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1	The SARS-CoV-2 protein NSP2 impairs the silencing capacity of the human 4EHP-GIGYF2
2	complex
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ABSTRACT 23

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There is an urgent need for a molecular understanding of how SARS-CoV-2 influences the machineries of the host cell. Herein, we focused our attention on the capacity of the SARS-CoV-2 protein NSP2 to bind 25 the human 4EHP-GIGYF2 complex, a key factor involved in microRNA-mediated silencing of gene 26 expression. Using *in vitro* interaction assays, our data demonstrate that NSP2 physically associates with both 27 4EHP and a central segment in GIGYF2 in the cytoplasm. We also provide functional evidence showing that 28 29 NSP2 impairs the function of GIGYF2 in mediating translation repression using reporter-based assays. Collectively, these data reveal the potential impact of NSP2 on the post-transcriptional silencing of gene 30 expression in human cells, pointing out 4EHP-GIGYF2 targeting as a possible strategy of SARS-CoV-2 to 31 32 take over the silencing machinery and to suppress host defenses.

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INTRODUCTION 34

Beta-coronaviruses (β -CoVs) are enveloped RNA viruses that infect a variety of vertebrate hosts, 35 including humans (Cui et al., 2019). In the last decades, two β -CoVs have caused epidemic diseases of the 36 respiratory tract: Severe Acute Respiratory Syndrome (SARS-CoV-1) in 2002 (Ksiazek et al., 2003, Drosten 37 38 et al., 2003) and Middle East Respiratory Syndrome (MERS) in 2012 (Zaki et al., 2012). A new β-CoV (SARS-CoV-2) emerged in 2019 that is the causative agent of coronavirus disease 2019 (COVID-19) pandemic (Zhou 39 et al., 2020). These three viruses possess single-stranded, positive-sense RNA genomes of nearly 30 kb in 40 length (Lu et al., 2020). The SARS-CoV-2 genome encodes 29 proteins with multiple functions in virus 41 42 replication and packaging, including 4 structural proteins (the nucleocapsid N, envelope E, membrane M, and 43 spike S proteins), 7 accessory proteins (ORF3a–ORF8) whose functions in SARS-CoV-2 pathogenesis remain 44 largely unknown, and 16 non-structural proteins (NSP1-NSP16) that encode the RNA-directed RNA 45 polymerase, helicase, protease, and other components required for virus replication (for review, see (V'Kovski 46 et al., 2021)).

Due to the urgent need to better understand SARS-CoV-2 biology, several CRISPR-Cas9 and 47 48 proteomic-based screening campaigns have investigated the landscape of host factors which are targeted by 49 the virus proteome (Hoffmann et al., 2021, Sadegh et al., 2020, Gordon et al., 2020b, Gordon et al., 2020a,

Davies et al., 2020). These screens identified more than 300 high-confidence protein-protein interactions between human and SARS-CoV-2 proteins, highlighting the intimate connection of SARS-CoV-2 proteins with multiple biological processes, including protein trafficking, transcription and mRNA translation. Among these interactions, the Non-Structural Protein 2 (NSP2) has been found to interact with key host proteins involved in vesicle trafficking (FKBP15, WASHC) and mRNA translation (4EHP, GIGYF2) which could be of therapeutic importance (Gordon et al., 2020b, Davies et al., 2020).

56 NSP2 exists in all coronaviruses studied to date, including SARS-CoV-1, SARS-CoV-2, MERS, and in their closely related β-CoVs infecting mammals. Although its role in the SARS-CoV-2 pathogenicity has 57 58 not been fully elucidated, the deletion of NSP2 in SARS-CoV-1 attenuates viral growth and RNA synthesis (Graham et al., 2005). Recent findings showed that SARS-CoV-2 NSP2 is undergoing positive nature selection 59 and could be thus essential to the virus (Angeletti et al., 2020, Flores-Alanis et al., 2021). At the structural 60 level, NSP2 has a complex multi-domain topology including an N-terminal domain with a highly-conserved 61 62 zinc binding site, and a C-terminal region rich in β -strands. With the exception of the zinc binding site, NSP2 displays a rapidly evolving surface with the presence of natural variations that could impact host-virus 63 interactions (Ma et al., 2021, Mompean et al., 2021, Gupta et al., 2021, Slavin et al., 2021). Among its host 64 interactors, SARS-CoV-2 NSP2 interacts with the 4EHP-GIGYF2 complex, a key machinery in translational 65 silencing and mRNA decay (Davies et al., 2020, Cornillez-Ty et al., 2009, Gordon et al., 2020b, Gordon et al., 66 2020a). It is worth noting that the NSP2/4EHP-GIGYF2 association is conserved across SARS-CoV-1 and 67 MERS, corroborating a functional importance for β-CoV infection in general (Gordon et al., 2020a, Cornillez-68 Ty et al., 2009). 69

70 The cap-binding eIF4E-Homologous Protein (4EHP) is an integral component of post-transcriptional 71 silencing mechanisms through competing with the eIF4F complex for binding to the mRNA 5'cap (Christie and Igreia, 2021). In complex with the GRB10-Interacting GYF (glycine-tyrosine-phenylalanine domain) 72 73 protein 2 (GIGYF2), the cap-binding activity of 4EHP is required for the optimal translational repression by microRNAs (miRNAs), as well as the RNA-binding proteins ZNF598 and Tristetraprolin (TTP) (Morita et al., 74 75 2012, Fu et al., 2016, Villaescusa et al., 2009, Cho et al., 2006, Cho et al., 2005, Chapat et al., 2017b, Chen 76 and Gao, 2017). In the case of miRNA-driven silencing, the recruitment of 4EHP-GIGYF2 is initiated by the 77 miRNA-induced silencing complex (miRISC), an assembly of argonaute and TNRC6/GW182 proteins. 4EHP

can be physically mobilized through the interaction of the GYF domain of GIGYF2 with a proline-prolineglycine-leucine (PPGL) motif in TNRC6/GW182 (Kryszke et al., 2016, Schopp et al., 2017). At the functional level, this 4EHP/miRNA axis is required to control ERK signaling, as well as to suppress IFN- β production by effecting the *miR-34a*-induced translational silencing of *Ifnb1* mRNA (Zhang et al., 2021, Jafarnejad et al., 2018).

83	Beyond miRNA action, 4EHP-GIGYF2 also forms a translation inhibitory complex with the RNA-
84	binding protein ZNF598, which functions in ribosome stalling on internally polyadenylated mRNAs during
85	ribosome quality control (Morita et al., 2012, Garzia et al., 2017). ZNF598 is also necessary for the repression
86	of TTP-targeted mRNAs that encode inflammatory cytokines. The 4EHP-GIGYF2-ZNF598 complex binds
87	TTP during an innate immune response in mouse macrophages to control the production of TTP-targeted
88	mRNAs such as TNF-α, Ier3, Csf2, and Cxcl10. In all cases, TNRC6/GW182, ZNF598 and TTP display a
89	comparable binding mode to GIGYF2, namely via the recognition of a proline stretch by the GYF domain of
90	GIGYF2 (Tollenaere et al., 2019, Fu et al., 2016).

A recent genetic screen has revealed that both 4EHP and GIGYF2 are necessary for infection by SARS-CoV-2 *in vitro*, while dispensable for seasonal coronaviruses (Hoffmann et al., 2021). The contribution of 4EHP-GIGYF2 into the pathogenicity of SARS-CoV-2 could therefore originate from their interaction with NSP2. In the present report, we focused on the physical and functional interplays existing between the 4EHP-GIGYF2 complex and the SARS-CoV-2 protein NSP2 in human cells. Combining interaction assays and reporter-based approaches, our data shed light on the negative impact of NSP2 on the 4EHP-GIGYF2-mediated translational silencing of gene expression.

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99 **RESULTS**

100 NSP2 binds the 4EHP-GIGYF2 complex *in cellulo*

Early large scale studies reported the capacity of SARS-CoV-2 NSP2 to bind the 4EHP-GIGYF2 complex using affinity-purification mass spectrometry (Gordon et al., 2020b, Gordon et al., 2020a). To validate the physical association of NSP2 with the GIGYF2-4EHP complex, we first sought to detect their interaction

104 using co-immunoprecipitation (co-IP). An inducible Flag-tagged version of NSP2 was stably expressed in HEK293 Flp-In T-REX cells and co-IPs were performed following tetracycline-induced expression of Flag-105 NSP2. Lysates prepared from control, non-induced, and induced cells were immunoprecipitated with anti-Flag 106 107 antibody. Subsequent Western blot (WB) analysis of the co-IP fraction showed that Flag-NSP2 efficiently binds both endogenous GIGYF2 and 4EHP (Figure 1A). This interaction was similarly detected in RNase A-108 treated lysates, indicating that the NSP2/4EHP-GIGYF2 interaction occurs in an RNA-independent manner. 109 The GIGYF2-associated protein ZNF598 was also found along with NSP2, while CNOT9, a subunit of the 110 CCR4-NOT complex known to bind GIGYF2, was not detected (Ajiro et al., 2009) (Figure 1A). Together, 111 these data demonstrate that NSP2 physically associates with the 4EHP-GIGYF2 complex, as well as their 112 interacting protein ZNF598. 113

To elucidate which protein is involved in the formation of this complex, GIGYF2- and 4EHP-Knock-114 Out (KO) HEK293 cells were used to conduct co-IPs with Flag-NSP2. Vectors expressing Flag-NSP2, or Flag 115 as a control, were then transiently transfected in these KO populations, as well as in their Wild-Type (WT) 116 counterpart. Following Flag IP in the 4EHP^{KO} cells, we observed that the NSP2/GIGYF2 interaction was still 117 detectable, while ZNF598 co-IP was reduced, indicating a plausible contribution of 4EHP in NSP2 binding. 118 119 By contrast, in the absence of GIGYF2, we could not detect any interaction of NSP2 with either 4EHP or ZNF598 (Figure 1B), supporting a central role of GIGYF2 in NSP2 binding. However, a contribution of 4EHP 120 cannot be excluded since its level is decreased in the GIGYF2^{KO} cells due to a co-stabilization effect (Figure 121 1B) (Morita et al., 2012). 122

We then performed immunofluorescence staining and confocal imaging to analyze the sub-cellular 123 distribution of NSP2. Following transient expression of Flag-tagged NSP2 in WT and GIGYF2^{KO} cells, 124 125 immunofluorescence staining was performed with antibodies raised against the Flag sequence and endogenous GIGYF2. In WT cells, a diffuse cytoplasmic signal was detected for both Flag-tagged NSP2 and GIGYF2 126 (Supplementary Figure S1A), indicating their mutual localization in the cytoplasm. By contrast, the GIGYF2 127 signal was lost in GIGYF2^{KO} cells while the distribution of Flag-NSP2 remained unchanged (Supplementary 128 Figure S1A), confirming the specificity of the anti-GIGYF2 antibody. The sub-cellular localization of 129 NSP2/GIGYF2 interaction was then examined using an in situ Proximity Ligation Assay (PLA). PLA was 130 conducted in Flag-NSP2-expressing cells using the anti-Flag and anti-GIGYF2 antibodies. Following confocal 131 imaging, a spot-like signal was abundantly detected in the cytoplasm of HEK293T cells expressing Flag-NSP2, 132

indicating the spatial proximity between endogenous GIGYF2 and Flag-NSP2 (Figure 1C). This PLA signal
 was not observed in cells transfected with a control plasmid. Similarly, the PLA signal showed a significant
 reduction when the Flag/GIGYF2 staining was performed in GIGYF2^{KO} cells expressing Flag-NSP2
 (Supplementary Figures S1B and S1C), supporting the specificity of the interaction.

In addition to its diffuse cytoplasmic localization, GIGYF2 is known to be found in P-bodies, a 137 subclass of RNA granules enriched in translationally repressed mRNAs and silencing factors (Amaya Ramirez 138 et al., 2018). We therefore tested whether NSP2 could also be detected in P-bodies by examining the 139 localization of Flag-tagged NSP2 alongside DDX6, a known resident of P-bodies. Using immunofluorescence 140 141 microscopy, we found that Flag-NSP2 did not form foci that co-localized with DDX6 in HEK293T cells (Supplementary Figure S1D). Intensity line scans were performed along the DDX6 foci to confirm the absence 142 of relationship between the spatial distribution of NSP2 and P-bodies, thus indicating that NSP2 could 143 preferentially bind the diffuse form of GIGYF2 instead of its P-body-associated counterpart. To confirm this 144 145 point, we took advantage of the properties of biotinylated-isoxazole (b-isox), a compound which selectively precipitates proteins located in RNA granules such as P-bodies and stress granules (Han et al., 2012, Kato et 146 al., 2012) (Supplementary Figure S1E). Extracts of HEK293T expressing Flag-NSP2 were exposed to 100 µM 147 of b-isox, or DMSO as a mock control. By comparing the level of proteins left in the soluble fraction after 148 149 precipitation (unbound) with the precipitated fractions (pellet), we confirmed that Flag-NSP2 was not recovered in the pellet whereas a significant proportion of endogenous GIGYF2 was selectively enriched in 150 the b-isox precipitate, alongside DDX6 (Supplementary Figure S1F). Since NSP2 could not be precipitated by 151 152 b-isox along with GIGYF2, we concluded that it only binds the diffuse form of GIGYF2 in the cytoplasm.

We next investigated which part of NSP2 binds the 4EHP-GIGYF2 complex. For this purpose, we 153 154 generated a collection of vectors encoding Flag-tagged truncated versions of NSP2 which were expressed in HEK293T for Flag IP (Figures 1D and 1E). Incremental terminal deletions revealed that the N-terminal half 155 of NSP2 is required for the maximal co-IP of the endogenous 4EHP-GIGYF2 complex as well as ZNF598. 156 While weakly expressed, the NSP2¹⁻³⁵⁰ fragment remained the minimal segment which was able to bind 4EHP-157 GIGYF2. Interestingly, deleting either the N-terminal extremity (fragment NSP2¹⁰⁷⁻³⁵⁰) or the middle region 158 (fragment NSP2¹⁻²¹²) impaired the NPS2/4EHP-GIGYF2 interaction, indicating a large interaction surface 159 between 4EHP-GIGYF2 and an intact N-terminal half of NSP2 (Figure 1E). 160

161 The NSP2/GIGYF2-4EHP interaction involves multiple binding sites

We then sought to delineate which part of the 4EHP-GIGYF2 complex is targeted by NSP2. Human 162 GIGYF2 is a 150 kDa scaffolding protein composed of several domains interspaced by intrinsically disordered 163 regions. These include the 4EHP-binding domain at the N-terminus, the so-called GYF domain, a putative 164 165 Single Alpha-Helix (SAH) and many glutamine-rich stretches (polyQ) at the C-terminus (Figure 2A) (Peter et al., 2017, Suveges et al., 2009, Morita et al., 2012, Kofler and Freund, 2006). V5-tagged fragments of GIGYF2 166 167 were designed to isolate these features, and expressed along with Flag-NSP2 for Flag IP (Figures 2A and 2B). Our WB analysis of the IP fractions revealed that NSP2 binds two distinct segments of GIGYF2, namely the 168 169 central putative SAH region (residues: 743-1,085), and to a smaller extent, the N-terminal extremity containing the 4EHP-binding motif (residues: 1-267; Figure 2B). The strong interaction between NSP2 and GIGYF2⁷⁴³⁻ 170 ^{1,085} prompted us to test their direct association. For this, full-length hexahistidine (His₆)-tagged NSP2 and 171 Glutathione S-transferase (GST)-fused GIGYF2^{743-1,085} were recombinantly expressed in *Escherichia coli* (E. 172 coli) and individually purified to perform a His pull-down assay. Because the untagged GIGYF2743-1,085 173 fragment appeared to be unstable following our two-step purification process, we tested whether this 174 recombinant protein could be recognized by an anti-GIGYF2 antibody raised against the 756-1,104 region. 175 Our Western blot assay confirmed that a predominant ~42-kDa band corresponding to an intact GIGYF2⁷⁴³⁻ 176 ^{1,085} is specifically detected by the anti-GIGYF2 antibody (Supplementary Figure S2A). Following incubation 177 of His6-NSP2 with untagged GIGYF2743-1,085 on a Ni-NTA resin, analysis of the eluates by SDS-PAGE and 178 Coomassie blue staining revealed a specific retention of GIGYF2^{743-1,085} with His₆-NSP2, confirming their 179 direct interaction in vitro (Figure 2C). Western blot analysis of these samples confirmed that the retained band 180 181 is specifically recognized by the anti-GIGYF2 antibody (Supplementary Figure S2B). GST alone was used as control and did not show any pull-down by His₆-NSP2, supporting the specificity of the NSP2-GIGYF2 182 interaction. We also successfully detected this interaction when both His₆-NSP2 and GST-GIGYF2^{743-1,085} were 183 co-expressed in E. coli. In this case, a GST pull-down assay was performed using an E. coli lysate and a 184 specific His₆-NSP2 retention on GST-GIGYF2743-1,085-bound beads was detected by both Coomassie blue 185 staining and Western blot (Supplementary Figure S2C). Similarly, we sought to test the capacity of His₆-NSP2 186 to bind the N-terminal region of GIGYF2 (residues: 1-267), but failed to obtain this recombinant GIGYF2 187 188 fragment with a sufficient yield and solubility (data not shown).

189 Our co-IP experiments pointed out the 1-350 region of NSP2 as the minimal segment required to bind GIGYF2 in cellulo (Figure 1E). In attempt to narrow down this region, we generated three recombinant His₆-190 tagged fragments of NSP2 covering the 1-350 region (residues: 1-115, 107-212 and 204-350) to test their direct 191 interaction with GIGYF2743-1,085. Using a His pull-down assay, we found that none of these fragments 192 successfully retained GIGYF2^{743-1,085}, with the exception of full-length His₆-NSP2 (Supplementary Figure 193 S2D), confirming a large interaction surface between NSP2 and GIGYF2. Since an interaction was detected 194 between NSP2 and the 4EHP-binding region of GIGYF2 (residues: 1-267) by co-IP, we also speculated that 195 4EHP could bind NSP2 independently of GIGYF2. To test this, we used a V5-tagged version of 4EHP carrying 196 197 the W95A substitution which is known to disrupt its interaction with GIGYF2 (Peter et al., 2017). HEK293T cells were co-transfected by vectors encoding Flag-NSP2 and V5-4EHP^{W95A}, or its WT counterpart. Using 198 Flag IPs, we found that both WT and W95A versions of 4EHP were co-immunoprecipitated by NSP2 to a 199 comparable extent (Figure 2D), indicating that 4EHP can bind NSP2 independently of GIGYF2. This result 200 prompted us to evaluate their direct association in vitro. As performed with GIGYF2743-1,085, full-length GST-201 fused 4EHP was expressed in E. coli, and the purified untagged protein was incubated with His₆-NSP2 to 202 perform a His pull-down assay. In agreement with our co-IP results, we observed a specific binding of 4EHP 203 204 to His₆-NSP2, thus confirming their interaction in a GIGYF2-independent manner (Supplementary Figure S2E). Conversely, we found that NSP2 can simultaneously bind both 4EHP and GIGYF2^{743-1,085} since the latter 205 two were retained in our His₆-NSP2 pull-down when all three recombinant proteins were incubated at the same 206 time (Figure 2E). 207

208 Gupta et al. recently reported that a natural variant of NSP2 carrying the G262V/G265V substitutions 209 showed a reduced interaction with 4EHP-GIGYF2 by affinity purification mass spectrometry (Gupta et al., 2021). We therefore tested whether these G to V substitutions could impact the integrity of the NSP2/4EHP-210 GIGYF2 complex by co-IP and *in vitro* interaction assays. Our co-IP experiment with a Flag-tagged NSP2 211 harboring the G262V/G265V variation confirmed that substituting G262 and G265 with valines reduced its 212 interaction with endogenous GIGYF2, 4EHP and ZNF598 (Supplementary Figure S3A). By contrast, when 213 214 introduced in the His6-NSP2 recombinant protein, these two substitutions did not change the capacity of NSP2 to retain both recombinant 4EHP and GIGYF2^{743-1,085} in a His pull-down assay (Supplementary Figure S3B). 215 Nevertheless, it is worth noting that the direct contribution of the G262 and G265 residues of SARS-CoV-2 216

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NSP2 in binding 4EHP-GIGYF2 remains unclear since both are not found in SARS-CoV-1 and MERS-CoV
while the NSP2/4EHP-GIGYF2 interaction is detected across these two β-CoVs (Supplementary Figure S3C).
Overall, these data suggest that the mode of interaction between NSP2 and the intact 4EHP-GIGYF2 complex
involves multiple interaction interfaces, and may be more sophisticated *in cellulo* than in our *in vitro* binding
assays.

222 NSP2 reduces the silencing capacity of GIGYF2

223 The role of 4EHP-GIGYF2 as a translational repressor has been described in various contexts (for 224 review, see (Christie and Igreja, 2021)). Having shown that NSP2 directly targets the 4EHP-GIGYF2 complex 225 through at least two contact points, namely the region 743-1,085 of GIGYF2 and 4EHP itself, we wished to assess whether the silencing capacity of 4EHP-GIGYF2 could be altered by NSP2. To address this point, we 226 used the λ N-BoxB tethering approach in HEK293T cells (Baron-Benhamou et al., 2004, Pillai et al., 2004). A 227 Renilla luciferase reporter mRNA containing five BoxB sequences (RLuc-5BoxB) in the 3' untranslated region 228 (3'UTR) was co-expressed with a plasmid expressing V5-tagged GIGYF2 fused to a λN peptide, which has a 229 high affinity for the BoxB sequences (Figure 3A). The silencing capacity of GIGYF2 was assessed following 230 231 the measurement of luciferase activity in HEK293T expressing Flag-NSP2 or Flag as control. The expression of Flag-NSP2 and λ N-GIGYF2 was also verified by WB (Figure 3C). As expected, we observed that the 232 recruitment of GIGYF2 to the 3' UTR markedly reduced luciferase activity in control cells (5-fold repression). 233 Interestingly, this λN -GIGYF2-mediated repression was reduced upon expression of Flag-NSP2 (3-fold 234 repression), indicating an inhibitory effect of NSP2 on GIGYF2 function in silencing (Figure 3A). 235

Several studies have reported that GIGYF2 has two distinct mechanisms of repression: one is 4EHP-236 dependent and affects translation; the other is 4EHP-independent and involves the deadenylase activity of the 237 CCR4-NOT complex (Amaya Ramirez et al., 2018). Our co-IP data showed that NSP2 did not interact with 238 CCR4-NOT (Figure 1A), suggesting that it should preferentially impair the 4EHP-dependent activity of 239 240 GIGYF2. To test this, λ N-GIGYF2 was tethered to a RLuc-5BoxB mRNA containing a self-cleaving hammerhead ribozyme (HhR) at the 3'-end to generate a poly(A) stretch of 114 nucleotides, followed by 40 241 242 nucleotides to block CCR4-NOT-dependent deadenylation (RLuc-5BoxB-A₁₁₄-N₄₀-HhR), as previously described (Chapat et al., 2017b). Tethering GIGYF2 to RLuc-5BoxB-A₁₁₄-N₄₀-HhR induced a 2.2-fold 243 repression of this reporter in cells transfected with a control vector (Figure 3B). Due to its inability to encounter 244

mRNA deadenylation, the silencing magnitude of this reporter remained ~40% lesser than the one induced
when GIGYF2 is tethered to RLuc-5BoxB. By contrast, we observed that NSP2 expression decreased the
GIGYF2-mediated repression of the reporter to 1.4-fold (Figure 3B), indicating a specific role of NSP2 in
blocking the deadenylation-independent silencing capacity of GIGYF2.

249 NSP2 impairs the contribution of 4EHP-GIGYF2 into miRNA-mediated silencing

250 The activity of the 4EHP-GIGYF2 complex is required for the optimal translation repression driven by several pathways in a large range of cellular processes (for review, see (Christie and Igreja, 2021)). In 251 particular, the miRNA-induced translational repression is effected by the 4EHP-GIGYF2 complex in human 252 cells (Chen and Gao, 2017, Chapat et al., 2017b, Schopp et al., 2017). To evaluate the impact of NSP2 in 253 254 miRNA-mediated silencing, we transiently transfected HEK293T cells with a Renilla luciferase construct either lacking (RLuc), or containing six bulged let7a miRNA binding sites in its 3' UTR (RLuc-6let7a), 255 together with a firefly luciferase construct (FLuc) as a transfection control (Figure 4A). Normalized RLuc 256 activity was markedly reduced by the presence of *let7a*-binding sites, with ~40-fold repression in cells 257 expressing a control vector. By contrast, expression of NSP2 resulted in a decreased repression magnitude of 258 259 the RLuc-*6let7a* reporter compared to RLuc (\sim 30-fold), indicating a reduced *let7a*-mediated silencing in the presence of NSP2 (Figure 4A). 260

Evidence suggests that the 4EHP-GIGYF2 complex contributes to miRNA action through the binding 261 of GIGYF2 to a proline-rich motif (PPGL) in the silencing domain (SD) of GW182, the scaffolding protein of 262 miRISC (Schopp et al., 2017). To investigate whether NSP2 could impair GW182-mediated silencing, the 263 264 silencing domain of GW182 was artificially tethered to the RLuc-5BoxB reporter in control and Flag-NSP2expressing cells. We found that tethering GW182^{SD} induced a ~40-fold repression of the RLuc-5BoxB level 265 in the control cells, while this silencing effect was reduced in NSP2-expressing cells (~30-fold; Figure 4B). 266 As a control, a GW182^{SD} mutant carrying a deletion of the GIGYF2-binding motif ($\Delta PPGL$) was used to 267 evaluate the contribution of GIGYF2. In control cells, tethering the Δ PPGL mutant of GW182^{SD} engendered 268 a silencing of RLuc-5BoxB that is \sim 35% lesser than the one induced by tethering WT GW182^{SD}, confirming 269 the importance of the GIGYF2/PPGL interaction for the GW182-mediated silencing. Tethering this Δ PPGL 270 mutant in cells expressing Flag-NSP2 showed that its silencing activity is not affected by NSP2, indicating 271 that NSP2 action on GW182^{SD}-mediated silencing is exerted through targeting GIGYF2 (Figure 4B). 272

Tethering GW182^{SD} to an mRNA is known to lead to translational repression, deadenylation and 273 degradation of the target mRNA, while 4EHP-GIGYF2 was only shown to participate to translational 274 repression (Jonas and Izaurralde, 2015, Schopp et al., 2017). To uncouple 4EHP-GIGYF2-driven translational 275 repression from mRNA destabilization effects, GW182^{SD} was tethered to the RLuc-5BoxB-A₁₁₄-N₄₀-HhR 276 reporter in control and Flag-NSP2-expressing cells. In this case, the GW182^{SD}-mediated repression was 277 reduced to ~12-fold in control cells due to the inability of RLuc-5BoxB-A₁₁₄-N₄₀-HhR to encounter mRNA 278 deadenylation, and to ~8-fold when the \triangle PPGL mutant was used (Figure 4C). Upon NSP2 expression, the 279 silencing activity of GW182^{SD} was decreased to ~8-fold but remains unchanged with its Δ PPGL version, 280 confirming a specific alteration of the 4EHP-GIGYF2-driven translational repression by NSP2. Altogether, 281 these results indicate that the GIGYF2-dependent repressive activity of GW182^{SD} is partially altered upon 282 expression of NSP2 in human cells. 283

284

285 **DISCUSSION**

Upon infection, SARS-CoV-2 impairs splicing, export, translation and degradation of host mRNAs 286 (Finkel et al., 2021, Banerjee et al., 2020). Here, we present evidence to support a new layer of complexity in 287 the post-transcriptional alteration of the host transcriptome by SARS-CoV-2. We propose that SARS-CoV-2 288 289 NSP2 directly targets the 4EHP-GIGYF2 complex to decrease its silencing capacity (Figure 4D). While our model lacks the context of other viral proteins that would be present in a bona fide infection, this mechanism 290 could nonetheless unveil the impact of NSP2 on the post-transcriptional silencing of gene expression of human 291 cells, pointing out 4EHP-GIGYF2 targeting as a possible strategy of SARS-CoV-2 to take over the silencing 292 machinery and to suppress host defenses. Further studies in a more physiological context, such as lung/airway 293 cell lines or SARS-CoV-2 infected samples, should help resolve this conundrum. 294

How does NSP2 impair 4EHP-GIGYF2 function? Combining co-IP experiments and *in vitro* binding assays with recombinant proteins, we concluded that NSP2 uses its N-terminal region encompassing its conserved zinc finger domain, to interact with the 4EHP-GIGYF2 complex. Our pull-down assays indicate the direct interaction of NSP2 with both 4EHP and two domains from GIGYF2, confirming a sophisticated mode of binding *in cellulo*. While we searched for the minimal region of NSP2 required for these interactions, we

300 failed to narrow down a fragment smaller than the 1-350 region since truncations at both extremities of this domain abrogate its binding to 4EHP-GIGYF2 (Figures 1E and S2D). Nevertheless, this remains in agreement 301 with Gupta et al. who pointed out that the G262V and G265V mutations located within this region of NSP2 302 303 reduced binding to 4EHP-GIGYF2 (Gupta et al., 2021). This natural variation occurs in a poorly conserved patch in NSP2 that is subsequently becoming more hydrophobic due to the G to V substitution. It is worth 304 305 noting that G262 and G265 are not conserved across the SARS-CoV-1 and MERS-CoV (Supplementary Figure S3C), while the NSP2/4EHP-GIGYF2 interaction exists among these viruses. The direct contribution of the 306 307 G262/G265 residues of NSP2 in binding 4EHP-GIGYF2 is therefore questionable. It is tempting to speculate 308 that the G to V variation could rather increase NSP2's affinity for host interactors that outcompete 4EHP-309 GIGYF2. This point is supported by the affinity purification mass spectrometry made by Gupta et al., showing that the G262V/G265V variation increases the affinity of NSP2 for factors such as the mitochondrial protein 310 UQCRC1, or the actin-nucleation-promoting protein WASHC5 (Gupta et al., 2021). This could explain why 311 the G262V/G265V variation reduces the NSP2/4EHP-GIGYF2 interaction in cellulo, but not in our in vitro 312 pull-down assay lacking the context of other host interactors of NSP2. Further investigation will be needed to 313 fully resolve the structural basis of the NSP2/4EHP-GIGYF2 complex and thus elucidate NSP2 action on 314 315 translation silencing. In particular, it now remains to be determined whether NSP2 binding induces conformational changes in 4EHP-GIGYF2 that impair either the cap-binding pocket of 4EHP, or influence the 316 recruitment of GIGYF2's co-factors such as CCR4-NOT and DDX6. 317

Global measurement of miRNA action showed that translational repression accounts for 6-26% of the 318 silencing of each mRNA target in mammalian cells, and 4EHP-GIGYF2-mediated translational repression is 319 320 observed at early time points of the silencing process (Schopp et al., 2017, Eichhorn et al., 2014). Consistent with these observations, the impact of NSP2 remains mild on the *let7a*-targeted RLuc reporter (Figure 4A). 321 322 The latter is also known to underestimate the contribution of the translational repression to the silencing process since mRNA destabilization is the dominant effect of miRNA-mediated silencing at steady state 323 (Eichhorn et al., 2014, Bethune et al., 2012). Our tethering assays with GW182^{SD} have proven helpful to 324 overcome this limitation. The derepression of the GW182^{SD}-induced silencing of RLuc-5BoxB-A₁₁₄-N₄₀-HhR 325 upon deletion of the PPGL motif supports the fact that our tethering assay faithfully recapitulates the 326 contribution of GIGYF2 into miRNA-induced translation repression. Consistently, these tethering assays 327

showed that the silencing capacity of $GW182^{SD}$ upon NSP2 expression equals the one of its $\Delta PPGL$ version in control cells (Figures 4B and 4C), indicating that the contribution of 4EHP-GIGYF2 into $GW182^{SD}$ mediated translation repression is fully targeted by NSP2. Indeed, this impact of NSP2 will need to be investigated in further more physiological studies using endogenous miRNAs and transcripts.

At the moment it is uncertain what the functional interplay between SARS-CoV-2 infection and 332 miRNA-mediated silencing is in human cells. Host miRNAs are known to be produced as a part of antiviral 333 response to counteract the infection by targeting viral transcripts, although SARS-CoV-2 infection was 334 recently shown to have minimal impact on the miRNA repertoire of its host cell (Cullen, 2006, Bruscella et 335 336 al., 2017, Pawlica et al., 2021). Computational analyses have predicted the presence of many putative miRNAbinding sites on the SARS-CoV-2 genome, suggesting that the SARS-CoV-2 genome could be actively 337 targeted by host miRNAs (Xie et al., 2021, Arisan et al., 2020, Chow and Salmena, 2020). Particularly worth 338 mentioning is the work of Xie et al. which recently identified let-7 binding sites in the coding sequence of S 339 340 and M proteins of SARS-CoV-2 genome, and experimentally confirmed that let-7 blocks SARS-CoV-2 replication by targeting S and M proteins (Xie et al., 2021). Through the NSP2/4EHP-GIGYF2 axis, SARS-341 CoV-2 could therefore escape from the host defense system by impairing the function of the effector machinery 342 of miRNAs. The recent discovery that 4EHP and GIGYF2 are needed for infection by SARS-CoV-2 could 343 reinforce this idea, although further research will be required to test this possibility (Hoffmann et al., 2021). 344

345 While the silencing capacity of miRISC is partially impeded upon NSP2 expression, there is no guarantee that miRNA action is the prime target of NSP2. The activity of 4EHP-GIGYF2 is mobilized by 346 several pathways, one of which may be more affected than miRNAs. These pathways include TTP- and 347 ZNF598-mediated mRNA silencing, as well as the repression of mRNAs with altered ribosome activity or 348 349 premature termination codons as part of the nonsense-mediated mRNA decay pathway (Christie and Igreja, 2021). Future studies are thus mandatory to evaluate the potential impact of NSP2 in modulating these 350 351 processes in human cells. In the case of miRNA, the alteration of *let-7a*-mediated inhibition by NSP2 could 352 be extrapolated to other miRNAs whose action relies on 4EHP, such as miR-145 or miR-34a (Jafarnejad et al., 353 2018, Zhang et al., 2021). Recent evidence demonstrated that the 4EHP/miR-34a axis is required for the translational repression of mRNAs encoding IFN-β through targeting the 3'UTR of *Ifnb1* mRNA (Zhang et 354 al., 2021). Beyond miRNA, 4EHP-GIGYF2 also controls the production of TTP-targeted mRNAs that encode 355

356 inflammatory cytokines such as TNF-α and IL-8 (Morita et al., 2012, Fu et al., 2016, Villaescusa et al., 2009, Cho et al., 2006, Cho et al., 2005). In this context, a possible consequence of NSP2 expression could be the 357 overproduction of early response pro-inflammatory cytokines. Exploring this point would be of utmost 358 359 importance since impaired type I interferon activity and inflammatory responses are detected in severe 360 COVID-19 patients (Hadjadj et al., 2020). To examine whether NSP2 could impact the function of 4EHP in regulating IFN- β expression, we expressed NSP2 along with a reporter construct containing the 3' UTR 361 of *Ifnb1* mRNA into HEK293T cells (Supplementary Figure S4A). Remarkably, the reporter expression was 362 repressed ~ 2.9 -fold in control cells, but only ~ 1.6 -fold in NSP2-expressing cells, indicating that NSP2 could 363 potentially unbalance the production of IFN- β through the *Ifnb1* 3' UTR (Supplementary Figure S4B). With 364 this in mind, further investigations into whether the NSP2/4EHP-GIGYF2 axis can dysregulate sustained 365 cytokine production may therefore prove useful. 366

In conclusion, our study raises the possibility that SARS-CoV-2 could target the human 4EHP-GIGYF2 complex to selectively modulate its capacity to effect translation repression. Our model may represent a novel framework to investigate the mechanisms underlying the pathogenicity of SARS-CoV-2 based on the interaction of NSP2 with 4EHP-GIGYF2. Ultimately, we hope that this study will be a primer for further more physiological research to evaluate the generalizability of our model.

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LIMITATIONS OF THE STUDY

Although our work depicts how the 4EHP-GIGYF2 complex is compromised by the SARS-CoV-2 373 protein NSP2, it is not without limitations. The molecular mechanism described in this article comes from *in* 374 vitro studies using the HEK293T cells, which were chosen to dissect the NSP2/4EHP-GIGYF2 interaction on 375 the basis of their capacity to express both NSP2 and 4EHP-GIGYF2 at a high level. However, our model will 376 377 need to be validated in a more physiological context, such as lung/airway cell lines or SARS-CoV-2 infected samples, to assess the functional relevance of this interaction. In this sense, whether other SARS-CoV-2 378 379 proteins interact with NSP2 and thereby prevent its interaction with GIGYF2/4EHP cannot be dismissed. Our conclusion that NSP2 impairs miRNA action is mainly based on the use of artificial reporters (miRNA and 380 tethering assays). To date, these assays have proven invaluable in dissecting the functions of numerous 381 silencing factors in human cells. Nevertheless, our data will need to be complemented by follow-up studies to 382

investigate the impact of NSP2 on endogenous transcripts. Future investigations that employ transcriptomic
 analysis of the NSP2-mediated post-transcriptional alterations will undoubtedly extend our view on the
 repertoire of endogenous miRNA/mRNA pairs which are affected by NSP2. Another potential limitation of
 our study is that we did not explore potential mechanisms for NSP2-mediated inhibition of 4EHP-GIGYF2
 and how they could cause the pathogenicity of SARS-CoV-2. Future studies will be needed to dissect the
 molecular basis of this mechanism using additional models of coronavirus infection.

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390 AUTHORS CONTRIBUTIONS

L.Z., C.M. and C.C. performed the experiments. L.Z., M.G. and C.C. designed research and analyzed the
experiments, and L.Z., C.M., M.G. and C.C. wrote the paper.

393

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408 **FIGURE LEGENDS**

409 Figure 1. NSP2 interacts with the 4EHP-GIGYF2 complex in the cytoplasm.

- 410 (A) Co-Immunoprecipitation (co-IP) between Flag-NSP2 and endogenous 4EHP-GIGYF2. A tetracycline-
- 411 inducible Flag-NSP2 construct was stably transfected in HEK293 Flp-In T-REx. Extracts from untransfected
- 412 (-), non-induced (NI) or tetracycline-induced (I) cells were immunoprecipitated with anti-Flag antibody. Total
- 413 lysates (input) and IP samples were analyzed by Western blot with the indicated antibodies.
- (B) Flag-NSP2 IP in 4EHP^{KO} or GIGYF2^{KO} cells. Vectors encoding Flag-NSP2, or Flag as control, were
 transiently transfected in the Wild-Type (WT), 4EHP^{KO} or GIGYF2^{KO} HEK293 cells, and Flag IPs were
 performed in RNase A-treated extracts, followed by Western blot with the indicated antibodies.
- 417 (C) Proximity Ligation Assay between Flag-NSP2 and endogenous GIGYF2. HEK293T cells were transfected
 418 with vector expressing Flag-NSP2, or Flag as control. PLA was performed using anti-Flag and anti-GIGYF2
 419 antibodies. Representative images of PLA (red), along with GIGYF2 immunofluorescence (IF; green) and
 420 DAPI (blue) are shown. Z-projection of 3 stacks (0.35 μm each). Scale bars, 5 μm.
- 421 (D) Representation of the NSP2 protein structure (PDB : 7MSW (Gupta et al., 2021)) and schematic cartoon
 422 of Flag-tagged NSP2 truncations used in panel (e).
- (E) The N-terminal half of NSP2 binds 4EHP-GIGYF2. The indicated constructs were expressed in HEK293T
 cells and Flag IPs were performed with RNase A-treated extracts. The starting material (Input) and bound
 fractions were analyzed by Western blot. EV: empty vector; FL: Full length.

426 Figure 2. The NSP2/GIGYF2-4EHP interaction involves multiple binding sites

- (A) Schematic cartoon of the V5-tagged GIGYF2 fragments used in panel (B). 4EHP-BM: 4EHP-binding
 motif; GYF: glycine-tyrosine-phenylalanine domain; SAH: putative single alpha helix; polyQ: glutamine-rich
 stretches.
- 430 (B) Western blot showing the interaction between Flag-NSP2 and two regions in GIGYF2. Vectors expressing
- 431 Flag-NSP2 and the indicated fragments of V5-tagged GIGYF2 were transiently transfected in HEK293T to
- 432 perform Flag IP. Extracts were RNase A-treated. Empty vectors were used as negative controls (-). FL: Full
- 433 length GIGYF2.

(D) Western blot showing the interaction between Flag-NSP2 and V5-4EHP in a GIGYF2-independent
manner. RNase A-treated extracts from cells expressing Flag-NSP2 along with V5-4EHP, WT or carrying the
W95A substitution (Mut), were used for Flag IP. Inputs and bound fractions were analyzed by Western blotting
using the indicated antibodies. Empty vectors served as negative controls (-).

(E) Ni-NTA pull-down assay showing the simultaneous interactions between recombinant His₆-NSP2 and both
 untagged 4EHP and GIGYF2^{743-1,085}. Incubations were performed with the indicated recombinant proteins. The
 starting material (Input) and bound (Ni-NTA pull-down) fractions were analyzed by SDS-PAGE followed by
 Coomassie blue staining.

445 Figure 3. NSP2 decreases the silencing capacities of GIGYF2 in cellulo

(A) Artificial tethering of GIGYF2 to the 3' UTR of a reporter mRNA. The upper panel shows a schematic of the λ N/BoxB tethering assay with the RLuc-5boxB reporter construct. Recruitment of GIGYF2 to the Renilla Luciferase (RLuc) mRNA was mediated by the fused λ N peptide. RLuc luminescence was normalized against Firefly luciferase (FLuc) level, and repression fold was calculated by dividing the relative luciferase activity of the cells transfected with the control pCI- λ NV5 vector (λ NV5) by the luciferase activity of λ N-GIGYF2 expressing cells. The mean values (±SD) from three independent experiments are shown and the *P* value was determined by two-tailed Student's t-test: (***) P < 0.001.

(B) Artificial tethering of GIGYF2 to the 3' UTR of a reporter mRNA which is refractory to deadenylation. The upper panel shows a schematic of the RLuc-5boxB-A₁₁₄-N₄₀-HhR reporter. HEK293T cells were cotransfected with vectors expressing either λ NV5-GIGYF2, or λ NV5 as a control, along with RLuc-5boxB-A₁₁₄-N₄₀-HhR and FLuc. Vectors encoding Flag-NSP2 or Flag (empty vector) were also added in the transfection mixture. RLuc luminescence was normalized against the FLuc level and analyzed as in (A).

458 (C) Extracts from the HEK293T cells used in (A) and (B) were analyzed by Western blot with the indicated459 antibodies. GAPDH was used as a loading control.

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460 Figure 4. NSP2 impairs miRNA-mediated silencing

461(A) NSP2 decreases *let7a*-mediated silencing. The upper panel shows a schematic of the RLuc-*6let7a* reporter462mRNA. HEK293T cells were co-transfected with RLuc or RLuc-*6let7a* plasmids, along with FLuc construct463to account for variations in transfection efficiency. Vectors encoding Flag-NSP2 or Flag (empty vector) were464also added in the transfection mixture. Repression fold was calculated by dividing the relative luciferase465activity of the cells transfected with the RLuc vector by the luciferase activity of RLuc-*6let7a* expressing cells.466Error bars indicate \pm SD (n = 3). (**) P < 0.01 (two-tailed Student's t-test).

467(B) Artificial tethering of GW182^{SD} to the 3' UTR of a reporter mRNA. The upper panel shows a schematic468of the λ N/BoxB tethering assay with the RLuc-5boxB reporter construct. HEK293T cells were co-transfected469with vectors expressing either λ NV5- GW182^{SD}, WT or a Δ PPGL mutant (Mut), or λ NV5 as a control, along470with RLuc-5boxB and FLuc. Vectors encoding Flag-NSP2 or Flag (empty vector) were also added in the471transfection mixture. RLuc luminescence was normalized against the FLuc level. The mean values (±SD) from472three independent experiments are shown and the *P* value was determined by two-tailed Student's t-test: (ns)473non-significant, (***) *P* < 0.001.</td>

474 (C) Artificial tethering of GW182^{SD} to the 3' UTR of a reporter mRNA which is refractory to deadenylation. 475 The upper panel shows a schematic of the RLuc-5boxB-A₁₁₄-N₄₀-HhR reporter. Transfections were performed 476 as in (b), except that GW182^{SD} was tethered on the RL-5*boxB*-A₁₁₄-N₄₀-HhR reporter. Data are presented as 477 mean \pm SD (n = 3). (***) *P* < 0.001 (two-tailed Student's t-test).

(D) Model of NSP2-mediated negative regulation of miRNA function. In absence of NSP2 (left panel),
miRISC recruits the 4EHP-GIGYF2 complex to effect translational silencing of the targeted mRNA. Following
NSP2 expression (right panel), the function of 4EHP-GIGYF2 is physically targeted by NSP2 and its silencing
capacity is impaired. The assembly of this complex subsequently alters the magnitude of miRNA-induced
silencing.

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486 STAR★Methods

487 **Resource availability**

- 488 Lead contact
- 489 Further information and requests for resources and reagents should be directed to and will be fulfilled by the
- 490 lead contact, Clément Chapat (clement.chapat@cnrs.fr).

491 Materials availability

492 All newly created cell populations generated in this study are available upon request.

493 **Data and code availability**

- All data reported in this paper will be shared by the lead contact upon request.
 - This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the
 lead contact upon request.
- 498 Experimental Model and Subject Details

499 Cell lines and culture

HEK293T cells (Sigma-Aldrich) were routinely maintained in high glucose Dulbecco's Modified 500 501 Eagle's Medium (DMEM) with GlutaMAX supplemented with 10 % fetal bovine serum (FBS) and 2 % penicillin/streptomycin in a humidified atmosphere of 5 % CO2 at 37 °C. Flp-In T-REx 293 cells (Thermo 502 Fisher Scientific) were grown in similar conditions supplemented with 100 µg/ml zeocin and 503 15 µg/ml blasticidin. The absence of mycoplasma contamination in cells was routinely tested. The cell line 504 inducibly expressing Flag-NSP2 was generated by co-transfecting pcDNA5-FRT-TO-FH-Nsp2 (Addgene 505 506 plasmid 157683) and pOG44 (Thermo Fisher Scientific) with a 1:10 ratio. Transfected cells were selected and maintained in media supplemented with 100 µg/ml hygromycin. Expression of Flag-tagged proteins was 507 induced for 24 h by addition of tetracycline to 1 µg/mL final concentration. 508

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510 Method details

511 CRISPR/cas9-mediated genome editing

CRISPR-Cas9-mediated genome editing of HEK293 cells was performed according to Ran et al. The following oligonucleotides encoding a small guide RNA cognate to the coding region of Gigyf2 gene were 513 used: 5'-CACCGGGAGGAACCCCTTCCACCAT and 5'-AAACATGGTGGAAGGGGTTCCTCCC. These 514 515 oligos which contain BbsI restriction sites were annealed creating overhangs for cloning of the guide sequence oligos into pSpCas9(BB)-2A-Puro (PX459) V2.0 (Addgene plasmid 62988) by BbsI digestion. To generate 516 KO HEK293 cells, we transfected 700,000 cells with the pSpCas9(BB)-2A-Puro plasmid. 24 hr after 517 transfection, puromycin was added in the cell medium to 1.5 µg/mL final concentration. After 72 h, puromycin-518 resistant cells were isolated into 96-well plates to obtain monoclonal colonies. Clonal cell populations were 519 analyzed by WB for protein depletion. 520

Plasmid cloning 521

For Flag-NSP2 IP, pcDNA5-FRT-TO-FH-Nsp2 (Addgene plasmid 157683) was used. Truncated 522 versions of NSP2 were generated by replacing the sequence encoding full-length NSP2 by PCR-amplified 523 524 fragments at the BamHI-XhoI sites of the pcDNA5-FRT-TO-FH vector. The full-length cDNAs encompassing the coding region of human 4EHP and GIGYF2 were obtained by RT-PCR using total RNA from HEK293T 525 cells and cloned into the pCI-Neo vector (Promega) at the XhoI-NotI sites in frame with a sequence encoding 526 a V5 tag inserted at the NheI-XhoI sites. To generate the pCI- λ NV5-GIGYF2 vector, a fragment containing 527 528 the GIGYF2 sequence was obtained by PCR and inserted into the pCI- λ NV5 at the XhoI-NotI sites. A similar strategy was used to generate the V5-tagged fragments of GIGYF2 using primers to isolate the following 529 domains encompassing residues: 1-267; 258-495; 486-752; 743-1,085; 1,076-1,320. To construct the pCI-530 λ NV5-GW182^{SD} vector, a fragment encoding the residues 1382-1690 was cloned by PCR as a XhoI-NotI 531 fragment from the pFRT/TO/FLAG/HA-DEST TNRC6C vector (Addgene plasmid 19885) (Landthaler et al., 532 2008) and inserted into the pCI-\u03c0NV5 at the XhoI-NotI sites. For recombinant GST-fused protein expression, 533 full-length 4EHP or GIGYF2743-1,085 coding sequences contained in PCR-amplified BamHI-NotI or XhoI-NotI 534 fragments, respectively, were cloned into a pGEX-6P-1 vector (Amersham) in frame with the GST coding 535 536 sequence. The pET-28b vector (EMD Biosciences) was used to express NSP2 as a recombinant protein fused to an His₆ tag at the N-terminus. For this, PCR-amplified fragments encoding full-length NSP2 or regions 1-537 115, 107-212 and 204-350 were inserted at the XhoI-BamHI sites of pET-28b-His₆-ZZ (Barbarin-Bocahu and 538

- Graille, 2022). Amino acid substitutions and deletions were introduced by site-directed mutagenesis using theQuikChange Kit (Agilent). Sequences of the primers used are listed in the Table S1.
- 541 Extract Preparation and Immunoprecipitation

Cells were resuspended in a lysis buffer containing 20 mM HEPES-KOH, pH 7.5, 100 mM NaCl, 2.5 542 mM MgCl2, 0.5 % NP40, 0.25 % sodium deoxycholate, supplemented with complete EDTA-free Protease 543 inhibitor and phosphatase inhibitor Cocktails (Roche), and incubated for 20 min on ice. The lysate was clarified 544 by centrifugation at $10,000 \times g$ for 10 min at 4 °C. One milligram of extract was used for immunoprecipitation 545 with the indicated antibodies. Thirty microliters of pre-equilibrated Anti-FLAG L5 Magnetic Beads (Thermo 546 547 Fisher Scientific, A36798) and RNase A (Thermo Fisher Scientific) were added, and the mixtures were rotated overnight at 4 °C. Beads were washed five times with lysis buffer and directly resuspended in protein sample 548 549 buffer for Western blot analysis.

550 Western blot and antibodies

Proteins were separated by SDS-PAGE on 4-15 % TGX gradient gels (Bio-Rad) and transferred onto 551 PVDF membranes. The membranes were blocked in PBS containing 5 % non-fat milk and 0.1 % Tween 20 552 for 30 min at room temperature. Blots were probed with the following antibodies: rabbit anti-eIF4E2/4EHP 553 (Proteintech, 12227-1-AP), rabbit anti-DDX6 (Proteintech, 14632-1-AP), rabbit anti-CNOT9 (Proteintech, 554 22503-1-AP), mouse anti-Flag M2 (Sigma-Aldrich, F1804), rabbit anti-GIGYF2 (Proteintech, 24790-1-AP), 555 mouse anti-GAPDH (Proteintech, 60004-1-Ig), mouse anti-V5 tag (Thermo Fisher Scientific, R96025), rabbit 556 anti-ZNF598 (Thermo Fischer Scientific, 703601) and mouse anti-6xHis Tag (Thermo Fischer Scientific, 557 MA1-21315-HRP). 558

559 **Biotinylated isoxazole (b-isox)-mediated precipitation**

560 HEK293T cells were resuspended in lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 0.1% NP-561 40, 1 mM EDTA, 2.5 mM EGTA, 10% glycerol, 1 μ M DTT) supplemented with complete EDTA-free protease 562 inhibitor, and phosphatase inhibitors, and incubated for 20 min on ice. The lysate was clarified by 563 centrifugation at 10,000 × g for 10 min at 4 °C. 50 μ g of the sample were mixed with 100 μ g of b-isox (Sigma-564 Aldrich) and rotated at 4°C for 90 min. The incubated reaction was then centrifuged at 10,000 × g for 10 min 565 to pellet the precipitates. The pellet was washed twice in the lysis buffer and resuspended in protein sample

buffer for Western blot analysis. Proteins in the supernatant fractions were precipitated by addition of four volumes of cold acetone, incubated for 1 h at -20° C and centrifuged at $15,000 \times g$ for 10 min to pellet the precipitates. The pellet was resuspended in protein sample buffer.

569 **Immunofluorescence**

WT and GIGYF2^{KO} cells were grown on an 8-well glass slide (Millicell EZ, Merck Millipore Ltd.) 570 and transfected with 100 ng of vector expressing Flag-NSP2. Cells were then fixed in 4 % paraformaldehyde 571 for 20 min, washed twice in PBS, permeabilized in PBS containing 0.1 % Triton X100 and 22.5 mg/mL glycine 572 for 5 min. After one wash in PBS, cells were incubated with a blocking solution (PBS containing 0.1 % Tween, 573 574 22.5 mg/mL glycine, and 1 % Bovine Serum Albumin) for 30 min at room temperature. Primary antibodies diluted in the blocking solution were then added for 1 h at 37 °C. After three washes with PBS containing 0.1 575 % Tween (PBS-T), the appropriate secondary antibodies were added for 30 minutes at 37°C, then washed in 576 PBS-T and mounted on glass slides in a mounting solution containing DAPI (Fluoroshield, Sigma-Aldrich). 577 578 Fluorescence was visualized under a LEICA-SP8ST-WS confocal microscope. Intensity line scans were 579 performed using Fiji.

580

PLA and confocal microscopy scanning

PLA was performed using the Duolink kit (Sigma-Aldrich) according to manufacturer's instructions. 581 Transfected cells (100,000) were fixed in 4 % paraformaldehyde for 20 min, washed twice in PBS, 582 permeabilized in PBS containing 0.1% Triton X100 for 5 min and incubated with primary antibodies for 1 h 583 at 37 °C in a pre-heated humidity chamber. After two washes in PBS-T, cells were incubated with the 584 585 appropriate PLA probes for 1 h at 37°C then washed in PBS-T and the ligation solution was added on the coverslips and incubated for 30 min at 37°C. Finally, the amplification solution containing a DNA polymerase 586 587 was added and incubated with the cells for 100 min at 37°C. For simultaneous immunofluorescence labelling of GIGYF2, secondary antibody coupled with Alexa Fluor 488 (Thermo Fisher Scientific) was added in the 588 amplification solution. After final washes, the cells were mounted on glass slides in a mounting solution with 589 DAPI, and imaging was performed on a LEICA-SP8ST-WS confocal microscope. Quantification of PLA dots 590 591 per cell was performed using the Fiji software as previously described (Nissan and Parker, 2008). Briefly, the quantification was accomplished by setting a threshold mask with the Otsu Thresholding Filter. Using the 592 "Invert LUT" and "Analyze Particles" tools of Fiji, the PLA dots were automatically counted on the 593

594 595 thresholded images. For each cell population, ten pictures with at least 20 cells per picture were used to calculate the mean values. The "Counter cells" plugin was used to analyze the number of cells in each image.

596 Expression and purification of His₆-NSP2

His₆-NSP2 was expressed in E. coli BL21 (DE3) Gold (Agilent). Large-scale expression was done in 1 597 L of auto-inducible terrific broth media (ForMedium AIMTB0260) supplemented with kanamycin (50 µg/ml), 598 599 first at 37 °C for 3 h and then at 18 °C overnight. The cells were harvested by centrifugation at 4,000 rpm for 30 min and the pellets were resuspended in 30 ml of lysis buffer (50 mM HEPES pH 7.5, 300 mM NaCl, 10 600 µM ZnCl₂, 2 mM MgCl₂ and 20 mM Imidazole) supplemented with 1 protease inhibitor tablet (Roche), 0.5 601 mM PMSF and 30 µL benzonase nuclease (Millipore Sigma). The cells were lysed by sonication on ice and 602 603 the lysate clearance was performed by centrifugation at 20,000 g for 30 min. The supernatant was applied on 604 Ni-NTA resin pre-equilibrated with the lysis buffer, and incubated at 4 °C on a rotating wheel for 1 h, followed by a washing step with 30 mL of washing buffer (50 mM HEPES pH 7.5, 1 M NaCl and 10 µM ZnCl₂). His₆-605 NSP2 was eluted by the addition of 15 ml of elution buffer (50 mM Tris/HCl pH 8.0, 300 mM NaCl, 10 µM 606 607 ZnCl₂, 2 mM MgCl₂ and 300 mM Imidazole), followed by concentrating up to 10 ml by a 30 kDa cutoff 608 concentrator. The sample was then diluted to 50 mL using Heparin buffer A (50 mM Tris/HCl pH 8.0, 5 mM β-mercaptoethanol, 10 μM ZnCl₂), followed by loading on a 5 mL Heparin HP column (Cytiva) and eluted 609 using a NaCl linear gradient from 75 mM (7.5 % Heparin buffer B: 50 mM Tris/HCl pH 8.0, 1 M NaCl, 5 mM 610 611 β-mercaptoethanol, 10 μM ZnCl₂) to 1 M (100 % Heparin buffer B). The fractions containing His₆-NSP2 protein were collected and concentrated up to 5 ml, followed by sample injection on a Superdex 200 increase 612 10/300 size-exclusion column (Cytiva) with Gel filtration buffer (50 mM HEPES pH 7.5, 250 mM NaCl, 5 613 mM β -mercaptoethanol and 10 μ M ZnCl₂). The fractions containing His₆-NSP2 were collected and 614 concentrated. 615

616 Expression and purification of 4EHP and GIGYF2^{743-1,085}

Expression of GST-4EHP and GST-GIGYF2^{743-1,085} was carried out in *E. coli* BL21 (DE3) Codon+ (Agilent) in 1 L of auto-inducible terrific broth media (ForMedium AIMTB0260) supplemented with ampicillin at 100 μ g/mL and chloramphenicol at 25 μ g/mL. When the OD_{600 nm} reached 0.6–0.8, cultures were incubated at 20 °C for 20 h. Bacteria were harvested by centrifugation and resuspended in lysis buffer (20 mM

621	HEPES pH 7.5, 200 mM NaCl, 5 mM β -mercaptoethanol). Cell lysis was performed by sonication on ice.
622	After centrifugation for 30 min at 20,000 g, 4°C, clarified samples were transferred to batch-bind with
623	Glutathione SepharoseTM 4B (Cytiva) resin for ~1 hr at 4°C followed by a washing step with 30 mL of
624	washing buffer (20 mM HEPES pH 7.5, 1 M NaCl and 5 mM β -mercaptoethanol) supplemented with 10 mM
625	ATP. 3C protease digestion was performed overnight at 4 °C to remove GST. The untagged GIGYF2 ^{743-1,085}
626	domain was present in the flowthrough and further purified on an HiTrap S FF column (Cytiva) using a linear
627	gradient of 92.5% Hitrap buffer A (20 mM HEPES pH7.5, 5 mM β -mercaptoethanol) to 100% Hitrap buffer
628	B (20 mM HEPES pH 7.5, 1 M NaCl and 5 mM β -mercaptoethanol). The peak fractions corresponding to
629	4EHP and GIGYF2 ^{743-1,085} were pooled, concentrated and used for pull-down assays.

630 Ni–NTA pull-down assays

Pull-down experiments were performed by incubating 1 nmol of His₆-NSP2, FL or truncated, with 631 equimolar amount of untagged GIGYF2743-1,085 or 4EHP, or GST used as control. All proteins were free of 632 nucleic acids according to the OD_{280 nm}/OD_{260 nm} ratio. Binding buffer (25 mM HEPES pH 7.5, 200 mM NaCl, 633 634 50 mM imidazole, 10% glycerol and 0.1 % triton X-100) was added to a final volume of 60 µL. The reaction mixtures were incubated on ice for 1 h. After that, 10 μ L was withdrawn and used as an input fraction for 635 SDS–PAGE analysis. The remaining 50 µL were incubated at 4 °C for 2 h with 40 µg of HisPur Ni–NTA 636 magnetic beads (Thermo Scientific) pre-equilibrated in binding buffer, in a final volume of 200 µL. After 637 638 binding, beads were washed three times with 500 μ L of binding buffer. Bound proteins were eluted with 50 μ L of elution buffer (20 mM HEPES pH 7.5, 200 mM NaCl, 250 mM imidazole, 10 % glycerol and 0.1 % triton 639 X-100). Samples were resolved on SDS–PAGE and visualized by Coomassie blue staining. 640

641 Co-expression and GST pull-down between His₆-NSP2 and GST-GIGYF2^{743-1,085}

Full-length His₆-NSP2 and GST-GIGYF2^{743-1,085} proteins were co-expressed in BL21 (DE3) Gold *E. coli* (Agilent technologies). Small-scale expression was done in 5 ml of auto-inducible terrific broth media (ForMedium, AIMTB0260) supplemented with ampicillin (100 µg/ml) and kanamycin (50 µg/mL), first at 37°C for 3 h and then at 18°C overnight. The cells were harvested by centrifugation at 4,000 rpm for 30 min and the pellets were resuspended in 750 µl of lysis buffer (50 mM HEPES pH 7.5, 200mM NaCl, 5 mM βmercaptoethanol). The cells were lysed by sonication on ice and the lysate clearance was performed by centrifugation at 13,000 g for 30 min. The supernatant was applied on Glutathione SepharoseTM 4B resin

649 (Cytiva) pre-equilibrated with the lysis buffer, incubated at 4°C on a rotating wheel for 1 h, followed by a
650 washing step with 1 ml of lysis buffer (50 mM HEPES pH 7.5, 200mM NaCl, 5 mM β-mercaptoethanol).
651 Retained proteins were eluted by 50 µl of elution buffer (50 mM HEPES pH 7.5, 200mM NaCl, 5 mM β652 mercaptoethanol, 20mM GSH), followed by SDS-PAGE and Coomassie blue staining or Western blot.

653 Tethering and luciferase assays

Tethering assays were performed as previously described (Chapat et al., 2017b, Chapat et al., 2017a). 654 Briefly, HEK293T cells were transfected with 20 ng of RLuc-5BoxB (or RLuc-5BoxB-A₁₁₄-N₄₀-HhR), 5 ng 655 of FLuc, and 100 ng of λ N-fusion constructs per well in a 24-well plate by using Lipofectamine 2000 (Thermo 656 657 Scientific, 11668019) according to the manufacturer's instructions. 100 ng of vectors encoding Flag-NSP2 or Flag (empty vector) were also added in the transfection mixture. Cells were lysed 24 h after transfection and 658 luciferase activities were measured with the Dual-Luciferase Reporter Assay System (Promega) in a GloMax 659 20/20 luminometer (Promega). RL activity was normalized to the activity of co-expressed FL, and the 660 661 normalized RL values are shown as repression fold relative to the indicated control. For experiments with miRNA reporters, HEK293T were co-transfected in a 24-well plate with 100 ng of vectors encoding Flag-662 NSP2, or Flag (empty vector) as control, 20 ng of RLuc-*6let7a* and 5 ng of FLuc plasmid. For the reporter 663 containing the 3'UTR of Ifnb1, 20 ng of psiCHECK2-RLuc-Ifnb1 3' UTR reporter (Zhang et al., 2021), or 664 665 empty psiCHECK2 (Promega), was added along with 100 ng of vectors encoding Flag-NSP2 or Flag (empty 666 vector).

667 Quantification and statistical analysis

Statistical analyses were performed with GraphPad Prism 9 software for Windows. All data were
calculated by two-tailed Student's t-test and presented as mean±SD (standard deviation). *P* values less than
0.001, 0.01 and 0.05 were assigned with ***, ** and *, respectively.

- 671 **Declaration of interests**
- The authors declare no competing interests.
- 673
- 674 **References**
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Figure 1







Figure 2









Figure 4

Highlights

- SARS-CoV-2 NSP2 binds the human 4EHP-GIGYF2 complex
- The NSP2/GIGYF2-4EHP interaction involves multiple binding sites
- NSP2 reduces the silencing capacity of GIGYF2
- NSP2 impairs microRNA-mediated silencing

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Figure S1. NSP2 associates with the cytoplasmic form of GIGYF2, related to Figure 1. (A) Immunofluorescence analysis of WT and GIGYF2^{KO} HEK293 cells expressing Flag-NSP2 and immunostained for the Flag tag (red) and GIGYF2 (green). Nuclei were stained with DAPI (blue). Images were acquired under identical conditions at objective X63. Z-projection of 3 stacks (0.35 µm each). Scale bar, 10 µm. (B) Proximity Ligation Assay between Flag-NSP2 and endogenous GIGYF2. WT and GIGYF2^{KO} HEK293 cells were transfected with vector expressing Flag-NSP2, and PLA was performed using anti-Flag and anti-GIGYF2 antibodies. Representative images of PLA (red), along with GIGYF2 immunofluorescence (IF; green) and DAPI (blue) are shown. Z-projection of 3 stacks (0.35 µm each). Scale bar, 15 µm. (C) Effect of GIGYF2 KO on PLA signals. The number of PLA dots per cell (n > 270) was quantified (mean \pm S.D.) in Flag-NSP2-expressing WT and GIGYF2^{KO} HEK293 cells using Fiji software. For each cell population, ten pictures with at least 20 cells per picture were used to calculate the mean values (\pm SD), and the P value was determined by two-tailed Student's ttest. (***) P < 0.001. (D) Confocal analysis of HEK293T cells expressing Flag-NSP2 and immunostained for the Flag tag (red) and DDX6 (green). Nuclei were stained with DAPI (blue). Insets show a higher magnification of a P-body (arrow). White lines in composite images show line scans used to determine the distribution of both DDX6 and Flag-NSP2 signals. Line plot graphs (bottom) show integrated intensity values for the two indicated DDX6 foci. Z-projection of 3 stacks (0.35 µm each). A.U., Arbitrary Unit. Scale bar, 10 µm. (E) Schematic overview of the biotinylated isoxazole (b-isox)-mediated precipitation of Intrinsically-Disordered Region (IDR)containing proteins. (F) B-isox precipitation of endogenous GIGYF2 in Flag-NSP2 expressing HEK293T cell extract. Cells transfected by an empty vector (Flag) were used as a negative control. Input (In), Precipitated (P) and Unbound (Un) fractions were analyzed by Western blot with the indicated antibodies. DMSO, used as the solubilizing agent for b-isox, served as a mock control.



Figure S2. His₆-NSP2 binds GIGYF2^{743-1,085} and 4EHP in vitro, related to Figure 2. (A) Recombinant GIGYF2^{743-1,085} is recognized by an antibody raised against GIGYF2. Recombinant GST and GIGYF2^{743-1,085} proteins were analyzed by SDS-PAGE followed by Coomassie blue staining (left panel), or by Western blot with an antibody raised against the 756-1,104 region of GIGYF2 (right panel). GST served as negative control. (B) Ni-NTA pull-down assay showing the interaction between recombinant His6-NSP2 and untagged GIGYF2743-1,085 by Western blot. The starting material (Input) and bound (Ni-NTA pull-down) fractions used in Figure 2c were diluted (1/30) and analyzed by Western blot with the indicated antibody. (C) GST pull-down assay showing the interactions between recombinant GST-fused GIGYF2743-1,085 and His6-NSP2. Both His6-NSP2 and GST-GIGYF2^{743-1,085} were co-expressed in *E. coli* and GST pull-down assays were performed using an *E. coli* lysate. The starting material (Input) and bound fractions were analyzed by SDS-PAGE followed by Coomassie blue staining and Western blot with the indicated antibodies. GST served as negative control. (D) Recombinant GIGYF2^{743-1,085} interacts with full-length His₆-NSP2, but not with truncated versions. The following regions of His₆-NSP2 were used: residues 1-115, 107-212 and 204-350. Ni-NTA pull-down assays were performed with the indicated recombinant proteins. The starting material (Input) and bound (Ni-NTA pull-down) fractions were analyzed by SDS-PAGE followed by Coomassie blue staining. (E) Ni-NTA pull-down assay showing the interaction between recombinant His₆-NSP2 and untagged full-length 4EHP. GST served as negative control and the indicated extracts were analyzed by SDS-PAGE followed by Coomassie blue staining.



Figure S3. The G262V/G265V natural variation of NSP2 reduces its interaction with 4EHP-GIGYF2 *in cellulo*, **not** *in vitro*, **related to Figure 2.** (A) The G262V/G265V natural variation of NSP2 impairs its interaction with endogenous 4EHP-GIGYF2 by co-IP. WT and G262V/G265V (Mut) Flag-NSP2 were transitory expressed in HEK293T, and Flag IP were performed with RNase A-treated extracts. The starting material (Input) and bound fractions were analyzed by Western blot. EV: empty vector. (B) Ni-NTA pull-down assay showing the interaction between the WT or G262V/G265V version (Mut) of His₆-NSP2 with both untagged 4EHP and GIGYF2^{743-1,085}. The starting material (Input) and bound (Ni-NTA pull-down) fractions were analyzed by SDS-PAGE followed by Coomassie blue staining. (C) Alignment of SARS-CoV-2 NSP2 (region: 232-281) with its SARS-CoV-1 (GenBank QJE50587.1) and MERS-CoV (GenBank QFQ59585.1) homologous versions. The sequences were aligned with Clustal Omega using default parameters, and colored in Jalview by identity.

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Figure S4. NSP2 derepresses the expression of a reporter harboring the *Ifnb1 3'* UTR, related to Figure 4. (A) Schematic of the psiCHECK2-RLuc-*Ifnb1 3'* UTR reporter. (B) HEK293T cells were co-transfected with psiCHECK2-RLuc-*Ifnb1 3'* UTR reporter or the psiCHECK2 reporter (as control). Vectors encoding Flag-NSP2 or Flag (empty vector) were also added in the transfection mixture. Luciferase activity was measured 24 h after transfection. RLuc values were normalized against FLuc levels, and the repression fold was calculated for the psiCHECK2-RLuc-*Ifnb1 3'* UTR reporter relative to the psiCHECK2 reporter level for each condition. Data are presented as mean \pm SD (n = 3). (***) P < 0.001 (two-tailed Student's t test).

Table S1. Oligonucleotides used in this study, related to STAR Methods.

Fragments		Sequences* (5'→3')
NEL CICVE2 N-4	Forward	GAG <u>CTCGAG</u> ATGGCAGCGGAAACGCAGAC
Anoi-GIGYF2-Nou	Reverse	GAG <u>GCGGCCGC</u> TCAGTAGTCATCCAACGTCTC
Nh - L CICVE21-267 NL-41	Forward	GAG <u>CTCGAG</u> ATGGCAGCGGAAACGCAGAC
Xnol-GIGYF2 ¹²⁰⁷ -Notl	Reverse	GAG <u>GCGGCCGC</u> CTACCAGCCTGCAGAACGAGGGC
VI-1 CICVE2258-495 N-41	Forward	GAG <u>CTCGAG</u> AGTCCTGATGGCCCTCGTTC
Allol-GIGTF2Noti	Reverse	GAG <u>GCGGCCGC</u> CTAACCAGGAGCACCTACAACTG
Xhol-GIGVF2486-752-NotI	Forward	GAG <u>CTCGAG</u> GTTGAAACACCAGTTGTAGG
Allo1-010112 -1100	Reverse	GAG <u>GCGGCCGC</u> CTACTTCTCTAGCTGCTGAAGCTG
Xhol-GIGVF2743-1,085-Notl	Forward	GAG <u>CTCGAG</u> GCCCTGGAACAGCTTCAGCAG
	Reverse	GAG <u>GCGGCCGC</u> CTAACTACTGACTAGGTCAGATGC
XhoI-GIGVE2 ^{1,076-1,320} -NotI	Forward	GAG <u>CTCGAG</u> AACCAGTGGGCATCTGACCT
Allo1-010112 -1100	Reverse	GAG <u>GCGGCCGC</u> TCAGTAGTCATCCAACGTCTC
BamHL-NSP2-Xhol	Forward	AAA <u>GGATCC</u> ATGGCATACACACGCTATGTTG
Damin-1031 2-XIIO1	Reverse	AA <u>CTCGAG</u> CTAGGCACCTCCCTTGAGCGTAA
RamHI NSD21-115 Yhol	Forward	AAA <u>GGATCC</u> ATGGCATACACACGCTATGTTG
Balliffi-NSF2 -Alloi	Reverse	AA <u>CTCGAG</u> CTAACCGTCCAGCTTCTTTTTCTCCAC
Damili NSD2107-212 Vhal	Forward	AAA <u>GGATCC</u> AGAGTGGAGAAAAAGAAGCTGGAC
Ballini-INSP2 -Alloi	Reverse	AA <u>CTCGAG</u> CTAACCAGATTCGTTGTGATATTCAGC
D	Forward	AAA <u>GGATCC</u> CTCGCTGAATATCACAACGAATCT
BamHI-NSP2	Reverse	AA <u>CTCGAG</u> CTAGAGTATGCTTTTCTGTTCACCAAT
D. HINGD21-212 VI. I	Forward	AAA <u>GGATCC</u> ATGGCATACACACGCTATGTTG
BamHI-NSP2 ¹ ²¹² -Xnoi	Reverse	AA <u>CTCGAG</u> CTAACCAGATTCGTTGTGATATTCAGC
Damali NED21-350 Vhal	Forward	AAA <u>GGATCC</u> ATGGCATACACACGCTATGTTG
BamHI-NSP2 200-Anol	Reverse	AA <u>CTCGAG</u> CTAGAGTATGCTTTTCTGTTCACCAAT
D III NGD21-509 XI I	Forward	AAAGGATCCATGGCATACACACGCTATGTTG
BamHI-NSP2 - Anol	Reverse	AA <u>CTCGAG</u> CTAACACAGAGCCAAGAACTTATTC
D III NICD2107-638 VI I	Forward	AAA <u>GGATCC</u> AGAGTGGAGAAAAAGAAGCTGGAC
BamHI-NSP2 ¹⁰⁷ 000-Anol	Reverse	AA <u>CTCGAG</u> CTAGGCACCTCCCTTGAGCGTAA
DIII NICD2204-638 VII	Forward	AAA <u>GGATCC</u> CTCGCTGAATATCACAACGAATCT
BamHI-NSP220-200-Anol	Reverse	AA <u>CTCGAG</u> CTAGGCACCTCCCTTGAGCGTAA
D III NGD2340-638 X1 I	Forward	AAA <u>GGATCC</u> GCTTGGAATATTGGTGAACAGAAA
BamHI-NSP2 ⁵¹⁰ 000-Anol	Reverse	AA <u>CTCGAG</u> CTAGGCACCTCCCTTGAGCGTAA
NED2 C2(2)//C2(5)/	Forward	GTGGTCGGGGAAGTGAGCGAGGTTCTGAATGATAACC
NSP2 G262 V/G263 V mutant	Reverse	GGTTATCATTCAGAACCTCGCTCACTTCCCCGACCAC
Damalii AEUD Na4i	Forward	GAG <u>GGATCC</u> AACAACAAGTTCGACGCTTTG
BamHI-4EHP-Noti	Reverse	GAGGCGGCCGCTCATGGCACATTCAACCGCG
VI J AFIID N. J	Forward	AACTCGAGAACAACAAGTTCGACGCTTTG
Anoi-4EHP-Noti	Reverse	AAGCGGCCGCTCATGGCACATTCAACCGC
	Forward	GCCTCTGTGGAGCAGTTCGCGAGGTTTTATAGCCACATG
4EHP W95A mutant	Reverse	CATGTGGCTATAAAACCTCGCGAACTGCTCCACAGAGGC
VI I CIWI 928D M (I	Forward	AACTCGAGAGCATCAACTGGCCCCCAG
Anol-GW182 ⁵⁰ -Notl	Reverse	AAGCGGCCGCTTACAGGGACTCCCCGCTGA
CW192SD ADDCL	Forward	CTGCACCCACGAGGCCAACCAATCCCAAG
Gw162 APPGL mutant	Reverse	CTTGGGATTGGTTGGCCTCGTGGGTGCAG

*Restriction sites in the oligonucleotides are underlined.

Key resources table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit anti-GIGYF2	Proteintech	Cat# 24790-1-AP; RRID:AB_2879727
Rabbit anti-DDX6	Proteintech	Cat# 14632-1-AP; RRID:AB_2091264
Rabbit anti-CNOT9	Proteintech	Cat# 22503-1-AP; RRID:AB_11232413
Rabbit anti-eIF4E2/4EHP	Proteintech	Cat# 12227-1-AP; RRID:AB_10642945
Mouse anti-GAPDH	Proteintech	Cat# 60004-1-Ig; RRID:AB_2107436
Rabbit anti-ZNF598	Thermo Fisher Scientific	Cat# 703601; RRID:AB_2815335
Mouse anti-Flag M2	Sigma-Aldrich	Cat# F1804; RRID:AB_262044
Mouse anti-V5 tag	Thermo Fisher Scientific	Cat# R960-25; RRID:AB_2556564
Mouse anti-6xHis	Thermo Fisher Scientific	Cat# MA1-21315-HRP; RRID:AB_2536989
Goat anti-Rabbit IgG (H+L) Cross- Adsorbed Secondary Antibody, Alexa Fluor™ 488	Thermo Fisher Scientific	Cat# A-11008; RRID:AB_143165
Goat anti-Mouse IgG (H+L) Cross- Adsorbed Secondary Antibody, Alexa Fluor 594	Thermo Fisher Scientific	Cat# A-11005; RRID:AB_2534073
Sheep anti-Mouse IgG - Horseradish Peroxidase	GE Healthcare	Cat# NA931; RRID:AB_772210
Goat anti-Rabbit IgG - Peroxidase	Sigma-Aldrich	Cat# A6154; RRID:AB_258284
Bacterial and virus strains		
BL21 (DE3) Gold competent ells	Agilent	Cat# 230132
BL21-CodonPlus (DE3) competent cells	Agilent	Cat# 230245
DH5a competent cells	Thermo Fisher Scientific	Cat# 18265017
Chemicals, peptides, and recombinat	nt proteins	
Recombinant His ₆ -NSP2	This paper	N/A
Recombinant His6-NSP21-115	This paper	N/A
Recombinant His6-NSP2 ¹⁰⁷⁻²¹²	This paper	N/A
Recombinant His6-NSP2204-350	This paper	N/A

Recombinant His6-NSP2G262/265V	This paper	N/A
Recombinant GIGYF2743-1,085	This paper	N/A
Recombinant 4EHP	This paper	N/A
Biotinylated isoxazole	Sigma-Aldrich	Cat# 900572
Critical commercial assays		
Duolink In Situ Red Kit	Sigma-Aldrich	Cat# DUO92101
Dual luciferase assay	Promega	Cat# E1960
Experimental models: Cell lines		C.
HEK293T cells	Sigma-Aldrich	Cat# 12022001-1VL
Flp-In T-REx HEK293 cells	Thermo Fisher Scientific	Cat# R78007
4EHP ^{KO} Flp-In T-REx HEK293 cells	(Jafarnejad et al., 2018)	N/A
GIGYF2 ^{KO} Flp-In T-REx HEK293 cells	This paper	N/A
Recombinant DNA	\mathcal{O}	
Plasmid: pFRT/TO/FLAG/HA-DEST TNRC6C	(Landthaler et al., 2008)	Addgene plasmid #19885
Plasmid: pcDNA5-FRT-TO-FH- NSP2	David Tollervey (Unpublished)	Addgene plasmid #157683
Plasmid: pcDNA5-FRT-TO-FH- NSP2 G262V/G265V	This paper	N/A
Plasmid: pcDNA5-FRT-TO-FH- NSP2 ¹⁻²¹²	This paper	N/A
Plasmid: pcDNA5-FRT-TO-FH- NSP2 ¹⁻³⁵⁰	This paper	N/A
Plasmid: pcDNA5-FRT-TO-FH- NSP2 ¹⁻⁵⁰⁹	This paper	N/A
Plasmid: pcDNA5-FRT-TO-FH- NSP2 ¹⁰⁷⁻⁶³⁸	This paper	N/A
Plasmid: pcDNA5-FRT-TO-FH- NSP2 ²⁰⁴⁻⁶³⁸	This paper	N/A
Plasmid: pcDNA5-FRT-TO-FH- NSP2 ³⁴⁰⁻⁶³⁸	This paper	N/A
Plasmid: pSpCas9(BB)-2A-Puro	(Ran et al., 2013)	Addgene plasmid #62988

Plasmid: pSpCas9(BB)-2A-Puro- <i>Gigyf2-</i> sgRNA	This paper	N/A
Plasmid: pET28-His ₆ -ZZ	(Barbarin-Bocahu and Graille, 2022)	N/A
Plasmid: pET28-His ₆ -ZZ-NSP2	This paper	N/A
Plasmid: pET28-His₀-ZZ-NSP2 G262V/G265V	This paper	N/A
Plasmid: pET28-His6-ZZ-NSP21-115	This paper	N/A
Plasmid: pET28-His ₆ -ZZ-NSP2 ¹⁰⁷⁻ 212	This paper	N/A
Plasmid: pET28-His6-ZZ-NSP2 ²⁰⁴⁻ 350	This paper	N/A
Plasmid: pCI-Neo	Promega	Cat# E1841
Plasmid: pCI-Neo-V5-GIGYF2	This paper	N/A
Plasmid: pCI-Neo-V5-GIGYF21-267	This paper	N/A
Plasmid: pCI-Neo-V5-GIGYF2 ²⁵⁸⁻⁴⁹⁵	This paper	N/A
Plasmid: pCI-Neo-V5-GIGYF2486-752	This paper	N/A
Plasmid: pCI-Neo-V5-GIGYF2 ⁷⁴³⁻ 1,085	This paper	N/A
Plasmid: pCI-Neo-V5-GIGYF2 ^{1,076-} 1,320	This paper	N/A
Plasmid: pCI-Neo-V5-4EHP	This paper	N/A
Plasmid: pCI-Neo-V5-4EHP W95A	This paper	N/A
Plasmid: pGEX-6P-1	Amersham	Cat# 27-4597-01
Plasmid: pGEX-6P-1-4EHP	This paper	N/A
Plasmid: pGEX-6P-1-GIGYF2 ⁷⁴³⁻ 1,085	This paper	N/A
Plasmid: pCI-Neo-λNV5-GIGYF2	This paper	N/A
Plasmid: pCI-Neo-λNV5-GW182 ^{SD}	This paper	N/A
Plasmid: pCI-Neo-λNV5-GW182 ^{SD} ΔPPGL	This paper	N/A
Plasmid: psiCHECK-2	Promega	Cat# C802A
Plasmid: psiCHECK-2-Ifnb1 3' UTR	(Zhang et al., 2021)	N/A

Plasmid: pCI-Neo-RLuc-5BoxB- A ₁₁₄ -N ₄₀ -HhR	(Chapat et al., 2017)	N/A
Plasmid: pCI-Neo-RLuc-5BoxB	(Pillai et al., 2004)	N/A
Plasmid: pCI-Neo-RLuc-6let7a	(Pillai et al., 2004)	N/A
Plasmid: pCI- Neo-FLuc	(Pillai et al., 2004)	N/A
Software and algorithms		
Fiji	Fiji	RRID: SCR 002285
	,	
Prism 9	GraphPad	RRID:SCR_002798
Prism 9 Illustrator CS6	GraphPad Adobe	RRID:SCR_002798 RRID:SCR_010279
Prism 9 Illustrator CS6 ChimeraX	GraphPad Adobe UCSF	RRID:SCR_002798 RRID:SCR_010279 RRID:SCR_015872
Prism 9 Illustrator CS6 ChimeraX Image Lab Software	GraphPad Adobe UCSF Bio-Rad	RRID:SCR_002798 RRID:SCR_010279 RRID:SCR_015872 RRID:SCR_014210

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