Cardiovirus leader proteins retarget RSK kinases toward alternative substrates to perturb nucleocytoplasmic traffic

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Abstract

Proteins from some unrelated pathogens, including viruses and bacteria can recruit and activate cellular p90-ribosomal protein S6 kinases (RSKs) through a common linear motif. Our data suggested that such pathogens' proteins might act as adapters to dock the kinase toward specific substrates. We explored this hypothesis using the *Cardiovirus* leader protein (L) as a paradigm. L is known to trigger phenylalanine-glycine nucleoporins (FG-NUPs) hyperphosphorylation and nucleocytoplasmic trafficking perturbation. Using a biotin ligase fused to either RSK or to L, we identified FG-NUPs as primary partners of the L-RSK complex in infected cells. Mutant analysis shows that L uses distinct motifs to recruit RSK and to dock the L-RSK complex toward the FG-NUPs. Using an analog-sensitive RSK2 mutant kinase, we show that, in infected cells, L can trigger RSK to use NUP98 and NUP214 as direct substrates. Our data illustrate a novel virulence mechanism where pathogens' proteins retarget cellular protein kinases toward specific substrates.

Introduction

Proteins encoded by several unrelated pathogens, including RNA viruses, DNA viruses and bacteria, were recently shown to use a common short linear motif (D/E-D/E-V-F, referred to as DDVF hereafter) to recruit members of the cellular p90-ribosomal S6 protein kinases (RSKs) family: RSK1, RSK2, RSK3 and RSK4 [1,2]. Interestingly, competition and cross-linking experiments, as well as crystallography data show that these pathogens' proteins, the leader (L) protein (cardioviruses), ORF45 (Kaposi sarcoma-associated herpes virus - KSHV) and YopM (*Yersinia*) use a common interface to recruit RSKs. Binding of the pathogens' proteins prevents RSK dephosphorylation by cellular phosphatases, thereby maintaining RSK

40 in an active state [1,2]. Although infection with all three pathogens leads to RSK activation, the
41 outcome of this activation differs according to the protein bound to RSK. YopM association
42 with RSK was proposed to inhibit the inflammasome and to lead to IL-10 production [3,4]; RSK
43 recruitment by ORF45 enhances lytic replication of KSHV [5,6], whereas RSK recruitment by
44 cardiovirus L protein leads to the inhibition of the antiviral eukaryotic initiation factor 2 alpha
45 kinase 2 (EIF2AK2), better known as PKR [1].
46 PKR inhibition by L was shown to depend on L interaction with RSK but also on L protein's
47 C-terminal domain. Indeed, the M60V mutation in the C-terminal domain of L does not affect
48 RSK recruitment but abolishes PKR inhibition. These observations led us to propose the
49 "model of the clamp" whereby pathogens' proteins would act as adaptor proteins that force a
50 given enzyme (here RSKs) to act on a specific substrate. Through their DDVF motif,
51 pathogens' proteins recruit and maintain RSKs in an activated state. Through another domain
52 (i.e. the C-terminal domain for L), they recruit proteins that could serve as substrate for
53 RSKs. After phosphorylation, such proteins would act as effectors to the benefit of the
54 pathogen (Fig 1A) [1]. In an alternative model, proteins interacting with RSK through the
55 conserved DDVF motif could directly act as preferential substrates for RSK-mediated
56 phosphorylation (Fig 1B), as was suggested for the cellular protein RHBDF1 (Rhomboid 5
57 homolog 1) [2].
58
59 Cardioviruses belong to the *Picornaviridae* family and include encephalomyocarditis virus
60 (EMCV), Theiler's murine encephalomyelitis virus (TMEV) and the human Saffold virus
61 (SAFV), closely related to TMEV. Despite its very small size (67-76 amino acids) and lack
62 of enzymatic activity, the L protein encoded by these viruses was shown to be
63 multifunctional as, beside inhibiting PKR, it blocks interferon gene transcription, and
64 triggers an extensive diffusion of nuclear and cytoplasmic proteins across the nuclear

65 membrane [1,7-16]. This nucleocytoplasmic trafficking perturbation was associated with the
66 hyperphosphorylation of phenylalanine-glycine-nucleoporins (FG-NUPs) such as NUP98,
67 NUP153 or NUP62 [17-20]. FG-NUPs are proteins of the nuclear pore complex (NPC) that
68 possess intrinsically disordered domains, rich in phenylalanine and glycine residues. These
69 domains form a mesh in the center of the nuclear pore, which enables interaction with
70 karyopherins, thus allowing selective transport of proteins and RNA through the NPC [21-24].
71 Molecular dynamic simulations predict that FG-NUP phosphorylation drastically decreases
72 the density of FG-NUPs inside the pore [25]. Accordingly, electron microscopy analysis of
73 EMCV-infected cells displayed such a density loss in the center of the NPC [20]. Through
74 perturbation of the NPC, cardioviruses trigger a diffusion of nuclear proteins to the cytoplasm
75 which can be used by the virus for viral replication and translation [16].
76
77 Preliminary data suggested that nucleocytoplasmic trafficking perturbation by cardiovirus L
78 proteins depended on RSK and on the recruitment of a cellular target by the L-RSK complex
79 (model of the clamp). The aim of this work was to assess whether RSK could indeed be
80 retargeted by L to a new substrate in infected cells and to identify such a substrate that could
81 trigger nucleocytoplasmic trafficking perturbation. Our data show that L can recruit both
82 RSK and FG-NUPs and that FG-NUPs can act as direct RSK substrates in infected cells. This
83 work provides strong support to the model of the clamp and elucidates a novel virulence
84 mechanism.
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Results

Cardiovirus L-mediated nucleocytoplasmic trafficking perturbation depends on RSK recruitment by L

We tested whether L-mediated redistribution of nuclear and cytoplasmic proteins across the nuclear envelope and the hyperphosphorylation of FG-NUPs depends on both RSK and L. In agreement with previous reports [14], cytosolic diffusion of the nuclear protein PTB occurred in HeLa cells infected for 10 hours with TMEV expressing a wild type L protein (L^{WT}) but not in non-infected cells or in cells infected with viruses carrying the M60V mutation in the C-terminal part of L (L^{M60V})(Figs 2A and B). In the latter cells, PTB however partly appeared in cytosolic punctae, likely corresponding to stress granules that were shown to form in the absence of PKR inhibition by L [12]. Importantly, L^{WT}-mediated PTB diffusion was almost abrogated in RSK-deficient HeLa cells (HeLa-RSK-TKO) (Figs 2B and C). Viral replication was also decreased in the absence of RSK, in agreement with increased PKR activation in these cells (Fig 2D). Interestingly, PTB diffusion was restored after transduction of HeLa-RSK-TKO cells with lentiviruses expressing any of the four human RSK isoforms (Figs 2B and C). Accordingly, NUP98 hyperphosphorylation was dramatically decreased in HeLa-RSK-TKO cells and restored in cells transduced to express any of the four RSK isoforms (Fig 2D). Similar observations were made in the case of EMCV as soon as 5 hours post-infection, although dependence on RSK was less pronounced (Figs 2E-H). Thus, PTB diffusion out of the nucleus and NUP98 hyperphosphorylation depend on both L and RSK.

In addition, TMEV and EMCV expressing L^{WT} but not mutant L proteins triggered an RSK-dependent disruption of nucleocytoplasmic trafficking of other proteins harboring canonical

113 nuclear export (NES) and import (NLS) sequences, as shown by diffusion of GFP-NES and
114 RFP-NLS proteins in live cells that stably express these proteins (Figs 3A-D). This
115 observation demonstrates that nucleocytoplasmic redistribution of proteins was not specific to
116 PTB. Again, RSK dependence of protein redistribution was slightly less prominent for
117 EMCV than for TMEV.
118 Ectopic expression of TMEV L^{WT} but not the L^{F48A} mutant (affected in the RSK-binding
119 motif) was sufficient to trigger nucleocytoplasmic redistribution of proteins (Figs 3E-G).
120 Also, expression of L^{M60V} or YopM did not affect nucleocytoplasmic trafficking although
121 these proteins can activate RSK [1]. Taken together, these data show that L-mediated
122 nucleocytoplasmic trafficking alteration does not dependent on virus replication per se or on
123 the expression of other viral proteins and requires both interaction with RSK and, at least for
124 TMEV L, integrity of the C-terminal part of the protein.
125

126 Identifying RSK partners in TMEV-infected cells using BioID2

127 According to the model of the clamp, TMEV L would recruit RSK through its central DDVF
128 motif and would recruit potential RSK substrates through its C-terminal domain. As
129 suggested above, the M60V mutation of L would prevent the recruitment of such a target,
130 without affecting RSK binding.
131 In order to identify target proteins that are recruited by L as potential RSK targets, we used
132 the modified bacterial biotin ligase BioID2 (BioID) which, when fused to a protein of
133 interest, allows promiscuous biotinylation of proximal proteins in the cell [26].
134 A BioID-RSK2 (BioID-RSK) fusion was constructed and expressed in RSK1/2-deficient
135 HeLa cells (HeLa-RSK-DKO) by lentiviral transduction to identify RSK's potential partners
136 during TMEV infection (Fig 4A). After testing that the BioID-RSK construct was activated
137 (i.e. phosphorylated) in the presence of L in infected cells and able to rescue L activities such

138 as L-mediated PKR inhibition (Fig S1A), we performed biotinylation experiments in order to
139 identify RSK partners in the context of infected cells. HeLa BioID-RSK cells were infected
140 with either L^{WT} or L^{M60V} viruses for 16 hours in presence of biotin. Biotinylated proteins
141 were pulled down using streptavidin beads and processed for mass spectrometry analysis.
142 Western blot detection of biotinylated proteins (Fig 4B) confirmed the concentration of
143 biotinylated proteins in the pulled down fraction and revealed the presence of a 100kDa band
144 specific to the L^{WT}-infected sample, suggesting that the L protein status indeed influenced
145 target biotinylation by the BioID-RSK fusion.
146

147 **Identifying L protein partners using BioID**

148 Several attempts to identify proteins that are recruited by TMEV L were made in our lab by
149 coimmunoprecipitation. However, beside RSK, no clear L partner was consistently identified.
150 It is not unlikely that proteins recruited by L would interact with L only transiently and with
151 low affinity. We thus used the BioID system to identify such proteins that may transiently
152 interact with L in infected cells and then serve as RSK substrates. However, due to packaging
153 limitations in picornaviruses, the BioID coding sequence (696 nt) would be too long to be
154 accommodated into the viral genome. We thus generated *trans*-encapsidated replicons where
155 a sequence coding for BioID-L and eGFP was substituted for the capsid-coding region (Fig
156 4C). These replicons were encapsidated in 293T cells stably expressing a capsid coding
157 sequence carrying synonymous mutations in the VP2 region that affect *CRE* function [27] to
158 prevents the selection of replication-competent wild type viruses that might emerge through
159 recombination. *Trans*-encapsidated replicons coding for BioID fused to L^{WT}, L^{M60V} and L^{F48A}
160 were produced (Fig 4A). In HeLa cells infected with these replicons, L activities (i.e. RSK
161 phosphorylation, PKR activation and NUP98 hyperphosphorylation) were as expected (Fig
162 S1B). HeLa cells were then infected with BioID-L replicons for 14 hours in presence of

163 biotin. Biotinylated proteins were pulled down in stringent conditions. Western blot
164 confirmed protein biotinylation in infected cell extracts and showed some bands occurring in
165 L^{WT} and L^{F48A} but not in L^{M60V} samples (Fig 4D). These results confirm specific biotinylation
166 of proteins by BioID-L.
167

168 **FG-NUPs are enriched in BioID-L and BioID-RSK proxeomes**

169 We expected that RSK targets recruited by L would be present in both BioID-RSK and
170 BioID-L screens. Pulled down biotinylated proteins from both screens (n=3 for each screen)
171 were identified by mass spectrometry (MS) and sorted according to their peptide spectrum
172 match (PSM) number (for more detail on the calculations see material and methods). For
173 BioID-RSK, proteins were sorted according to their abundance in L^{WT}-infected cells relative
174 to that in non-infected cells and in cells infected with the L^{M60V} mutant virus (Fig 5A, vertical
175 axis). For BioID-L (replicons), proteins were sorted according to their abundance in L^{WT} and
176 L^{F48A} samples relative to that in L^{M60V}-infected and in non-infected samples (Fig 5A,
177 horizontal axis). Hence, the proteins that would be recruited by the C-terminal domain of L
178 are those that have high ratios in both screens. Interestingly, many of these proteins were FG-
179 NUPs (Figs 5A and B). ProDA (Probabilistic Dropout Analysis) statistical analysis [28] was
180 used to identify proteins whose abundance differs in pairwise comparisons: L^{WT} versus L^{M60V}
181 for the BioID-RSK experiments, and L^{WT} versus L^{M60V} or L^{F48A} versus L^{M60V} for the BioID-L
182 experiment. The table in Fig 5B shows adjusted P-values obtained from this analysis for the
183 20 best-ranked proteins. In the BioID-RSK experiments, FG-NUPs had the lowest adjusted P-
184 values (though not significant) and highest fold change (Figs 5B and C). In the BioID-L
185 experiments many FG-NUPs reached significant scores when pairwise comparisons were
186 made between L^{WT} and L^{M60V} or between L^{F48A} and L^{M60V} (Figs 5B and C). Most
187 importantly, FG-NUPs that had previously been shown to be hyperphosphorylated during

188 cardiovirus infection: NUP62, NUP98, NUP153 and NUP214 [17-20] exhibited statistical
189 significance (Fig 5B). From these results we conclude that L recruits RSK through its DDVF
190 motif and then docks RSK toward FG-NUPs when the C-terminal domain of L is intact.
191

192 **Proteins biotinylated by BioID-RSK and BioID-L are localized at the NPC**

193 We analyzed the subcellular localization of proteins that were biotinylated by BioID-RSK
194 and by BioID-L in infected cells. To this end, biotinylation experiments were performed as
195 above but infected cells were fixed after 10h of infection, permeabilized and stained them
196 with Alexa Fluor conjugated streptavidin. For BioID-RSK, mock-infected cells show staining
197 of biotinylated proteins in the nucleus, in agreement with the mostly nuclear localization of
198 RSKs [29] (Figs 6A-C). In cells infected with the L^{WT} virus, biotinylated proteins form a faint
199 rim around the nucleus that colocalizes with the FG-NUP NUP98, in addition to the diffuse
200 nuclear staining, showing that part of the (BioID)-RSK molecules are recruited to the nuclear
201 envelope. In cells infected with the L^{M60V} mutant virus, this nuclear rim is lost, in agreement
202 with a lack of L^{M60V} interaction with nucleoporins.
203

204 For BioID-L replicons, staining occurs in the nucleus and at the nuclear envelope for L^{WT}, in
205 agreement with an interaction of L with both RSK and NUPs (Figs 7A-C). Proteins
206 biotinylated by BioID-L^{M60V} show diffuse staining in the nucleus, in agreement with L^{M60V}
207 interaction with RSK but not with nucleoporins. Proteins biotinylated by BioID-L^{F48A} show
208 extensive colocalization with nucleoporin POM121 at the nuclear envelope (Fig 7C), in
209 agreement with the lack of interaction with RSK, which would likely sequester part of the L
210 protein in the nucleoplasm. In conclusion, labeling of biotinylated proteins support our model
211 where L interacts with RSK in the nucleus and recruits a portion of RSK molecules to the
212 NPC, via an interaction between the C-terminal domain of L and NUPs.

213

214 **FG-NUPs are direct substrates for RSK in cardiovirus infected cells**

215 Since FG-NUPs are known to be hyperphosphorylated during cardiovirus infection and since
216 our results showed that RSK is in their close proximity, we aimed to test if RSK can directly
217 phosphorylate these FG-NUPs in infected cells. For this, we used the « analog-sensitive
218 kinase system » developed by the group of Shokat [30]. This system is based on the use of an
219 ATP analog: N6-alkylated ATP-γ-S (A*TP-S), bulkier than ATP, that only fits in ATP
220 binding pockets of kinases that have been mutated to accommodate such bulkier analogs. The
221 advantage of this technique is that the mutated kinase (RSK in our case) is the only kinase in
222 the cell that can use A*TP-S and will therefore thiophosphorylate its substrates. After
223 alkylation, such target proteins can be recognized using a specific anti-thiophosphate ester
224 antibody (Fig 8A). We identified Leu147 as the RSK2 gatekeeper residue (i.e. residue in the
225 ATP-binding pocket that, when mutated to a smaller residue, allows the access of the A*TP-
226 S) by homology to Thr338 in the consensus sequence defined for c-SRC [31] (Fig 8B). Leu147
227 was mutated either to Gly (As1-RSK) or to Ala (As2-RSK). To test for ATP analog usage by
228 the modified RSK kinases, WT, As1- and As2-RSKs were stably expressed in HeLa-RSK-
229 TKO cells by lentiviral transduction, yielding WT-, As1- and As2-RSK cells. Expression of
230 both As1- and As2-RSK allowed L functions (i.e. PKR inhibition, RSK activation and
231 NUP98 hyperphosphorylation) (Fig S2A). The ATP analog N6-Bn-ATP-γ-S was better
232 incorporated than N6-PhEt-ATP-γ-S by the As-RSKs (Fig S2B). The As2-RSK kinase was
233 better expressed than As1-RSK and readily accommodated this analog (Figs S2A and B).
234 Therefore, As2-RSK cells and N6-Bn-ATP-γ-S were chosen for further experiments. HeLa
235 WT-RSK or As2-RSK cells were then infected with L^{WT} and L^{M60V} viruses for 8h30min.
236 Cells were then permeabilized with digitonin and treated with A*TP-S (N6-Bn-ATP-γ-S) for
237 1 hour. Then, NUP98 was immunoprecipitated and its thiophosphorylation status was

238 analyzed, after alkylation, by western blot with the anti-thiophosphate ester antibody. The
239 blot shown in Fig 8C shows a band highly enriched in the immunoprecipitated sample,
240 migrating at the expected molecular mass for NUP98 (98kDa). This band was most
241 prominent in the samples from As2-RSK cells, confirming the specificity of thiophosphate
242 ester detection. Importantly, this band was detected much more in samples of cells infected
243 with the L^{WT} virus than in samples of cells that were not infected or infected with the L^{M60V}
244 virus. These data indicate that NUP98 can be directly phosphorylated by RSK and that, as
245 expected, this phosphorylation only occurs after infection with the L^{WT} virus. For EMCV, as
246 this virus replicates much faster than TMEV, NUP98 was immunoprecipitated after 3h30 of
247 infection. Thiophosphorylation of immunoprecipitated proteins was tested by western blot
248 using the anti-thiophosphate ester antibody (Fig 8D). As for TMEV, a ~100kDa protein likely
249 corresponding to NUP98 was thiophosphorylated by As2-RSK only in L^{WT} infected
250 conditions. It is noteworthy that additional bands were detected, which likely correspond to
251 other FG-NUPs such as NUP62, NUP153 and NUP214 that coimmunoprecipitated with
252 NUP98.
253 In order to confirm FG-NUPs thiophosphorylation by As2-RSK in infected cells, experiments
254 were performed the other way around. Therefore, thiophosphorylated proteins were
255 immunoprecipitated from cells infected with TMEV variants, using the thiophosphate ester
256 antibody. Then the IP fraction (i.e. thiophosphorylated proteins) was analyzed by western
257 blot for the presence of NUP98 (Fig 8E) and NUP214 (Fig S2C). Again, these NUPs were
258 most prominently detected in the conditions where the L protein was WT and RSK was
259 mutated (As2-RSK). These data confirm that RSK directly phosphorylates FG-NUPs when
260 the L^{WT} protein is present.
261

262 Taken together, these results confirm that part of the cellular RSKs can be redirected to the
263 nuclear pore complex by the L protein, where RSKs phosphorylate FG-NUPs. By interacting
264 with RSK through its DDVF motif and with FG-NUPs through its C-terminal domain, L
265 serves as a viral adapter protein to modulate RSK's activity and redirects these kinases
266 toward new substrates thus supporting the model of the clamp. Hyperphosphorylation of FG-
267 NUPs by RSK likely triggers a global perturbation of nucleocytoplasmic trafficking and may
268 therefore facilitate cytoplasmic replication of cardioviruses.
269

270 # Discussion

271 Recent work showed that some proteins expressed by unrelated viruses and bacteria hijack
272 host RSK kinases through a conserved DDVF linear motif that likely emerged in those
273 proteins by convergent evolution [1,2]. Occurrence of the DDVF motif in RHBDF1, a known
274 target of RSKs suggests that some proteins, either from pathogens or from the host cell, can
275 associate with RSK through the DDVF motif to promote their own phosphorylation by RSKs
276 [2]. In the case of TMEV L protein, a point mutation in the C-terminal domain of the protein
277 (L^{M60V}) abrogated L activities although this mutation affected neither L-RSK interaction nor
278 RSK activation by L [1,32]. The phenotype of this mutant supported the model of the clamp
279 where the pathogens' protein would act to bridge RSK to specific substrates. The two models
280 are not mutually exclusive.
281 Our data show that L proteins from TMEV and EMCV indeed act by redirecting part of the
282 cellular RSK kinases toward the nuclear pore complex where RSKs directly phosphorylate
283 FG-NUPs.
284 Bacteria of the genus *Yersinia* use a type III secretion system to inject Yop proteins into
285 contacted eukaryotic cells [33]. YopM was shown to interact with RSK through a C-terminal

286 DDVF motif [1] and with protein kinase PKNs through central leucin-rich motifs. Formation of
287 the tripartite (YopM-RSK-PKN) complex results in the phosphorylation and activation of
288 PKN, which requires active RSK [34,35], suggesting that RSK might also be redirected toward
289 PKN as a specific substrate. This scenario is very likely although direct phosphorylation of
290 PKN by RSK was not formally proven.
291 Another question regarding the clamp model is whether the substrates to which RSKs are
292 redirected by the pathogens' proteins are physiological RSK substrates, the phosphorylation
293 of which is increased by their forced association with RSKs or whether they represent
294 unconventional RSK substrates. From the blots of analog-sensitive RSK experiments (Figs
295 8C-E and S2C), it appears that basal thiophosphorylation of NUP98 or NUP214 might occur,
296 even in non-infected cells that do not contain the L protein. Also, given that the main binding
297 site of pathogens' proteins is located in a surface-exposed loop of RSK, relatively remote
298 from the catalytic site, it is unlikely that this interaction can modify the catalytic site enough
299 to accommodate structurally divergent substrates. Thus, the role of the bridging proteins is
300 likely to increase the frequency of encounters between RSKs and specific substrates, and
301 possibly also to drive these kinases to specific subcellular locations.
302 Viruses of the *Picornaviridae* family disturb nucleocytoplasmic traffic, likely to recruit host
303 nuclear RNA-binding proteins to the cytoplasm of the infected cells, where their genome
304 translation and replication takes place. Perturbation of the traffic was also reported to inhibit
305 the activation of innate immunity genes such as genes coding for interferon or chemokines,
306 because these genes rely on the translocation of transcription factors like IRF3/7 or NFκB
307 [16,36]. Enteroviruses encode proteases $2A^{pro}$ and $3C^{pro}$, which directly cleave FG-NUPs [37],
308 thereby opening the central channel of the nuclear pore complex [20,38]. Cardioviruses,
309 however, induce the hyperphosphorylation of FG-NUPs, thus mimicking a process occurring
310 during mitosis, which ends up in the dismantling of the NPCs and the free diffusion of

311 nuclear and cytoplasmic proteins. Here, we show that TMEV L promotes direct FG-NUPs
312 phosphorylation by RSKs. During mitosis, FG-NUP phosphorylation was proposed to
313 involve mostly cyclin-dependent kinase 1 (CDK1), polo-like kinase 1 (PLK1) and NIMA-
314 related kinases [23,39]. A phosphoproteomic analysis by Kosako et al. [40] also suggested the
315 involvement of the ERK pathway, ERK1/2 being the upstream kinase of RSK. We
316 hypothesize that RSKs might indeed contribute, to some extent, to NUP phosphorylation
317 during mitosis and that L triggers a stronger RSK-mediated FG-NUP phosphorylation during
318 infection.
319 Our work illustrates a new virulence mechanism whereby pathogens' proteins not only
320 activate but also redirect host kinases toward specific substrates and decipher how
321 cardioviruses trigger RSK-mediated FG-NUP hyperphosphorylation to perturb
322 nucleocytoplasmic trafficking in the host cell.
323

324 Material & methods

325

326 **Cells**

327 The subclone of HeLa cells used in this work were HeLa M cells kindly offered by R.H.
328 Silvermann [41].
329 HeLa-RSK-DKO, (RSK1- and RSK2-deficient) and HeLa-RSK-TKO (RSK-1, RSK2- and
330 RSK3-deficient) cells were obtained from HeLa M cells using the CRISPR-Cas9 technology
331 [1]. HeLa-LVX and HeLa-RSK-TKO-LVX were obtained by transduction of HeLa M cells and
332 of HeLa-RSK-TKO cells with pLVX-EF1alpha-2xGFP:NES-IRES-2xRFP:NLS. Clones
333 showing regular 2xGFP:NES and 2xRFP:NLS expression levels under the fluorescent
334 microscope were selected for further use. HeLa BioID-RSK cells were obtained by

335 transduction of HeLa-RSK-DKO cells with BLP10 and transduced cell populations were then
336 selected with 1mg/ml of G418 (Roche). HeLa WT-RSK, HeLa As1-RSK and HeLa As2-RSK
337 cells were obtained by transduction of HeLa-RSK-TKO cells with TM1117, BLP20 and
338 BLP21 respectively and transduced cell populations were selected with 1mg/ml of G418
339 (Roche).
340 HeLa-RSK-TKO cells were transduced with the empty lentiviral vector TM952 or with
341 TM1116-19 derivatives expressing the four isoforms of human RSK. Transduced cell
342 populations were selected with 2mg/ml of G418.
343 BHK-CL13 and 293T-CL13 cells were obtained by lentiviral transduction of CL13.
344 mCherry-positive cells were then sorted by FACS.
345 293T cells were kindly provided by F. Tangy (Pasteur Institute, Paris). Both HeLa M and
346 293T cells and their derivatives were maintained in Dulbecco's modified Eagle medium
347 (DMEM) (Lonza) supplemented with 10% of fetal calf serum (FBS, Sigma), 100 U/ml
348 penicillin and 100µg/ml streptomycin (Thermo Fisher). BHK-21 cells (ATCC), were
349 maintained in Glasgow's modified Eagle medium (GMEM) (Gibco) supplemented with 10%
350 newborn calf serum (Gibco), 100 U/ml penicillin, 100µg/ml streptomycin and 2.6g/l tryptose
351 phosphate broth (Difco). All cells were cultured at 37°C, in 5% CO2.
352
353 **Viruses**
354 TMEV viruses used in this study are derivatives of KJ6, a variant of the persistent DA strain
355 (DA1 molecular clone) adapted to grow on L929 cells [42]. FB09 carries the M60V mutation in
356 L (L^{M60V}), which was shown to abrogate L toxicity [12,32]. KJ6 and FB09 are indicated as
357 TMEV L^{WT} and L^{M60V} respectively.
358 EMCV viruses used is this study are derived from pMC24 , a Mengo virus molecular clone
359 carrying a shortened (24C) polyC tract [43]. The virus denoted $EMCV^{WT}$ (used in Fig 8) is the

360 virus produced from the pMC24 plasmid. Viruses denoted EMCV L^{WT} and L^{Zn} (used in Figs
361 2 and 3) are produced from plasmids pFS269 and pTM1098 and express N-terminally Flag-
362 tagged L proteins. L^{Zn} carries two point mutations (C19A and C22A) in the zinc finger of L,
363 which likely affect the overall structure of the protein [44].
364 These viruses were produced by reverse genetics from plasmid constructions containing the
365 full-length viral cDNA sequences. To this end, BHK-21 cells were electroporated (1500V,
366 25µFd, no shunt resistance) using a Gene pulser apparatus (Bio-Rad) with viral RNA
367 produced by *in vitro* transcription (RiboMax P1300, Promega). Supernatants were collected
368 48-72h after electroporation, when cytopathic effect was complete. After 2 to 3 freeze-thaw
369 cycles, the supernatants containing the virus were clarified by centrifugation at 1258g for 20
370 min and viruses were stored at -80°C. Viruses were titrated by plaque assay in BHK-21 cells.
371
372 **Trans-encapsidated viral replicons**
373 FW12 is the replicon encoding for BioID2-HA-linker(3xGGGGS)-L^{WT}. This replicon derives
374 from TMEV DA1. It was constructed by replacing the capsid-coding region of the virus by a
375 sequence coding sequentially (5'-to-3'), in a single frame, for: BioID2-HA-
376 linker(3xGGGGS)-L, the L/VP4 boundary cleaved by protease 3C, eGFP, the VP2 coding
377 segment encompassing the CRE sequence, the 3' end of the 2A coding region and the
378 following part of the viral genome (see Fig 4). The BioID-L^{M60V} (FW16) and BioID-L^{F48A}
379 (FW17) viral replicons are derivates of FW12 carrying the indicated mutation. Trans-
380 encapsidation of the replicon occurred in 293T-CL13 to stably express the viral capsid
381 precursor protein. C-terminal residues of L were inserted in the construct to restore a genuine
382 L/VP4 clivage site for protease 3C, which allows the capsid protein to start with an N-
383 terminal glycine, potentially undergoing myristoylation [45]. Importantly, 4 silent mutations
384 were introduced in the VP2 coding sequence corresponding to the *CRE* region to prevent the

385 replication of recombinant wild type viruses that would emerge through recombination
386 between replicon RNA and capsid-coding mRNA in packaging cells.
387 293T-CL13 cells were seeded (600,000/well) in 6-well plates. 24h after seeding, cells were
388 transfected (TransIT-mRNA, Mirus) with 5µg of replicon RNA previously obtained by *in*
389 *vitro* transcription. Six hours post-transfection, medium was changed to DMEM without
390 serum. The infected cells were passaged 8 times and reseeded with part of the collected
391 supernatant to amplify viral production. Then, 150ml of replicon-containing supernatant was
392 collected and filtered (0.45µm). SDS was then added to a final concentration of 0.5% and,
393 after 2h incubation at RT, supernatants were centrifuged for 5min, at 1258g at 20°C. Next,
394 13ml of clarified supernatant was added to a 2.6ml sucrose 30% cushion in polyallomer tubes
395 (Beckman) and centrifuged for 16h at 20°C, 25000rpm in a SW28 swinging bucket rotor.
396 After ultracentrifugation, pellets containing the replicons were resuspended in 150µl of
397 10mM Tris-HCl pH 7.5 and further dialyzed in 10mM Tris-HCl pH 7.5. Replicons were then
398 titrated by plaque assay using BHK-21 cells stably expressing viral capsid proteins (BHK-21
399 CL13), and kept at -80°C.
400
401 **Lentiviral vectors and cell transduction**
402 - pLVX-EF1alpha-2xGFP:NES-IRES-2xRFP:NLS was a gift from Fred Gage (Addgene
403 plasmid # 71396) [46]
404 Other lentiviral vectors used for protein expression were derived from
405 pCCLsin.PPT.hPGK.GFP.pre. [47]
406 - TM945 is a $Prom_{CMV}$-MCS-IRES-mCherry construct [48]
407 - TM952 is a $Prom_{CMV}$-MCS-IRES-neo construct [49]
408 - BLP10 is a TM952 derivative coding for BioID2-HA-linker(3xGGGS)-MuRSK2. This
409 construct expresses murine RSK2, which differs from human RSK2 by a single amino acid.

410 - BLP20 is a TM952 derivative coding for As1-RSK2 (hu-RSK2 with Analog-sensitive
411 kinase 1 (As1) mutation (Leu 147 → Gly).
412 - BLP21 is a TM952 derivative coding for As2-RSK2 (hu-RSK2 with Analog-sensitive
413 kinase 2 (As2) mutation (Leu 147 → Ala).
414 - CL13 is a TM945 derivative expressing the 10 C-terminal aminoacids of L followed by the
415 entire capsid precursor of virus DA1.
416 - TM1116, TM1117, TM1118 and TM1119 are TM952 derivatives carrying the sequences
417 coding for 3xHA-Human RSK1, RSK2, RSK3 and RSK4 respectively [1]. They were
418 constructed using the Gateway technology (Invitrogen) from donor plasmids Hs.RPS6KA1,
419 Hs.RPS6KA2, Hs.RPS6KA3 and Hs.RPS6KA6 kindly provided by Dominic Esposito
420 through the Addgene collection (Addgene refs: 70573, 70575, 70577, and 70579,
421 respectively)
422 Lentiviruses were produced in 293T cells by co-transfection of the following plasmid, using
423 TransIT-LT1 reagent (Mirus Bio): 2.5 µg of lentiviral vector, 0.75 µg of pMD2-VSV-G
424 (VSV-glycoprotein), 1.125 µg of pMDLg/pRRE (Gag-Pol), and 0.625 µg of pRSV-Rev
425 (Rev). DNA quantities are for transfection of 1 well of a 6-well plate. Supernatants were
426 typically collected 72h post transfection and filtered (porosity: 0.45µM). For transduction,
427 cells were typically seeded in a 24-well plate as of 5,000 - 10,000 cells/well and infected 1 or
428 2 times with 100µL of filtered lentivirus.
429
430 **Biotinylation experiments**
431 BioID-RSK : HeLa BioID-RSK cells were seeded in 6-well plates at a density of 90,000 cells
432 per well. Two 6-well plates were used per condition. 24h after seeding the cells, the medium
433 was changed to OptiMEM (Gibco) depleted from biotin (previously incubated with
434 streptavidin beads for 2h at 4°C, then filtered). Cells were kept in OptiMEM without biotin

435 for 48h, then they were infected with 600µl of virus per well at an MOI of 2.5. One hour
436 post-infection 2ml/well of DMEM containing 5µM biotin was added. Infection proceeded for
437 16h. For immunostaining, same protocol with these differences: 1,000 HeLa BioID-RSK
438 cells were seeded in wells of a 96-well plate. Cells were infected with 50µL of virus per well
439 at an MOI of 5 and infection proceeded for 10h.
440 BioID-L : HeLa cells were seeded in 6-well plates as of 160,000 cells per well. Two 6-well
441 plates were used per condition. 24h after seeding, the cells were infected with 600µl of
442 BioID-L replicons at an MOI of 2.5. One hour post-infection 2ml/well of DMEM containing
443 5µM biotin was added. Infection proceeded for 14h. For immunostaining, same protocol with
444 these differences: 3,500 HeLa cells were seeded in wells of a 96-well plate. Cells were
445 infected with 50µL of virus per well at an MOI of 5 and infection proceeded for 10h.
446
447 **Streptavidin Pulldown**
448 Cells were washed with PBS 3 times and lysed with 200µL/well of stringent lysis buffer
449 (50mM Tris-HCl pH 7.6, 500mM NaCl, 0.4% SDS, 1mM DTT, 1 tablet of Pierce
450 phosphatase/protease inhibitor (Thermo Scientific) per 10ml of lysis buffer) for 15 min at
451 room temperature (RT). Lysates were then diluted twice by addition of 200µl/well of 50mM
452 Tris-HCl pH7.6 and homogenized by 10 passages through 21G needles. Lysates were then
453 cleared by centrifugation at 12,000g for 10 min at RT. Supernatants were transferred to new
454 tubes, 200µL (per condition) of protein A/G magnetic beads (Pierce) were added to remove
455 non-specific binding and incubated for 30 min at RT. Supernatants were then transferred to a
456 new tube and a sample of 160µL per condition was mixed with 80µL of 3x Laemmli buffer
457 (cell lysate control). The rest of the supernatant was incubated for 2h at RT with 260µL (per
458 condition) of Streptavidin magnetic beads (Pierce). Streptavidin beads were then washed
459 once with 2% SDS, and twice with "normal lysis buffer" (50mM Tris-HCl pH 7.5, 100mM

460 NaCl, 2mM EDTA, 0.5% NP40, 1 tablet of phosphatase/protease inhibitor (Thermo
461 Scientific) per 10ml of lysis buffer) for 5 min at RT. Beads were resuspended in 40µl of 1x
462 Laemmli buffer and heated for 5min at 100°C to allow protein's separation from the beads.
463 Supernatants were then conserved at -20°C.
464
465 **Immunostaining**
466 Immunostaining was performed as described previously [49]

Antibody	**Dilution**	**Reference**
Anti-TMEV 3D polymerase (rabbit)	1/1000	kindly provided by M.Brahic
Anti-2A (rabbit)	1/1200	Home-made
Anti-NUP98 (rat)	1/400	N1038 - Sigma
Anti-POM121 (rabbit)	1/400	15645-1-AP - Proteintech
Anti-PTB (mouse)	1/400	324800 - Invitrogen
Anti-rabbit Alexa Fluor 594 (chicken)	1/800	A21442 - Molecular Probes
Anti-rabbit Alexa Fluor 488 (Goat)	1/800	A11008 - Molecular Probes
Anti-rat Alexa Fluor 594 (chicken)	1/800	A21471 - Molecular Probes
Anti-mouse Alexa Fluor 488 (Goat)	1/400	A11029 - Molecular Probes
Streptavidin Alexa Fluor 488	1/500	S32354 - Invitrogen
Streptavidin Alexa Fluor 594	1/500	S32356 - Invitrogen

467
468 **Western blotting**
469 Western blots were performed as described previously [49]

Antibody	**Dilution**	**Reference**

Anti-2A (rabbit)	1/4000	Home-made
Anti-Phospho-S380-RSK (rabbit)	1/4000	CST11989
anti-PKR (rabbit)	1/4000	18244-1-AP
anti-Phospho-T446-PKR (rabbit)	1/4000	AB32036
anti-ß-actin (mouse)	1/10000	A5441- Sigma
anti-TMEV 3D polymerase (rabbit)	1/2000	kindly provided by M.Brahic
anti-NUP98 (rat)	1/2000	N1038 - Sigma
anti-HA (mouse)	1/4000	MMS101P - Covance
anti-phospho-Akt/RSK-substrates:RxxS*/T* (rabbit)	1/1000	CST9614
anti-thio-P-ester (rabbit)	1/1000	NBP2-67738
anti-TMEV capsid (mouse)	1/2000	Home-made
anti-FLAG M2	1/5000	F1804 - Sigma
Anti-rabbit-HRP (Goat)	1/5000	P0448 – Dako
Anti-mouse-HRP (Goat)	1/5000	P0447 – Dako
Anti-rat-HRP (Rabbit)	1/5000	P0162 – Dako
Streptavidin-HRP	1/5000	P0397 – Dako

470

471

472 **Mass spectrometry**

473 Mass spectometry analysis was performed as described previously [50,51]. Streptavidin
474 pulldown samples were resolved using a 10% Tris-Glycine SDS gel run until 6mm migration
475 in the separating gel. Proteins were colored using PageBlue (Thermo Scientific, 24620). The
476 6mm bands containing whole proteins were cut into 3 different slices and trypsin digested (50

477 ng/µl in 50 mM NH4HCO3 buffer, pH 8.0). The peptides were analyzed by capillary LC-
478 tandem mass spectrometry using an Orbitrap Fusion Lumos tribrid ion trap mass
479 spectrometer (ThermoFisher Scientific). The resulting MS/MS data was processed using
480 Sequest HT search engine within Proteome Discoverer 2.5 SP1 against a *Homo Sapiens*
481 protein database obtained from Uniprot (78 787 entries) and containing the sequences of viral
482 proteins and BioID2-RSK. Trypsin was specified as cleavage enzyme allowing up to 2
483 missed cleavages, 4 modifications per peptide and up to 5 charges. Mass error was set to 10
484 ppm for precursor ions and 0.1 Da for fragment ions. Oxidation on Met (+15.995 Da),
485 phosphorylation on Ser, Thr and Tyr (+79.966 Da), conversion of Gln (-17.027 Da) or Glu (-
486 18.011 Da) to pyro-Glu at the peptide N-term, biotinylation of Lys (+ 226.077) were
487 considered as variable modifications. False discovery rate (FDR) was assessed using
488 Percolator and thresholds for protein, peptide and modification site were specified at 1%.
489 PSM Ratio calculations were as followed:

490 $$BioID - RSK: \frac{PSM(L^{WT})}{PSM(L^{M60V}) + PSM(mock) + 1} \qquad BioID - L: \frac{PSM(L^{WT}) + PSM(L^{F48A})}{PSM(L^{M60V}) + PSM(mock) + 1}$$

491

492 ***In vitro* kinase assay**

493 293T cells were seeded (500,000 cells/well) in 6-well plates. 24h after seeding, cells were
494 transfected with TransIT-LT1 reagent (Mirus Bio) with 2.5 µg of plasmids pTM1117,
495 pBLP20 and pBLP21. 6 hours post-transfection, cells were treated with 32nM PMA over-
496 night. HA-RSKs were then immunoprecipitated with anti-HA magnetic beads and
497 resuspended in 30µl of TBS, 1mM PMSF, 1mM Na_3VO_4, 1 tablet of phosphatase/protease
498 inhibitor (Thermo Scientific) per 10ml.
499 For one *in vitro* kinase reaction we put: 2.5µg of GST-S6 (recombinant substrate), 10µL of
500 kinase buffer 5x (125mM Hepes pH 7.5, 250mM NaCl, 100mM ß-glycerophosphate, 5mM
501 DTT, 100mM $MgCl_2$, 500µM Na_3VO_4), 0.5 µL of 10mM ATP (Roth, HN35.1) or A*TP

502 analogs (BioLog), and water to a final volume of 30µL. To this we added 10µL of the
503 immunoprecipitated RSK and incubated everything at 30°C for 30 min with shacking.
504 Following this, the samples were alkylated with PNBM (Abcam) at a final concentration of
505 2.5mM for 2 hours at room temperature with shacking. Reaction was stopped by the addition
506 of Laemmli buffer 3x. Samples were then heated at 100°C for 5 min, separated from the
507 magnetic beads and kept at -20°C.
508
509 **Thiophosphorylation in permeabilized cells**
510 1.5 x10^6 HeLa-RSK-TKO TM1117 or BLP21 cells were seeded in 10-cm dishes, 1 dish per
511 condition. The next day, cells were infected at an MOI of 5 for 8h30min (for TMEV) and
512 3h30min (for EMCV). After that, cells were permeabilized with 500µL of the analog-kinase
513 buffer: 20mM Hepes pH 7.5, 100mM KOAc, 5mM NaOAc, 2mM $MgOAc_2$, 1mM EGTA,
514 20µg/ml digitonin, 10mM $MgCl_2$, 0.5mM DTT, 1x phosphatase inhibitor cocktail 2 (P5726,
515 Merck), 1x cOmplete protease inhibitor (REF), 57µg/ml creatin kinase (Calbiochem 23895),
516 5mM Creatin phosphate (Calbiochem, 2380), 0.1mM ATP, 0.1 mM N6-Bn-A*TP analog
517 (BioLog), 3mM GTP (Roth, K056.4) in order to get the ATP analog inside the cells and the
518 thiophosphorylation reaction to happen. Reaction proceeded for 1h at 37°C, 5% CO2 with the
519 dishes on a rocking plate.
520
521 **Immunoprecipitation after thiophosphorylation reactions.**
522 <u>NUP98 IP:</u> cells were lysed by adding 500µL of 2x salty lysis buffer (100mM Tris-HCl pH8,
523 800mM NaCl, 2% Triton X-100, 4mM EDTA, 2mM DTT, 1 tablet of phosphatase/protease
524 inhibitor (Thermo Scientific) per 10ml of lysis buffer) to the analog-kinase buffer for 15 min
525 at 4°C. Lysates were diluted 4x by addition of 3ml of regular lysis buffer (50mM Tris-HCl
526 pH8, 100mM NaCl, 0.5% NP40, 2mM EDTA, 1 tablet of phosphatase/protease inhibitor

527 (Thermo Scientific) per 10ml of lysis buffer). Lysates were then homogenized by 10 passages
528 through 21G needles and cleared by centrifugation at 12,000g for 10min at 4°C. Supernatants
529 were then transferred to new tubes, 30µL (per condition) of protein A/G magnetic beads
530 (Pierce) were added to remove non-specific binding and incubated for 30 min at 4°C.
531 Supernatants were then transferred to a new tube and a sample of 200µL/ condition was
532 alkylated with PNBM at a final concentration of 2.5mM for 2h at RT.Alkylation reaction was
533 stopped by addition of 3x Laemmli buffer (cell lysate control). The rest of the supernatant
534 was incubated O/N at 4°C with 8µg (per condition) of anti- NUP98 (rat, N1038 – Sigma).
535 62.5µl of A/G beads per condition were added and incubated for 2h at 4°C. A/G beads were
536 then washed three times with regular lysis buffer for 5 min at 4°C. Beads were resuspended
537 in 40µl of kinase buffer 1x. The IP samples were then alkylated with PNBM at a final
538 concentration of 2.5mM for 2h at RT. Alkylation reaction was stopped by the addition of 3x
539 Laemmli, and immunoprecipitated proteins were separated from the beads after heating 5min
540 at 100°C.
541 Thiophosphate ester IP: cells were lysed by adding 500µL of 2x lysis buffer (100mM Tris-
542 HCl pH8, 200mM NaCl, 1% NP40, 40mM EDTA, 1 tablet of phosphatase/protease inhibitor
543 (Thermo Scientific) per 10ml of lysis buffer) for 15min at 4°C. Lysates were then
544 homogenized by 10 passages through 21G needles and cleared by centrifugation at 12,000g
545 for 10min at 4°C. Supernatants were then alkylated with PNBM at a final concentration of
546 2.5mM for 1h30min at RT. As PNBM inhibits immunoprecipitation, lysates were run through
547 PD10 columns to remove PNBM prior to the immunoprecipitation procedure. Fractions
548 containing protein were kept and incubated with 30µl per condition of A/G magnetic beads
549 (to avoid non-specific binding) for 30 min at 4°C. Supernatants were then transferred to a
550 new tube and 1/10th of the lysate (200µl) was mixed with 100µl of 3x Laemmli (cell lysate
551 control). The rest of the supernatant was incubated with 8µg per condition of anti-

552 thiophosphate ester antibody (rabbit, NBP2-67738 – Novusbio) for 2h at 4°C. Then 62.5µl
553 per condition of A/G magnetic beads were added and incubated for 2h at 4°C. A/G beads
554 were then washed three times with regular lysis buffer for 5 min at 4°C and resuspended in
555 60µl of Laemmli 1x. Immunoprecipitated proteins were separated from the beads after
556 heating 5min at 100°C.
557
558 **Data processing and statistical analysis (for MS)**
559 Protein identification and label-free quantitation were performed with MaxQuant version
560 1.6.7.0.[52]. Database searching was performed against the UniProt FASTA database, using a
561 false-discovery rate at the peptide and protein level was set to 0.01 and allowing a maximum
562 of two missed cleavages. All search parameters are available in the parameter file available in
563 the study repository (https://github.com/UCLouvain-CBIO/2022-RSK-Nups-VIRO). After
564 filtering out of identified contaminants, identified reverse sequences and proteins having
565 missing values in all samples, Max Quant protein intensities were log-2 transformed and
566 normalized using sample median alignment. In order to cope with the numerous drop-outs in
567 protein intensities, the proDA method, as described in [28] was applied.
568 For the analysis of the BioID-L experiment, the backbone linear model included one indicator
569 variable representing a potential batch effect between 2 groups of replicates, and 2 condition
570 indicator variables, representing the effect of resp. WT and F48A conditions with respect to
571 the reference condition, i.e. M60V condition. Potential batch effect was tested for each
572 protein using two-sided Wald test on the batch indicator variable. Since adjusted p-values
573 were > 0.2 for all proteins, this indicator variable was subsequently removed from the model.
574 Then, the effects of WT and F48 conditions (w.r.t. M60V) were tested, for each protein,
575 using one-sided Wald tests, since the anticipated sign of the effect was known. All p-values
576 were adjusted using Benjamini-Hochberg corrections [53].

577 For the analysis of the BioID-RSK experiment, the backbone linear model included one
578 indicator variable representing a potential batch effect between 2 groups of replicates, and 1
579 condition indicator variable, representing the effect of WT condition with respect to the
580 reference condition, i.e. M60V condition. Potential batch effect was tested for each protein
581 using two-sided Wald test on the batch indicator variable. Since adjusted p-values were > 0.6
582 for all proteins, this indicator variable was subsequently removed from the model. Then, the
583 effect of WT condition (w.r.t. M60V) was tested, for each protein, using a one-sided Wald
584 test, since the anticipated sign of the effect was known. All p-values were adjusted using
585 Benjaminin-Hochberg corrections.
586
587 **Statistical analysis on immunofluorescence**
588 Statistical analysis on immunofluorescence experiments was done using GraphPad Prism v9
589 A one-way ANOVA test was used. The number of independent experiments (n) and
590 statistical comparison groups are indicated in the Figures and Figure legends.
591
592 **Materials & Correspondence**
593 Further information and requests for resources and reagents should be directed to and will be
594 fulfilled by the lead contact, Thomas Michiels (thomas.michiels@uclouvain.be). All reagents
595 generated in this study are available upon request after completion of a Materials Transfer
596 Agreement
597
598 **Data and code availability**
599 The mass spectrometry proteomics data have been deposited to the ProteomeXchange
600 Consortium via the PRIDE partner repository with the dataset identifier PXD034604".

601 MaxQuant outputs are available in the study repository https://github.com/UCLouvain-
602 CBIO/2022-RSK-Nups-VIRO
603 Codes: Processed data and reproducible proDA analyses scripts are available in the study
604 repository at https://github.com/UCLouvain-CBIO/2022-RSK-Nups-VIRO

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Writing – original draft: B.L.P.

Writing – review & editing: B.L.P., D.V., F.S. and T.M.

Supervision: T.M.

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Funding acquisition: T.M.

Competing interests

The authors declare no conflicts of interests

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Figure legends

Fig 1. Pathogen's proteins hijack RSK kinases.

(A) Indirect substrate recruitment - model of the clamp: a pathogen's protein acts as an adaptor protein, which binds and activates RSK through its DDVF motif and recruits a target

801 protein to be phosphorylated by RSK through another domain. (B) Direct substrate
802 recruitment. A pathogen's protein interacts with RSK through its DDVF motif and is directly
803 phosphorylated by RSK.
804
805 **Fig 2. PTB diffusion and NUP98 hyperphosphorylation induced by cardioviruses**
806 **depends on both the L protein and RSK kinases.**
807 (A-C and E-G) PTB diffusion out of the nucleus depends on both L and RSK. (A and E)
808 Confocal microscopy images of HeLa WT cells infected with TMEV for 10h (A) or with
809 EMCV for 4h (E). (B and F) Graphs showing the percentage of cells with PTB diffusion
810 (mean ± SD), among cells infected by TMEV (3D-positive) for 10h (B) or by EMCV (capsid-
811 positive) for 5h (F). Counts: 25-50 infected cells per experiment (n=4). **** denotes
812 significant differences (One-way ANOVA, $p < 0.0001$) between HeLa-RSK-TKO cells
813 transduced with an empty vector (HeLa TKO) and cells re-expressing indicated RSK
814 isoforms, for L^{WT}-infected samples. (C and G) Confocal microscopy images of HeLa-WT or
815 HeLa RSK-TKO cells transduced with an empty vector (HeLa TKO) or re-expressing the
816 indicated RSK isoforms. Cells were infected with TMEV for 10h (C) or EMCV for 4h (G).
817 3D and 2A were stained as controls of TMEV and EMCV infection respectively. (D and H)
818 Western blots showing the RSK-dependent hyperphosphorylation of NUP98 (shift upwards)
819 or the inhibition of PKR activation induced by TMEV (D) or EMCV (H). 3D or 2A were
820 detected as control of infection, and ß-actin as loading control. Dashed lines between lanes
821 indicate deletion of irrelevant sections from the same membrane.
822
823 **Fig 3. Nucleocytoplasmic traffic perturbation in live cells depends on both the L protein**
824 **and RSK kinases.**

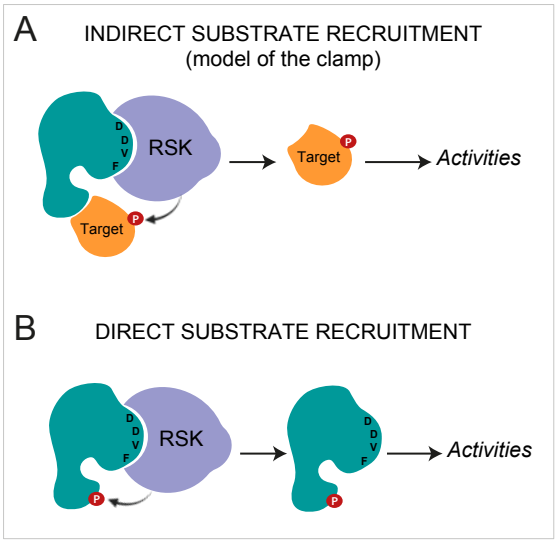
825 (A-D) Graphs and confocal microscopy pictures showing the quantification of RFP-NLS and
826 GFP-NES diffusion (mean ± SD) in live HeLa-LVX cells infected with TMEV for 10h (A-B)
827 or EMCV for 4h30 (C-D). HeLa-LVX cells were either WT, or RSK-TKO cells transduced
828 with an empty vector (HeLa-TKO) or re-expressing RSK1. Counts: 50 cells per experiment
829 (n=4). (E-G) Impact of L on nucleocytoplasmic traffic. HeLa-LVX cells transfected with an
830 empty plasmid or with plasmids expressing L^{WT}, L mutants or YopM. (E) Confocal
831 microscopy of HeLa-LVX cells 24hours post-transfection. (F) Western blot of HeLa-LVX
832 cells 24h post-transfection. FLAG staining shows the expression of L proteins and YopM.
833 (G) Graph showing the percentage of cells with GFP-NES and RFP-NLS diffusion (mean ±
834 SD). Counts: 70±5 cells per experiment (n=6). One-way ANOVA test was used to compare
835 all samples to L^{WT}. **** denotes significant differences (One-way ANOVA, $p < 0.0001$).
836 Scale bar: 20µm
837
838 **Fig 4. BioID-RSK and BioID-L fusion proteins biotinylate specific proteins during**
839 **cardiovirus infection.**
840 (A) Cartoon showing the expected biotinylation by BioID-RSK or BioID-L fusion proteins of
841 a target recruited by the C-terminal domain of L. (B) Western blot of proteins biotinylated by
842 BioID-RSK in lysates and pulled down samples of TMEV infected cells. HeLa BioID-RSK
843 cells were incubated for 2 days without biotin. Cells were then infected for 16h (MOI 2.5)
844 with L^{WT} or L^{M60V} viruses in medium containing 5µM biotin. Biotinylated proteins were
845 pulled-down using streptavidin-magnetic beads. (C) Schematic representation of BioID-L
846 TMEV replicon constructs. Capsid protein sequences were replaced by BioID-L and GFP
847 sequences. The beginning of the VP4 sequence was kept in order to allow BioID-L protein
848 processing by viral protease 3C, and the CRE sequence (localized in VP2) was added to
849 allow replication. (D) Western blot of proteins biotinylated by BioID-L in lysates and pulled

850 down samples of TMEV infected cells. HeLa cells were infected for 14h (MOI 2.5) with
851 BioID-L^{WT}, BioID-L^{M60V} or BioID-L^{F48A} replicons in medium containing 5µM biotin.
852 Biotinylated proteins were then pulled-down using streptavidin-magnetic beads.
853
854 **Fig 5. BioID-RSK and BioID-L proxeomes identify FG-NUPs as targets recruited by the**
855 **L-RSK complex.**
856 (A) Proteins identified in the proximity of BioID-RSK (y axis) and BioID-L (x axis). Y-axis
857 shows proteins detected after infection with L^{WT} but not L^{M60V} viruses (=PSMs in
858 L^{WT}/(L^{M60V}+NI+1)). X-axis shows proteins detected after infection with BioID-L^{WT} or
859 BioID-L^{F48A} but not BioID-L^{M60V} replicons (= PSMs in (L^{WT}+ L^{F48A})/(L^{M60V}+NI+1)). FG-
860 NUPs are identified by green dots. (B) Table showing the adjusted P-values for the 20 best
861 ranked proteins. Ranking was attributed by multiplying the BioID-RSK ratio by the BioID-L
862 ratio. Statistical analysis of pairwise comparisons made between L^{WT} and L^{M60V} for BioID-
863 RSK and between L^{WT} and L^{M60V} or L^{F48A} and L^{M60V} for BioID-L. Adjusted P-values < 0.05
864 are colored in red, FG-NUPs are colored in green. (C) Volcano plots showing the same
865 pairwise comparisons as in B. Proteins having an adjusted P-value < 0.05 and a Log_2 fold
866 change (LFC) > 1 are colored in red (= proteins in Q2). FG-NUPs are colored in green.
867
868 **Fig 6. Subcellular localization of proteins biotinylated by BioID-RSK.**
869 (A) Confocal microscopy images of biotinylated proteins (green) and 3D viral polymerase
870 (red) detected in BioID-RSK expressing HeLa cells infected with L^{WT} or L^{M60V} viruses for
871 10h (MOI 5). (B) Quantification of cells showing a visible nuclear rim of biotinylated
872 proteins in HeLa BioID-RSK cells infected with L^{WT} or L^{M60V} viruses (mean ± SD). Counts:
873 42 ± 10 infected cells per experiment (n=4). One-way ANOVA tests were used to compare
874 all samples with each other, **** denotes significant differences (p < 0.0001). (C) BioID-

875 RSK biotinylates proteins that colocalize with the FG-NUP NUP98. Confocal microscopy
876 images of biotinylated proteins (green), 3D viral polymerase (red), and NUP98 (orange) in
877 HeLa BioID-RSK cells infected with L^{WT} or L^{M60V} viruses for 10h (MOI 5). 3D viral
878 polymerase was stained as a control of infection. White arrows point to examples of cells
879 exhibiting a nuclear rim staining. Scale bar: 10µm.
880
881 **Fig 7. Subcellular localization of proteins biotinylated by BioID-L.**
882 (A) Confocal microscopy images of biotinylated proteins stained with streptavidin-Alexa
883 Fluor 594 (orange) and 3D viral polymerase (green) in HeLa cells infected for 10h with
884 BioID-L^{WT}, BioID-L^{M60V} or BioID-L^{F48A} replicons (MOI 5). (B) Quantification of nuclear
885 rim staining in HeLa cells infected with BioID-L^{WT}, BioID-L^{M60V} or BioID- L^{F48A} viruses
886 (mean ± SD). Counts: 31 ± 11 infected cells per experiment (n=4). One-way ANOVA tests
887 were used to compare all samples with each other, **** denotes significant differences ($p <$
888 0.0001). 3D viral polymerase was stained as a control of infection. (C) BioID-L biotinylates
889 proteins that colocalize with the FG-NUP POM121. Confocal microscopy images of
890 biotinylated proteins (orange) and POM121 (green) in HeLa cells infected with BioID-L^{WT},
891 BioID-L^{M60V} or BioID-L^{F48A} viruses for 10h (MOI 5). White arrows point to examples of
892 cells exhibiting a nuclear rim staining. Scale bar: 10µm.
893
894 **Fig 8. RSK directly phosphorylates NUP98 in TMEV and EMCV infected cells.**
895 (A) Cartoon showing the principle of the analog-sensitive kinase system. The kinase mutated
896 in the ATP binding pocket (As-RSK) can use regular ATP as well as a bulkier ATP analog
897 (A*TP-S). Use of the ATP analog induces the thiophosphorylation of the substrate. After an
898 alkylation reaction with paranitrobenzylmesylate (PNBM), thiophosphates are converted to
899 thiophosphate esters, which are specifically recognized by an anti-thiophosphate ester

900 antibody. All other wild-type kinases in the cell, have normal ATP binding pockets that
901 cannot accommodate the ATP analog. (B) Identification of the gatekeeper residue in RSK2.
902 The gatekeeper residue of RSK2 was identified by aligning the sequences of c-SRC (P00523)
903 with those of the N-terminal kinase domain of RSKs (RSK1: Q15418, RSK2: P51812, RSK3:
904 Q15349, RSK4: Q9UK32). Typically, the gatekeeper residue aligns with T338 of c-SRC, and
905 is preceded by two hydrophobic amino acids and followed by an acidic and another
906 hydrophobic amino acid. RSK2 gatekeeper residue was thereby identified as L147. (C-E)
907 HeLa cells expressing As2-RSK or WT-RSK were infected with TMEV for 8h (MOI 5) (C
908 and E) or with EMCV for 3h30min (MOI 5) (D). Cells were then permeabilized with
909 digitonin, and N6-Bn-ATP-γ-S was added for 1 hour. Cells were then lysed and either
910 NUP98 (C and D) or thio-phosphate-ester containing proteins were immunoprecipitated (E).
911 (C and D) Immunoblots showing a concentrated amount of NUP98 in the
912 immunoprecipitation samples and thiophosphate-ester proteins. (E) Immunoblots showing
913 NUP98 in the thiophosphate ester IP fraction when L^{WT} is present. Detection of viral capsid
914 in the lysates was made as control for infection.

Fig. 1


A
INDIRECT SUBSTRATE RECRUITMENT
(model of the clamp)
D
D
V
F
RSK
Target
P
Target
P
Activities
B
DIRECT SUBSTRATE RECRUITMENT
D
D
V
F
RSK
P
D
D
V
F
P
Activities

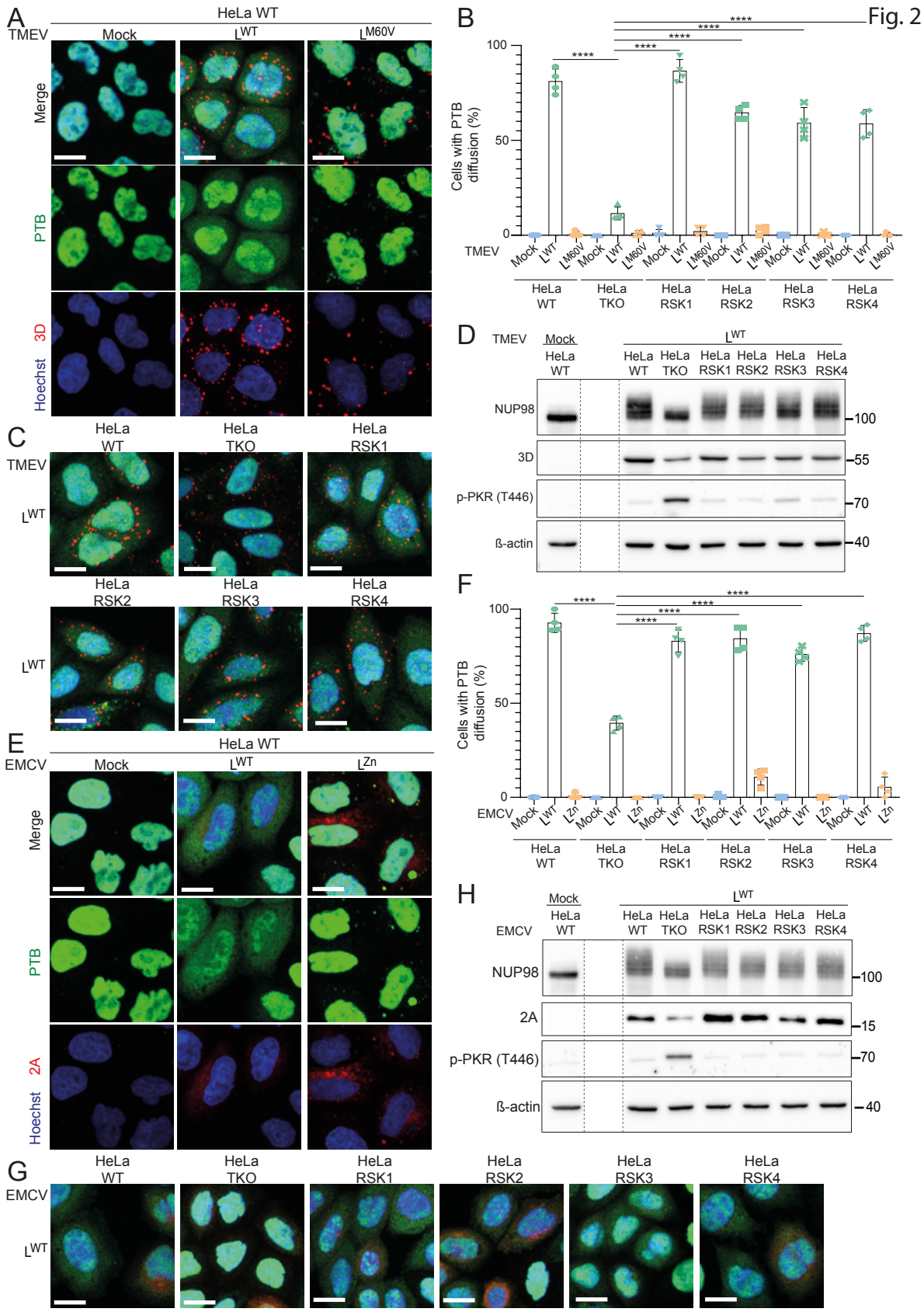


Fig. 2
A
HeLa WT
TMEV
Mock
LWT
LM60V
Merge
PTB
3D
Hoechst
B
Cells with PTB diffusion (%)
100
50
0
****
TMEV
Mock
LWT
LM60V
HeLa WT
HeLa TKO
HeLa RSK1
HeLa RSK2
HeLa RSK3
HeLa RSK4
C
HeLa WT
HeLa TKO
HeLa RSK1
HeLa RSK2
HeLa RSK3
HeLa RSK4
TMEV
LWT
D
TMEV
Mock
LWT
HeLa WT
HeLa TKO
HeLa RSK1
HeLa RSK2
HeLa RSK3
HeLa RSK4
NUP98
3D
p-PKR (T446)
ß-actin
100
55
70
40
E
HeLa WT
EMCV
Mock
LWT
LZn
Merge
PTB
2A
Hoechst
F
Cells with PTB diffusion (%)
100
50
0
EMCV
Mock
LWT
LZn
HeLa WT
HeLa TKO
HeLa RSK1
HeLa RSK2
HeLa RSK3
HeLa RSK4
H
Mock
LWT
EMCV
HeLa WT
HeLa TKO
HeLa RSK1
HeLa RSK2
HeLa RSK3
HeLa RSK4
NUP98
2A
p-PKR (T446)
ß-actin
100
15
70
40
G
HeLa WT
HeLa TKO
HeLa RSK1
HeLa RSK2
HeLa RSK3
HeLa RSK4
EMCV
LWT

Fig. 3

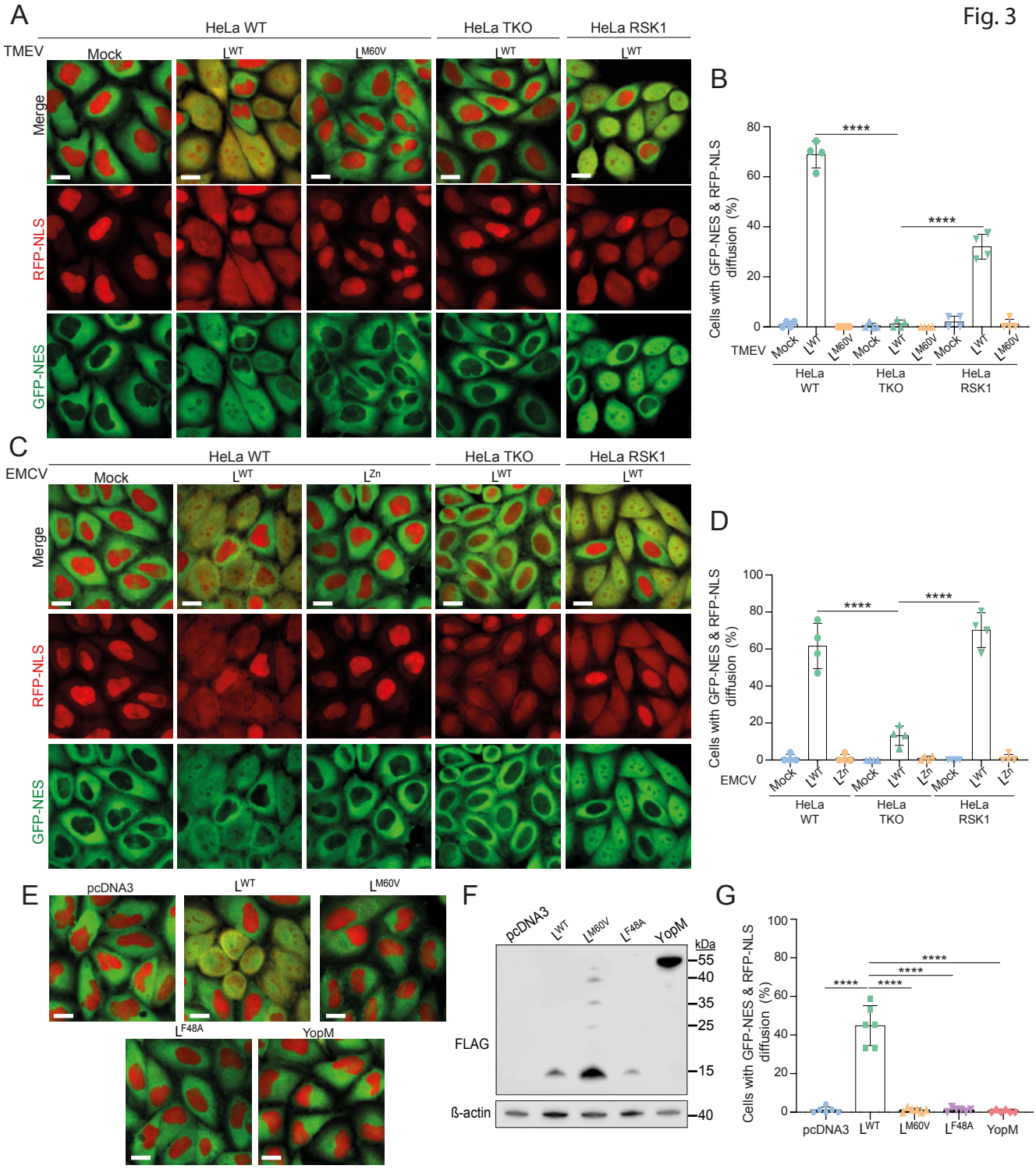
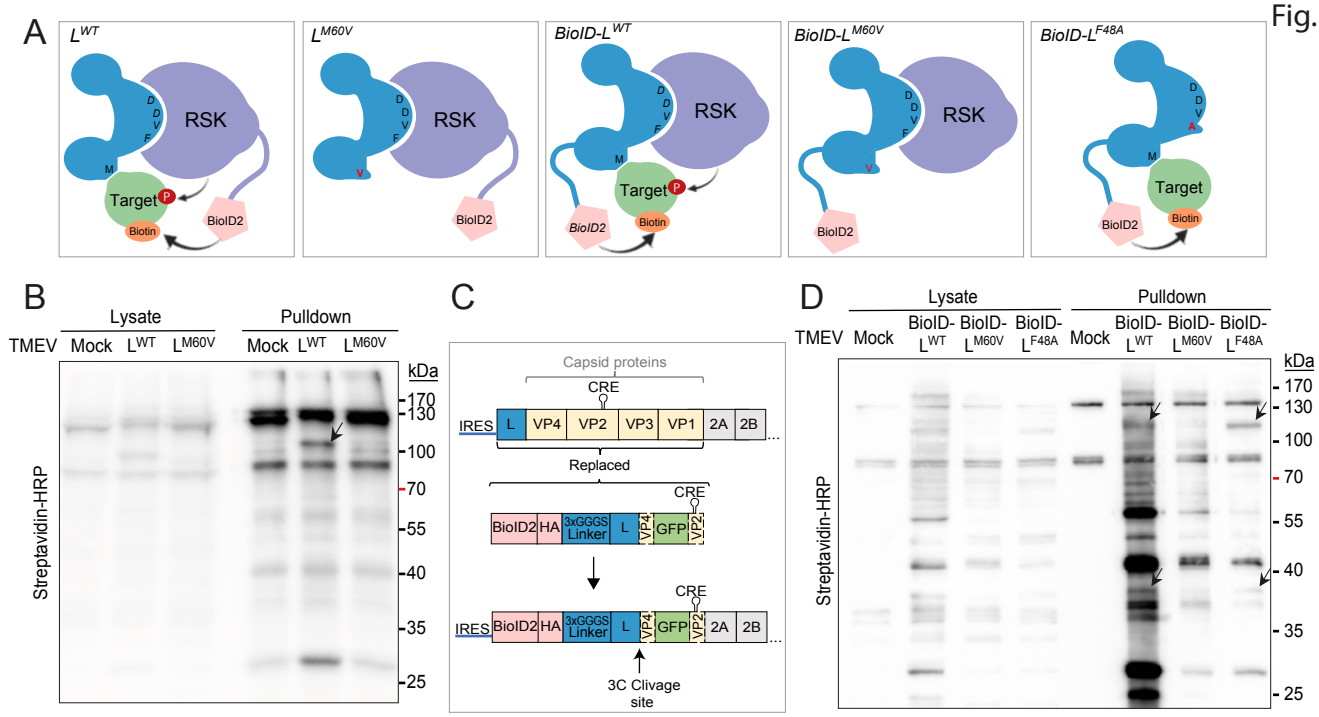
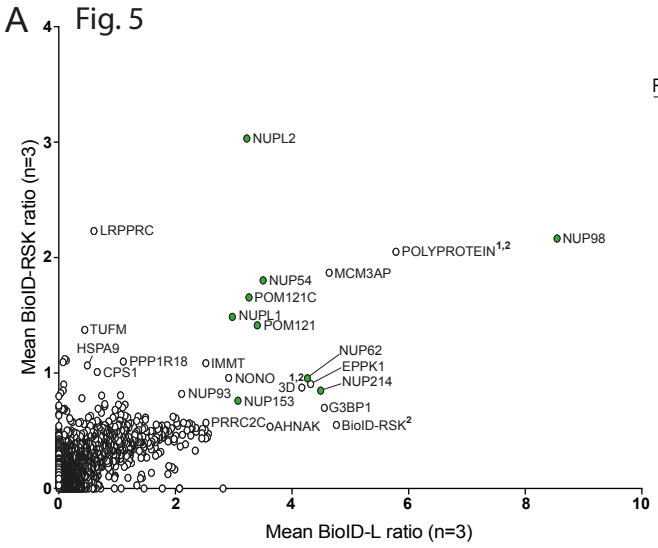

A
HeLa WT
HeLa TKO
HeLa RSK1
TMEV
Mock
L^WT
L^M60V
Merge
RFP-NLS
GFP-NES
B
Cells with GFP-NES & RFP-NLS diffusion (%)
****
TMEV
HeLa WT
HeLa TKO
HeLa RSK1
C
EMCV
L^Zn
D
E
pcDNA3
L^F48A
YopM
F
kDa
FLAG
ß-actin
G

Fig. 4


A
L WT
L M60V
BioID-L WT
BioID-L M60V
BioID-L F48A
RSK
Target
BioID2
Biotin
B
Lysate
Pulldown
TMEV
Mock
L WT
L M60V
kDa
170
130
100
70
55
40
35
25
Streptavidin-HRP
C
Capsid proteins
CRE
IRES
L
VP4
VP2
VP3
VP1
2A
2B
Replaced
BioID2
HA
3xGGGS Linker
GFP
3C Clivage site
D
BioID-L WT
BioID-L M60V
BioID-L F48A

A Fig. 5

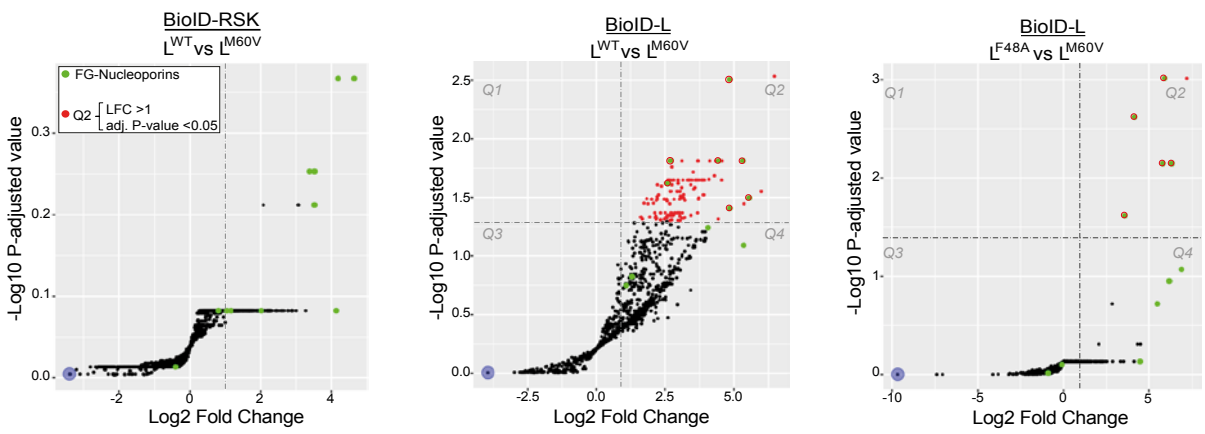


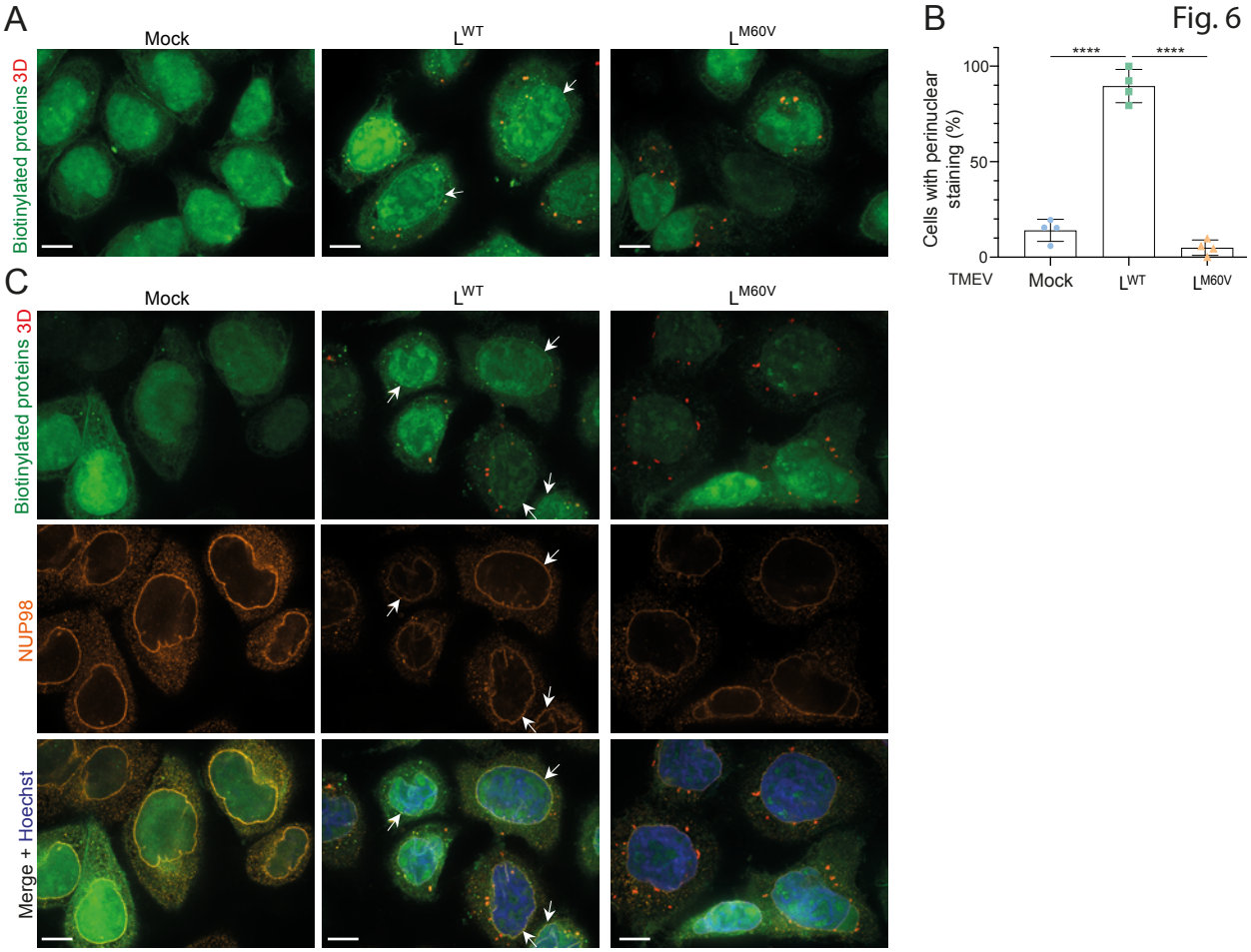
B

Ranking	BioID-RSK ratio x BioID-L ratio	Gene name	BioID-RSK ratio (mean)	BioID-L ratio (mean)	Adj. P-value BioID-RSK L^{WT} vs L^{M60V}	Adj. P-value BioID-L L^{WT} vs L^{M60V}	Adj. P-value BioID-L L^{F48A} vs L^{M60V}
1	18.51	NUP98	2.17	8.55	0.558	0.003	0.001
2	11.85	POLYPROTEIN[1,2]	2.05	5.78	NA	NA	NA
3	9.78	NUPL2	3.03	3.22	0.429	0.081	0.740
4	8.67	MCM3AP	1.87	4.64	0.614	0.003	0.001
5	6.32	NUP54	1.80	3.51	0.827	0.039	0.112
6	5.40	POM121C	1.66	3.26	0.429	0.057	0.192
7	4.81	POM121	1.41	3.40	0.827	0.032	0.085
8	4.43	NUPL1	1.49	2.98	0.614	0.015	0.007
9	4.07	NUP62	0.95	4.26	0.558	0.015	0.007
10	3.91	EPPK1	0.90	4.32	0.827	0.033	0.975
11	3.81	NUP214	0.85	4.49	0.827	0.015	0.002
12	3.64	3D[1,2]	0.87	4.17	NA	NA	NA
13	3.18	G3BP1	0.70	4.55	0.827	0.015	0.493
14	2.79	NONO	0.96	2.91	0.827	0.062	0.839
15	2.74	IMMT	1.09	2.52	0.827	0.028	0.740
16	2.62	BIOID-RSK2[2]	0.55	4.76	NA	NA	NA
17	2.34	NUP153	0.76	3.07	0.827	0.024	0.024
18	1.94	AHNAK	0.54	3.62	0.903	0.237	0.813
19	1.73	NUP93	0.82	2.11	0.827	0.081	0.493
20	1.44	PRRC2C	0.57	2.52	0.827	0.045	0.916

[1] Viral proteins
[2] Data of identified peptides belonging to shared protein groups.

C




A
Mock
L^WT
L^M60V
Biotinylated proteins 3D
B
Fig. 6
Cells with perinuclear staining (%)
100
50
0
****
****
TMEV
Mock
L^WT
L^M60V
C
Mock
L^WT
L^M60V
Biotinylated proteins 3D
NUP98
Merge + Hoechst

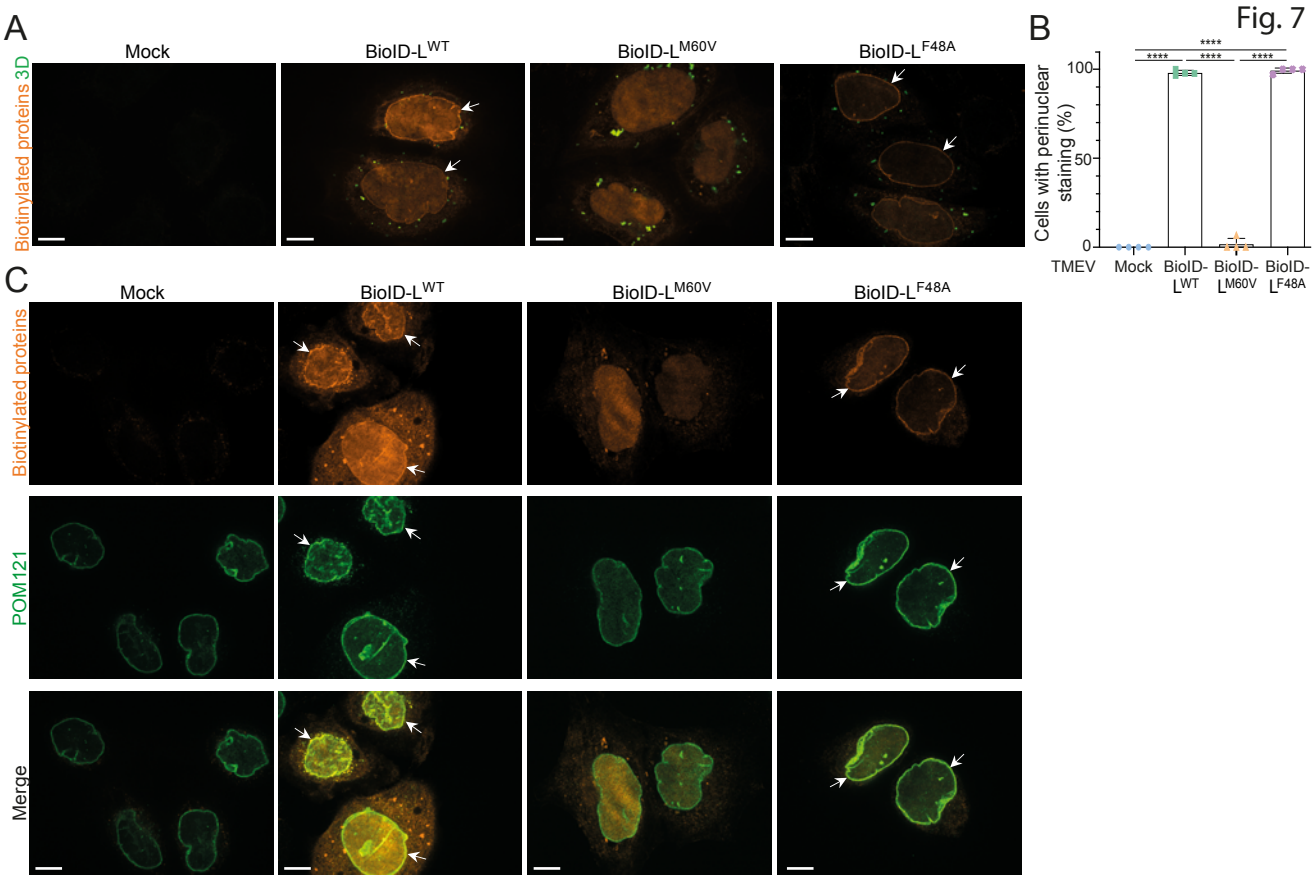
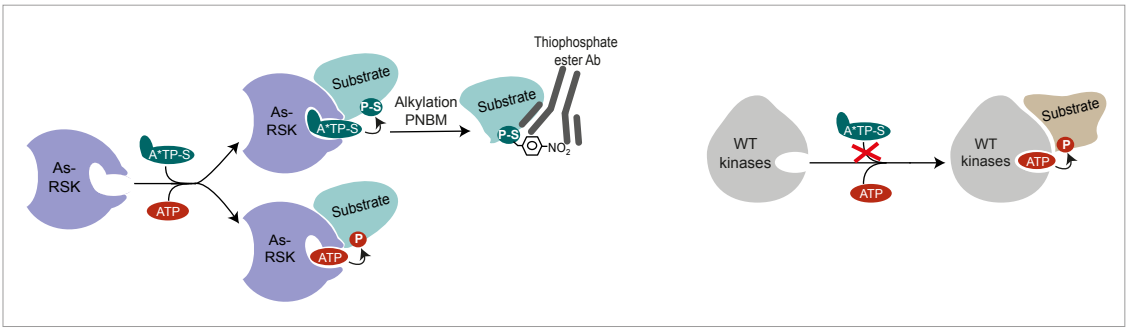


Fig. 7
A
Mock
BioID-L^WT
BioID-L^M60V
BioID-L^F48A
Biotinylated proteins 3D
B
Cells with perinuclear staining (%)
100
50
0
****
TMEV
Mock
BioID-L^WT
BioID-L^M60V
BioID-L^F48A
C
Mock
BioID-L^WT
BioID-L^M60V
BioID-L^F48A
Biotinylated proteins
POM121
Merge

Fig. 8

A

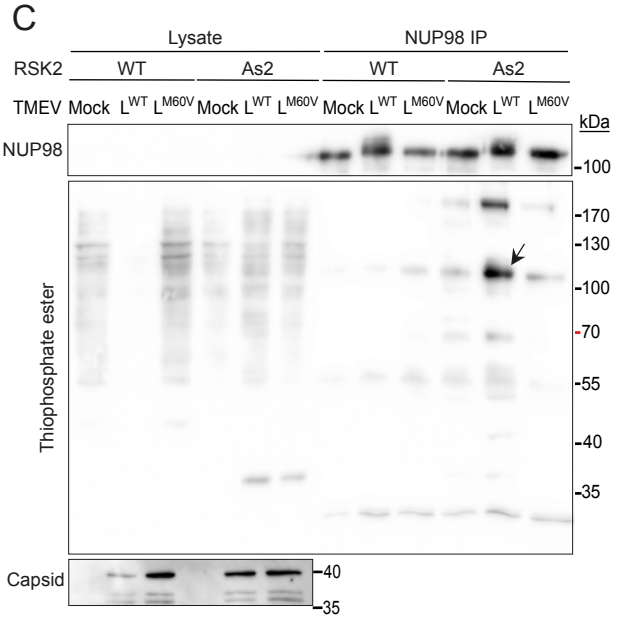


B

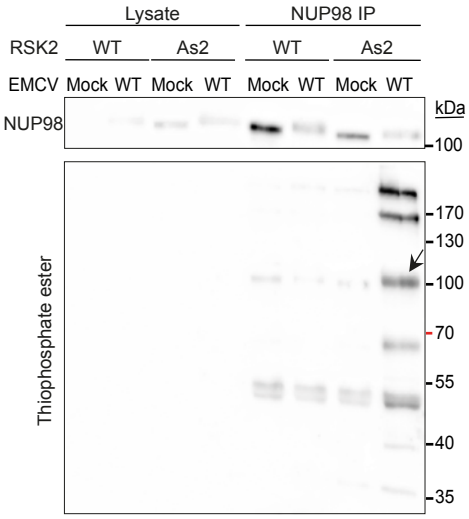
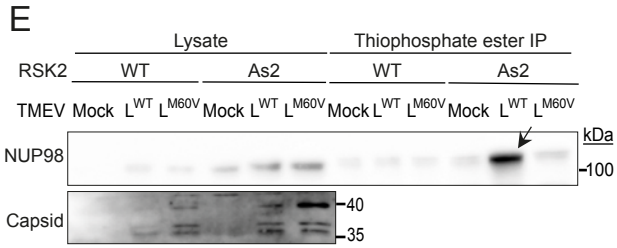
Gatekeeper residue

```
c-SRC   318 RHEKLV-QLYAVVSEEPIYIVTEYMSKGSLLDFLKGE-------MGKYLRLPQLVDMAAQ
RSK4    131 NHPFIVKLHYAFQTEGKLYLILDFLRGGDVFTRLSKEVLFTEEDVKFYLAE---------
RSK1    117 NHPFVVKLHYAFQTEGKLYLILDFLRGGDLFTRLSKEVMFTEEDVKFYLAE---------
RSK2    126 NHPFIVKLHYAFQTEGKLYLILDFLRGGDLFTRLSKEVMFTEEDVKFYLAE---------
RSK3    120 NHPFIVKLHYAFQTEGKLYLILDFLRGGDLFTRLSKEVMFTEEDVKFYLAE---------
```

C



D



E