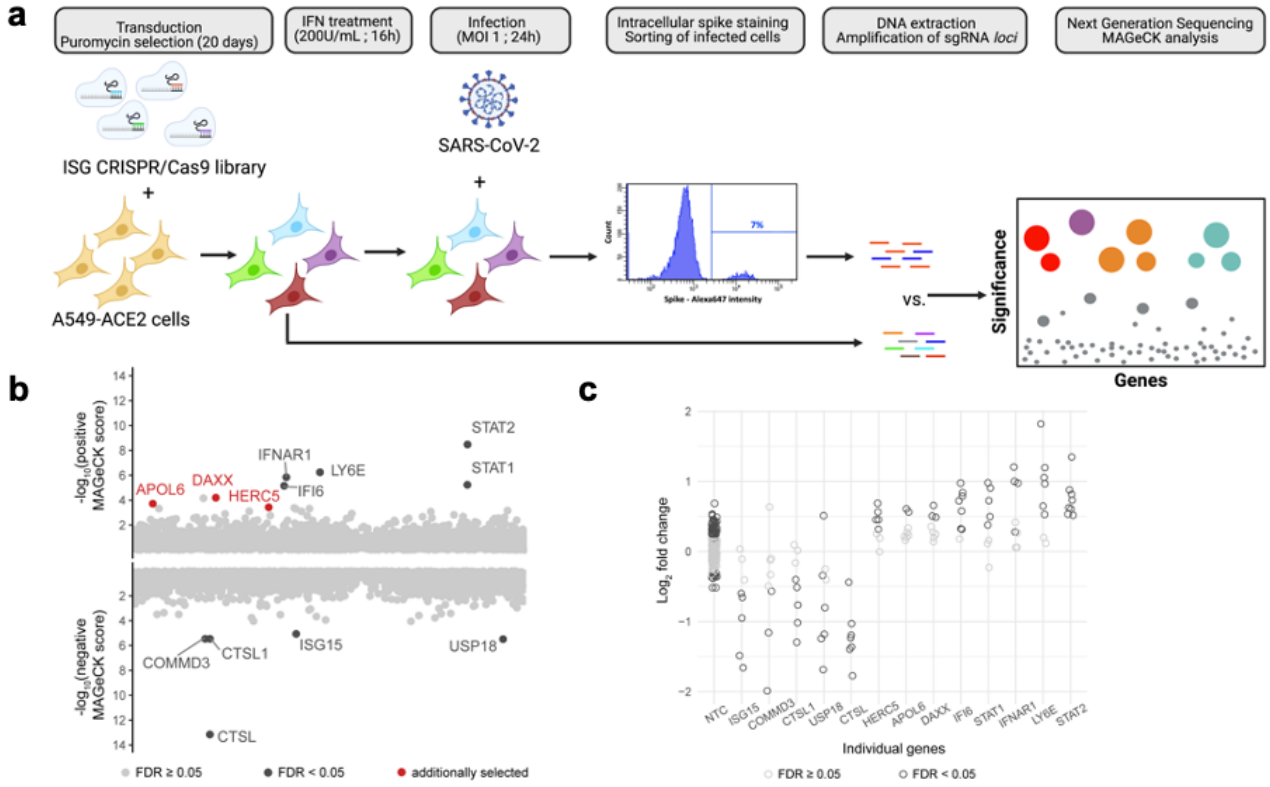
Yellow Fever Virus and Measles Virus infection assays. Cells were infected with YFV (at an MOI of 0.3) or MeV-GFP (MOI of 0.2) in DMEM without FBS for 2h in small volume of medium to enhance contacts with the inoculum and the cells. After 2h, the viral inoculum was replaced with fresh DMEM 10% FBS 1% P/S. FACS analysis were performed at 24h p.i. Cells were fixed and permeabilized using BD Cytofix/Cytoperm (Fisher Scientific, # 15747847) for 30 min on ice (all the following steps were performed on ice and centrifuged at 4°C) and then washed tree times with wash buffer. Cells infected with YFV were incubated with the pan-flavivirus anti-Env 4G2 antibody for 1h at 4°C and then with Alexa 488 anti-mouse IgG secondary antibodies (Thermo Fisher, #A28175) for 45 min at 4°C in the dark. Non-infected, antibody-stained samples served as controls for signal background. The number of cells infected with MeV-GFP were assessed with the GFP signal, using non-infected cells as controls. Data were acquired with an Attune NxT Acoustic Focusing Cytometer (Life technologies) and analyzed using FlowJo software.

Hit validation. $2.5x10^4$ A549-ACE2 KO cells were seeded in 96-well plates 18h before the experiment. Cells were treated with IFNα and infected as described above. At 72h post-infection, supernatants and cellular monolayers were harvested in order to perform qRT-PCR and plaque assay titration. Infectious supernatants were heat-inactivated at 80°C for 10 min. For intracellular RNA, cells were lysed in a mixture of Trizol Reagent (Invitrogen #15596018) and PBS at a ratio of 3:1. Total RNA was extracted using the Direct-zol 96 RNA kit (Zymo Research #R2056) or the Direct-zol RNA Miniprep kit (Zymo Research #R2050). For SARS-CoV-2 detection, qRT-PCR was performed either directly on the inactivated supernatants or on extracted RNA using the Luna Universal One-Step RT-qPCR Kit (NEB #E3005E) in a QuantStudio 6 thermocycler (Applied Biosystems) or in a StepOne Plus thermocycler (Applied Biosystems). The primers used are described in **Table S4**. Cycling conditions were the following: 10 min at 55°C, 1 min at 95°C and 40 cycles of 95°C for 10s and 60°C

436 for 1 min. Results are expressed as genome copies/mL as the standard curve was performed by diluting a
437 commercially available synthetic RNA with a known concentration (EURM-019, JRC). For SARS-CoV and
438 MERS-CoV, qRT-PCR were performed using FAM-labelled probes (Eurogentech) and the Superscript III
439 Platinum One-Step qRT-PCR System (Thermo Fisher Scientific, #11732020). The cycling conditions were the
440 following: 20 min at 55°C, 3 min at 95°C and 50 cycles of 95°C for 15 s and 58°C for 30 s. The primers used are
441 described in **Table S4**. Standard curves were performed using serial dilutions of RNA extracted from and SARS-
442 CoV and MERS-CoV viral culture supernatants of known infectious titer. For plaque assay titration, VeroE6 cells
443 were seeded in 24-well plates (10^5 cells per well) and infected with serial dilutions of infectious supernatant
444 diluted in DMEM during 1h at 37°C. After infection, 0.1% agarose semi-solid overlays were added. At 72h post-
445 infection, cells were fixed with Formalin 4% (Sigma #HT501128-4L) and plaques were visualized using crystal
446 violet coloration. Time-course experiments were performed the same way except that supernatants and cellular
447 monolayers were harvested at 0h, 2h, 4h, 6h, 8h, 10h and 24h post-infection.
448
449 **Overexpression assay.** $2x10^5$ 293T-ACE2 cells were seeded in a 24-well plate 18h before experiment. Cells
450 were transfected with 500 ng of plasmids expressing HA-DAXX WT, HA-DAXX 15KR and HA-DAXXΔSIM
451 plasmids, using Fugene 6 (Promega # E2691), following the manufacturer's instructions. HA-NBR1 was used as
452 negative control. After 24h cells were infected at the indicated MOI in DMEM 2% FBS. When indicated, cells
453 were treated with 10 mM of remdesivir (MedChemExpress #HY-104077) at the time of infection. For flow
454 cytometry analysis, cells were fixed with 4% formaldehyde and permeabilized in a PBS 1% BSA 0.025% saponin
455 solution for 30 min prior to staining with corresponding antibodies for 1h at 4°C diluted in the permeabilization
456 solution. Samples were acquired on a BD LSR Fortessa and analyzed using FlowJo. Total RNA was extracted
457 using a RNeasy Mini kit and submitted to DNase treatment (Qiagen). RNA concentration and purity were
458 evaluated by spectrophotometry (NanoDrop 2000c, ThermoFisher). In addition, 500 ng of RNA were reverse
459 transcribed with both oligo dT and random primers, using a PrimeScript RT Reagent Kit (Takara Bio) in a 10 mL
460 reaction. Real-time PCR reactions were performed in duplicate using Takyon ROX SYBR MasterMix blue dTTP
461 (Eurogentec) on an Applied Biosystems QuantStudio 5 (ThermoFisher). Transcripts were quantified using the
462 following program: 3 min at 95°C followed by 35 cycles of 15s at 95°C, 20s at 60°C, and 20s at 72°C. Values for
463 each transcript were normalized to expression levels of RPL13A. The primers used are indicated in **Table S4**.
464
465 **Microscopy Immunolabeling and Imaging.** 293T-ACE2 cells were cultured and infected with SARS-CoV-2 as
466 described above. When indicated, cells were treated with 50 μM of GRL0617 (MedChemExpress #HY-117043)
467 at the time of infection. Cultures were rinsed with PBS and fixed with 4% paraformaldehyde (electronic
468 microscopy grade; Alfa Aesar) in PBS for 10 min at room temperature, treated with 50 mM NH4Cl for 10 min,
469 permeabilized with 0.5% Triton X-100 for 15 min, and blocked with 0.3% BSA for 10 min. Cells were incubated
470 with primary and secondary antibodies for 1h and 30 min, respectively, in a moist chamber. Nuclei were labeled
471 with Hoechst dye (Molecular Probes). Images were acquired using a LSM700 (Zeiss) confocal microscope
472 equipped with a 63X objective or by Airyscan LSM800 (Zeiss). Image analysis and quantification was performed
473 using ImageJ.
474
475 **Western blot.** 293T-ACE2 cells were transfected with the indicated plasmids or treated with the indicated
476 concentrations of GRL0617; with 10 μM of Masitinib (MedChemExpress #HY-10209); or with 10μM of MG132
477 (SIGMA #M7449), an inhibitor of the proteasome and infected with SARS-CoV-2. Cell lysates were prepared
478 using RIPA lysis and extraction buffer (ThermoFisher #89901). Protein concentration was determined using
479 Bradford quantification. Proteins were denaturated using 4X Bolt LDS Sample Buffer (Invitrogen) and 10X Bolt
480 Sample Reducing Agent (Invitrogen). 40 µg of proteins were denatured and loaded on 12% ProSieve gel and
481 then subjected to electrophoresis. Gels were then transferred (1h, 90V) to Western blotting membranes,
482 nitrocellulose (GE Healthcare #GE10600002) using Mini Trans-Blot Electrophoretic Transfer Cell (Biorad
483 #1703930EDU). Membranes were blocked with 5% BSA in PBS (blocking buffer) and incubated with primary
484 antibodies diluted in blocking buffer. Membranes were washed and incubated with secondary antibodies diluted
485 in blocking buffer. Chemiluminescent acquisitions were performed on a Chemidoc™ MP Imager and analysed
486 using Image Lab™ desktop software (Bio-Rad Laboratories).
487
488 **Flow cytometry.** For flow cytometry analysis, all cells were fixed with 4% formaldehyde. For intracellular
489 staining, cells were permeabilized in a PBS/1% BSA/0.025% saponin solution for 30 min prior to staining with
490 corresponding primary antibodies for 1h at 4°C and then secondary antibodies for 45min at 4°C, diluted in the
491 permeabilization solution. Acquisition was done with Fortessa Cytometer and analyses with FlowJo software
492 (Treestar Inc., Oregon, USA).
493

494 **Single-cell RNAseq analysis.** Single cell RNAseq analysis were performed in the BioTuring Browser Software
495 (v2.8.42) developed by BioTuring, using a dataset made available by Liao *et al.* (43) (ID: GSE145926). All
496 processing steps were done by BioTuring Browser (71). Cells with less than 200 genes and mitochondrial genes
497 higher than 10% were excluded from the analysis.
498
499 **Statistical analysis.** GraphPad Prism was used for statistical analyses. Linear models were computed using
500 Rstudio.

501 **Figures.**



502
503
504 **Figure 1: ISG-focused CRISPR/Cas9 screening approach to identify restriction factors for**
505 **SARS-CoV-2. a: CRISPR/Cas9 screen outline**. A549-ACE2 cells were transduced with lentivectors
506 encoding the ISG CRISPR/Cas9 library and selected by puromycin treatment for 20 days. Library cells
507 were then pre-treated with 200 U/mL of IFNα for 16 hours, and infection with SARS-CoV-2 at MOI 1.
508 At 24h p.i., infected cells were fixed with formalin treatment, permeabilized by saponin treatment and
509 stained with a monoclonal anti-spike antibody. After secondary staining, infected cells were sorted and
510 harvested. Non-infected, non-IFNα treated cells were harvested as a control. DNA was extracted from
511 both cellular fractions and sgRNA *loci* amplification was carried out by PCR. Following NGS, bio-
512 informatic analysis using the MAGeCK package was conducted. **b: Screen results**. By taking into
513 account the enrichment ratios of each of the 8 different sgRNAs for every gene, the MAGeCK analysis
514 provides a positive score for KO enriched in infected cells (*i.e.* restriction factor, represented in the top
515 fraction of the graph) and a negative score for KO depleted in infected cells (*i.e.* proviral factors,
516 represented in the bottom portion of the graph). Gene with an FDR < 0.05 are represented in black. 3
517 genes with a FDR > 0.05, but with a p-value < 0.005 were additionally selected and are represented in
518 red. **c: Individual sgRNA enrichment.** For the indicated genes, the enrichment ratio of the 8 sgRNAs
519 present in the library was calculated as the MAGeCK normalized read counts in infected cells divided
520 by those in the original pool of cells and is represented in log2 fold change. As a control, the
521 enrichment ratios of the 200 non-targeting control (NTCs) is also represented.

522

Gene	% of alleles edited
LY6E	96 ± 1.73
DAXX	79,67 ± 2.52
APOL6	99 ± 0
HERC5	97 ± 0
CTSL	91 ± 1
IFI6	88,33 ± 0.58
IFNAR1	76,67 ± 3.21

523
524 **Table 1: Gene editing efficiency**. The frequency of editing was determined using Sanger sequencing
525 and ICE analysis. Values are represented as mean ± SD (n=3).

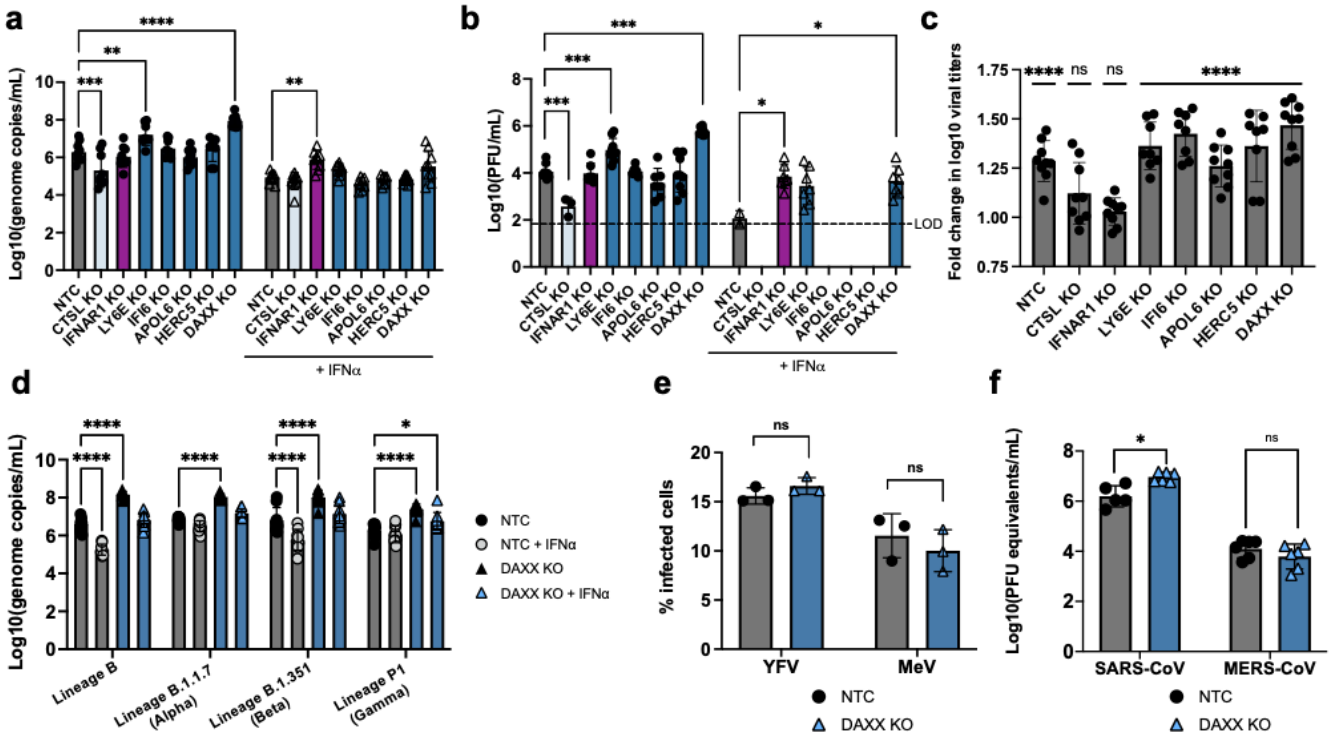
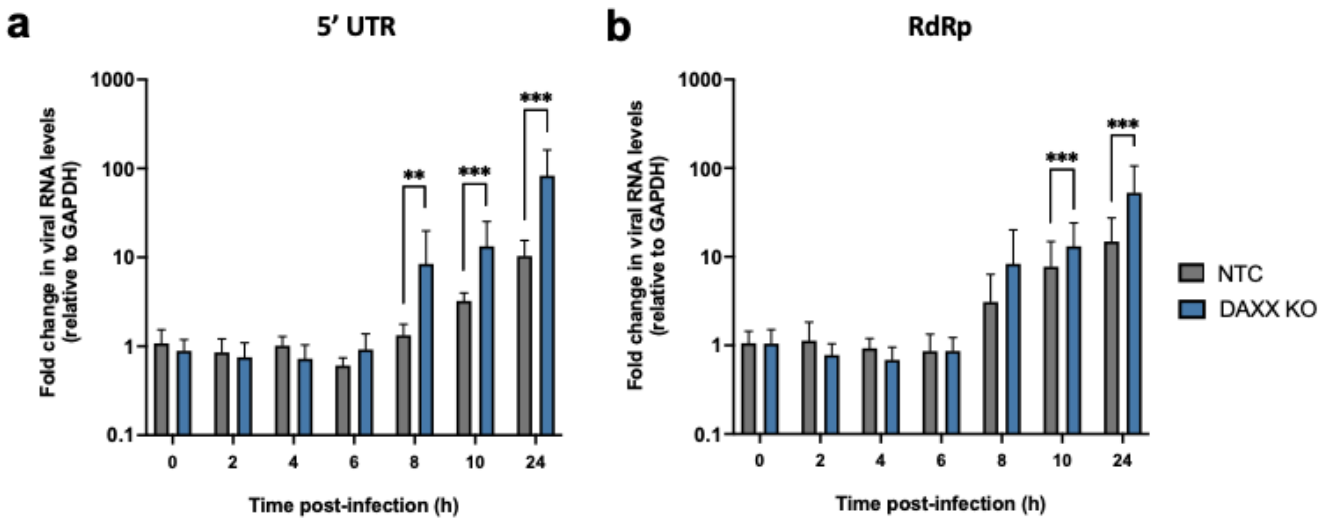


Figure 2: DAXX is a restriction factor for SARS-CoV-2. a-c: Antiviral activity of ISGs against SARS-CoV-2. A549-ACE2 knocked-out for the indicated genes were generated using a multi-guide approach, leading to pools of KO cells with a high frequency of indels. KO cells were pre-treated with 0 (circles) or 200 (triangles) U/mL of IFNα 24h prior to triplicate infection with SARS-CoV-2 (MOI 0.1). Supernatants were harvested at 72h p.i. The mean of three independent experiments, with infections carried out in triplicate, is shown. **a:** For the titration of RNA levels, supernatants were heat inactivated prior to quantification by qRT-PCR. Genome copies/mL were calculated by performing serial dilutions of a synthetic RNA with a known concentration. Statistics: 2-way ANOVA using Dunnett's test, * = p-value < 0.05, ** = p-value < 0.01, *** = p-value < 0.001, **** = p-value < 0.0001. **b:** For the titration of infectious virus levels by plaque assay, supernatants were serially diluted and used to infect VeroE6 cells. Plaques formed after 3 days of infection were quantified using crystal violet coloration. Statistics: Dunnett's test on a linear model, * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001. **c:** For each of the indicated KO, the data shown in **a.** is represented as fold change in log10 titers (*i.e.* the triplicate log10 titers of the non-treated condition divided by the mean of the triplicate log10 titers IFNα-treated condition, n=3). Statistics: 2-way ANOVA using Sidak's test, ns = p-value > 0.05, **** = p-value < 0.001. **d-f: Antiviral activity of DAXX against SARS-CoV-2 variants and other viruses. d:** A549-ACE2 WT or DAXX KO cells were infected in triplicates at an MOI of 0.1 with the following SARS-CoV-2 strains: Lineage B (original strain); Lineage B.1.1.7. (Alpha variant); Lineage B.1.35.1 (Beta variant); Lineage P1 (Gamma variant). Supernatants were harvested at 72h p.i. Supernatants were heat inactivated prior to quantification by qRT-PCR. Genome copies/mL were calculated by performing serial dilutions of a synthetic RNA with a known concentration. The mean of three independent experiments, with infections carried out in triplicate, is shown. **e:** A549-ACE2 WT or DAXX KO cells were infected in triplicates with Yellow Fever Virus (YFV, Asibi strain, MOI of 0.3) or with Measles Virus (MeV, Schwarz strain expressing GFP, MOI of 0.2). At 24h p.i., the percentages of cells positive for viral protein E (YFV) or GFP (MeV) was assessed by flow cytometry. The mean of 3 independent experiments is represented. **f:** WT or DAXX KO cells were infected in triplicates at an MOI of 0.1 with SARS-CoV or MERS-CoV. Supernatants were harvested at 72h p.i. Supernatants were heat inactivated prior to quantification by qRT-PCR. Serial dilutions of a stock of known infectious titer was used as a standard. The mean of 2 independent experiments is represented. Statistics: 2-way ANOVA using Dunnett's test, * = p-value < 0.05, *** = p-value < 0.001, **** = p-value < 0.0001



558
559 **Figure 3: DAXX restricts SARS-CoV-2 transcription.** A549-ACE2 WT or DAXX KO were infected at
560 MOI 1 in triplicates. Cell monolayers were harvested at the indicated time points, and total RNA was
561 extracted. The levels of viral RNA (**a:** 5' UTR; **b:** RdRp) were determined by qRT-PCR and normalized
562 against GAPDH levels. The mean of 3 independent experiments is represented. Statistics: Dunnett's
563 test on a linear model, * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001.

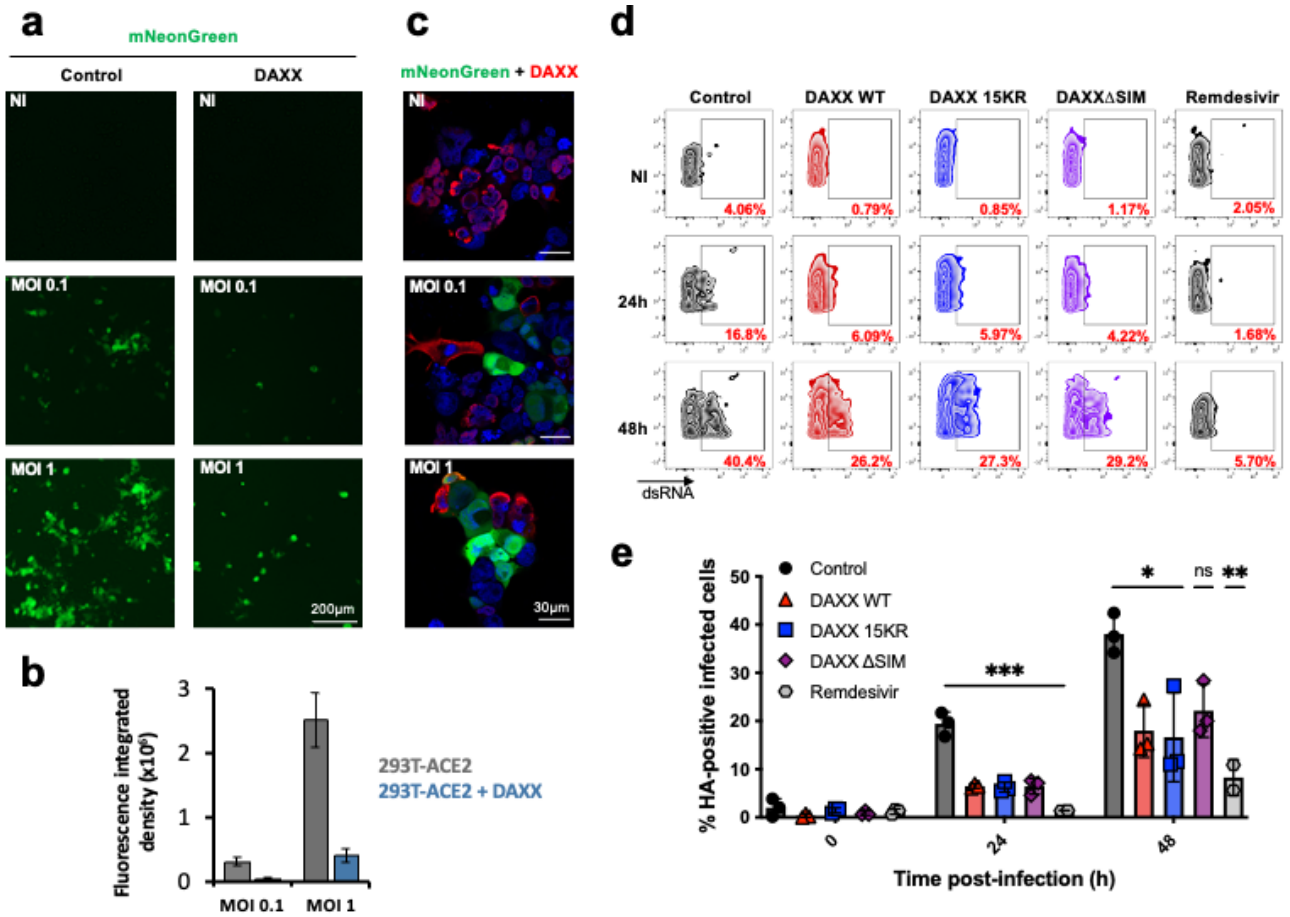
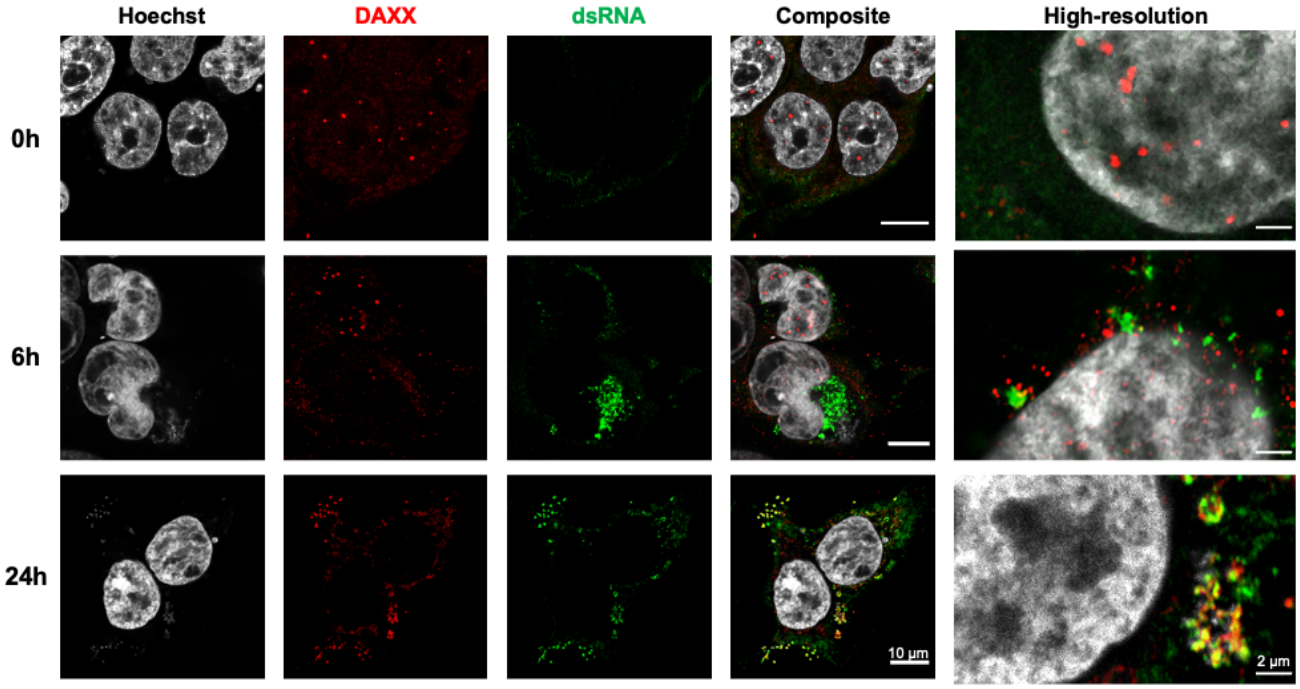


Figure 4: DAXX restriction of SARS-CoV-2 is SUMOylation independent. a-c: DAXX overexpression restricts SARS-CoV-2. 293T-ACE2 cells were transfected with DAXX WT. 24h after transfection, cells were infected with the mNeonGreen fluorescent reporter SARS-CoV-2 at the indicated MOI. Cells were either visualized with an EVOS fluorescence microscope (**a-b**) or stained with an HA-antibody detecting DAXX and imaged by confocal microscopy (**c**). Scale bars correspond to 200 µm (**a)** and 30 µm (**c**). Images shown in (**a**) were quantified using ImageJ software (**b**). Data show the mean +/- SD of Fluorescence integrated densities. The analysis was performed on around 200 cells from 3 different fields. **d-e: DAXX mutants are still able to restrict SARS-CoV-2**. 293T-ACE2 cells were transfected with HA-DAXX WT; HA-DAXX 15KR; HA-DAXXΔSIM; or with HA-NBR1 as negative control plasmid. 24h after transfection, cells were infected with SARS-CoV-2 at an MOI of 0.1. When indicated, cells were treated with remdesivir at the time of infection. After 24h or 48h, infected cells were double-stained recognizing dsRNAs (to read out infection) and HA (to read out transfection efficiency) and acquired by flow cytometry. The percentage of infected cells among HA-positive (transfected) cells for one representative experiment is shown in **d**, for the mean of 3 independent experiments in **e**. Statistics: one-way ANOVA using Dunnett's test, Holm corrected, ns = p-value > 0.05, * = p-value < 0.05, ** = p-value < 0.01, *** = p-value < 0.001.



582
583
584 **Figure 5: SARS-CoV-2 infection induces DAXX cytoplasmic re-localization to sites of viral**
585 **replication.** 293T-ACE2 cells were infected with SARS-CoV-2 at the indicated MOI 1. 24h post-
586 infection, cells were labelled with Hoescht and with antibodies against dsRNA (detecting viral RNA, in
587 green) and HA (detecting DAXX, in red). When indicated, the high-resolution Airyscan mode was
588 used. Scale bars correspond to 10 µm for confocal images, and 2 µm for the high-resolution images.

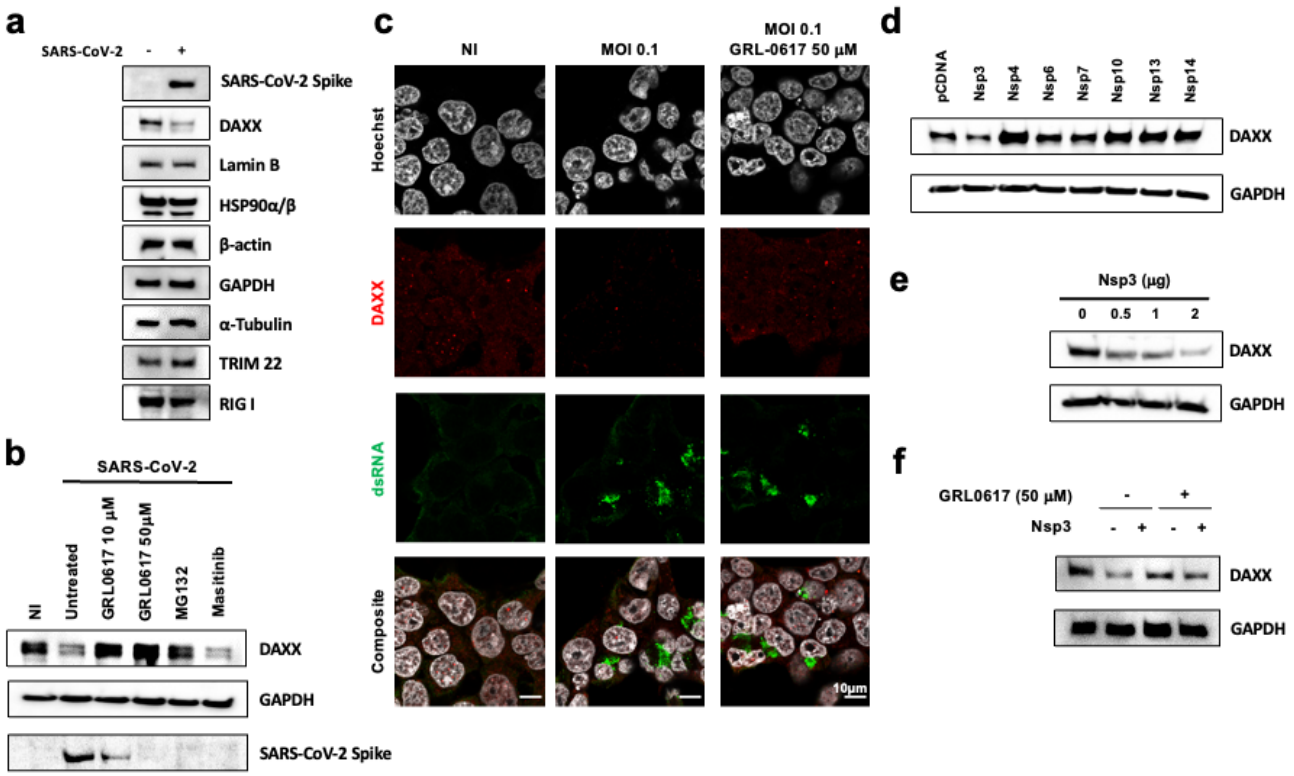


Figure 6: SARS-CoV-2 PLpro induces the proteasomal degradation of DAXX. a: DAXX degradation after infection. 293T-ACE2 cells were infected with SARS-CoV-2 at MOI 0.1. After 24h, cells were harvested and levels of DAXX, Lamin B, HSP90, Actin, GAPDH, Tubulin, TRIM22, RIG-I and of the viral protein spike were analyzed by Western Blot. **b: GRL0617 and MG132 treatment restores DAXX expression.** 293T-ACE2 cells were infected with SARS-CoV-2 at MOI 0.1. When indicated, cells were pretreated 2h before infection with GRL0617 (at the indicated concentrations), or with MG132 (10 µM), a proteasome inhibitor, or Masitinib (10 µM) a 3CL inhibitor. After 24h, cells were harvested and levels of DAXX, GAPDH and of the viral protein spike were analyzed by Western Blot. **c: GRL0617 treatment restores DAXX localization.** 293T-ACE2 cells were infected with SARS-CoV-2 at MOI 0.1. 24h post-infection, cells were labelled with Hoescht and with antibodies against dsRNA (detecting viral RNA, in green) and HA (detecting DAXX, in red). When indicated, cells were treated with 50 µM of GRL0617 at the time of infection. Scale bars correspond to 10 µm. **d-f: Nsp3 induces DAXX degradation. D:** 293T-ACE2 cells were transfected with 1 µg of the indicated viral proteins. After 24h, the levels of DAXX and GAPDH were analyzed by Western Blot. **E:** 293T-ACE2 cells were transfected with the indicated amounts of Nsp3. After 24h, the levels of DAXX and GAPDH were analyzed by Western Blot. **f:** 293T-ACE2 cells were transfected with 1 µg of Nsp3. 6 hours post transfection, cells were also, when indicated, treated with 50 µM of GRL0617. 24h after transfection, the levels of DAXX and GAPDH were analyzed by Western Blot.

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Data availability: Raw NGS data was deposited to the NCBI GEO portal and is accessible with the number GSE173418.

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