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## Depletion of TAX1BP1 amplifies innate immune responses during respiratory syncytial virus infection

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1 **Depletion of TAX1BP1 amplifies innate immune responses during respiratory**  
2 **syncytial virus infection**

3

4 **Running title: Revealing the role of TAX1BP1 during RSV infection**

5

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21

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31 **ABSTRACT**

32 Respiratory syncytial virus (RSV) is the main cause of acute respiratory infections in young children,  
33 and also has a major impact on the elderly and immunocompromised people. In the absence of a  
34 vaccine or efficient treatment, a better understanding of RSV interactions with the host antiviral  
35 response during infection is needed. Previous studies revealed that cytoplasmic inclusion bodies (IBs)  
36 where viral replication and transcription occur could play a major role in the control of innate immunity  
37 during infection by recruiting cellular proteins involved in the host antiviral response. We recently  
38 showed that the morphogenesis of IBs relies on a liquid-liquid phase separation mechanism  
39 depending on the interaction between viral nucleoprotein (N) and phosphoprotein (P). These scaffold  
40 proteins are expected to play a central role in the recruitment of cellular proteins to IBs. Here, we  
41 performed a yeast two-hybrid screen using RSV N protein as a bait, and identified the cellular protein  
42 TAX1BP1 as a potential partner of this viral protein. This interaction was validated by pulldown and  
43 immunoprecipitation assays. We showed that TAX1BP1 suppression has only a limited impact on RSV  
44 infection in cell cultures. However, RSV replication is decreased in TAX1BP1-deficient mice  
45 (TAX1BP1<sup>KO</sup>), whereas the production of inflammatory and antiviral cytokines is enhanced. *In vitro*  
46 infection of wild-type or TAX1BP1<sup>KO</sup> alveolar macrophages confirmed that the innate immune  
47 response to RSV infection is enhanced in the absence of TAX1BP1. Altogether, our results suggest  
48 that RSV could hijack TAX1BP1 to restrain the host immune response during infection.

49

50 **Importance**

51 Respiratory syncytial virus (RSV), which is the leading cause of lower respiratory tract illness in  
52 infants, still remains a medical problem in the absence of vaccine or efficient treatment. This virus is  
53 also recognized as a main pathogen in the elderly and immunocompromised people, and the  
54 occurrence of co-infections (with other respiratory viruses and bacteria) amplifies the risks of  
55 developing respiratory distress. In this context, a better understanding of the pathogenesis associated  
56 to viral respiratory infections, which depends on both viral replication and the host immune response,  
57 is needed. The present study reveals that the cellular protein TAX1BP1, which interacts with the RSV  
58 nucleoprotein N, participates in the control of the innate immune response during RSV infection,  
59 suggesting that N-TAX1BP1 interaction represents a new target for the development of antivirals.

60

61 **INTRODUCTION**

62 Respiratory syncytial virus (RSV) is the main pathogen responsible for acute respiratory infections and  
63 bronchiolitis in children (1). Almost all children are infected by the age of two. A systemic multisite  
64 study on the cause of infant's pneumonia in hospitalized children in Asia and Africa recently revealed  
65 that RSV is the main etiological agent of severe pneumonia, accounting for over 30% of infections (2).  
66 In the United States, RSV is estimated to be responsible for the hospitalization of 86,000 children per  
67 year, with a related cost of 394 million dollars (3). Furthermore, RSV infections in early childhood is  
68 recognized to later increase the susceptibility to chronic asthma (4, 5). Reinfections occur throughout  
69 life and if healthy adults generally present symptoms of bad cold, RSV infections are associated with  
70 significant morbidity and mortality in the elderly and immunocompromised people (6-9). Indeed, RSV  
71 is estimated to cause over 17,000 deaths per year in the United States, 78% of which occur in adults  
72 over 65 years of age, and is responsible for 5% of total hospital admissions in the elderly (10).  
73 Although RSV has a major impact on human health and the economy, there is still no vaccine  
74 available. The development of vaccines has been hampered by the repercussions of a failed vaccine  
75 trial using a formalin-inactivated virus in the 1960s, which resulted in an exacerbation of the pathology  
76 upon infection and led to two deaths (11). The current standard of care consists of prophylactic  
77 treatment of at-risk infants with a monoclonal antibody (Palivizumab), but its use is limited by its  
78 moderate effectiveness and high cost (12).

79 The pathology associated with RSV infection results from both viral replication and the host's immune  
80 response (13). RSV infection triggers an early immune response mediated by the production of type I  
81 interferons (IFN-I) which induces the transcription of IFN-stimulating genes (ISG) and the production of  
82 proinflammatory mediators (14-17). On the other hand, RSV has developed multiple strategies to  
83 hijack cellular pathways controlling the IFN-I and NF- $\kappa$ B (Nuclear Factor kappa B) pathway in order to  
84 blunt the host antiviral response (17-19). In particular, the two nonstructural viral proteins NS1 and  
85 NS2 are known to suppress IFN-I production and cell signaling during infection (20). Although IFN-I  
86 are major players in viral clearance and are essential to induce an appropriate immune response (21),  
87 they could also contribute to RSV pathogenesis with potentially different roles in infants and adults  
88 (17, 22-26). Indeed, high levels of IFN-I and inflammatory cytokines usually correlate with severity as  
89 this reflects the inability of the immune response to control the virus. It is thus essential to better

90 characterize the complex interactions between RSV and the host immune response to decipher  
91 pathogenesis and design effective treatments.

92 RSV belongs to the *Mononegavirales* (MNV) order and the *Pneumoviridae* family (27). It is an  
93 enveloped virus with a non-segmented negative strand RNA genome containing 10 genes that encode  
94 11 proteins. The two surface glycoproteins G and F are involved in the initial steps of infection, *i.e.*  
95 attachment and fusion with the cell membrane. The viral membrane, which also contains the small  
96 hydrophobic protein SH, is lined by the matrix protein M that drives virus assembly. The genome is  
97 encapsidated by the nucleoprotein N, forming a helical nucleocapsid (28). The polymerase complex  
98 composed of the large polymerase (L) and its main cofactor the phosphoprotein P, is associated with  
99 this ribonucleoprotein complex (RNP) which serves as a template for viral transcription and replication  
100 (29). The viral transcription factor M2-1 is also present in the viral particle. After cell entry, RSV  
101 replicates in the cytoplasm of host cells within viro-induced spherical cytoplasmic granules called  
102 inclusion bodies (IBs). These structures are viral factories where all the viral proteins of the  
103 polymerase complex concentrate to perform the replication and transcription of the viral genome (30).  
104 These structures also play a role in viral escape from the innate immune system by limiting the  
105 recognition of viral RNAs by cytoplasmic pattern recognition receptors (PRRs) such as RIG-I (Retinoic  
106 acid-Inducible Gene I) and MDA5 (Melanoma Differentiation-Associated gene 5). Once stimulated,  
107 these PRRs activate the transcription factors NF- $\kappa$ B and interferon regulatory factor 3 and 7 (IRF3/7)  
108 (31). The function of IBs in the modulation of the host innate immune response was further supported  
109 by a study showing that MDA5 interacts with the RSV-N protein. In addition, MDA5 and the  
110 downstream signaling molecule MAVS (Mitochondrial AntiViral Signaling) both colocalize to IBs as  
111 soon as 12 hours post-infection, leading to downregulation of *IFN $\beta$*  mRNA expression (32). More  
112 recently, a study also revealed the sequestration of the NF- $\kappa$ B subunit p65 in RSV IBs (33). It is thus  
113 now recognized that the recruitment of cellular proteins into IBs participates not only in viral replication  
114 but is also involved in the control of cellular responses (34).

115 We recently showed that RSV IBs display hallmarks of liquid-liquid phase separation, and that the N  
116 and P proteins are at the core of the RSV IBs biogenesis (35). Their role as scaffold proteins suggest  
117 that N and P are directly involved in the partitioning of cellular proteins to IBs. However, their  
118 interactions with cellular factors are still poorly characterized. Here we report the identification of Tax1-  
119 binding protein 1 (TAX1BP1) as an interactor of RSV-N. TAX1BP1 was initially identified as a partner

120 of the Tax protein from Human T-lymphotropic virus 1 (HTLV-1) (36). Since then, TAX1BP1 was  
121 shown to interact with viral proteins from Papillomaviruses (37), measles virus (MeV) (38) and  
122 Mammarenaviruses (39). Among the described activity of TAX1BP1, this protein was involved in the  
123 negative regulation of NF- $\kappa$ B and IRF3 signaling by editing the ubiquitylation of its catalytic partner, the  
124 protein A20 (40, 41). We thus investigated the role of TAX1BP1 in both RSV replication and control  
125 the host antiviral response using *in vitro* and *in vivo* infection models. Altogether our results suggest  
126 that TAX1BP1 is recruited by RSV to inhibit the host antiviral response.

127

## 128 RESULTS

### 129 ***Identification of TAX1BP1 interaction with the viral nucleoprotein N***

130 To identify cellular interactors of the RSV-N protein, we first performed a yeast two-hybrid (Y2H)  
131 screen. Yeast cells were transformed with a vector encoding the RSV-N protein fused to GAL4 DNA  
132 binding domain (GAL-BD) in order to use it as bait in the Y2H system. Surprisingly, no yeast clones  
133 were obtained, suggesting that RSV-N is toxic. This could be due to the non-specific RNA-binding  
134 properties of N (42). We thus decided to use as a substitute the N protein harboring the K170A/R185A  
135 mutations that were previously shown to impair the interaction of N with RNA. This mutant is  
136 expressed as a monomeric RNA-free N, named N<sup>mono</sup>, which can mimic the natural N<sup>0</sup> form (42). When  
137 yeast cells were transformed with a vector encoding N<sup>mono</sup> fused to GAL4-BD, growing colonies were  
138 obtained on selective medium as expected. Yeast cells expressing N<sup>mono</sup> were then mated with yeast  
139 cells transformed with a human spleen cDNA library or a normalized library containing 12,000 human  
140 ORFs fused to the GAL4 activation domain (GAL4-AD; prey libraries). Yeast diploids were grown on  
141 appropriate medium for the selection of bait-prey interactions, and positive colonies were analyzed by  
142 PCR and sequencing for identifying human proteins captured by N<sup>mono</sup> in the Y2H system. This screen  
143 allowed us to identify, among others, the protein TAX1BP1 as an interactor of the N<sup>mono</sup> protein (Table  
144 1). For this specific interaction, 40 positive yeast colonies were obtained, and the alignments of the  
145 reads from the PCR products showed that the C-terminal part of TAX1BP1 (residues 401-789),  
146 including half of the central coiled-coil domain involved in TAXBP1 dimerization and the C-terminal  
147 zinc fingers (ZF), is involved in the interaction with N (Figure 1A). None of the cDNA clones expressed  
148 full-length TAX1BP1. This probably reflects the fact that isolated domains often better perform than full

149 length proteins in the Y2H system as the reconstitution of a functional GAL4 transcription factor is  
150 usually facilitated (43).

151 To validate the interaction between TAX1BP1 and the RSV-N protein, we then performed pulldown  
152 assays using recombinant proteins. Analysis of purified GST-TAX1BP1 by SDS-PAGE stained with  
153 Coomassie blue revealed two main bands of equivalent intensity with apparent MW close to 120 kDa  
154 (Figure 1B). Mass spectrometry analysis of these products allowed to identify the higher migrating  
155 band as full length GST-TAX1BP1 (theoretical mass, 112 kDa). The lower band corresponds to GST-  
156 TAX1BP1 deleted from the last 77 residues of TAX1BP1 (data not shown), which include the two C-  
157 terminal ZF of the protein (Figure 1A). This analysis revealed the strong instability of TAX1BP1 C-  
158 terminal domain when expressed alone in bacteria. When co-incubated with Sepharose-glutathione  
159 beads bound to either GST or GST-TAX1BP1, recombinant N protein was specifically captured in the  
160 presence of GST-TAX1BP1 (Figure 1B). This result confirmed that RSV-N and TAX1BP1 can directly  
161 interact. Finally, we investigated the capacity of RSV-N protein to interact with TAX1BP1 in  
162 mammalian cells. Cells were co-transfected with plasmids encoding RSV-N and Flag-tagged  
163 TAX1BP1 or the Flag-tag alone as a control, and an immunoprecipitation assay was performed using  
164 an anti-Flag antibody. As shown on figure 1C, the RSV-N protein co-precipitated specifically with Flag-  
165 TAX1BP1. Altogether, if our results indicate that the RSV-N protein can interact directly with  
166 TAX1BP1, further characterization of the domain of TAX1BP1 involved in the interaction should be  
167 required to validate the potential role of the oligomerization and the ZF domains in N binding.

168

#### 169 ***Downregulation of TAX1BP1 expression has limited impact on RSV replication in human cells***

170 TAX1BP1 was recently shown to control the cellular antiviral response during RSV infection (44). We  
171 thus determined whether downregulation of TAX1BP1 expression has an impact on RSV replication in  
172 cell culture (45). Human epithelial A549 cells were transfected with control siRNA (siCT) or siRNA  
173 targeting TAX1BP1 (siTAX1BP1). After 24 h of culture, cells were infected with recombinant strains of  
174 human RSV expressing either the fluorescent protein mCherry (rHRSV-mCherry) or the  
175 bioluminescent enzyme firefly luciferase (rHRSV-Luc). After 48 h of culture, mCherry and luciferase  
176 expression were determined as a proxy for viral infection. Lower signals were observed in siTAX1BP1-  
177 treated cells, thus suggesting a role of TAX1BP1 in RSV replication (Figure 2A). Western-blot analysis  
178 of cell lysates confirmed that TAX1BP1 expression is suppressed at this time point (Figure 2B).

179 Somewhat unexpectedly, RSV-N expression in siTAX1BP1-treated cells was similar to control cells  
180 (Figure 2B), suggesting that TAX1BP1 has no major impact on viral replication in this cell culture  
181 system. We then further assessed the consequence of TAX1BP1 downregulation on viral shedding by  
182 quantifying virions in culture supernatants of infected cells. As shown on figure 2C, viral titers in  
183 supernatants of siTAX1BP1-treated cells were similar to siCT-treated controls. These results  
184 corroborate those of Martin-Vicente *et al.* (44), showing only a weak reduction of virus titer upon  
185 downregulation of TAX1BP1 expression. Altogether, these results led to the conclusion that although a  
186 slight decrease of RSV replication was detected using quantitative approaches based on fluorescent  
187 or luminescent reporter proteins, TAX1BP1 does not have a strong impact on RSV replication. This  
188 suggested a more indirect effect of TAX1BP1 on RSV replication that could depend on its regulatory  
189 role on the innate immune response.

190

#### 191 ***Depletion of TAX1BP1 impairs RSV replication in mice***

192 Given the complexity of the immune response triggered upon RSV infection, we assessed the impact  
193 of TAX1BP1 depletion directly *in vivo* using TAX1BP1-deficient (TAX1BP1<sup>KO</sup>) mice. These mice being  
194 generated in 129-strain mice (40), we first investigated the kinetics of rHRSV-Luc replication in this  
195 genetic background. Although luminescence was shown to be correlated to viral replication by direct  
196 measurement on live animals in BALB/c mice using the IVIS system (45, 46), the skin pigmentation of  
197 129 mice impaired luminescence detection. We thus decided to monitor viral replication in infected  
198 animals by measuring the luciferase activity in lung homogenates. Wild-type 129 mice were either instilled  
199 with mock control (Mock) consisting of HEp2 cell culture supernatant, or infected with  $1.87 \times 10^5$  pfu of  
200 rHRSV-Luc *via* intranasal (IN) inoculation. The viral replication was quantified the first 4 days post-  
201 infection (p.i.). The bioluminescence in lung homogenates was detected at day 1 p.i., and viral  
202 replication in the lungs increased from day 2 to day 4 p.i. (Figure 3A, left). In parallel, expression of *N*-  
203 RSV gene in the lung lysates was quantified by qRT-PCR (Figure 3A, right). Data showed that *N*-RSV  
204 mRNA could be detected from day 2 p.i., and that the peak of infection was reached at day 3 and 4 p.i..  
205 These results revealed a correlation between bioluminescence intensity and *N*-RSV mRNA expression  
206 in line with previous reports (45), with a clear detection of RSV replication at day 3 and 4 p.i.. Of note,  
207 this kinetics of replication is similar to the one described in BALB/c mice, a reference mouse strain to  
208 study RSV infection (45, 46).



209 Based on these results, we decided to compare rHRSV-Luc replication in wild-type (WT) and  
210 TAX1BP1<sup>KO</sup> 129 mice. We chose to quantify bioluminescence in the lung of mock-treated and HRSV-  
211 infected animals at day 2 and day 4 p.i. in order to compare viral replication at an early time point and  
212 at the peak of infection. Our results showed a strong reduction in RSV replication in TAX1BP1<sup>KO</sup> mice  
213 compared to WT mice, at both day 2 p.i. and 4 p.i. (Figure 3B). In order to confirm these results, viral  
214 replication in the lungs of infected mice at day 4 p.i. was assessed by quantification of *N-RSV* gene  
215 expression in the lungs by qRT-PCR, and of virions production in the lungs using a plaque assay approach.  
216 As shown in figure 3C, the amount of N-RSV mRNA was significantly lower in TAX1BP1<sup>KO</sup> mice compared  
217 to wild-type mice. Once again, these results reveal that *in vivo* quantification of viral replication by  
218 bioluminescence correlate with viral load, as previously reported (45). However, we didn't manage to  
219 recover virus from lungs' lysates to quantify virions production. Altogether our results revealed a  
220 supportive role of TAX1BP1 on RSV replication *in vivo*.

221

#### 222 ***Depletion of TAX1BP1 favors antiviral and inflammatory responses during RSV infection***

223 As mentioned, among the various functions of TAX1BP1, this cellular protein acts as a cofactor of the  
224 A20 protein, which is a negative regulator of NF- $\kappa$ B and IRF3/7 pathways that are respectively  
225 involved in inflammatory and antiviral responses. In the mouse model of RSV infection, the induction  
226 of inflammatory cytokines and IFN-I in the first hours post-exposure to the virus are well documented  
227 (47-50). We thus assessed if the inhibition of RSV replication upon TAX1BP1 depletion could be  
228 associated with a modulation of the antiviral and inflammatory responses in the lungs of infected mice  
229 at early time point post-infection. Mice were mock-treated or infected with  $1.87 \times 10^5$  pfu of rHRSV-Luc  
230 and at day 1 p.i., expression levels of IFN-I (IFN- $\alpha$  and IFN- $\beta$ ) and of the inflammatory cytokines IL-6  
231 and TNF- $\alpha$  were determined from lung lysates of WT or TAX1BP1<sup>KO</sup> mice. As shown on Figure 4, RSV  
232 infection of WT mice induced the production of IFN- $\alpha$  and IFN- $\beta$  in all the animals. Of note, one of the  
233 WT infected mice that presented a strong induction of IFN- $\alpha$  and IFN- $\beta$  also displayed an induction of  
234 IL-6 and TNF- $\alpha$ . TAX1BP1<sup>KO</sup> mice were infected in parallel, and higher levels of IFN- $\alpha$  and TNF- $\alpha$  were  
235 detected in the lungs of TAX1BP1<sup>KO</sup> mice compared to WT mice (Figure 4A and D). On the contrary,  
236 IFN- $\beta$  induction by RSV was unchanged (Figure 4B). Although IL6 was induced in only one of the  
237 infected WT animals, this cytokine was induced in all TAX1BP1<sup>KO</sup> infected mice (Figure 4C). However,  
238 IL6 expression levels were not statistically significant when comparing TAX1BP1<sup>KO</sup> to WT mice. Of

239 note, all groups of animals showed comparable levels of RSV infection at this early time point (1 d.p.i.)  
240 as assessed by bioluminescence quantification in the lung homogenates (not shown). Because the  
241 measurements were performed in whole lung lysates, the quantified cytokines and chemokines are  
242 probably produced by several cell populations (*i.e.* both epithelial and immune cells). We thus decided  
243 to specifically focus on alveolar macrophages (AMs) which are major actors in the antiviral response to  
244 RSV (48). AMs were isolated from WT and TAX1BP1<sup>KO</sup> mice after repeated bronchoalveolar lavages  
245 and cultured for 24 h before incubation for another 24 h in the presence of either rHRSV-mCherry or  
246 UV-inactivated rHRSV-mCherry (MOI = 5). Culture supernatants were collected, and IFN- $\alpha$ , IFN- $\beta$ , IL-  
247 6 and TNF- $\alpha$  were quantified by immunoassay. A strong induction of both anti-viral (Figure 5A and B)  
248 and inflammatory cytokines (Figure 5C and D) was detected in the supernatant of AMs exposed to  
249 RSV, whereas a much weaker induction of these molecules was observed for AMs exposed to  
250 inactivated RSV, thus validating an efficient infection of AMs. Of note, although AMs can be infected  
251 by RSV, these cells do not productively replicate the virus (51). Most interestingly, the production of  
252 IFN- $\alpha$ , IFN- $\beta$ , IL-6 and TNF- $\alpha$  was enhanced in AMs derived from TAX1BP1<sup>KO</sup> mice compared to AMs  
253 isolated from WT mice (Figure 5). Altogether, these results demonstrate that TAX1BPA1 is a key  
254 factor involved in the inhibition of the antiviral and inflammatory responses in the lungs of RSV-  
255 infected animals and in isolated AMs.

256

## 257 **DISCUSSION**

258 Previous studies using microarray and proteomic approaches have provided key information on RSV-  
259 host interactions (52, 53), but the interactome of RSV proteins still remains poorly characterized. Due  
260 to their pivotal role during virus entry, replication and assembly, it is expected that components of the  
261 viral polymerase complex, and especially the N protein, are involved in various interactions with  
262 cellular factors. The objective of this study was to find new cellular partners of RSV-N by performing a  
263 yeast two-hybrid screen. Using this approach, we captured 6 cellular proteins using RSV-N as bait,  
264 among which TAX1BP1 was overrepresented. We thus focused on TAX1BP1 as TAX1BP1 depletion  
265 has recently been shown to favor the innate immune response to RSV infection and to impair viral  
266 replication in cell culture (44). In addition, TAX1BP1 is already known to interact with different viral  
267 proteins including the N protein of measles virus that belongs to *Mononegavirales* order (36-39), like  
268 RSV, suggesting that this protein is often hijacked by viruses. TAX1BP1 is a homodimer of about 90

269 kDa and is organized into three main structural domains. The N-terminal SKIP carboxyl homology  
270 (SKICH) domain (54) was recently shown to interact with the adaptor protein NAP1, allowing the  
271 recruitment of the TANK-binding kinase 1 (TBK1), which is involved in selective autophagy of invading  
272 pathogens and damaged mitochondria but is also critical to the induction of IFN-I by RIG-I, MDA5 and  
273 STING (55-59). It is followed by a LC3-interacting region (LIR) that can bind different LC3/GABARAP  
274 orthologs (60) involved in the recruitment of TAX1BP1 to autophagosomes. The central part of  
275 TAX1BP1 exhibits coiled coils forming the oligomerization domain that interacts with TRAF6 protein  
276 (61), and is followed by two C-terminal zinc fingers (UBZ1 and UBZ2) (62). These zinc fingers were  
277 shown to interact with ubiquitylated proteins, with myosin VI, and with the protein A20 (63-65).  
278 Here, the alignment of the PCR reads obtained from the 40 yeast clones that expressed TAX1BP1 in  
279 the two-hybrid screen revealed that the C-terminal part of this protein is involved in the interaction with  
280 RSV-N. Based on our results, it is expected that the TAX1BP1 binding site to RSV-N is located within  
281 the oligomerization domain and/or the C-terminal zinc finger domains. The N-TAX1BP1 interaction  
282 was validated first by pulldown using recombinant TAX1BP1 and RSV-N proteins, and then by  
283 immunoprecipitation when co-expressing the two proteins in human cells. Noteworthy, we managed to  
284 purify the recombinant TAX1BP1 protein to validate the direct interaction with the RSV-N protein.  
285 However, the purification of this protein was challenging as TAX1BP1 tends to be cleaved at its C-  
286 terminus, and this hampered affinity study with RSV-N by biophysical approaches. To gain structural  
287 and functional insights on this interaction that could represent a new therapeutic target, a precise  
288 characterization of TAXBP1 binding domains to RSV-N is required. The structure of the C-terminal  
289 UBZ domain of TAXBP1 either alone or in complex with Myosin VI has already been resolved (62, 65).  
290 The crystal structure of RSV nucleocapsid-like structures consisting of rings containing 10 N  
291 protomers and RNA of 70 nucleotides has been determined (66). Recently, a recombinant RSV N<sup>0</sup>-P  
292 complex has also been characterized (67). The reconstitution of a recombinant complex of RSV-N  
293 (monomeric or oligomeric form) bound to the C-terminal fragment of TAX1BP1 could thus provide key  
294 structural information on this interaction. Finally, given the strong homology between the N proteins of  
295 RSV and human Metapneumovirus (hMPV), another pneumovirus also responsible of acute  
296 respiratory infections, the potential interaction between hMPV-N and TAX1BP1, and its functional  
297 relevance during infection should also be investigated.

298 We then investigated the potential role of TAX1BP1 in RSV infection. TAX1BP1 suppression showed a  
299 limited or no impact on viral protein expression in cell culture, and the production of new viral particles  
300 was unaffected. However, a model of RSV-infected TAX1BP1<sup>KO</sup> mice revealed the critical role of  
301 TAX1BP1 in RSV infection *in vivo*, the depletion of TAX1BP1 leading to a nearly 3-fold decrease in  
302 viral replication in the lungs of infected mice. We also showed that RSV-infected TAX1BP1<sup>KO</sup> mice  
303 present higher levels of IFN- $\alpha$  and TNF- $\alpha$  in the lungs compared to WT mice at day 1 p.i.. Besides,  
304 RSV-infected AMs isolated from TAX1BP1<sup>KO</sup> mice produced higher levels of IFN-I (IFN- $\alpha$  and  $\beta$ ) and  
305 inflammatory cytokines (IL-6 and TNF- $\alpha$ ) compared to those isolated from WT mice.

306 These results reveal that TAX1BP1 participates to the attenuation of the host antiviral and  
307 inflammatory responses during RSV infection *in vivo* and especially in AMs. Altogether, this suggests  
308 that TAXBP1 recruitment by RSV-N indirectly promotes RSV growth by inhibiting the innate immune  
309 response. It is noteworthy that this interaction could compete with the interaction of TAX1BP1 with  
310 another partner. Overall, this conclusion is consistent with the recent study by Martín-Vicente *et al.*  
311 (44) but significant differences should be highlighted. Indeed, they found that the production of  
312 infectious RSV particles in A549 cells decreases when silencing TAX1BP1 or interacting co-factors  
313 A20, ABIN1 and ITCH. In our hands, the effect of TAX1BP1 silencing on RSV infection was striking  
314 only *in vivo*. At this point, we don't have an explanation to this discrepancy as we both used the same  
315 *in vitro* model of A549-infected cells. Besides, they found in their study that A549 cells silenced for  
316 TAX1BP1 express higher levels of ISG15, IL-6 and IL-8 upon RSV infection, but IFN- $\beta$  and TNF- $\alpha$   
317 expression were not significantly affected. On the contrary, we found that TAX1BP1-deficient AMs  
318 express higher level of TNF- $\alpha$ , IL6, IFN- $\beta$  and IFN- $\alpha$  when infected by RSV. The use of distinct cellular  
319 models and TAX1BP1-depletion methods could account for these differences. Indeed, TAX1BP1 is  
320 directly involved in the regulation of innate immune pathways, but is also an adaptor for autophagy  
321 (63) which is required for the induction of an optimal antiviral response in RSV-infected macrophages  
322 (68). Thus, the role of TAX1BP1 in the regulation of the innate immune response induced upon RSV  
323 infection could vary between epithelial and immune cells depending on the relative contribution of  
324 autophagy in the activation of the innate immune response. Finally, it should be noticed that TAX1BP1  
325 has been previously described to regulate B cell differentiation (69). It would thus be interesting to  
326 study whether TAX1BP1 could also be involved in acquired immune responses in the context of RSV  
327 infection *in vivo*, and in particular the production of antibodies.

328 As TAX1BP1 works an adaptor protein in different processes, it is essential to characterize TAX1BP1  
329 partners in different cell lines when infected by RSV. During our study, we investigated the cellular  
330 localization of TAX1BP1 in the context of viral infection or overexpression of N, in order to determine in  
331 particular if TAX1BP1 could be recruited to IBs, as previously shown for MDA5 and MAVS (32), or if  
332 TAXBP1 could recruit RSV-N to specific cellular compartments. However, we were not able to clearly  
333 detect TAX1BP1 by immunolabeling using commercial antibodies. Furthermore, upon overexpression  
334 of Flag- or GFP-tagged TAX1BP1 in cells, TAX1BP1 was shown to concentrate into cytoplasmic  
335 granules and to induce cell death, thus precluding further analysis (data not shown).

336 In conclusion, we have shown that TAX1BP1 is suppressing the innate immune response to RSV *in*  
337 *vivo* and in AMs. Results also suggest that RSV hijacks this mechanism through a direct physical  
338 interaction with RSV-N. Although the precise role of TAX1BP1 in RSV infection needs to be further  
339 characterized, this interaction helps understanding the pathology associated with the infection and  
340 represents new target for antiviral approaches.

341

342

## 343 **MATERIALS AND METHODS**

344

### 345 ***Plasmids and siRNA***

346 The plasmid pFlag-TAX1BP1 encoding for TAX1BP1 in fusion with a N-terminal Flag tag was kindly  
347 provided by Dr C. Journo (ENS, Lyon, France). The plasmid pFlag was obtained by inserting a stop  
348 codon in the pFlag-TAX1BP1 vector, using the Quickchange site-directed mutagenesis kit  
349 (Stratagene). The already described p-N (70) was used for cell transfection and immunoprecipitation  
350 assay.

351 The pGEX-4T-3 vector was used to produce recombinant Glutathione S-transferase protein (GST).  
352 The pGEX-TAX1BP1 plasmid expressing the GST in fusion with the N-terminus of TAX1BP1 was  
353 obtained by cloning the TAX1BP1 sequence between BamHI and XhoI sites of the pGEX-4T-3  
354 plasmid. For purification of recombinant N protein, the pET-N and pGEX-PCT plasmids already  
355 described (42) were used. For yeast two-hybrid screening, the DNA sequence encoding the N<sup>mono</sup>  
356 (monomeric N mutant K170A/R185A) was cloned by *in vitro* recombination (Gateway technology;  
357 Invitrogen) from pDONR207 into the yeast two-hybrid vector pPC97-GW for expression in fusion

358 downstream of the GAL4 DNA-binding domain (GAL4-BD). The control siRNA and a pool of TAX1BP1  
359 siRNA (Ambion) were used for TAX1BP1 silencing experiments.

360

### 361 **Antibodies**

362 The following primary antibodies were used for immunoprecipitation assay and/or immunoblotting:  
363 a mouse anti-Flag and a mouse anti-Flag-HRP antibody (Sigma), a rabbit anti-N antiserum (71),  
364 and a mouse monoclonal anti- $\beta$ -tubulin antibody (Sigma). Secondary antibodies directed against  
365 mouse and rabbit Ig G coupled to HRP (P.A.R.I.S) were used for immunoblotting.

366

### 367 **Cell lines**

368 BHK-21 cells (clone BSRT7/5), hamster kidney cells constitutively expressing the T7 RNA polymerase  
369 (72), HEp-2 cells (ATCC number CCL-23), and human lung carcinoma epithelial A549 cells were  
370 grown in Dulbecco Modified Essential Medium (Lonza) supplemented with 10% fetal calf serum (FCS),  
371 2 mM glutamine, and 1% penicillin-streptomycin. The transformed human bronchial epithelial cell line  
372 BEAS-2B (ATCC) was maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal  
373 bovine serum (FBS, Invitrogen), 1% L-glutamine, and 1% penicillin-streptomycin.

374

### 375 **Viruses**

376 Recombinant RSV viruses rHRSV-mCherry and rHRSV-Luc corresponding to RSV Long strain  
377 expressing either the mCherry or the Luciferase proteins were amplified on HEp-2 cells and titrated  
378 using a plaque assay procedure as previously described (45). Briefly for titration cells were infected  
379 with serial 10-fold dilutions of viral supernatant in complete minimum essential medium (MEM). The  
380 overlay was prepared with microcrystalline cellulose Avicel RC581 (FMC Biopolymer) at a final  
381 concentration of 0.6% in complete MEM containing 1% foetal calf serum. After 6 days at 37°C and 5%  
382 CO<sub>2</sub>, plaques were revealed by 0.5% crystal violet with 20% ethanol solution staining of the cell  
383 layers, and the number of plaque-forming unit (pfu) per well was counted.

384

### 385 **Yeast Two-Hybrid Screening**

386 Yeast two-hybrid screens were performed following the protocol described in Vidalain et al. (73).  
387 AH109 yeast cells (Clontech; Takara, Mountain View, CA, USA) were transformed with pGAL4-BD-

388 N<sup>mono</sup> using a standard lithium-acetate protocol. Screens were performed on a synthetic medium  
389 lacking histidine (-His) and supplemented with 3-amino-1,2,4-triazole (3-AT) at 10 mM. A mating  
390 strategy was used to screen two different prey libraries with distinct characteristics: a human spleen  
391 cDNA library, and a normalized library containing 12,000 human ORFs (74). All libraries were  
392 established in the yeast two-hybrid expression plasmid pPC86 to express prey proteins in fusion  
393 downstream of the GAL4 transactivation domain (GAL4-AD). After six days of culture, colonies were  
394 picked, replica plated, and incubated over three weeks on selective medium to eliminate potential  
395 contamination with false positives. Prey proteins from selected yeast colonies were identified by PCR  
396 amplification using primers that hybridize within the pPC86 regions flanking the cDNA inserts. PCR  
397 products were sequenced, and cellular interactors were identified by multi-parallel BLAST analysis.

398

#### 399 **Expression and purification of recombinant proteins**

400 *E. coli* BL21 bacteria (DE3) (Novagen, Madison, WI) transformed with pGEX-4T-3 and pGEX-  
401 TAX1BP1 plasmids were grown at 37°C for 2-3 h in 200 mL of Luria Bertani (LB) medium containing  
402 100 µg/mL ampicillin until the OD<sub>600nm</sub> reached 0.6. Protein expression was then induced by addition of  
403 1 mM of isopropyl-β-D-thio-galactoside (IPTG) in the presence of 50 mM ZnSO<sub>4</sub> during 4 h at 37°C  
404 before harvesting by centrifugation. Expression and purification of the recombinant N protein was  
405 previously described (66, 75). Briefly, BL21 bacteria co-transformed with pET-N- pGEX-PCT plasmids  
406 were grown in LB medium containing kanamycin (50 µg/mL) and ampicillin for 8 h at 37°C. Then, the  
407 same volume of fresh LB was added and protein expression was induced by adding IPTG at 80 µg/ml  
408 to the culture. The bacteria were incubated for 15 h at 28°C and then harvested by centrifugation. For  
409 GST-fusion proteins purification, bacterial pellets were re-suspended in lysis buffer (50 mM Tris-HCl  
410 pH 7.8, 60 mM NaCl, 1 mM EDTA, 2 mM DTT, 0.2% Triton X-100, 1 mg/mL lysozyme) supplemented  
411 with complete protease inhibitor cocktail (Roche, Mannheim, Germany), incubated for 1 hour on ice,  
412 sonicated, and centrifuged at 4°C for 30 min at 10,000 g. Glutathione-Sepharose 4B beads (GE  
413 Healthcare, Uppsala, Sweden) were added to clarified supernatants and incubated at 4°C for 15 h.  
414 Beads were then washed two times in lysis buffer and three times in PBS 1X, then stored at 4°C in an  
415 equal volume of PBS. To isolate the recombinant N protein, beads containing bound GST-PCT+N  
416 complex were incubated with thrombin (Novagen) for 16 h at 20°C. Purified recombinant N proteins



417 were loaded onto a Superdex 200 16/30 column (GE Healthcare) and eluted in 20 mM Tris/HCl pH  
418 8.5, 150 mM NaCl.

419

420 **Pull-down assays.** Purified recombinant N protein was incubated in the presence of GST or the GST-  
421 TAX1BP1 fusion protein fixed on beads in a final volume of 100  $\mu$ L in buffer Tris 20 mM, pH 8.5, NaCl  
422 150 mM. After 1 h under agitation at 4°C, the beads were extensively washed with 20 mM Tris (pH  
423 8.5)–150 mM NaCl, boiled in 30  $\mu$ L Laemmli buffer, and analyzed by SDS-PAGE and Coomassie blue  
424 staining.

425

426 **Coimmunoprecipitation assay.** BSRT-7 cells were cotransfected with pFlag or pFlag-TAX1BP1 and  
427 pN for 36 h. Transfected cells were then lysed for 30 min at 4°C in ice-cold lysis buffer (Tris HCl 50  
428 mM, pH 7.4, EDTA 2 mM, NaCl 150 mM, 0.5% NP-40) with a complete protease inhibitor cocktail  
429 (Roche), and coimmunoprecipitation experiments were performed on cytosolic extracts. Cell lysates  
430 were incubated for 4 h at 4°C with an anti-Flag antibody coupled to agarose beads (Euromedex). The  
431 beads were then washed 3 times with lysis buffer and 1 time with PBS, and proteins were eluted in  
432 Laemmli buffer at 95°C for 5 min and then subjected to SDS-PAGE and immunoblotting.

433

#### 434 **siRNA transfection and infection**

435 Freshly passaged A549 cells were transfected with the indicated siRNA at a final concentration of 10  
436 nM by reverse transfection into 48 wells plates, using Lipofectamine RNAiMAX (ThermoFischer)  
437 according to the manufacturer's instructions. Briefly, a mixture containing Opti-MEM (Invitrogen),  
438 lipofectamine RNAiMAX and siRNA was incubated for 5 min at room temperature before depositing at  
439 the bottom of the wells. The cells in DMEM medium without antibiotics were then added dropwise  
440 before incubation at 37°C, 5% CO<sub>2</sub>. After 24 h of transfection in the presence of siRNA, the medium  
441 was removed and the cells were infected with recombinant rHRSV-mCherry or rHRSV-Luc viruses at a  
442 MOI of 0.5 in DMEM medium without phenol red and without SVF, for 2 h at 37°C. The medium was  
443 then replaced by DMEM supplemented with 2% SVF and the cells were incubated for 48 h at 37°C.  
444 For cells infected with the rHRSV-mCherry virus, the quantification of replication was performed by  
445 measuring the mCherry fluorescence (excitation: 580 nm, emission: 620 nm) using a Tecan Infinite  
446 M200 Pro luminometer. For HRSV-Luc replication quantification, cells were lysed in luciferase lysis



447 buffer (30 mM Tris pH 7.9, 10 mM MgCl<sub>2</sub>, 1 mM DTT, 1% Triton X-100, and 15% glycerol). After  
448 addition of luciferase assay reagent (Promega), luminescence was measured using a Tecan Infinite  
449 M200 Pro luminometer. Non-infected A549 cells were used as standards for fluorescence or  
450 luminescence background levels. Each experiment was performed in triplicates and repeated at least  
451 three times. For each experiment, cells treated in the same conditions were lysed and protein  
452 expression was analyzed by Western blotting.

453

#### 454 **RSV infection of mice and luciferase measurement.**

455 TAX1BP1-deficient (TAX1BP1<sup>KO</sup>) 129 mice were created by gene targeting, as previously described  
456 (40). TAX1BP1<sup>KO</sup> mice and wild-type 129 co-housed control animals were bred and housed under  
457 SPF conditions in our animal facilities (IERP, INRAE, Jouy-en-Josas). Wild type (WT) and TAX1BP1<sup>KO</sup>  
458 female and male mice at 8 weeks of age (n=11 per group) were anesthetized with of a mixture of  
459 ketamine and xylazine (1 and 0.2 mg per mouse, respectively) and infected by intranasal  
460 administration of 80  $\mu$ L of recombinant RSV expressing luciferase (rHRSV-Luc, 2.34 x 10<sup>6</sup> pfu/mL)  
461 (45, 76, 77) or cell culture media as mock-infection control. Mice were then sacrificed at different  
462 timepoints by intraperitoneal (I.P.) injection of pentobarbital and lungs were frozen.

463

#### 464 **Viral N-RNA gene expression by RT-qPCR**

465 Frozen lungs were homogenized in NucleoSpin®RNA XS Kit (Macherey-Nagel) lysis buffer with a  
466 Precellys 24 bead grinder homogenizer (Bertin Technologies, St Quentin en Yvelines, France). Total  
467 RNA was extracted from lungs or infected cells using NucleoSpin® RNA kit (Macherey-Nagel) and  
468 reverse transcribed using the iScript™ Reverse Transcription Supermix for RT-qPCR kit (Bio-Rad)  
469 according to the manufacturer's instructions. The primers (Sigma-Aldrich) used are listed below. The  
470 qPCRs were performed with the MasterCycler RealPlex (Eppendorf) and SYBRGreen PCR Master  
471 Mix (Eurogenetec) and data analyzed with the Realplex software (Eppendorf) to determine the cycle  
472 threshold (Ct) values. Results were determined with the formula  $2^{-\Delta Ct}$  with  $\Delta Ct = Ct_{\text{gene}} - Ct_{\text{HPRT}}$ . The  
473 primers (Sigma-Aldrich) used are listed below: HPRT (hypoxanthine-guanine  
474 phosphoribosyltransferase), Forward primer 5'-CAGGCCAGACTTTGTTGGAT-3' and Reverse primer  
475 5'- TTGCCTCATCTTAGGCTTT-3'; and N-RSV, Forward primer 5'-

476 AGATCAACTTCTGTCATCCAGCAA-3' and Reverse primer 5'-  
477 TTCTGCACATCATAATTAGGAGTATCAAT-3'.

478

#### 479 **Luciferase expression in lung lysates**

480 Frozen lungs were weighed and then homogenized in 300  $\mu$ L of Passive Lysis Buffer (PLB) (1 mM  
481 Tris pH 7.9; 1 mM MgCl<sub>2</sub>; 1% Triton  $\times$  100; 2% glycerol; 1 mM DTT) with a Precellys 24 bead grinder  
482 homogenizer (Bertin Technologies, St Quentin en Yvelines, France) and a cycle of 2  $\times$  15 s at 4 m/s.  
483 Lung homogenates were clarified by centrifugation 5 min at 2000 g and distributed on microplates (50  
484  $\mu$ L). Then, 50  $\mu$ L of luciferase assay reagent (Promega) were added on each well. The detection of  
485 firefly luciferase activity was measured by photon emission using an *In Vivo* Imaging System (IVIS-  
486 200, Xenogen, Advanced Molecular Vision) and Live Imaging software (version 4.0, Caliper Life  
487 Sciences). Data were expressed in radiance (photons/sec/cm<sup>2</sup>/sr) and normalized to weight lungs.

488

#### 489 **RSV infection of AMs**

490 A cannula was inserted in trachea from mice and repeated bronchoalveolar lavages (BALs) were  
491 made with PBS. AMs were isolated after centrifugations of the BALs of 5 mice per group, pooled, and  
492 1  $\times$  10<sup>5</sup> AMs were plated in 96-well cell culture plates in RPMI supplemented with L-glutamine 2 mM,  
493 FCS 5% and antibiotics for 24 h to allow for adhesion, as previously described (78). AMs were then  
494 exposed to rHRSV-mCherry or ultra-violet (UV)-inactivated rHRSV-mCherry (the same batch exposed  
495 20 min to UV) at MOI 5 or Hep2 cell culture supernatant (Mock). After 24 h, supernatants were  
496 collected and were frozen for cytokine quantification.

497

#### 498 **Cytokine quantification**

499 IFN- $\alpha$  and IFN- $\beta$  or IL-6 and TNF- $\alpha$  were measured in supernatants of AMs or lung lysates using IFN  
500 alpha/IFN beta 2-Plex Mouse ProcartaPlex™ immunoassay (ebiosciences) or Milliplex MAP Mouse™  
501 assay (Merck), respectively. Data were acquired using a MagPix multiplex system (Merck) in order to  
502 determine the mean of fluorescent intensities (MFIs) and results were analyzed on Bio-Plex Manager™  
503 software. The concentrations were normalized to lungs weight.

504

505 **Ethics statement.** The *in vivo* work of is study was carried out in accordance with INRAE guidelines in  
506 compliance with European animal welfare regulation. The protocols were approved by the Animal  
507 Care and Use Committee at “Centre de Recherche de Jouy-en-Josas” (COMETHEA) under relevant  
508 institutional authorization (“Ministère de l’éducation nationale, de l’enseignement supérieur et de la  
509 recherche”), under authorization number 2015060414241349\_v1 (APAFIS#600). All experimental  
510 procedures were performed in a Biosafety level 2 facility.

511

#### 512 **Statistical analysis**

513 Nonparametric Mann-Whitney (comparison of two groups,  $n \geq 4$ ) was used to compare unpaired  
514 values (GraphPad Prism software). Significance is represented: \* $p < 0.05$ ; \*\* $p < 0.01$  and \*\*\* $p < 0.001$ .

515

516

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532

533 **Conflict of interest:** The authors declare that they have no conflicts of interest with the contents of  
534 this article.

535

536 **Author contributions:** DD, AMV, JFE, POV and MG designed experiments. APO, SM, LG, JF, FB  
537 and MG performed molecular and cellular assays. SM, CD, VP, QM, EB, HI and DD performed mice  
538 experiments, samples' treatment and analysis of *in vivo* experiments. APO, FT and POV performed  
539 two hybrid screens. MG, DD, POV and JFE wrote the paper. MG edited the manuscript. All authors  
540 commented on the manuscript.

541

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#### Figure legends

821 **Figure 1: Identification and validation of TAX1BP1-N interaction.** (A) Multiple alignment of  
 822 sequencing reads obtained from the 40 yeast colonies matching TAXBP1. As the cDNA library used in  
 823 the screen was built by oligo-dT priming, TAX1BP1 fragments captured in the screen extend from the  
 beginning of the sequencing reads (thick green line) to the end of the TAX1BP1 sequence. The

824 shortest TAX1BP1 fragment captured with N<sup>mono</sup> is depicted in blue. Below the alignment, a scheme  
825 of TAX1BP1 structural organization is presented, with numbers indicating residues of TAX1BP1:  
826 SKIP carboxyl homology domain (SKICH), LC3-interacting region (LIR), central coiled coils  
827 constituting the oligomerization domain, and the two C-terminal zinc fingers (ZF). **(B)** Validation of N-  
828 TAX1BP1 interaction by GST-pulldown with recombinant proteins. GST and GST-TAX1BP1 proteins  
829 were purified on glutathione-Sepharose beads and incubated in the presence of recombinant N  
830 protein, and interactions was analyzed by SDS-PAGE and Coomassie blue staining. The asterisks  
831 indicate the product of degradation of GST-TAX1BP1 corresponding to the deletion of the C-terminal  
832 domain. Molecular masses (MW) corresponding to the ladder's bands are indicated. **(C)** Western blot  
833 analysis of the TAX1BP1-N interaction after immunoprecipitation assay. Cells were transiently  
834 transfected with constructs allowing the expression of Flag tag alone or the Flag-TAX1BP1 fusion  
835 protein with N protein. Immunoprecipitations (IP) were performed with an anti-Flag antibody.

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837 **Figure 2: Impact of TAX1BP1 depletion on RSV replication in cells.** A549 cells were transfected with  
838 siRNAs control (siCT) or targeting TAX1BP1 (siTAX1BP1) and then infected 24 h later with either rHRSV-  
839 mCherry or rHRSV-Luc, at a MOI of 0.5. **(A)** RSV replication was quantified 48 h post-infection by  
840 measurement of fluorescence (left) and luminescence (right) expressed in arbitrary unit (A.U.) in cell  
841 lysates. Data are representative of three experiments made in quadruplicates. Data are mean  $\pm$  SEM,  
842 \* $p < 0.05$ . **(B)** Western blot analysis of TAX1BP1 silencing and RSV N expression in cells infected  
843 with either rHRSV-mCherry or rHRSV-Luc, 48 h post-infection. **(C)** Titration of virions released in the  
844 culture media of cells treated with siCT (left) and siTAX1BP1 (right) and infected with rHRSV-mCherry  
845 (upper panel) or rHRSV-Luc (lower panel). Calculated viral titers in plaque-forming unit per ml (pfu/ml)  
846 are indicated.

847  
848 **Figure 3: TAX1BP1-deficient mice infected with RSV present a reduced virus replication in the**  
849 **lungs. (A)** Kinetics of RSV infection in 129 mice. Wild-type (WT) strain 129 mice were infected with Hep2-  
850 supernatant (Mock,  $n = 1$ ) or rHRSV-Luc ( $n = 4$ ). (Left) Luciferase activity associated to viral replication was  
851 measured at different days post-infection (d.p.i.) in lung lysates, by quantification of photon emission  
852 (radiance in photon/sec/cm<sup>2</sup>/sr) and normalized to the amount of lysed tissue. (Right) In parallel, *N*-RSV  
853 gene expression was measured in the lung lysates by RT-qPCR and calculated by the formula  $2^{-\Delta Ct}$  with  
854  $\Delta Ct = Ct_{N-RSV} - Ct_{HPRT}$ . Data are mean  $\pm$  SEM, \* $p < 0.05$ . **(B)** WT or TAX1BP1<sup>KO</sup> 129 mice were infected

855 with HEp2-supernatant (Mock) or rHRSV-Luc. Luciferase activity associated to viral replication was  
 856 measured at 2 or 4 d.p.i. (left and right respectively) in lung lysates, by quantification of photon emission  
 857 (radiance in photon/sec/cm<sup>2</sup>/sr) and normalized to the amount of lysed tissue. Data are mean ± SEM from  
 858 two independent experiments with *n* = 7 for RSV infected WT mice and *n* = 11 for RSV infected  
 859 TAX1BP1<sup>KO</sup> mice. **(C)** Quantification of *N*-RSV gene expression at 4 d.p.i. in RSV-infected WT or  
 860 TAX1BP1<sup>KO</sup> mice (*n* = 4). *N*-RSV gene expression was measured in the lung lysates by RT-qPCR and  
 861 calculated by the formula  $2^{-\Delta Ct}$  with  $\Delta Ct = Ct_{N-RSV} - Ct_{HPRT}$  (right). Data are mean ± SEM, \**p* < 0.05.

862

863 **Figure 4: Study of antiviral/inflammatory immune responses in the lungs of infected TAX1BP1<sup>KO</sup>**  
 864 **mice.** WT or TAX1BP1<sup>KO</sup> mice were infected with HEp2-supernatant (Mock) or rHRSV-Luc. **(A, B)** The  
 865 productions of IFN-α and IFN-β were measured 24 h post-infection in lung lysates using ProcartaPlex  
 866 immunoassay. **(C, D)** The productions of IL-6 and TNF-α were measured 24 h post-infection in lung lysates  
 867 using MilliPlex MAP immunoassay. The concentrations were normalized to weight lungs. Data are mean  
 868 ± SEM, \**p* < 0.05; \*\**p* < 0.01, and are representative of two independent experiments with *n* = 5-6 mice  
 869 per group.

870

871 **Figure 5: Deletion of TAX1BP1 enhances the production of type I IFN and inflammatory cytokines in**  
 872 **AMs following RSV infection.** AMs from WT or TAX1BP1<sup>KO</sup> mice were either not infected (mock, black  
 873 triangle) or exposed to rHRSV-mCherry (RSV, inverted black triangle symbol) or UV-inactivated rHRSV-  
 874 mCherry (UV-RSV, white circle) at MOI of 5 for 2 h. **(A, B)** The productions of IFN-α and IFN-β were  
 875 measured 24h post-infection in supernatants using ProcartaPlex immunoassay. **(C, D)** The productions of  
 876 IL-6 and TNF-α were measured 24 h post-infection in supernatants using MilliPlex MAP immunoassay.  
 877 Data are mean ± SEM from two independent experiments, \*\*\**p* < 0.001.

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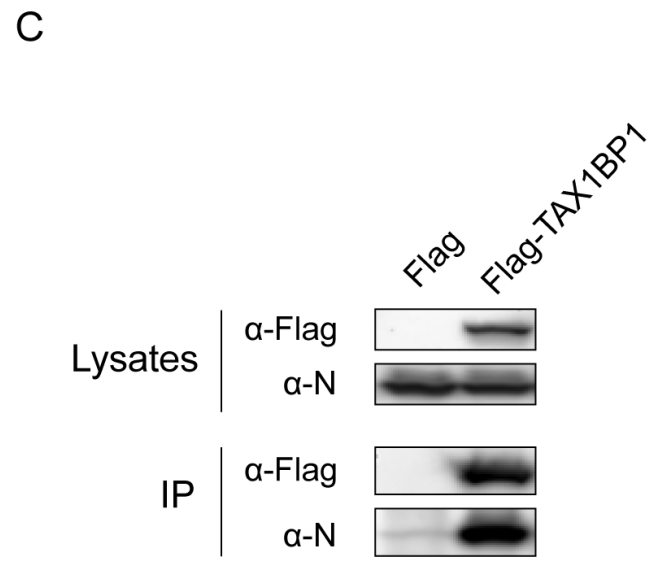
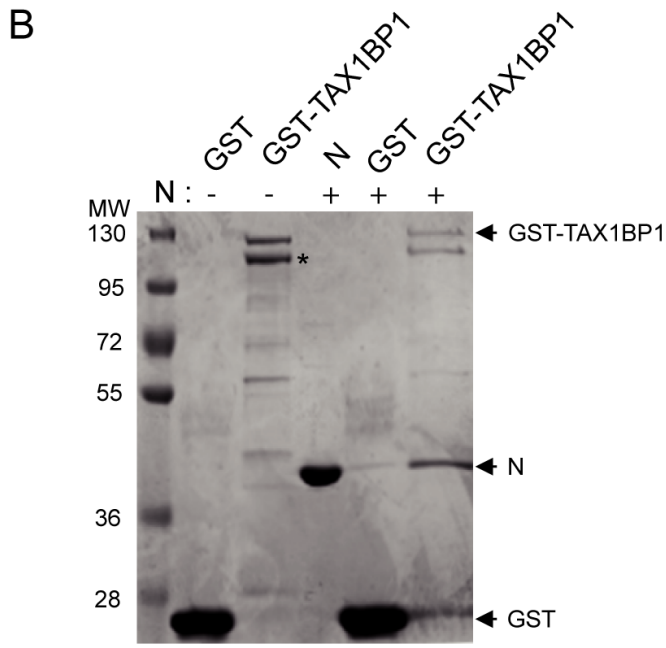
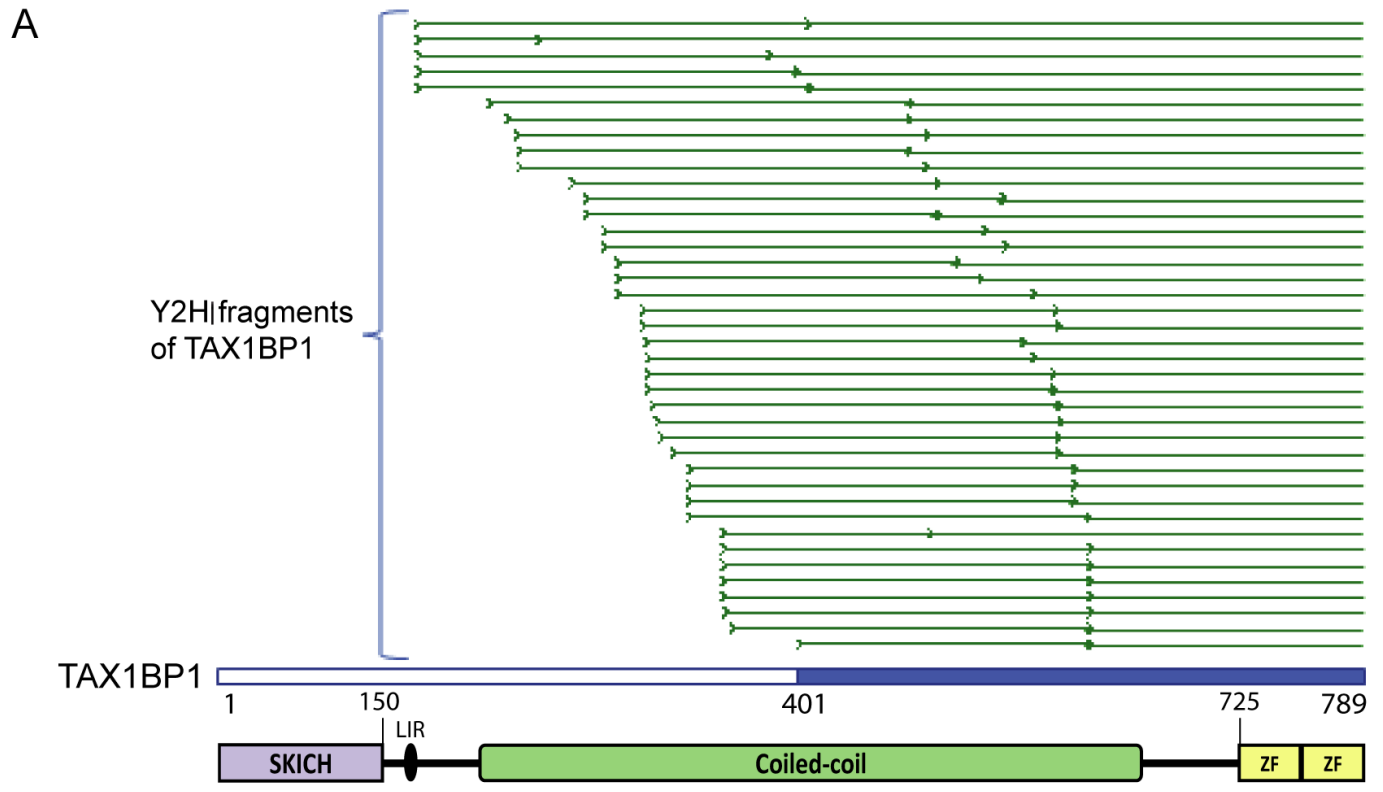
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880 **Table 1. Cellular proteins interacting with RSV N<sup>mono</sup> identified by Y2H screening.**

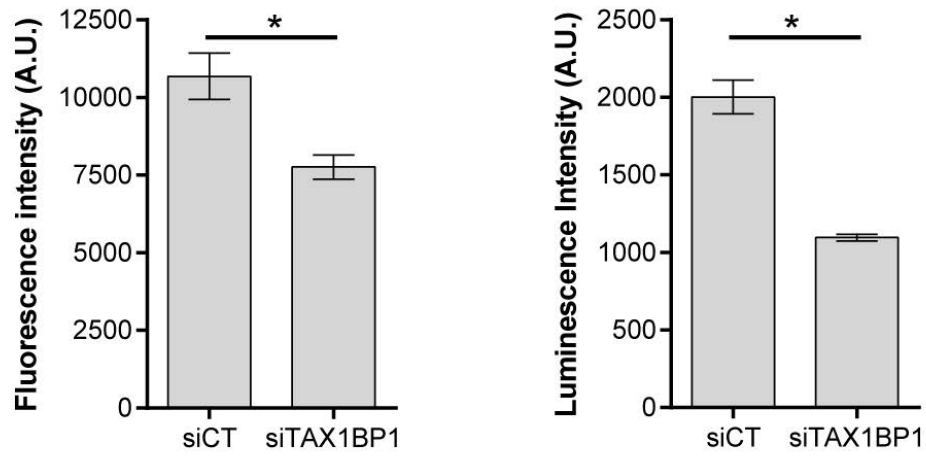
Gene	Gene ID	Hits	Functional annotation (GO Biological process)
MAGEA11	4110	2/67	Undetermined
TAX1BP1	8887	40/0	Negative regulation of NF-κB transcription factor activity Negative regulation of type I interferon production Negative regulation of apoptotic process
TMCC3	57458	11/0	Undetermined
IHO1	339834	5/0	Synapsis Regulation of homologous chromosome segregation

			DNA recombination Spermatogenesis Oogenesis Meiotic DNA double-strand break formation
BEND7	222389	0/4	Undetermined
CCDC102B	79839	4/0	Undetermined

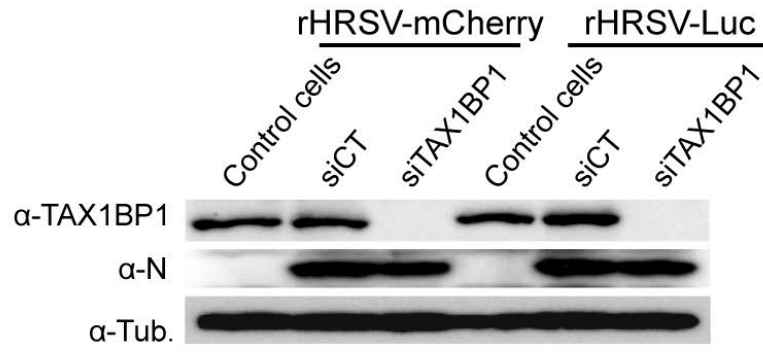
881 The first and second columns correspond respectively to the canonical gene names and gene IDs of interacting  
882 cellular proteins. Column 3 shows the number of positive yeast colonies (Hits) obtained for each cellular protein  
883 when screening the human spleen cDNA or the human ORFeome library. Columns 4 provides information on the  
884 roles of the corresponding proteins using the Gene Ontology annotation (79, 80).  
885



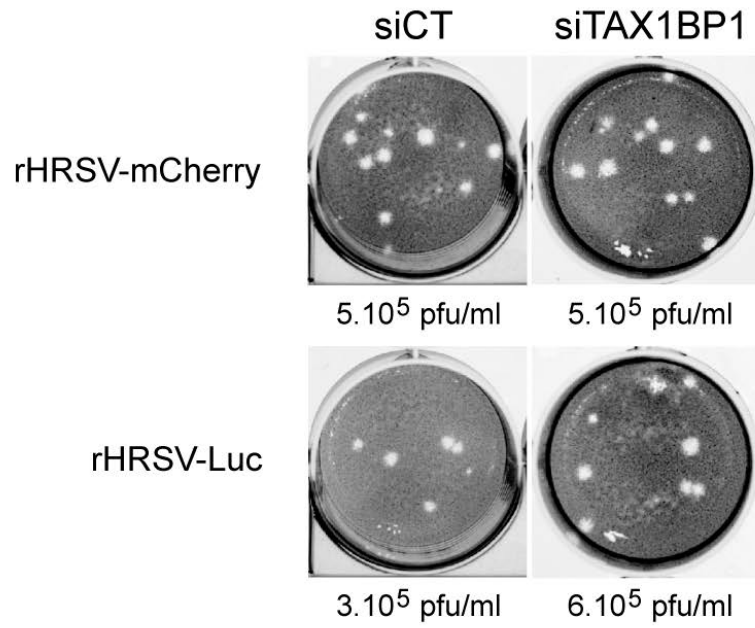
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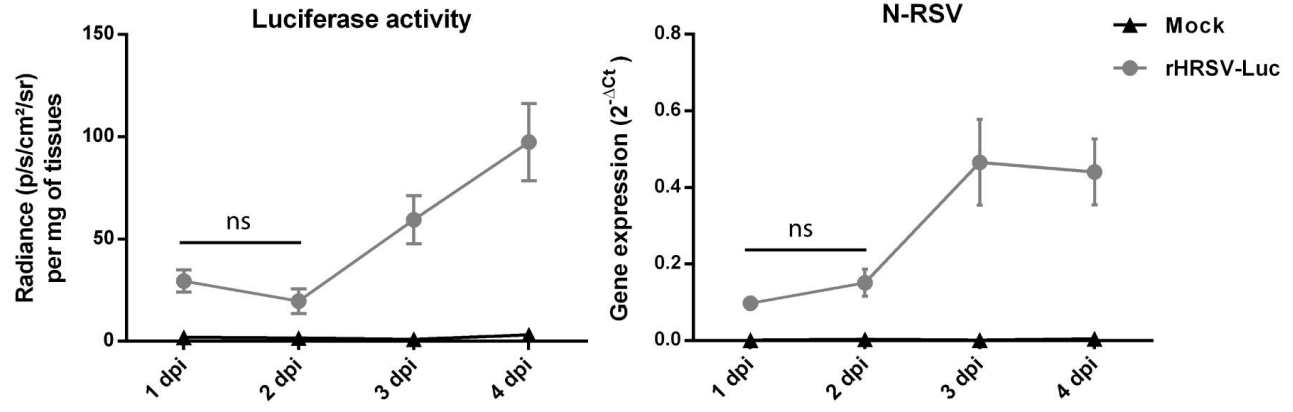
B



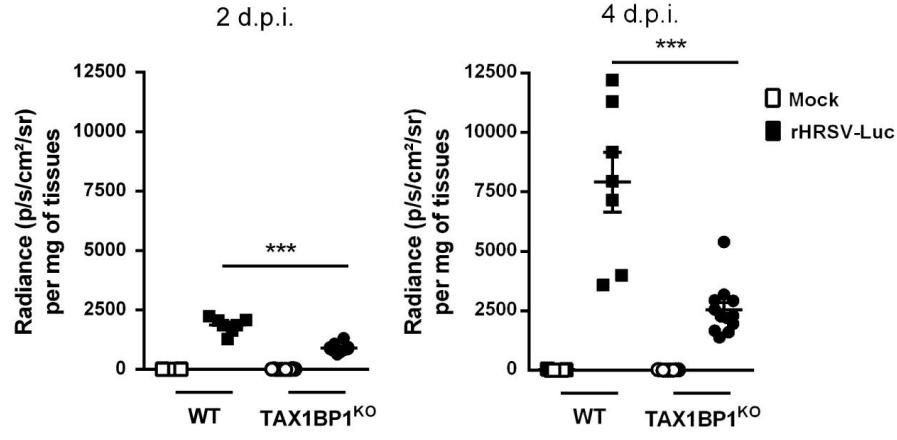
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