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# Ral GTPases promote breast cancer metastasis by controlling biogenesis and organ targeting of exosomes

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38 **Abstract:**

39 Cancer extracellular vesicles (EVs) shuttle at distance and fertilize pre-metastatic niches facilitating  
40 subsequent seeding by tumor cells. However, the link between EV secretion mechanisms and their capacity to  
41 form pre-metastatic niches remains obscure. Using mouse models, we show that GTPases of the Ral family  
42 control, through the phospholipase D1, multi-vesicular bodies homeostasis and tune the biogenesis and  
43 secretion of pro-metastatic EVs. Importantly, EVs from RalA or RalB depleted cells have limited organotropic  
44 capacities *in vivo* and are less efficient in promoting metastasis. RalA and RalB reduce the EV levels of the  
45 adhesion molecule MCAM/CD146, which favors EV-mediated metastasis by allowing EVs targeting to the  
46 lungs. Finally, RalA, RalB and MCAM/CD146, are factors of poor prognosis in breast cancer patients.  
47 Altogether, our study identifies RalGTPases as central molecules linking the mechanisms of EVs secretion and  
48 cargo loading to their capacity to disseminate and induce pre-metastatic niches in a CD146 dependent  
49 manner.

50

51

## 52 Introduction

53 The communication between tumor cells and their neighboring stromal cells is essential to sustain tumor  
54 growth and promote invasion and metastasis (Becker et al., 2016; Follain et al., 2020). Notably, this  
55 communication allows tumors to indoctrinate their microenvironment and switch the phenotypes of various  
56 cell types, such as endothelial cells, fibroblasts or immune cells to the benefit of tumor growth, invasion,  
57 immune escape and metastasis. Such communication occurs with organs distant of the primary tumors and  
58 favors the formation of pre-metastatic niches where the modified microenvironment can help settling  
59 metastatic tumor cells (Peinado et al., 2017). Seeding of this favorable metastatic environment can be  
60 mediated by soluble molecules (Kaplan et al., 2005; Wang et al., 2017) or by extracellular vesicles (EVs)  
61 secreted by tumor cells (Costa-Silva et al., 2015; Hoshino et al., 2015; Jung et al., 2009; Peinado et al., 2012).  
62 EVs are lipid bilayered vesicles of nanometric diameters containing a complex mixture of RNA and protein  
63 cargoes, including a repertoire of surface receptors (Mathieu et al., 2019). They can be directly secreted from  
64 the plasma membrane and called microvesicles or originate from an endosomal compartment, the multi-  
65 vesicular body (MVB), and then called exosomes (van Niel et al., 2018). The levels of circulating tumor EVs  
66 tend to correlate with tumor progression (Baran et al., 2010; Galindo-Hernandez et al., 2013; Logozzi et al.,  
67 2009). Accordingly, inhibition of key components of the EV secretion machinery often correlates with  
68 decreased metastasis (Hyenne et al., 2017). For instance, Rab27a, which directs exosome secretion by  
69 controlling the docking of MVBs to the plasma membrane (Ostrowski et al., 2010), promotes breast and  
70 melanoma tumor growth and metastasis in mice (Bobrie et al., 2012; Peinado et al., 2012) and predicts poor  
71 survival in human pancreatic cancer (Wang et al., 2015). In addition to the levels of secreted tumor EVs, their  
72 content, and in particular their set of surface adhesion proteins equally orchestrates metastasis formation. For  
73 instance, the presence of tetraspanins CD151 and Tspan8 on the surface of pancreatic adenocarcinoma EVs  
74 favors metastasis in rats by enhancing their adhesive capacities and controlling their biodistribution (Yue et  
75 al., 2015). Moreover, integrin receptors exposed by tumor EVs dictate their organotropism and thereby  
76 tune/control the seeding of a premetastatic niche in specific and distant organ (Hoshino et al., 2015).  
77 Therefore, accumulating evidence show that both the levels and the content of secreted tumor EVs are  
78 instrumental in promoting metastasis.

79 However, the molecular mechanisms coordinating these processes remain elusive. In particular, how the  
80 machinery governing EV secretion can impact the pro-metastatic properties of tumor EVs deserves in-depth  
81 characterization. To address this issue, we focused on the members of the Ral family, RalA and RalB  
82 (collectively referred to as RalA/B), acting downstream of RAS and promoting metastasis of different tumor  
83 types in both mice and human (Gentry et al., 2014; Yan and Theodorescu, 2018). We recently found that these  
84 versatile proteins are evolutionarily conserved regulators of exosome secretion (Hyenne et al., 2015). We  
85 originally observed that, in the nematode *C. elegans*, the Ral GTPase ortholog RAL-1 controls exosome  
86 secretion by acting on the biogenesis of MVBs. Importantly, we further showed that RalA/B modulate the  
87 levels of secreted EVs in models that are relevant to human breast cancer (Hyenne et al., 2015) suggesting that  
88 these GTPases could influence disease progression through EVs release. Here, we exploited 4T1 cells, an  
89 aggressive mammary tumor model that mimics human triple-negative breast cancer (Kaur et al., 2012) to



90 further decipher how RalA/B tune EV secretion mechanisms and thereby control metastatic progression of the  
91 disease.

92 In this study, we first provide a detailed dissection of the impact of the Ral GTPases on EV secretion levels and  
93 unravel the mechanisms by which they control the homeostasis of MVBs. We have discovered that RalA/B  
94 directly acts through the phospholipase D1 (PLD1), which, as we show, also promotes EVs secretion, to favor  
95 the maturation of MVBs. We further demonstrate that RalA and RalB promote lung metastasis without  
96 affecting the invasive potential of breast carcinoma. Importantly, RalA/B are crucial for the organ targeting of  
97 tumor EVs, and, as a consequence, for the seeding of pre-metastatic niches. Finally, we identify the adhesion  
98 protein CD146/MCAM as a key EV cargo controlled by RalA and RalB and demonstrate that it conveys, in part,  
99 the pro-metastatic function to EVs by controlling the lung tropism of breast cancer EVs.

100

## 101 **Results**

### 102 **RalA and RalB control exosome secretion levels through the homeostasis of MVBs**

103 We have previously shown that RalA and RalB control EV secretion in aggressive 4T1 mammary tumor cells  
104 (Hyenne et al., 2015) that reliably mimics the aggressive phenotype of human triple-negative breast cancer.  
105 We thus built on this relevant tumor model and decided to test the hypothesis that RalA and RalB could  
106 orchestrate pro-metastatic functions by tuning the molecular mechanisms driving the secretion levels and  
107 nature of EVs. We first confirmed our initial observations with the nanoparticle tracking analysis (NTA) of EVs  
108 released by 4T1 cells and isolated by ultracentrifugation (100.000g pellet). Stable depletion of RalA or RalB by  
109 shRNA reduces by 40% the amount of secreted EVs (Figure 1a, Figure 1- Figure Supplement 1a), with no  
110 impact on their average size (Figure 1- Figure Supplement 1b). RBC8 and BQU57, two previously described  
111 specific chemical inhibitors of Ral GTPases (Yan et al., 2014) significantly reduced EV secretion levels in mouse  
112 and human mammary tumor cell lines (4T1, MDA-MB231, D2A1 and MCF7 cells) as well as in two other cancer  
113 cell lines, human melanoma (A375) and pancreatic carcinoma (Panc1) cells (Figure 1b and Figure 1- Figure  
114 Supplement 1c). Together with evidence previously obtained in *C. elegans* (Hyenne et al., 2015), this  
115 demonstrates that the mechanisms by which RalA/B GTPases tune EV secretion levels are conserved  
116 throughout evolution and are notably at play in various cancer cell lines.

117 To better understand how Ral GTPases could impact EVs secretion, we first characterized their intracellular  
118 distribution in 4T1 cells. Endogenous RalA and RalB localize mostly within CD63-positive endosomal  
119 compartments (MVBs and late endosomes), as well as at the plasma membrane (Figure 1c). Similarly, GFP-  
120 tagged RalA and RalB localize both in late endosomal compartments positive for LysoTracker and at the  
121 plasma membrane (Figure 1c). Therefore, in 4T1 cells, Ral GTPases localize both at biogenesis sites of  
122 microvesicles (plasma membrane) and exosomes (MVBs). To further determine whether Ral GTPases affect  
123 MVBs as previously observed in *C. elegans*, we performed thorough electron microscopy (EM) analysis of  
124 endosomal compartments in 4T1 cells. In a first analysis of cells that were processed upon chemical fixation,  
125 we quantified the densities of i) MVBs and ii) endolysosomes, as well as iii) the diameter of MVBs, iv) the  
126 number and v) the diameter of intraluminal vesicles (ILVs) per MVB. Strikingly, we found RalA or RalB  
127 depletion leads to a 40% decrease in the number of MVB per cytoplasmic surface in 4T1 cells (Figure 1d and

128 Figure 1- Figure Supplement 2a), with no impact on the density of endolysosomes (Figure 1- Figure  
129 Supplement 2b). Further analysis of LysoTracker positive compartments using FACS confirmed that RalA/B  
130 depletion has no significant effect on the late endosome-lysosome pathway (Figure 1- Figure Supplement 2c).  
131 Besides, EM analysis revealed no differences in ILV numbers per MVB surface (Figure 1- Figure Supplement  
132 2d), nor in MVB diameters (Figure 1- Figure Supplement 2e). However, since chemical fixation is known to  
133 affect the morphology of endosomal compartments, we took our EM analysis one step forward by  
134 implementing high-pressure freezing (HPF) of cells, which better preserves the ultrastructure of endosomes  
135 (Klumperman and Raposo, 2014). A similar decrease in the number of MVBs per cytoplasmic surface in RalA  
136 and RalB knockdown cells was observed in these conditions (Figure 1- Figure Supplement 2a). Upon HPF, we  
137 further observed a slight decrease in the number of ILVs per MVB surface (Figure 1- Figure Supplement 2d)  
138 that could be, in part, explained by a slight increase in MVB diameters (Figure 1- Figure Supplement 2e). In  
139 conclusion, depletion of either RalA or RalB significantly reduces MVB number, while the remaining MVBs are  
140 slightly bigger. Overall, thorough EM analysis of intracellular compartments using both chemical fixation and  
141 HPF clearly demonstrates that both RalA and RalB control MVB homeostasis in breast mammary tumor cells.

142

#### 143 **A RalA/B-PLD1-PA axis governs exosome biogenesis**

144 We further investigated the molecular mechanisms controlling MVB homeostasis downstream of RalA/B  
145 GTPases. We decided to focus on phospholipases D (PLDs), which catalyzes the hydrolysis of  
146 phosphatidylcholine (PC) into phosphatidic acid (PA), for three reasons: 1) PLD1 and PLD2 are two well-known  
147 targets of RalA and RalB (Jiang et al., 1995; Luo et al., 1998; Vitale et al., 2005), 2) PLD2 controls exosome  
148 secretion in breast cancer cells (Ghossoub et al., 2014) and 3) PLDs impact cancer progression (Bruntz et al.,  
149 2014). We first verified that both PLD1 and PLD2 are expressed in 4T1 cells by RT-qPCR (Figure 2- Figure  
150 Supplement 1a). In the absence of efficient anti-PLD antibody for immunofluorescence, we decided to assess  
151 the subcellular localization of PLD-GFP fusion proteins. PLD1 mostly localizes to endosomal compartments  
152 positive for RalA, RalB and LysoTracker, whereas PLD2 mostly localizes to the plasma membrane (Figure 2a  
153 and Figure 2- Figure Supplement 1b). Therefore, we tested whether PLDs could function downstream of  
154 RalA/B to control MVBs homeostasis and exosome secretion using two chemical inhibitors, CAY10593 for  
155 PLD1 and CAY10594 for PLD2 (Lewis et al., 2009; Scott et al., 2009). EM analysis of 4T1 cells revealed that  
156 inhibition of PLD1, but not of PLD2, induces a 40% decrease in the number of MVBs per cytoplasmic surface  
157 (Figure 2b). This phenotype is consistent with PLDs respective localizations and suggests that PLD1 functions  
158 in the RalA/B exosome secretion pathway. Further NTA analysis of treated cells showed that both inhibitors  
159 reduce EV secretion levels in 4T1 cells (Figure 2c), suggesting that both PLD isoforms regulate EV secretion  
160 potentially through distinct mechanisms. Importantly, PLD1 inhibition fully phenocopies the effect of RalA/B  
161 GTPases depletion, both on the cellular density of MVBs and on the level of EV secretion. To determine  
162 whether PLD1 acts downstream of RalA/B, we looked at its localization in the absence of RalA or RalB.  
163 Confocal analysis revealed that in 40% of shRalA or shRalB cells, PLD1 is uniformly cytoplasmic instead of  
164 being endosomal (Figure 2d). By contrast, RalA/B depletion had no major impact on PLD2 localization at the  
165 plasma membrane (also its trafficking might be altered) (Figure 2- Figure Supplement 1c). This shows that

166 RalA/B GTPases are required for PLD1 localization on endosomes. To further investigate if PLD activity is  
167 involved in Ral GTPases dependent EV secretion, we performed a lipidomic analysis of secreted EVs. As PLD  
168 converts PC into PA, we focused on these two lipid species. Importantly, RalA/B depletion significantly  
169 reduces the PA/PC ratio of secreted EVs (Figure 2e). In particular, the PA/PC ratio made of mono- and di-  
170 unsaturated lipid species (36:1, 36:2, 38:1 and 38:2), known to be PLD product/target, respectively, showed a  
171 tendency to be decreased although not reaching statistical significance (Figure 2- Figure Supplement 1d). This  
172 further implies that PLD's main product, PA, plays a crucial role in MVB homeostasis. Altogether, these results  
173 suggest that Ral GTPases control PLD1 localization on MVBs, which is required for local PA accumulation and  
174 ultimately for MVB homeostasis and exosome secretion (Figure 2f).

175

### 176 **RalA and RalB promote metastasis non-cell autonomously**

177 Having identified RalA and RalB as important regulators of EV secretion in breast cancer cells, we next  
178 investigated whether such a function could impact metastasis. At first, we analyzed public databases to  
179 interrogate a potential correlation between RalA/B expression levels and metastatic progression. Using a large  
180 cohort of breast cancer patients with metastatic progression from the Cancer Genome Atlas (TCGA), we found  
181 that high expression of either RalA or RalB is significantly correlated with reduced survival (Figure 3a).  
182 Automated quantification of RalA/B expression levels by immunohistochemistry in primary tumors of breast  
183 cancer patients unraveled overexpression of both proteins in tumors from patients with metastasis (Figure 3b).  
184 These results prompted us to investigate in depth the role of RalA/B in a syngeneic mouse model of aggressive  
185 breast cancer, which is highly relevant to the human pathology.

186 Therefore, we conducted a careful and exhaustive longitudinal analysis of metastatic progression of mammary  
187 tumors in syngeneic Balb/c mice. Briefly, 4T1 cells depleted or not for RalA or RalB were orthotopically grafted  
188 in mammary ducts, and several criteria were tracked over time. First, RalA and RalB have antagonist effects on  
189 tumor growth measured *in vivo* over time and *ex vivo* after 41 days: while RalA depletion significantly  
190 increased tumors growth, RalB depletion induced the opposite effect when compared to control tumors  
191 (Figure 3c). Neither RalA, nor RalB affected apoptosis, using caspase3 as a read-out (Figure 3- Figure  
192 Supplement 1a-b). In contrast, 4T1 cells depleted of RalA and RalB show increased growth rate *in vitro* and a  
193 decreased proportion of cells in sub-G1 phase of the cell cycle (Figure 3- Figure Supplement 1c-d). A similar  
194 increase in proliferation rates was observed *in vivo* in the absence of RalA (Figure 3d). Therefore, while  
195 depletion of RalA favors *in vivo* tumor growth by enhancing 4T1 proliferation potential, it is likely that  
196 additional non-cell autonomous factors are responsible for the decreased tumor growth observed upon RalB  
197 depletion.

198 We obtained the most striking result when carefully assessing the lung metastasis burden of these mice after  
199 41 days. We measured the number and the surface covered by metastatic foci in serial lung sections and  
200 observed that RalA or RalB depletion in mammary tumors drastically reduced their metastatic potency (Figure  
201 3e). When compared to the tumor growth rate, the most dramatic reduction of metastasis was observed in the  
202 case of RalA depletion. These experiments show that although RalA and RalB have antagonist effects on  
203 primary tumors, they both promote metastasis. To dissect this phenotype, we tested whether RalA or RalB

204 could impact inherent cell migration and invasion potential of 4T1 cells, as it had been reported for RalB  
205 (Oxford et al., 2005; Zago et al., 2018). We performed 2D (Figure 3f) and 3D (Figure 3g) *in vitro* invasion assays  
206 and observed no effect of RalA or RalB expression levels on motility potential of 4T1 cells. Therefore, RalA/B  
207 seem to promote metastasis independently of cell invasion and are likely to promote metastasis of aggressive  
208 breast cancer cells non-cell autonomously by inducing pro-metastatic micro-environmental changes.

209

### 210 **RalA and RalB dependent EVs induce endothelial permeability**

211 Since RalA and RalB promote metastasis independently of their cell-intrinsic properties, we wondered  
212 whether they could control secreted factors that are likely to induce micro-environmental alterations. In  
213 addition to EVs, tumor cells secreted soluble factors can promote metastasis by modulating the  
214 microenvironment, notably by promoting the formation of a metastatic niche (Ombrato et al., 2019). To test  
215 this possibility, we examined the impact of RalA and RalB on the soluble secretome of 4T1 cells. Depletion of  
216 RalA or RalB had no drastic effect on the soluble factors secreted by 4T1 cells (Figure 3- Figure Supplement 2).  
217 However, the secretion of one protein known to promote metastasis (Ombrato et al., 2019), WISP1/CCN4, is  
218 significantly decreased in shRalA/B cells (Figure 3- Figure Supplement 2). Thus, RalA and RalB are likely to  
219 enhance metastatic potency by promoting the secretion of EVs and possibly as well through WISP1/CCN4.  
220 Furthermore, in addition to enhancing the levels of secreted EVs, RalA/B could alter their functionality. To test  
221 this possibility, we challenged the pro-tumoral function of RalA/B EVs in an *in vitro* functional assay.

222 Since tumor EVs are known to induce vascular permeability in the vicinity of tumors as well as in distant  
223 organs (Tominaga et al., 2015; Treps et al., 2016; Zhou et al., 2014), we tested the capacity of RalA/B  
224 dependent EVs to promote endothelial permeability *in vitro*. When added to a monolayer of endothelial cells,  
225 4T1 EVs increased its permeability in a dose-dependent manner (Figure 3- Figure Supplement 1e). We then  
226 tested the impact of EV content on vascular permeability by subjecting endothelial cells to similar amounts of  
227 EVs derived from 4T1 cells expressing or not RalA/B. Interestingly, endothelial monolayers became less  
228 permeable when treated with a similar amount of EVs derived from shRalA or shRalB cells. Similarly, such EVs  
229 fail to disrupt adherent and tight junctions by contrast to EVs derived from 4T1 control cells (Figure 4b)  
230 suggesting that EVs from RalA/B knockdown cells have reduced pro-permeability abilities. Therefore,  
231 depletion of RalA/B reduces secretion levels of EVs and leads to the secretion of EVs whose effect on vascular  
232 leakiness is hampered. The important observation that vascular permeability could be reduced upon depletion  
233 of RalA or RalB, and with a similar amount of EVs, prompted us to further dissect whether RalA or RalB could  
234 tune the priming of pre-metastatic niches.

235

### 236 **RalA and RalB dependent EVs are pro-metastatic and lung tropic**

237 Here, we thus explored whether RalA and RalB synergistically impact the pro-metastatic functions of EVs by  
238 tuning their secretion levels as well as their content. Since on one hand RalA and RalB positively control the  
239 levels and the functionality of secreted tumor EVs (Figure 1 and 4a), and on the other hand they promote  
240 metastasis (Figure 3), we tested a direct impact of RalA/B-dependent EVs on the promotion of lung  
241 metastasis. For this, we decided to directly assess the role of 4T1 EVs in priming lung metastatic niches *in vivo*,

242 as previously described for other tumor EVs (Costa-Silva et al., 2015; Hoshino et al., 2015; Peinado et al., 2012;  
243 Zhou et al., 2014). Priming of lungs with control EVs significantly enhances lung metastasis over 14 days when  
244 compared to PBS (Figure 4c). In striking contrast, priming of mouse lungs with a similar number of EVs derived  
245 from Ral-depleted cells did not promote metastasis. This key experiment demonstrates that RalA/B confer  
246 pro-metastatic functions to EVs, in addition to controlling their secretion levels. Indeed, the decreased  
247 metastasis observed in absence of RalA/B can result from either drastically reduced EVs secretion or  
248 diminished pro-metastatic potential of EVs. To unravel why EVs from RalA/B depleted cells are unable to  
249 promote metastasis, we first determined their capacity to efficiently reach the lungs and prime pre-metastatic  
250 niches by tracking the dissemination of fluorescently labeled EVs that were injected in the blood circulation of  
251 Balb/c mice. We found that one hour after injection 4T1 EVs mostly accumulate in the lungs, as well as the liver  
252 and brain (Figure 4d and Figure 4- Figure Supplement 1a). These three organs are the main metastatic organs  
253 of 4T1 cells, and breast carcinoma, showing that the organotropism of 4T1 EVs mirrors the metastatic  
254 organotropism of their parental cells and further validates the relevance of our model to human pathology  
255 (Kaur et al., 2012; Lou et al., 2008). Through a careful analysis of cell types that internalize EVs in these  
256 conditions, we observed that 4T1 EVs mostly accumulate in endothelial cells, macrophages and fibroblasts of  
257 the lung parenchyma (Figure 4- Figure Supplement 1b). Importantly, EVs derived from RalA or RalB depleted  
258 cells failed to efficiently reach the lungs, even though similar amounts were injected in all conditions (Figure  
259 4d, e). Similar results were observed for EVs reaching the liver (Figure 4- Figure Supplement 1c). Hence, we  
260 can conclude at this stage that RalA/B control the pro-metastatic properties of EVs by tuning their ability to  
261 reach vascular regions and local parenchyma and efficiently reach metastatic organs, thereby modulating the  
262 formation of a pre-metastatic niche.

263 The latter results raised the exciting hypothesis that metastasis impairment could be, in part, explained by a  
264 general defect in adhesion of circulating EVs at the vascular wall. We recently showed that EVs target specific  
265 vascular regions by first arresting at the surface of endothelial cells (Hyenne et al., 2019). We used two  
266 complementary models that allow careful tracking of single EVs and assessed early events of EVs  
267 internalization in endothelial cells. Using microfluidics, we found that internalization of 4T1 EVs within  
268 endothelial cells is decreased after one hour when they originate from RalA/B-depleted cells (Figure 4f).  
269 Similarly, upon tracking of fluorescent EVs injected in the circulation of zebrafish embryos, we observed that  
270 endothelial arrest/internalization of EVs from RalA/B knockdown cells is significantly hampered (Figure 4g).  
271 Altogether, these experiments suggest that RalA/B knockdown significantly reduced the adhesive properties  
272 of EVs to the endothelium, establishing a potential link with their failure to accumulate in mice lungs.  
273 Furthermore, our results support a model in which RalA/B GTPases, in addition to promoting EV secretion,  
274 also control the pro-metastatic function of these EVs, likely by modulating their content.

275

### 276 **RalA/B promote CD146 EV loading for efficient lung targeting and pre-metastatic niche priming**

277 These functional experiments (Figure 4) suggest that the content of EVs can directly influence metastasis  
278 formation and that such content is likely to be impacted by RalA/B. Therefore, we carried out a careful and  
279 thorough molecular comparison of the cargo content of EVs derived from RalA/B-tuned cells. We first

280 analyzed the RNA content of EVs using RNAseq and found that a large proportion of the RNAs present in EVs  
281 from shRal cells were different from the control (30-50%) (Figure 5a; Supplementary File 1). Accordingly, GO  
282 terms associated with mRNA enriched in each EV type showed important differences in biological processes,  
283 molecular function or cellular components (Figure 5- Figure Supplement 1). In addition, EVs from shRalA cells  
284 differed from control or shRalB EVs in the nature of the RNA they contain, as shRalA EVs showed an important  
285 increase in non-coding RNA (Figure 5b). Overall, this experiment reveals that RalA/B have a profound impact  
286 on the content of RNA in 4T1 EVs.

287 We further analyzed the protein content of 4T1 EVs by mass spectrometry. As shown in Figure 5c, 4T1 EVs  
288 contain a large number of proteins usually found in small EVs (77 of the top 100 proteins from Exocarta are  
289 found in 4T1 EVs; Supplementary File 2), such as tetraspanins, integrins, ESCRT proteins or small GTPases,  
290 such as RalA/B themselves. Importantly, many of these proteins are known to localize to endosomes,  
291 suggesting that some of these EVs are *bona fide* exosomes. Unexpectedly, comparison of the proteome of EVs  
292 secreted by RalA or RalB knockdown cells did not reveal major differences, as no protein is exclusive to one  
293 type of EVs. Instead, a small proportion of proteins showed differential expression levels (Figure 5d;  
294 Supplementary File 2). Regarding their protein content, we noted that EVs from control cells are closer to EVs  
295 from shRalB cells (97 proteins with differential expression) than to EVs from shRalA cells (217 proteins with  
296 differential expression). We then focused on the five proteins over-expressed in EVs from shCtl cells compared  
297 to both EVs from shRalA and EVs from shRalB cells. These proteins are CD146/MCAM, Clic4, Glypican 4,  
298 BDKRB2 and Abcg2. We verified the expression levels of CD146/MCAM, Clic4 and Glypican 4 by western blot  
299 of identical number of EVs (Figure 5e). While Clic4 and Glypican 4 are significantly under-expressed in EVs  
300 from shRalA or shRalB cells, the long isoform of CD146/MCAM (Figure 5- Figure Supplement 2a) showed a  
301 significant decrease in EVs from shRalA cells, and a tendency to decrease in EVs from shRalB cells, which was  
302 confirmed by anti-CD146 ELISA (Figure 5- Figure Supplement 2b). The hypothesis that Ral GTPases could  
303 control CD146 EV loading is further sustained by colocalization analysis. Indeed, by immunofluorescence, we  
304 observed that CD146 localizes both at the plasma membrane and in CD63 positive MVB/late endosomes in  
305 4T1 cells, similarly to Ral GTPases (Figure 5- Figure Supplement 2c). Altogether, content analysis reveals that  
306 depletion of either RalA or RalB deeply affects the EV RNA loading and changes the levels of several key  
307 proteins.

308 We next interrogated whether the impact of RalA/B on the lung targeting and priming potential of EVs could  
309 be explained by its effect on the EV levels of MCAM/CD146. MCAM/CD146 (also known as Mel-CAM, Muc18,  
310 S-endo1, Gicerin) is an adhesion receptor overexpressed in various cancer types, including breast cancer,  
311 where it was shown to promote invasion and tumor progression (Garcia et al., 2007; Zeng et al., 2011, 2012). In  
312 addition, MCAM/CD146 is present on endothelial cells where it mediates the adhesion of several cell types,  
313 including the transendothelial migration of monocytes (Bardin et al., 2009). Given, the known function of  
314 MCAM/CD146 in cell adhesion (Wang and Yan, 2013), we hypothesized that it may, at least in part, be  
315 responsible for the lung tropism defects observed with EVs derived from RalA/B-depleted cells. To test the  
316 involvement of MCAM/CD146 in EVs adhesion, we treated 4T1 EVs with an anti-mouse MCAM/CD146  
317 blocking antibody before injection in zebrafish or mouse circulation. EVs pretreated with MCAM/CD146

318 blocking antibody failed to successfully arrest on endothelial walls of zebrafish embryos (Figure 5f) and  
319 inefficiently reached the lungs in our mouse model (Figure 5g). Finally, we assessed the functional role of EV-  
320 bound CD146 in priming of pre-metastatic niches. To do this, 4T1 EVs were pre-treated with MCAM/CD146  
321 blocking antibody (or with an isotype control) and injected intra-venously, preceding tail-vein injection of 4T1  
322 luciferase cells. Blocking CD146 on EVs significantly reduced their pro-metastatic potential. Therefore,  
323 inhibition of MCAM/CD146 precludes their lung accumulation and the subsequent formation of metastasis and  
324 thereby phenocopies RalA/B knockdown. These results demonstrate that MCAM/CD146, whose presence at  
325 the surface of EVs is tuned by RalA/B, is, at least partly responsible of the adhesion and lung tropism of 4T1  
326 EVs. It further explains why EVs from RalA knockdown cells, which have reduced levels of MCAM/CD146, fail  
327 to reach the lungs efficiently. The pro-metastatic role of MCAM/CD146 is further confirmed by the analysis of  
328 a human cohort of breast cancer showing that its high expression is associated with worsened prognosis  
329 (Figure 5h). Altogether, our work demonstrates that RalA/B, by controlling MVB homeostasis, promote the  
330 secretion CD146-enriched EVs, whose lung tropism sustains efficient metastasis (Figure 5i).

331

## 332 **Discussion**

333 The therapeutic limitations of breast cancer metastasis warrant a deeper understanding of its molecular  
334 machinery. Our findings highlight the exosome-mediated priming of metastatic niches by Ral GTPases as a  
335 critical requisite for lung metastasis during breast cancer progression. We show that RalA and RalB promote  
336 the secretion of exosomes by maintaining a high number of multi-vesicular bodies, likely through the PLD1-PA  
337 axis. Furthermore, we demonstrate that RalGTPases favor the secretion of CD146-rich exosomes, which  
338 accumulate in metastatic organs, notably in lungs, where they establish premetastatic niches (Figure 5i).  
339 Finally, we show that high levels of RalA and RalB correlated with poor prognosis suggesting a unified  
340 mechanism for human breast cancer metastasis.

341 This work, together with our previous study of RAL-1 in *C. elegans* (Hyenne et al., 2015), establishes Ral  
342 GTPases as major evolutionarily conserved mediators of exosome secretion. Our experiments suggest that  
343 RalA/B contribute to exosome secretion in several tumor cell lines, of different origins, implying that they  
344 might function pleiotropically over various cancers. Our results suggest that RalA/B and their effector PLD1  
345 affect the levels of secreted exosomes by tuning the levels of cytoplasmic MVBs. While Ral GTPases, partially  
346 localized at the plasma membrane, could also affect microvesicle secretion, our data indicate that they  
347 function in exosome biogenesis upstream of PLD1. Similarly, a direct correlation between MVB density and  
348 levels of secreted EVs was recently suggested by studies showing that chemical or electric stimulation of MVB  
349 biogenesis results in increased EV secretion (Kanemoto et al., 2016; Yang et al., 2020). The formation of MVBs  
350 results from dramatic biochemical transformations of endosomes involving multiple protein and lipid switches  
351 (Huotari and Helenius, 2011; Scott et al., 2014). Understanding the steps at which RalA/B and PLD affect this  
352 endosome maturation program is critical and remains to be fully deciphered. Our results from mice and *C.*  
353 *elegans* suggest that biogenesis of ILVs, which is a key step in MVB maturation and the initial phase of the  
354 exosome secretion pathway, could as well be controlled by RalA/B. Our work further identifies PLD as an  
355 effector acting downstream of Ral to control exosome secretion. Whether other Ral effectors contribute to EV

356 secretion remains to be addressed. Interestingly, while PLD2 was found to impact exosome secretion by  
357 governing ILV biogenesis in a different breast carcinoma cell line (Ghossoub et al., 2014), our data rather  
358 suggest that PLD1 controls exosome biogenesis in 4T1 cells. Indeed, PLD1 localizes on MVBs and its inhibition,  
359 but not the inhibition of PLD2, decreases MVB density. Nevertheless, it should be noted that we measured EV  
360 secretion levels and MVB density based on PLD inhibition at previously-published high concentrations of the  
361 inhibitors (compared to their respective IC<sub>50</sub>) and that off-target effect can not be ruled out. By contrast,  
362 PLD2 is essentially localized at the plasma membrane of 4T1 cells and its inhibition reduces EV secretion  
363 suggesting that PLD2 could rather promote microvesicle secretion in 4T1 cells. Therefore, we speculate that  
364 RalA/B-PLD1 control ILV biogenesis in 4T1 cells, possibly through the regulation of PA levels. Alternatively,  
365 they could impact the homeostasis of a subclass of MVBs, for instance by controlling their stability or their  
366 degradation.

367 Priming of metastatic niches by (soluble or) EV-mediated factors takes central stages in cancer progression  
368 (Gao et al., 2019; Peinado et al., 2017) and identification of molecular machineries that underlie this condition  
369 could point to new therapeutic or diagnostic targets. Our study demonstrates that Ral GTPases enhance the  
370 formation of lung metastasis in mouse models, by promoting the secretion of exosomes within primary  
371 tumors, while RalA/B expression levels correlates with metastasis in human breast cancer. While the pro-  
372 tumoral activity of Ral GTPases was so far mostly attributed to their capacity to promote anchorage-  
373 independent cell growth (for RalA) or cell invasion (for RalB) (Yan and Theodorescu, 2018), we now show that  
374 Ral GTPases have additional non-cell autonomous functions, and that these functions are important  
375 contributors to metastasis. Indeed, in 4T1 cells, depletion of either RalA or RalB alters the levels, content and  
376 functionality of secreted EVs, without decreasing cell migration or proliferation. Depending on the cell type or  
377 the biological process, RalA and RalB can display redundant, synergistic or even antagonist activities (Gentry  
378 et al., 2014). Since RalA and RalB mostly share similar phenotypes regarding EV secretion, content and  
379 function, they likely function in the same pathway. Interestingly, both Ral proteins appear to be essential for  
380 exosome secretion, revealing that their functions are not fully redundant. Therefore, both GTPases are  
381 required for the generation of a specific subpopulation of EVs with enhanced pro-metastatic properties and  
382 further work is needed to fully unravel the downstream molecular pathways. With this work, RalA and RalB  
383 add to the list of proteins known to control exosome secretion and to affect tumor progression, such as  
384 Rab27a (Bobrie et al., 2012; Kren et al., 2020; Peinado et al., 2012), Alix (Monypenny et al., 2018), syntenin  
385 (Das et al., 2019) and components of the ESCRT machinery (Mattissek and Teis, 2014). These studies  
386 demonstrate that the number of EVs secreted by a primary tumor is an essential element determining the  
387 efficiency of metastasis. However, it is important to keep in mind that all these proteins regulating EV  
388 trafficking, including RalA/B, contribute to tumor progression through both exosome dependent and exosome  
389 independent functions. Altogether, despite pointing to additional functions of RAL GTPases, our study is the  
390 first to identify new molecular machinery from its function in EV biogenesis up to its pro-metastatic function in  
391 breast cancer lung metastasis.

392 Priming of metastatic niches by EVs has, so far, mostly been attributed to increased levels of pro-metastatic  
393 EVs with pro-metastatic functions (Becker et al., 2016; Bobrie et al., 2012; Peinado et al., 2012). In addition to



394 controlling the levels of secreted EVs, we show that RalA/B affect their function by enhancing their capacity to  
395 induce endothelial permeability *in vitro* and pre-metastatic niches *in vivo*. These two observations could be  
396 linked, as RalA/B dependent EVs could promote endothelial permeability locally in the primary tumor or at  
397 distance in lungs, thereby favoring both tumor intravasation and extravasation. Content analysis revealed that  
398 RalA/B control the identity and levels of RNAs and proteins present in secreted EVs. Interestingly, Ras, which  
399 is known to activate RalA/B (Gentry et al., 2014), also controls the protein and RNA cargo of tumor EVs (Cha et  
400 al., 2015; Demory Beckler et al., 2013; McKenzie et al., 2016), although its effect on the levels of secreted EVs  
401 is unclear (Demory Beckler et al., 2013; McKenzie et al., 2016). As McKenzie and collaborators identified a  
402 MEK-ERK-Ago2 pathway downstream of Ras (McKenzie et al., 2016), it would be interesting to determine how  
403 this pathway connects with the Ral-PLD-PA axis described in our study. Among the few proteins significantly  
404 enriched in RalA/B dependent EVs, we identified CD146, a molecule known to modulate cell-cell adhesion  
405 (Wang and Yan, 2013). We showed, using functional inhibition, that CD146 present on pro-metastatic EVs  
406 controls their lung targeting efficiency thereby impacting their biodistribution and niche-promoting function.  
407 Accordingly, we and others show that high expression of CD146 correlates with poor prognosis in human  
408 breast carcinoma (Garcia et al., 2007; Zeng et al., 2012). CD146 functions as an adhesion molecule involved in  
409 homophilic and heterophilic interactions (Wang and Yan, 2013), promoting for instance monocyte  
410 transmigration (Bardin et al., 2009). CD146 can perform trans-homophilic interactions via its immunoglobulin-  
411 like extracellular domain (Taira et al., 1994, 2005). It also binds to extracellular matrix proteins or other  
412 transmembrane proteins, such as VEGFR2 (Wang and Yan, 2013). Therefore, it is tempting to speculate that  
413 CD146 affects the biodistribution and organ targeting efficiency of circulating tumor EVs by mediating their  
414 interaction with specific ligands present on the luminal side of endothelial cells of metastatic organs. Other  
415 adhesion molecules, such as integrins and tetraspanins were shown to affect the biodistribution of tumor EVs  
416 and ultimately the formation of metastasis (Hoshino et al., 2015; Yue et al., 2015). Therefore, it is likely that  
417 the combination of these receptors at the surface of tumor EVs, combined with the differential expression of  
418 their ligands on endothelial cells throughout the organism will dictate their homing. More work will be needed  
419 to characterize this organ specific EV zip code and to identify relevant endothelial ligands for circulating EVs  
420 and develop inhibitory strategies to impair their arrest and uptake at metastatic sites. In addition, the  
421 presence of other cell types in the circulation, such as patrolling monocytes, which take up large amounts of  
422 circulating EVs, could also contribute to the accumulation of tumor EVs in specific organs (Hyenne et al., 2019;  
423 Plebanek et al., 2017). Finally, other factors, such as the vascular architecture and hemodynamic patterns  
424 could be involved (Follain et al., 2020; Hyenne et al., 2019) and the interplay between these mechanical cues  
425 and the surface repertoire of metastatic EVs should be a fertile ground for future research. Precisely dissecting  
426 the mechanisms by which tumor EVs reach specific organs would allow to understand the priming of  
427 premetastatic niches.

428 Overall, our study identifies RalA/B GTPases as a novel molecular machinery that regulates the formation and  
429 shedding of pro-metastatic EVs. We also discovered CD146 as an EV cargo whose targeting could inspire new  
430 therapeutic strategies to impact the progression of metastatic breast cancer.

431

432 **Methods**

433 **Cell culture**

434 The establishment of 4T1 cell lines stably expressing shRNA against RalA, RalB, or a scramble sequence has  
435 been described previously (Hyenne et al., 2015). 4T1-Luciferase (RedLuc) cells were purchased from Perkin-  
436 Elmer. All 4T1 cell lines were cultured in RPMI-1640 medium, completed with 10% fetal bovine serum (FBS,  
437 Hyclone) and 1% penicillin-streptomycin (PS) (GIBCO). 4T1 shRNA cell lines were maintained in medium  
438 containing 1 µg/ml puromycin, except during experiments, and regularly checked for the stability of  
439 knockdown by western blots. Human Umbilical Vein Endothelial Cells (HUVEC) (PromoCell) were grown in  
440 ECGM (PromoCell) supplemented with a supplemental mix (PromoCell C-39215) and 1% PS. Human  
441 A375 melanoma and human MDA-MB-231, MCF7 and SKBR3 breast cancer (ATCC) cell lines were grown in  
442 high-glucose Dulbecco's modified Eagle's Medium (DMEM, Gibco Invitrogen Corporation) supplemented with  
443 10% (FBS) and 1% PS. D2A1 cell were grown in high-glucose Dulbecco's modified Eagle's Medium (DMEM,  
444 Gibco Invitrogen Corporation) supplemented with 5% (FBS), 5% new born calf serum, 1% Non-essential amino  
445 acids and 1% PS. Human Panc-1 pancreatic adenocarcinoma cell line was grown in RPMI-1640 supplemented  
446 with 10% FBS, and 50 µg/ml gentamicin sulfate (Gibco/Life Technologies). All cell lines were cultured in a  
447 humidified atmosphere containing 5% CO<sub>2</sub> at 37°C and checked regularly for absence of mycoplasma by PCR  
448 (Venor®GeM, Clinisciences).

449 Plasmid transfections: Cells at 50–70% confluency were transfected with 1 µg of plasmid using JetPRIME  
450 (PolyPlus, Illkirch, France) according to the manufacturer's instructions. The following plasmids were used:  
451 pGFP-PLD1, pGFP-PLD2 (Corrotte et al., 2006), pLenti CMV:tdtomato-RalA and pLenti CMV:tdtomato-RalB.

452 Drug treatment: Cells were incubated with the following drugs in the appropriate medium: RalA/B inhibitors  
453 BQU57 (10 µM; Sigma) and RBC8 (10 µM; Sigma), PLD1 inhibitor CAY10593 (10 µM; Santa Cruz  
454 Biotechnology) or PLD2 inhibitor CAY10594 (10 µM; Santa Cruz Biotechnology). Cells were treated for 18h  
455 before processing for EV isolation or cell analysis.

456

457 **qRT-PCR Analysis**

458 Total RNA was extracted from cells using TRI Reagent (Molecular Research Center) according to the  
459 manufacturer's instructions. For qRT-PCR, RNA was treated with DNase I and reverse transcribed using the  
460 High Capacity cDNA RT Kit. qRT-PCR was performed using the Power SYBR Green PCR Master Mix or TaqMan  
461 Gene Expression Master Mix using a 7500 Real Time PCR machine (Applied Biosystems). All compounds were  
462 purchased from Life Technologies (St Aubin, France). Data were normalized using a Taqman mouse probe  
463 against GADPH as endogenous control (4333764T, Life Technology) and fold induction was calculated using  
464 the comparative Ct method (-ddCt).

465

466 **Western blot**

467 Cell or EV extracts were denatured in Laemmli buffer and incubated at 95 °C for 10 min. 10µg of protein  
468 extract (for cell lysates) or equal number of EVs (8.50x10<sup>8</sup> EVs per lane, measured by NTA) were loaded on  
469 4%–20% polyacrylamide gels (Bio-Rad Laboratories, Inc.). The following antibodies were used: CD9 (Rat,

470 553758; BD Biosciences), RalA (mouse, 610221; BD Biosciences), RalB (mouse, 04037; Millipore), Glypican 4  
471 (Rabbit, PA5-97801; Thermo Fisher Scientific), antibodies specifically recognizing the short and long isoforms  
472 of CD146 were previously described (Kebir et al., 2010), Clic4 (mouse, 135739; Santa Cruz Biotechnology),  $\alpha$ -  
473 tubulin (mouse, CP06; Millipore) and Secondary horseradish peroxidase -linked antibodies: anti-Rat (GE  
474 healthcare; NA935), anti-Mouse (GE healthcare; NA 931) and anti-rabbit (GE healthcare; NA934). Acquisitions  
475 were performed using a PXi system (Syngene). Intensities were measured using the Fiji software.

476

#### 477 **Elisa**

478 Elisa was performed according to the manufacture's instruction (RayBiotech) by loading equal number of EVs  
479 ( $7 \times 10^8$  -  $9.5 \times 10^9$ ) per well (2 experiments in triplicate).

480

#### 481 **Electron microscopy**

482 Chemical fixation: Cells were fixed with 2,5% glutaraldehyde/2,0% paraformaldehyde (PFA) (Electron  
483 Microscopy Sciences) in 0.1M Cacodylate buffer at room temperature for 2h, then rinsed in 0.1M Cacodylate  
484 buffer (Electron Microscopy Sciences) and post-fixed with 1% OsO<sub>4</sub> (Electron Microscopy Sciences) and 0.8%  
485 K<sub>3</sub>Fe(CN)<sub>6</sub> (Sigma-Aldrich) for 1h at 4°C. Then, samples were rinsed in 0.1M Cacodylate buffer followed by a  
486 water rinse and stained with 1% uranyl acetate, overnight at 4°C. The samples were stepwise dehydrated in  
487 Ethanol (50%, 70% 2x10min, 95% 2x15min and 100% 3x15min), infiltrated in a graded series of Epon  
488 (Ethanol100%/Epon 3/1, 1/1, 1h) and kept in Ethanol100%/Epon 1/3 overnight. The following day, samples were  
489 placed in pure Epon and polymerized at 60°C. 100 nm thin sections were collected in 200 copper mesh grids  
490 and imaged with a Philips CM12 transmission electron microscope operated at 80 kV and equipped with an  
491 Orius 1000 CCD camera (Gatan).

492 High-pressure freezing: HPF was performed using an HPF COMPACT 03 high pressure freezing machine  
493 (Wohllwend), using 3mm diameter Aclar film disks (199µm thickness), as cell carriers. Subsequent freeze  
494 substitution in acetone was performed using an automatic FS unit (Leica AFS), including 0.25% OsO<sub>4</sub> staining,  
495 and Epon embedding. Sections were contrasted on grids with 1% uranyl acetate followed with 0,4% lead  
496 citrate (Sigma-Aldrich). Imaging was performed similarly to chemical fixation.

497 The number of MVBs and lysosomes per surface of cytoplasm were quantified using the Fiji software. MVBs  
498 and lysosomes were distinguished based on their morphology: MVBs have one or more ILVs and lysosomes  
499 contain ILVs but are also electron dense and contain irregular membrane curls.

500

#### 501 **FACS analysis**

502 For cell cycle analysis,  $10^6$  cells were fixed using the FoxP3 Staining Kit (00-5523-00 eBioscience) for 30min at  
503 room temperature in the dark. Samples were then resuspended in permeabilization buffer containing 20 µg  
504 of RNase A (R6513 Sigma) and 1 µg of propidium iodide (PI) (130-093-233 Miltenyi Biotech) for 30 min. PI  
505 fluorescence was analyzed using a BD Accuri<sup>TM</sup> C6 cell analyzer with BD CSampler<sup>TM</sup> Analysis Software.  
506 Results were analyzed with FlowJo software version 10 (TreeStar).

507 For lysosomal analysis, confluent cells were incubated with 1  $\mu$ M LysoTracker Green DND 26 (L7526-Thermo  
508 Fischer) diluted in complete RPMI medium for 30 minutes at 37°C. Cells were then detached by addition of  
509 TrypLE (12604021, ThermoFischer), washed in PBS 2% (v/v) FCS, and stained with 0.1  $\mu$ M DAPI in PBS 2% (v/v)  
510 FCS immediately before analysis. Samples were processed on a Gallios Flow Cytometer (Beckman Coulter).  
511 Dead cells and doublets were excluded from analysis respectively by the selection of DAPI negative cells and  
512 co-analysis of integral vs time-of-flight side scatter signals. Data were analyzed on FlowJo software (BD  
513 Bioscience). Mean Fluorescence intensities (MFI) of lysotracker in each condition were normalized by  
514 performing a ratio with MFI of an unstained condition in the same channel.

515

### 516 **Migration assays**

517 For 2D migration assays, 4T1 mammary tumor cells were plated on 35-mm plastic dishes (6 well plates) and  
518 grown for 2 days until reaching 90% confluence. The cells were then grown for 16h in serum-free medium  
519 before wounding of the monolayer by scraping from the middle of the plate. Cells were incubated in complete  
520 RPMI medium and sequential images of the wound were collected with a 10X objective at 0, 8 and 24h after  
521 wounding. Percentage of wound closure over time was analyzed and quantified using the Fiji software.

522 3D Organotypic invasion assays were conducted as previously described (Timpson et al., 2011; Vennin et al.,  
523 2017). Briefly, rat tail tendon collagen was extracted with 0.5 mol/L acetic acid to a concentration of 2.5 mg/ml.  
524  $8.4 \times 10^4$  telomerase immortalized fibroblasts (TIFs) were embedded into the neutralized collagen in the  
525 presence of 1 x MEM and 8.8% FBS. Matrices were allowed to contract over a 12-day period in DMEM (1% P/S,  
526 10% FBS). Following contraction TIFs were removed with puromycin (2  $\mu$ g/ml) for 72 hours before  $8 \times 10^4$  4T1  
527 cells were seeded on the contracted matrices and allowed to grow to confluence for 48 hours in RPMI (1% P/S,  
528 10% FBS). The matrices were then transferred to an air-liquid interface on a metal grid and cells allowed to  
529 invade for 15 days with media changes every 2 days. Following the invasion, organotypic matrices were fixed  
530 in 10% buffered formalin and processed for histochemical analysis. The invasive index was measured in 3  
531 representative fields of view per matrix with three matrices per replicate for three replicates.

$$\text{Invasive Index} = \frac{\text{Number of cells} > 200 \mu\text{m depth}}{\text{Cells on top of the matrix}}$$

532

### 533 ***In vitro* permeability assay**

534 Transwell filter inserts (pore size 1.0  $\mu$ m, 12 mm diameter, polyester membrane, Corning, New York, USA)  
535 were coated with fibronectin (10 $\mu$ g/ml; Sigma). Then, HUVECs were seeded ( $0.3 \times 10^6$  cells/well) and grown on  
536 transwell filters for 48 h until reaching confluency. Confluent monolayers of HUVEC cells were treated with  
537 similar amounts (10-100 $\mu$ g) of 4T1-EVs, PBS (as a negative control) or with 100ng/ml TNF- $\alpha$  (as a positive  
538 control) overnight. FITC-dextran (MW ~70,000; Sigma) was added to the top well at 25 mg/ml for 20 min at  
539 37°C, and fluorescence was measured in the bottom well using a fluorescence plate reader (Berthold Tris Star  
540 2; 485 nm excitation and 520 nm emission). Cells were washed for 3 times and were fixed for  
541 immunofluorescence (described below).

542

543 **Secretome analysis**

544 Cell culture supernatants were collected and centrifuged for 15 minutes at 300 g. Supernatants were incubated  
545 with Mouse XL Cytokine Array membranes (R&D Systems) according to the manufacturers' instructions.  
546 Three independent experiments were performed. Intensities were measured using the Fiji software.

547

548 ***in vitro* proliferation assay**

549 Briefly, cells were seeded in 96-well plates at the density of 2000 cells per well with 200µl of complete culture  
550 medium and cultured for 24, 48 and 72h at 37°C. Culture medium without cells was used as the blank control  
551 group. To avoid the edge effect, the peripheral wells were filled with sterile PBS. For the proliferation test, a  
552 total of 20 µl MTS solution was added to each well, followed by incubation for 2h at 37°C. Optical density was  
553 measured at 490nm using a Berthold Tristar device.

554

555 **EVs isolation and characterization**

556 Cells were cultured in EV depleted medium (obtained by overnight ultracentrifugation at 100,000g, using a  
557 Beckman, XL-70 centrifuge with a 70Ti rotor) for 24h before supernatant collection. The extracellular medium  
558 was concentrated using a Centricon Plus-70 centrifugal filter (10k; Millipore) and EVs were isolated by  
559 successive centrifugation at 4°C: 15 minutes at 300 g, 10 minutes at 2,000 g, 30 minutes at 10,000 g and 70  
560 minutes at 100,000 g (using a Beckman XL-70 centrifuge with a SW28 rotor). EVs pellets were washed in PBS,  
561 centrifuged again at 100,000 g for 70 minutes, resuspended in PBS and stored at 4°C. For all functional  
562 experiments, EVs were used immediately after isolation or stored overnight at 4°C and injected the next day.  
563 For content analysis, EVs were frozen at -80°C. After EV isolation, EVs numbers and size distribution were  
564 measured by NTA using a ZetaView (Particle Metrix, Meerbusch, Germany).

565 For *in vivo* mouse experiments, EVs were isolated the using the iZON qEV2 size exclusion column (Izon  
566 science, Cambridge MA) according to the manufacturer's instructions. After rinsing the columns with PBS,  
567 2 ml of concentrated extracellular medium were applied on top of a qEV column (Izon Science) and 6 ml  
568 fractions were collected. For organotropism experiments, four EV-rich fractions (F<sub>2</sub>, F<sub>4</sub>, F<sub>6</sub>, and F<sub>8</sub>) were  
569 pooled, then ultracentrifuged for 1 h at 100,000 ×g, 4°C with a SW28 rotor in a Beckman XL-70 centrifuge or  
570 concentrated using an Amicon Ultra-4 10 kDa centrifugal filter device (Merck Millipore). Pellets were  
571 resuspended in 500 µL PBS. For priming experiment, the most EV-rich fraction was used (F<sub>4</sub>).

572 For fluorescent labeling, isolated EVs were incubated with MemBright-Cy3 or Cy5 (Collot et al., 2018) at  
573 200nM (zebrafish) and 500nM (mice) (final concentration) in PBS for 30 minutes at room temperature in the  
574 dark. Labeled EVs were then rinsed in 15ml of PBS, centrifuged at 100,000g with a SW28 rotor in a Beckman  
575 XL-70 centrifuge and pellets were resuspended in 50 µL PBS. EVs were used immediately after isolation or  
576 stored for a maximum of one night at 4°C before use.

577

578 **Mass spectrometry-based proteomics experiments**

579 Sample preparation of EVs Proteins. 20 mg samples were denatured at 95 °C for 5 min in Laemmli buffer and  
580 concentrated in one stacking band using a 5% SDS-PAGE gel. The gel was fixed with 50% ethanol/3%

581 phosphoric acid and stained with colloidal Coomassie Brilliant Blue. The gel bands were cut, washed with  
582 ammonium hydrogen carbonate and acetonitrile, reduced and alkylated before trypsin digestion (Promega).  
583 The generated peptides were extracted with 60% acetonitrile in 0.1% formic acid followed by a second  
584 extraction with 100% acetonitrile. Acetonitrile was evaporated under vacuum and the peptides were  
585 resuspended in 10  $\mu$ L of H<sub>2</sub>O and 0.1% formic acid before nanoLC-MS/MS analysis.

586 NanoLC-MS/MS analysis. NanoLC-MS/MS analyses were performed on a nanoACQUITY Ultra-Performance  
587 LC system (Waters, Milford, MA) coupled to a Q-Exactive Plus Orbitrap mass spectrometer (ThermoFisher  
588 Scientific) equipped with a nanoelectrospray ion source. The solvent system consisted of 0.1% formic acid in  
589 water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). Samples were loaded into a Symmetry C18  
590 precolumn (0.18 x 20 mm, 5  $\mu$ m particle size; Waters) over 3 min in 1% solvent B at a flow rate of 5  $\mu$ L/min  
591 followed by reverse-phase separation (ACQUITY UPLC BEH130 C18, 200 mm x 75  $\mu$ m id, 1.7  $\mu$ m particle size;  
592 Waters) using a linear gradient ranging from 1% to 35% of solvent B at a flow rate of 450 nL/min. The mass  
593 spectrometer was operated in data-dependent acquisition mode by automatically switching between full MS  
594 and consecutive MS/MS acquisitions. Survey full scan MS spectra (mass range 300-1800) were acquired in the  
595 Orbitrap at a resolution of 70K at 200 m/z with an automatic gain control (AGC) fixed at  $3.10^6$  and a maximal  
596 injection time set to 50 ms. The ten most intense peptide ions in each survey scan with a charge state  $\geq 2$  were  
597 selected for fragmentation. MS/MS spectra were acquired at a resolution of 17,5K at 200 m/z, with a fixed first  
598 mass at 100 m/z, AGC was set to  $1.10^5$ , and the maximal injection time was set to 100 ms. Peptides were  
599 fragmented by higher-energy collisional dissociation with a normalized collision energy set to 27. Peaks  
600 selected for fragmentation were automatically included in a dynamic exclusion list for 60 s. All samples were  
601 injected using a randomized and blocked injection sequence (one biological replicate of each group plus pool  
602 in each block). To minimize carry-over, a solvent blank injection was performed after each sample. EVs mass  
603 spectrometry was performed in triplicate.

604 Data interpretation. Raw MS data processing was performed using MaxQuant software<sup>1</sup> v1.6.7.0 (Cox et al.,  
605 2014). Peak lists were searched against a database including *Mus musculus* protein sequences extracted from  
606 SwissProt (09-10-2019; 17 007 sequences, Taxonomy ID= 10 090). MaxQuant parameters were set as follows:  
607 MS tolerance set to 20 ppm for the first search and 5 ppm for the main search, MS/MS tolerance set to 40  
608 ppm, maximum number of missed cleavages set to 1, Carbamidomethyl (C) set as a fixed modification,  
609 Oxidation (M) and Acetyl (Protein N-term) set as variable modifications. False discovery rates (FDR) were  
610 estimated based on the number of hits after searching a reverse database and were set to 1% for both peptide  
611 spectrum matches (with a minimum length of seven amino acids) and proteins. All other MaxQuant  
612 parameters were set as default. Protein intensities were used for label free quantification. The imputation of  
613 the missing values (DetQuantile imputation) and differential data analysis were performed using the open-  
614 source ProStaR software(Wieczorek et al., 2017). A Limma moderated t-test was applied on the dataset to  
615 perform differential analysis. The adaptive Benjamini-Hochberg procedure was applied to adjust the p-values  
616 and FDR values under 1% were achieved.

617 Complete dataset has been deposited to the ProteomeXchange Consortium via the PRIDE partner repository<sup>5</sup>  
618 with the dataset identifier PXD020180(Deutsch et al., 2020).

619

## 620 **RNA sequencing**

621 EV pellets were treated with proteinase K (0.05µg/µl) for 10 min at 37°C. Roche Cocktail Inhibitor was then  
622 added to the sample for 10 min at room temperature followed by incubation at 85 °C for 5 min. Samples were  
623 then incubated with RNase A (0.5µg/µl) for 20 min at 37°C to degrade unprotected RNA. Total RNAs of  
624 isolated EVs was extracted using TRI Reagent (Molecular Research Center). Total RNA Sequencing libraries  
625 were prepared with SMARTer® Stranded Total RNA-Seq Kit v2 - Pico Input Mammalian (TaKaRa) according to  
626 the manufacturer's instructions. Libraries were pooled and sequenced (paired-end 2\*75bp) on a NextSeq500  
627 using the NextSeq 500/550 High Output Kit v2 according to the manufacturer's instructions (Illumina, San  
628 Diego, CA, USA). Raw sequencing data generated by the Illumina NextSeq500 instrument were mapped to the  
629 mouse reference genome using the hisat2 software(Kim et al., 2015). For every sample, quality control was  
630 carried out and assessed with the NGS Core Tools FastQC  
631 (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). Read counts were generated with the htseq-  
632 count tool of the Python package HTSeq (Anders et al., 2015). Differential analysis was performed by the  
633 DESEQ2 (Love et al., 2014) package of the Bioconductor framework. Detection of significantly up- and down-  
634 regulated genes between pairs of conditions based on their log<sub>2</sub>FC and functional enrichment analyses were  
635 performed using STRING v11 (Szklarczyk et al., 2019). EVs RNA sequencing was performed in triplicate.

636

637

## 638 **Lipidomics**

639 EVs were extracted with 2ml of chloroform/methanol 2/1 v/v and 1ml water, sonicated for 30 s, vortexed, and  
640 centrifuged. Lower organic phase was transferred to a new tube, the upper aqueous phase was re-extracted  
641 with 2ml chloroform. Organic phases were combined and evaporated to dry. Lipid extracts were resuspended  
642 in 50µL of eluent A. Synthetics internal lipid standards (PA 14:1/17:0, PC 17:0/14:1 and PS 17:0/17:0) from  
643 Avanti Polar Lipids was added. LC-MS/MS (MRM mode) analyses were performed with a MS model QTRAP®  
644 6500 (ABSciex) coupled to an LC system (1290 Infinity II, Agilent). Analyses were achieved in the negative (PA)  
645 and in positive (PC) mode; nitrogen was used for the curtain gas (set to 20), gas 1 (set to 20) and gas 2 (set to 1  
646 0). Needle voltage was at - 4, 500 or 5, 500 V without needle heating; the declustering potential was adjusted  
647 set at - 172 V or + 40 V. The collision gas was also nitrogen; collision energy is set to - 46 or + 47 eV. The dwell  
648 time was set to 30 ms. Reversed phase separations were carried out at 50 °C on a Luna C8 150x1 mm column,  
649 with 100 Å pore size, 5 µm particles (Phenomenex). Eluent A was isopropanol/CH<sub>3</sub>OH/H<sub>2</sub>O (5/1/4) +0.2 %  
650 formic acid+0.028 % NH<sub>3</sub> and eluent B was isopropanol+0.2 % formic acid+0.028 % NH<sub>3</sub>. The gradient elution  
651 program was as follows: 0-5 min, 30-50 % B; 5 - 30 min, 50-80 % B; 31-41 min, 95 % B; 42-52 min, 30 % B. The  
652 flow rate was set at 40 µL/min; 15 µL sample volumes were injected. The areas of LC peaks were determined  
653 using MultiQuant software (v3.0, ABSciex) for PA and PC quantification. EVs lipid analysis was performed in  
654 triplicate.

655

## 656 **Animal experiments**

657 All animals were housed and handled according to the guidelines of INSERM and the ethical committee of  
658 Alsace, France (CREMEAS) (Directive 2010/63/EU on the protection of animals used for scientific purposes).  
659 Animal facility agreement number: #C67-482-33. Experimental license for mice:  
660 Apafis #4707-2016032416407780; experimental license for zebrafish: Apafis #16862-2018121914292754.  
661 Mouse experiments: 6-8 weeks-old female BalB/c mice (Charles River) were used in all experiments.  
662 Orthotopic breast tumor experiments: Syngenic BalB/c mice were injected in the left fourth mammary gland  
663 with 250.000 4T1 mammary tumor cells stably expressing either scramble control shRNA, RalA shRNA, or RalB  
664 shRNA and diluted in 50  $\mu$ l PBS. When tumors became palpable, tumor volume was assessed by caliper  
665 measurements using the formula  $(width^2 \times length)/2$  ( $mm^3$ ) twice a week for 41 days. At the endpoint of the  
666 experiment, tumors and lungs were harvested, weighted and fixed in formaldehyde. Alternatively, organs  
667 were embedded in OCT and frozen at  $-80^\circ C$ . In this case, lungs were inflated with OCT before dissection.  
668 Priming experiments: Mice were injected retro-orbitally with  $1.5 \times 10^8$  EVs isolated from 4T1-shControl, shRAlA  
669 and shRAlB cells. Two injections of EVs were performed two days apart. PBS was used as a negative control.  
670 Subsequently, 4T1-luciferase cells (90.000) were injected via tail vein one day after EV pre-conditioning. After  
671 cells injection, the extent of lung metastasis was measured every 3 days for 12 days using non-invasive  
672 imaging with IVIS Lumina III (Perkin Elmer). In brief, a D-luciferin solution (purchased from Perkin Elmer and  
673 used at 150 mg/kg, according to manufacturer's instructions) was injected intraperitoneally to the isoflurane  
674 (Zoetis) anesthetized mice. 5 min after luciferin injection, a bioluminescence image was acquired with an IVIS  
675 Lumina III (Perkin Elmer) imaging system and then analyzed using the Living Image software (Perkin Elmer).  
676 The rate of total light emission of the lung metastatic area was calculated and expressed as radiance photons  
677 counted during the whole acquisition time (5 min) and normalized to the initial radiance photon  
678 (photon/second/ $cm^2/sr$ ) measured immediately after 4T1- luciferase cells injection for each mouse (to).  
679 EV biodistribution. Mice were injected via retro-orbital venous sinus with  $1.4 \times 10^8$  MenBright-Cy3-labelled EVs  
680 freshly isolated from 4T1-shControl, shRAlA and shRAlB cells. PBS was used as a negative control. Mice were  
681 sacrificed 1h post-injection to quantify the fluorescence intensity of the organs *ex-vivo* with IVIS Lumina III  
682 (Perkin Elmer). Average of fluorescent photons per lung were quantify as radiant efficiency  
683 [photon/second/ $cm^2/sr$ ] / [ $\mu W/cm^2$ ]. For experiment testing the role of CD146 in EV biodistribution, isolated  
684 EVs were incubated with CD146 blocking antibody (EPR3208; Abcam; 12  $\mu g/ml$ ) for 30 min at room  
685 temperature before injection. For metastasis priming experiments, CD146 was blocked similarly and a rabbit  
686 IgG isotype was used as control (Abcam) at an equivalent concentration.  
687 Zebrafish experiments: At 48h post-fertilization (hpf), Tg(Fli1 :GFP) zebrafish embryos were dechorionated  
688 and mounted in 0.8% low melting point agarose pad containing 650 mM of tricaine (ethyl-3-aminobenzoate-  
689 methanesulfonate). Embryos were injected in the duct of Cuvier with 27,6 nL of Membright Cy5- labeled EVs  
690 (at  $10^{10}$  EVs/ml) freshly isolated from 4T1-shControl, shRAlA and shRAlB cells with a Nanoject microinjector 2  
691 (Drummond) under a M205 FA stereomicroscope (Leica), using microforged glass capillaries (25 to 30 mm  
692 inner diameter) filled with mineral oil (Sigma). Embryos were imaged with confocal right after injection. For  
693 experiment testing the role of CD146, 4T1isolated EVs were incubated with CD146 blocking antibody (12  
694  $\mu g/ml$ ) for 30 min at room temperature before injection.



695 **Tissue section and staining**

696 Mouse lungs were incubated overnight in 4% PFA, dehydrated in 100% ethanol for 24 h, embedded in paraffin,  
697 cut in 7µm thick sections, dewaxed and rehydrated with 100% Toluene (2 washes of 15 min) then incubated in  
698 100%-70% alcohol solutions (10 min each) followed by final staining with hematoxylin (Surgipath) for 5 min  
699 and washing with tap water. Sections were further processed with differentiation solution (1% HCl in absolute  
700 ethanol, for 7 s), followed by washing under tap water for 10 min. Sections were then incubated in eosin  
701 (Harris) for 10 s, rinsed and dehydrated in 70% - 100% alcohol baths with rapid dips in each bath before a final  
702 wash in toluene for 15 min and embedded in Eukitt solution (Sigma). 2 random distanced sections taken in  
703 each of the 5 lung were analyzed for each mouse. Stitching imaging was performed using an AxioImager  
704 (Zeiss) with a 10x objective. Metastatic surfaces and whole lung surfaces were measured using the Fiji  
705 software.

706

707 **Caspase 3/7 assay**

708 Mouse tumor samples stored at -80°C are disrupted in a buffer containing Tris HCl pH 7.5, 50mM, NaCl  
709 150mM, NP40 1% + Protease Inhibitors cocktail (Complete from Roche) in the presence of 4 zirconium beads,  
710 using the Precellis system (Bertin instruments) with 2 pulses (10'') at 5000 rpm. Protein concentration was  
711 measured using Bradford kit (BioRad) and 5µg was analyzed using the Caspase 3/7 glo kit (Promega) according  
712 to manufacturer's instructions. Photons production generated by the luciferase was measured using a  
713 luminometer (Berthold Tris Star 2).

714

715 **Immunofluorescence**

716 For immunofluorescence on cultured cells, cells were fixed with 4% PFA for 15min, permeabilized in PBS-  
717 Triton 0.1% (Sigma) for 10 min and incubated in 5% normal goat serum for 1 h. The following primary  
718 antibodies were used: ZO-1 (Rabbit, 61-7300; Thermo Fisher Scientific), VE-Cadherin (mouse, 348502;  
719 BioLegend), CD63 (rat, D623-3; MBL), Ra1A (mouse, 610221; BD), Ra1B (mouse, 04037; Millipore), CD146  
720 (Mouse, P1H12, ThermoFisher). The following secondary antibodies were used: goat anti-mouse/rat/rabbit  
721 coupled with Alexa Fluor 488, Alexa 555, or Alexa 647 (Invitrogen). Cells were mounted with DAPI-containing  
722 Vectashield (Vector Laboratories).

723 For immunofluorescence on tissue sections, tissues were cut in 7µm thick sections, dewaxed for paraffin-  
724 embedded tissues and air-dried for frozen tissues. Sections were incubated first in 5% normal goat serum for  
725 2h in a humidified container. The following antibodies were used: CD31 (Mouse, 37-0700; Thermo Fisher  
726 Scientific), S100A4 A gift from Nona Ambartsumian (Institut for Cancer Biology, Copenhagen, DK-2100,  
727 Denmark.), F4/80 (Rat, ab6640; abcam), rabbit monoclonal antibody against Ki67 (Rabbit, RM-9106-So;  
728 Thermo Fisher Scientific) and caspase-3 (Mouse, 966S1; Cell Signaling Technology). Secondary antibodies  
729 were similar to the ones used with cells. Nuclei were stained with DAPI (Sigma).

730

731 **Imaging and Analysis**

732 Imaging on fixed samples. Tissue and cell sections were imaged with a Zeiss Imager Z2 with a 40X objective  
733 (N.A. 1.4) or with an SP5 confocal (Leica) with a 63X objective (N.A. 1.25). Image analysis and processing were  
734 performed using the Fiji software. For endothelial adherent and tight junction analysis, 10 random junctions  
735 were analyzed per image (5 images per sample) measuring junction width. For Ki67 and Caspase 3 imaging, 15  
736 random fields of view were quantified per sample. For EVs imaging, 40 to 60 random fields of view were  
737 imaged on 3 to 4 sections per mouse.

738 Live-cell imaging. For live-cell imaging, cells were seeded on 3.5 cm diameter glass-bottom dishes (MatTek  
739 Corporation, Ashland, MA) pre-coated with fibronectin (10µg/ml; Sigma). Nuclei were labeled with NucBlue™  
740 Live Ready Probe (Life Technologies, Grand Island, NY). In some experiments, cells were incubated with  
741 LysoTracker Deep Red (Thermo Fisher Scientific) at 1µM for 30 min before imaging. Cells were imaged by  
742 confocal microscopy (SP5, Leica) equipped with a thermostated chamber at 37°C with 5% CO<sub>2</sub>. Image analysis  
743 and processing were performed using the Fiji software.

744 HUVEC cells were seeded in fibronectin (10µg/ml; Sigma) pre-coated glass bottom culture chambers (LabTek  
745 I, Dutscher 055082). Confluent cells were incubated with 2x10<sup>8</sup> MemBright-labeled EVs in ECGM EV-free  
746 medium for 1h. Nucleus were labeled using NucBlue™ (Life Technologies, Grand Island, NY). Cells were  
747 imaged by confocal microscopy (SP5 Leica) in a thermostated chamber at 37°C with 5% CO<sub>2</sub>.

748 Zebrafish imaging: Confocal imaging was performed on the caudal plexus of zebrafish embryos right after  
749 injection with an inverted TCS SP5 with HC PL APO 20X/0,7 IMM Corr CS objective (Leica). Image analysis and  
750 processing were performed using the Fiji software.

751

## 752 **Human samples**

753 Human databases: Kaplan-Meier survival curves and statistical analysis of overall survival and gene expression  
754 was assessed on the TCGA breast invasive carcinoma cohort (1097 patients) using data generated by the  
755 TCGA Research Network: <https://www.cancer.gov/tcga>.

756 Immunohistochemistry: Paraffin sections of 4 µm from metastatic and non-metastatic breast tumours were  
757 obtained from CRB-Tumorothèque of the Institut de Cancérologie de l'Ouest (ICO, Saint-Herblain, France)  
758 (Heymann et al., 2020). Immunohistochemistry was performed using RalA (BD Transduction #610222, 1/100)  
759 and RalB (Sigma WH0005899, 1/400) antibodies on MicroPICell facility (Nantes, France) Citrate buffer pH6  
760 was used for antigen retrieval 20min à 96°C (Target Retrieval solution low pH, Dako) and DAB and  
761 Hematoxylin staining were revealed using ImPath detection kit (DAB OB Sensitive Detection Kit, ImPath).  
762 Whole slides were scanned on Hamamatsu scanner using Nanozoomer Digital Pathology software.  
763 Automated computer quantification of DAB staining in perinuclear zones (brown intensity measurement)  
764 after automatic nuclei detection with hematoxylin staining in the whole biopsies was performed using Qupath  
765 open source software for digital pathology image analysis (Bankhead et al., 2017) on MicroPICell platform  
766 (Nantes, France). Quantification was further confirmed by manual blinded arbitrary scoring of DAB brown  
767 intensity in tumoral zones was performed using a score of 1 for low staining to score of 3 for intense staining.

768

## 769 **Statistical analyses**

770 All results were confirmed in at least two independent experiments. Statistical significance of results was  
771 analyzed using the GraphPad Prism program version 5.04. The Shapiro-Wilk normality test was used to  
772 confirm the normality of the data. The statistical difference of Gaussian data sets was analyzed using the  
773 Student unpaired two-tailed t test, with Welch's correction in case of unequal variances and the one-way  
774 ANOVA test followed by a Bonferonni multiple comparison post-test was used for multiple data comparison.  
775 For data not following a Gaussian distribution, the Mann-Whitney test was used, and the Kruskal-Wallis test  
776 followed by Dunn's Multiple Comparison post-test was used for multiple data comparison. Two Way Anova  
777 was used to compare more than 1 parameters followed by Bonferonni post-test. For analyzing data containing  
778 only 3 measurements, One Way Anova permutation test followed pairwise permutation test with false  
779 detection rate (fdr) correction, using R software (version 3.6.2) was used. Illustrations of these statistical  
780 analyses are displayed as the mean +/- standard deviation (SD). p-values smaller than 0.05 were considered as  
781 significant. \*,  $p < 0.05$ , \*\*,  $p < 0.01$ , \*\*\*,  $p < 0.001$ , \*\*\*\*,  $p < 0.0001$ .  
782  
783

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798

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800

801

## 802 **Figures**

803 **Figure 1: RalA and RalB control exosome secretion and MVB homeostasis.** a-b) Nanoparticle tracking  
804 analysis of EVs isolated by ultracentrifugation (100.000g pellet) from the supernatant of shCtl, shRalA or  
805 shRalB 4T1 cells (a) or from various cell types treated with Ral inhibitors RBC8 (b, left) or BQU57 (b, right). 231:  
806 MDA-MB-231 cells. Each dot represents one experiment (a: 10 independent experiments; one Way Anova  
807 followed by Bonferroni's Multiple Comparison Test; b: 4 to 5 independent experiments, Mann Whitney test). c)  
808 Representative confocal images of 4T1 cells showing endogenous expression of RalA, RalB and CD63 by  
809 immunofluorescence (left) and overexpression of GFP-RalA and GFP-RalB in cells incubated with LysoTracker  
810 (right). Scale bar: 10  $\mu\text{m}$ ; zoom: 2  $\mu\text{m}$ . d) Representative electron micrographs of 4T1 shCtl, shRalA and shRalB  
811 cells, with zoom on MVBs; Scale bar: 1  $\mu\text{m}$ ; zoom: 200 nm. Violin plots show quantification of the number of  
812 MVB per cytoplasm surface. Each dot represents one field of view; horizontal bars represent the average (76 to  
813 88 fields of view; Kruskal-Wallis test followed by Dunn's Multiple Comparison Test).

814  
815 **Figure 2: The RalA/B-PLD1-PA axis governs exosome secretion.** a) Representative confocal images of 4T1  
816 cells co-transfected with PLD1-GFP and tdTomato-RalA (upper panels) or tdTomato-RalB (Lower panels) and  
817 incubated with LysoTracker. Scale bar: 10  $\mu\text{m}$ ; zoom: 2  $\mu\text{m}$ . b) Electron microscopy analysis of 4T1 cells treated  
818 with PLD1 or PLD2 inhibitor. Scale bar: 1  $\mu\text{m}$ . Violin plots show quantification of the number of MVB per  
819 cytoplasmic surface. Each dot represents one field of view; horizontal bar represents the average (180 to 194  
820 fields of view; Kruskal-Wallis test followed by Dunn's Multiple Comparison Test). c) Nanoparticle tracking  
821 analysis of EVs isolated by ultracentrifugation (100.000g pellet) from the supernatant of 4T1 cells treated with  
822 PLD1 (CAY10593) or PLD2 (CAY10594) inhibitor. Each dot represents one experiment (3 independent  
823 experiments; One Way Anova permutation test followed by fdr multi-comparison permutation test). d)  
824 Representative confocal images of shControl, shRalA and shRalB 4T1 cells transfected with PLD1-GFP. Scale  
825 bar: 10  $\mu\text{m}$ ; zoom: 2  $\mu\text{m}$ . Graph shows the percentage of cells with high (>5) number of PLD1-GFP cytoplasmic  
826 puncta. (Each dot represents one experiment. 5 independent experiments; Number of cells analyzed: shCtl  
827 (136), shRalA (170), shRalB (244); Kruskal-Wallis test followed by Dunn's Multiple Comparison Test). e)  
828 Quantification of the Phosphatidic Acid (PA) / PhosphatidylCholine (PC) ratio in EVs isolated from shControl,  
829 shRalA and shRalB cells (each dot represents one experiment; 3 independent experiments; One Way Anova  
830 permutation test followed by fdr multi-comparison permutation test;  $\text{fdr} < 0,1$ ). f) Model showing how RalA and  
831 RalB could control PLD1 localization on MVBs, thereby inducing the PA accumulation on MVBs, promoting  
832 MVB homeostasis and controlling exosome secretion.

833  
834 **Figure 3: RalA and RalB promote lung metastasis in a non-cell autonomous fashion.** a) Kaplan-Meier curve,  
835 obtained from TCGA 1097 cohort, showing the survival probability of patients with tumor breast invasive  
836 carcinoma having high or low RalA (pvalue: 5,15 e-03; pAdj: 1,35e-01) or RalB (pvalue: 1,77 e-05; pAdj: 5,99e-  
837 03) expression levels. b) Representative images of immunohistochemistry against RalA or RalB performed on  
838 mammary primary tumors from patients with or without metastasis. Scale bar: 500  $\mu\text{m}$  Graphs represent  
839 automated scoring of DAB staining. Each dot represents one patient; 10 patients per group; Student t-test. c)

840 Orthotopic injection of shControl, shRalA and shRalB  $4T1$  cells in syngenic mice. Representative images of  
841 primary tumors at day 41. Scale bar: 1cm. Graphs showing the primary tumor growth over time (Left) and the  
842 primary tumor weight at day 41. Each dot represents one mouse. (Two independent experiments; Left: Two  
843 way Anova followed by Bonferonni post-test, Right: Kruskal-Wallis test followed by Dunn's Multiple  
844 Comparison Test). **d**) Representative confocal images of primary tumors stained with anti-Ki67 antibody.  
845 Scale bar: 50  $\mu\text{m}$ . Graph indicates the % of Ki67 positive nuclei. Each dot represents one mouse. (6 mice taken  
846 from 2 independent experiments; Kruskal-Wallis test followed by Dunn's Multiple Comparison Test. **e**)  
847 Analysis of lung metastasis in mice from the orthotopic experiment presented in (c). Representative images of  
848 lung sections (Day 41) stained with hematoxilin eosin. Scale bar: 1mm. Graphs show the number of metastatic  
849 foci per section (upper, One Way Anova followed by Bonferroni's Multiple Comparison Test) and the  
850 metastatic surface per lung surface (lower; Kruskal-Wallis test followed by Dunn's Multiple Comparison Test).  
851 Each dot represents one section **f**) Pictures of wound healing closure at different time points. Scale bar: 150  
852  $\mu\text{m}$ . Graph represents the percentage of wound closure at 16h (3 independent experiments; Kruskal-wallis test  
853 followed by Dunn's multiple comparison test). **g**) Pictures of 3D invasion assay after 15 days. Graph represents  
854 the invasive index. Scale bar: 100  $\mu\text{m}$ .

855

856 **Figure 4: RalA and RalB control lung tropism of pro-metastatic tumor EVs.** **a**) Effect of a similar amount of  
857 EVs on HUVEC monolayer permeability *in vitro*. The graph represents the normalized amount of fluorescent  
858 dextran that crossed the endothelial barrier. Each dot represents one experiment (8 independent experiments;  
859 One Way Anova followed by Bonferroni's Multiple Comparison Test). **b**) Representative epifluorescence  
860 images of VE-cadherin (upper panels) and ZO1 (Lower panel) stainings on HUVECS cells treated with similar  
861 amounts of EVs. Scale bar: 20  $\mu\text{m}$ ; zoom: 2  $\mu\text{m}$ . Graphs represent the disorganization of adherent (up) and  
862 tight (low) junctions (Three independent experiments; up; Kruskal-Wallis test followed by Dunn's Multiple  
863 Comparison Test)). **c**) Metastasis priming experiment, Balb/c mice are first injected twice with tumor equal  
864 number of EVs ( $1,5 \times 10^8$  EVs), then intravenously with  $4T1$  luciferase cells and metastasis is then followed over  
865 time. Graph shows metastasis progression over time in mice pre-injected with PBS, or with equal number of  
866 EVs from shControl, shRalA or shRalB cells (7-10 mice per group; merge of two independent experiments; Two  
867 way Anova followed by Bonferonni multiple comparison post test; stars indicate statistically significant  
868 differences at day 14). Right: *In vivo* and *ex vivo* representative images of mice and lungs at day 14. Scale bars:  
869 1 cm. **d-e**) Lung accumulation of equal number of fluorescent-labeled EVs ( $3 \times 10^8$  EVs), from shControl, shRalA  
870 or shRalB cells injected intravenously. **d**) Representative *ex vivo* images and graph showing the total lung  
871 fluorescence 1h post-injection. Each dot represents one mouse. (8 mice taken from 2 independent  
872 experiments; Kruskal-Wallis test followed by Dunn's Multiple Comparison Test. **e**) Representative confocal  
873 lung sections images and graph showing the percentage of EVs positive fields. Each dot represents one  
874 section (3 mice; Kruskal-Wallis test followed by Dunn's Multiple Comparison Test). Scale bar: 5  $\mu\text{m}$ . **f-g**) Arrest  
875 and internalization of equal number of EVs from shControl, shRalA and shRalB cells on endothelial cells *in vitro*  
876 and *in vivo*. **f**) Representative confocal Z-stacks of equal number of EVs after 1h or incubation with HUVEC  
877 monolayer. Scale bar: 25  $\mu\text{m}$ . Each dot represents one field of view (each dot represents one field of view

878 from 3 independent experiments; Kruskal-Wallis test followed by Dunn's Multiple Comparison Test). **g**)  
879 Representative confocal Z-stacks the caudal plexus of Tg(Fli1:GFP) zebrafish embryos, where GFP is expressed  
880 in the endothelium, injected with similar number of EVs and imaged right after injection. Each dot represents  
881 one zebrafish (31 to 53 embryos from 4 independent experiments; Kruskal-Wallis test followed by Dunn's  
882 Multiple Comparison Test). Scale bar: 20  $\mu$ m.

883

884 **Figure 5: CD146/MCAM is under-expressed in RalA/B knockdown EVs and mediates their lung tropism. a)**  
885 Venn diagram representing the RNA present in the EVs isolated from shControl, shRalA or shRalB cells (with a  
886 minimum of 10 reads per sample; RNA sequencing performed in triplicate). **b)** Type of RNA associated  
887 identified in EVs isolated from shControl, shRalA or shRalB cells. Left: RNA exclusively present in one type of  
888 EVs. Right: enriched RNAs ( $\log_2$  fold change  $>2$ ;  $p(\text{adj.}) < 0,05$ ). **c)** GO terms of the proteins identified in EVs  
889 isolated from 4T1 cells by ultracentrifugation (100.000g pellet) and illustration of some proteins known to be  
890 present in EVs. **d)** Comparison of the protein content of EVs isolated from shControl, shRalA and shRalB cells.  
891 The venn diagram represents proteins having different expression levels (Mass spectrometry performed in  
892 triplicate; FDR  $< 1\%$ ). **e)** Analysis of the expression of CD146/MCAM, Clic4 and Glypican 4 in EVs isolated from  
893 shControl, shRalA and shRalB cells by western blots. Each dot represents one experiment (4 to 6 independent  
894 experiments; Kruskal-Wallis test followed by Dunn's Multiple Comparison Test). **f-g)** Arrest, internalization  
895 and organotropism of EVs treated with an anti-CD146 antibody and injected in the circulation of zebrafish  
896 embryos (**f**) or mouse (**g**). **f)** Representative confocal Z-stacks the caudal plexus of Tg(Fli1:GFP) zebrafish  
897 embryos, where GFP is expressed in the endothelium, injected with equal number of EVs and imaged right  
898 after injection. Scale bar: 20  $\mu$ m; Zoom scale bar: 5  $\mu$ m. Each dot represents one zebrafish (46 embryos from 4  
899 independent experiments; Mann Whitney test). **g)** Representative confocal images of lung sections and graph  
900 showing the percentage of EVs positive fields. Scale bar: 10  $\mu$ m. Each dot represents one mouse (8 mice from  
901 2 independent experiments; Mann Whitney test). **h)** Metastasis priming experiment, Balb/c mice are injected  
902 twice with tumor equal number of EVs ( $1,5 \times 10^8$  EVs), pre-incubated with CD146 blocking antibody or isotype  
903 control, and then intravenously injected with 4T1 luciferase cells and metastasis is followed over time. Graph  
904 shows metastasis progression over time (14 mice per group; merge of two independent experiments; Two way  
905 Anova followed by Bonferonni multiple comparison post test; stars indicate statistically significant differences  
906 at day 14). *In vivo* and *ex vivo* representative images of mice and lungs at day 14. Scale bars: 1 cm. **i)** Kaplan-  
907 Meier curve, obtained from TCGA 1097 cohort, showing the survival probability of patients with tumor breast  
908 invasive carcinoma having high or low MCAM/CD146 expression levels (pvalue: 3,42 e-02; pAdj: 5,67e-01). **j)**  
909 Model describing the role of RalA/B dependent EVs in metastatic formation.

910

## 911 **Supplementary Figures**

912 **Figure 1 – Figure Supplement 1: a)** Representative western blots showing tubulin, RalA (left) and RalB (right)  
913 expressions in 4T1 shControl, shRalA and shRalB cells. **b)** Graph showing the average diameter of the EVs  
914 isolated from 4T1 shControl, shRalA and shRalB cells measured by nanoparticle tracking analysis. Each dot  
915 represents one experiment (12 independent experiments; One Way Anova followed by Bonferroni's Multiple

916 Comparison Test). c) Nanoparticle tracking analysis of EVs isolated by ultracentrifugation (100.000g pellet)  
917 from the supernatant of various breast cancer cell lines treated with the Ral inhibitor RBC8. Each dot  
918 represents one experiment (4 independent experiments, Mann Whitney test, p value indicated on the graph)

919

920 **Figure 1 – Figure Supplement 2: Electron microscopy analysis of endosomes in the absence of RalA or**  
921 **RalB a-b)** Graph showing the number of MVB (a) or endolysosomes (b) per cytoplasm surface in electron  
922 microscopy analysis performed by chemical fixation or high pressure freezing on 4T1 shControl, shRalA and  
923 shRalB cells. Each dot represents one field of view. Kruskal-Wallis test followed by Dunn's Multiple  
924 Comparison Test. c) FACS analysis of 4T1 shControl, shRalA and shRalB cells stained with Lyotracker. Each  
925 dot represents one experiment (5 independent experiments; Kruskal-Wallis test followed by Dunn's Multiple  
926 Comparison Test). d) Representative electron micrographs of MVBs in 4T1 shControl, shRalA and shRalB cells  
927 after chemical fixation or high-pressure freezing. Scale bar: 200nm. e-f) Number of ILV per MVB surface (e)  
928 and MVB diameter (f) measured in electron microscopy analysis performed by chemical fixation or high  
929 pressure freezing on 4T1 shControl, shRalA and shRalB cells. Each dot represents one MVB; Kruskal-Wallis test  
930 followed by Dunn's Multiple Comparison Test.

931

932 **Figure 2 – Figure Supplement 1: PLD1 and PLD2 in 4T1 cells. a)** PLD1 and PLD2 expression in 4T1 shControl  
933 cells quantified by qRT-PCR and normalized by *gapdh* expression. b) Representative confocal images showing  
934 PLD1-GFP and PLD2-GFP sub-cellular localization in 4T1 cells stained with lysotracker. Scale bars: 10 µm c)  
935 Representative confocal images of PLD2-GFP localization in shControl, shRalA and shRalB cells. Scale bar: 10  
936 µm d) PA/PC ratio of species known to be targeted by PLD1 identified in EVs isolated from shControl, shRalA  
937 and shRalB cells. Each dot represents one experiment; 3 independent experiments.

938

939 **Figure 3 – Figure Supplement 1: Proliferation and apoptosis of 4T1 cells and tumors. a)** Graph showing the  
940 percentage of cells expressing caspase 3 assessed by immunofluorescence on breast primary tumors. Each dot  
941 represents one mouse (8 mice taken from 2 independent experiments; one Way Anova followed by  
942 Bonferroni's Multiple Comparison Test). b) Graph showing the caspase3/7 expression levels on protein extracts  
943 from breast primary tumors. Each dot represents (12 to 19 mice from 2 independent experiments; Kruskal-  
944 Wallis test followed by Dunn's Multiple Comparison Test) c) Graph showing the proliferation of 4T1 shControl,  
945 shRalA and shRalB cells over time in an *in vitro* assay Two-way ANOVA followed by Bonferroni's Multiple  
946 Comparison Test (3 independent experiments with n=5). d) Graph showing the cycle analysis of 4T1 shControl,  
947 shRalA and shRalB cells. e) Graph showing the effect of high and low doses of 4T1 EVs on permeabilisation of  
948 a HUVEC monolayer *in vitro*. The graph represents the normalized amount of fluorescent dextran that crossed  
949 the endothelial barrier. Each dot represents one experiment (3 independent experiments; One Way Anova  
950 permutation test followed with *fdr* multicomparison test).

951



952 **Figure 3 – Figure Supplement 2: Soluble secretome** of 4T1 shControl cells compared to 4T1 shRalA or 4T1  
953 shRalB cells (3 independent experiments; One Way Anova permutation test followed with pairwise  
954 permutation test with fdr correction).

955  
956 **Figure 4 – Figure Supplement 1: 4T1 EVs organotropism.** (a) Organs (left) and lungs sections (right)  
957 harvested from mice injected with PBS-MB or 4T1 MB-EVs 1h after intravenous injection. Scale bars: 10 µm.  
958 (b) Representative confocal images showing the identity of lung cells accumulating fluorescently labeled 4T1  
959 EVs. Scale bars: 10 µm. Graphs show the percentage of endothelial cells, fibroblasts, macrophages and cells of  
960 unknown identity among EV positive cells (108 positive cells from 6 mice taken from 2 independent  
961 experiments). c) Graph showing liver accumulation of fluorescent EVs isolated from shControl, shRalA and  
962 shRalB cells. Equal numbers of EVs were injected intravenously one hour before organ harvesting (8 mice  
963 taken from 2 independent experiments; Kruskal-Wallis test followed by Dunn's Multiple Comparison Test).

964  
965 **Figure 5 – Figure Supplement 1: RNA content of EVs from shControl, shRalA and shRalB cells** a) Volcano  
966 plots comparing the mRNA present in EVs isolated from shControl and shRalA cells. b) Volcano plots  
967 comparing the mRNA present in EVs isolated from shControl and shRalB cells. c) GO term analysis of the  
968 mRNA present in EVs from shControl, shRalA and shRalB cells.

969  
970 **Figure 5 – Figure Supplement 2: 4T1 cells and EVs express CD146/MCAM long isoform.** a) Western blots on  
971 4T1 cell and EVs extracts using antibodies selectively recognizing CD146/MCAM short (left) and long (right)  
972 isoforms. b) Anti-CD146 Elisa on EVs isolated from shControl, shRalA and shRalB cells. Each dot represents  
973 one replicate. Horizontal line represents the median. c) Representative confocal images of 4T1 cells showing  
974 endogenous expression of CD146 and CD63 by immunofluorescence. Scale bars: 10 µm; zoom: 2 µm.

975

976 **Supplementary File 1: EVs RNA analysis.**

977 Sheet a: RNAs overexpressed in EVs from 4T1 shCtl cells Vs EVs from shRalA cells

978 Sheet b: RNAs overexpressed in EVs from 4T1 shRalA cells Vs EVs from shCtl cells

979 Sheet c: RNAs overexpressed in EVs from 4T1 shCtl cells Vs EVs from shRalB cells

980 Sheet d: RNAs overexpressed in EVs from 4T1 shRalB cells Vs EVs from shCtl cells

981

982 **Supplementary File 2: EVs proteomic analysis.**

983 Sheet a: Proteins identified in EVs from 4T1 shCtl cells

984 Sheet b: Proteins overexpressed in EVs from 4T1 shCtl cells Vs EVs from shRalA cells

985 Sheet c: Proteins overexpressed in EVs from 4T1 shRalA cells Vs EVs from shCtl cells

986 Sheet d: Proteins overexpressed in EVs from 4T1 shCtl cells Vs EVs from shRalB cells

987 Sheet e: Proteins overexpressed in EVs from 4T1 shRalB cells Vs EVs from shCtl cells

988 Sheet f: Proteins overexpressed in EVs from 4T1 shCtl cells Vs EVs from shRalA cells and EVs from

989 shRalB cells

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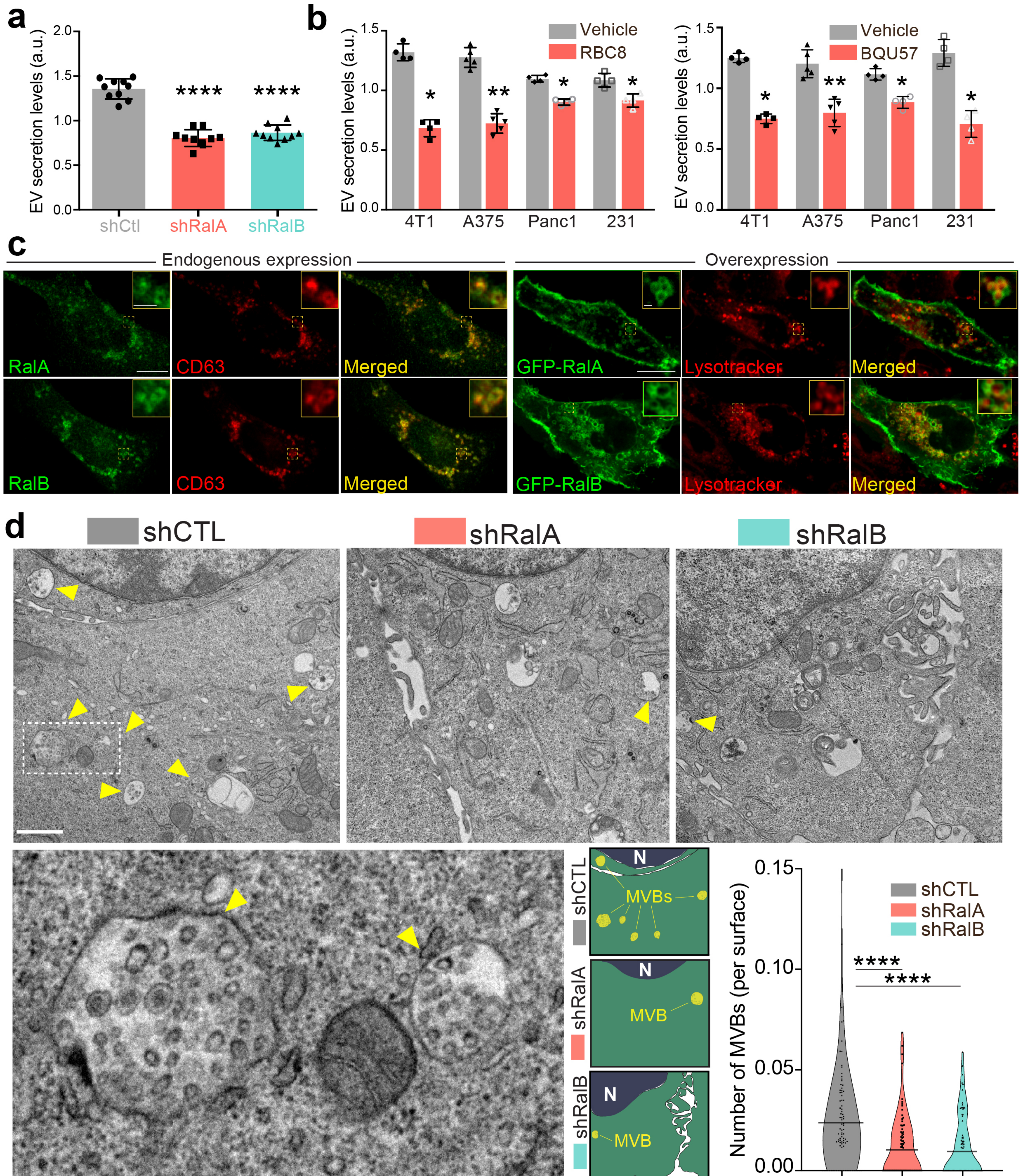


Figure 1\_Ghoroghi *et al.*



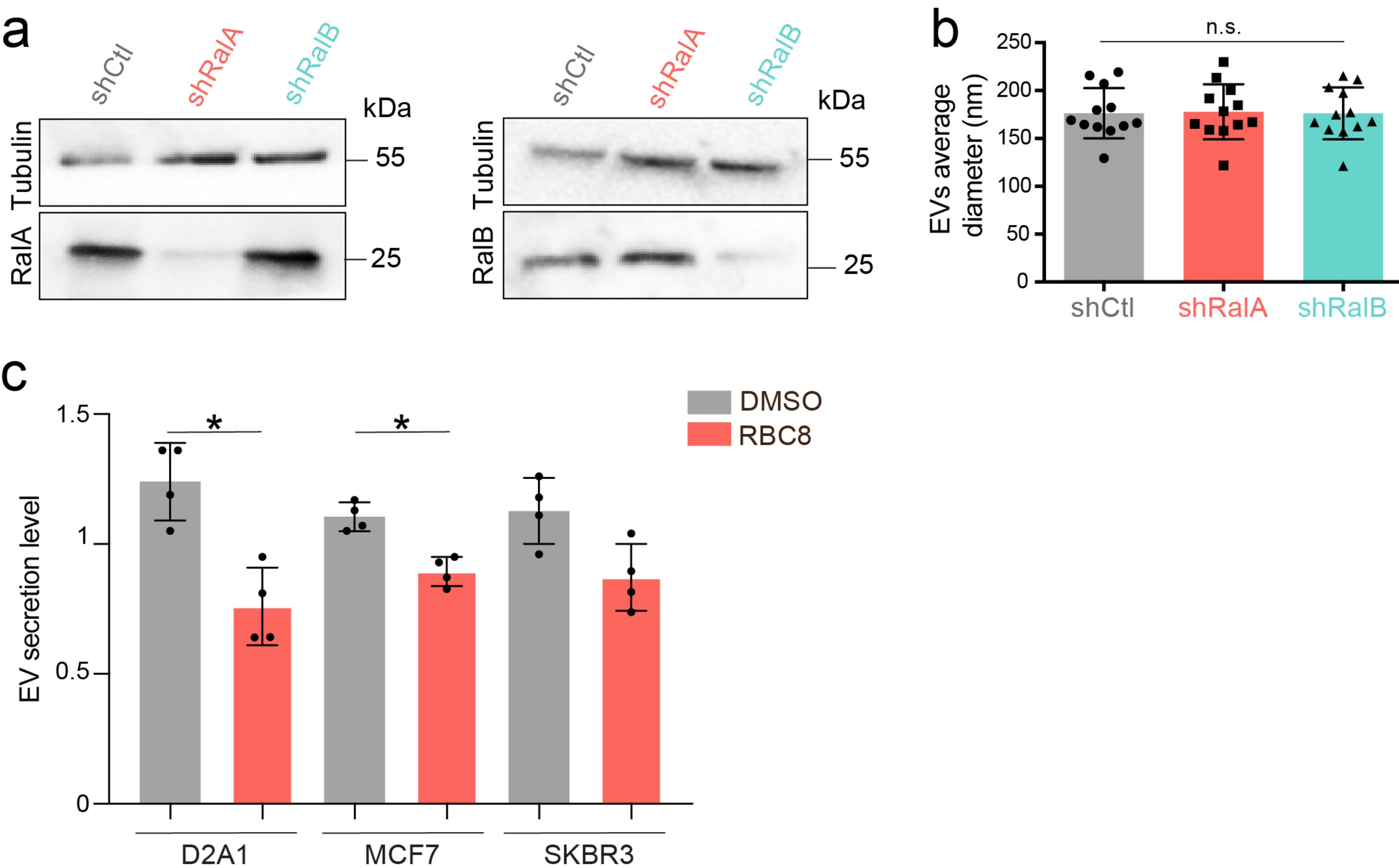


Figure 1 - Figure Supplement 1\_Ghoroghi *et al.*



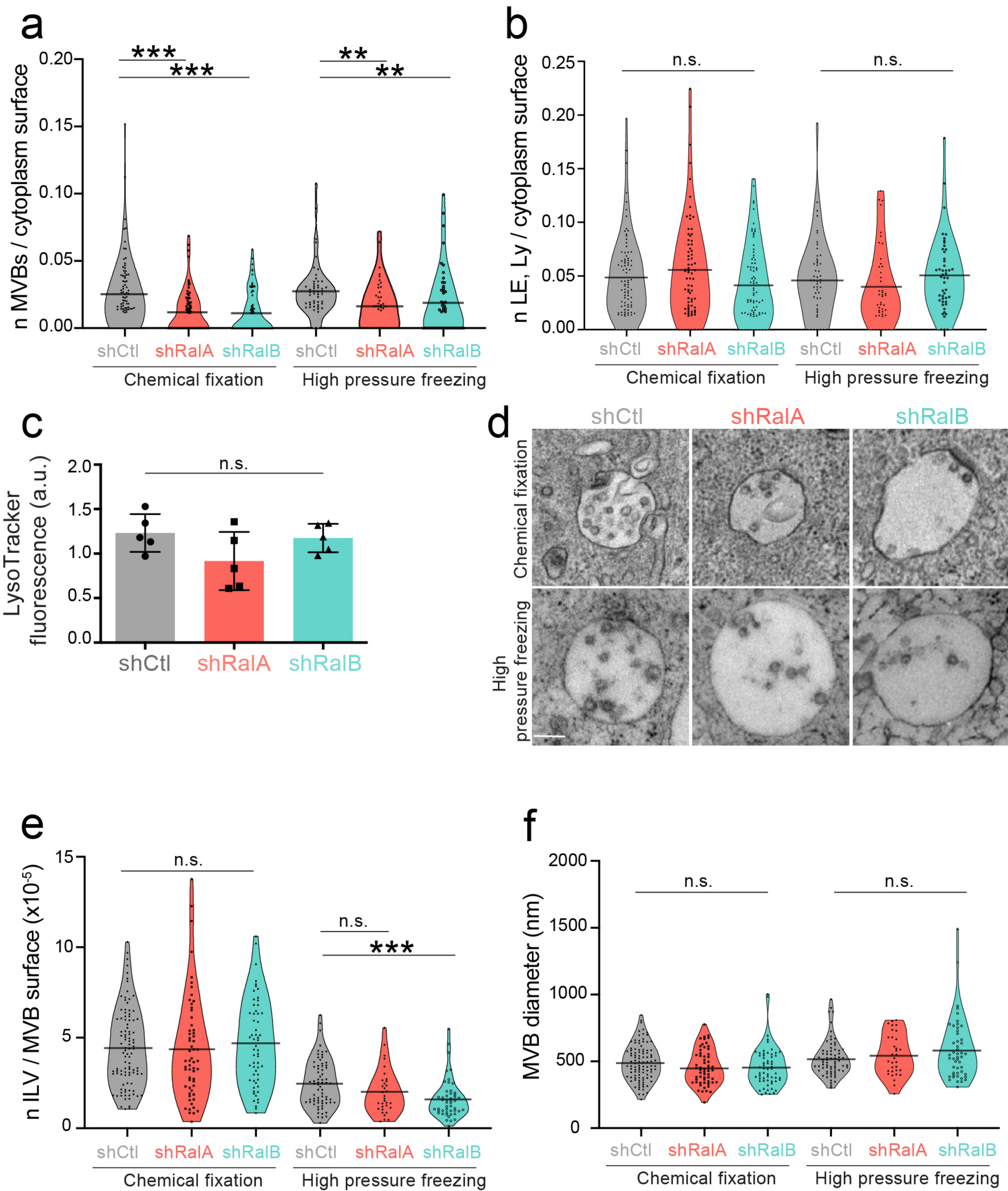


Figure 1 - Figure Supplement 2\_Ghoroghi *et al.*



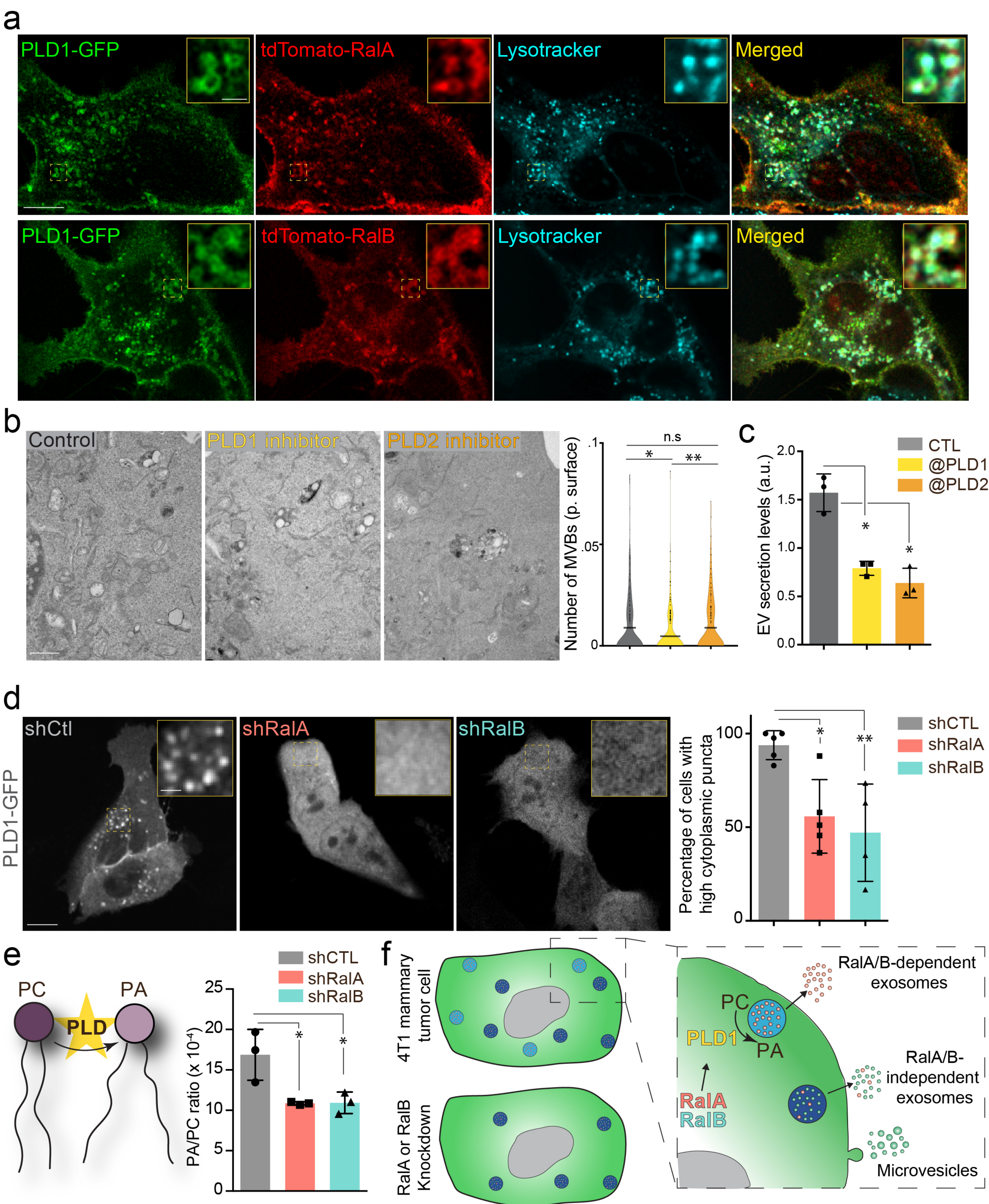


Figure 2\_Ghoroghi *et al.*



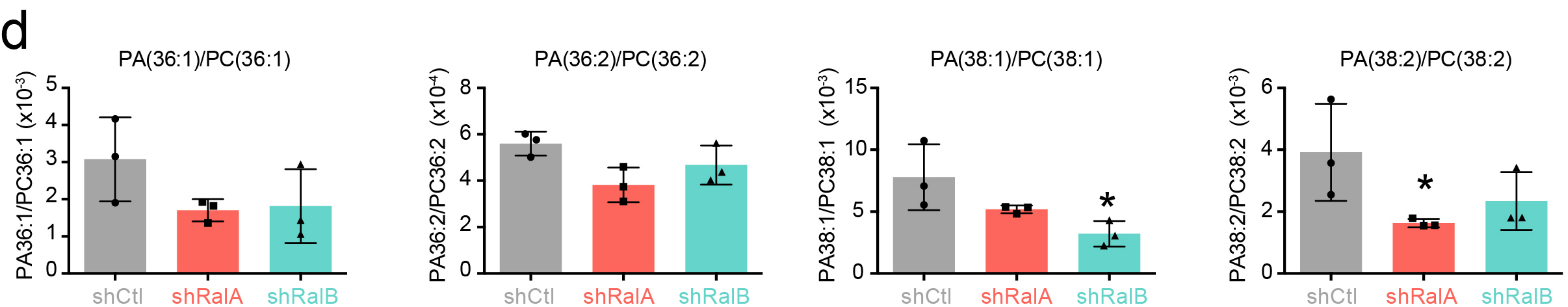
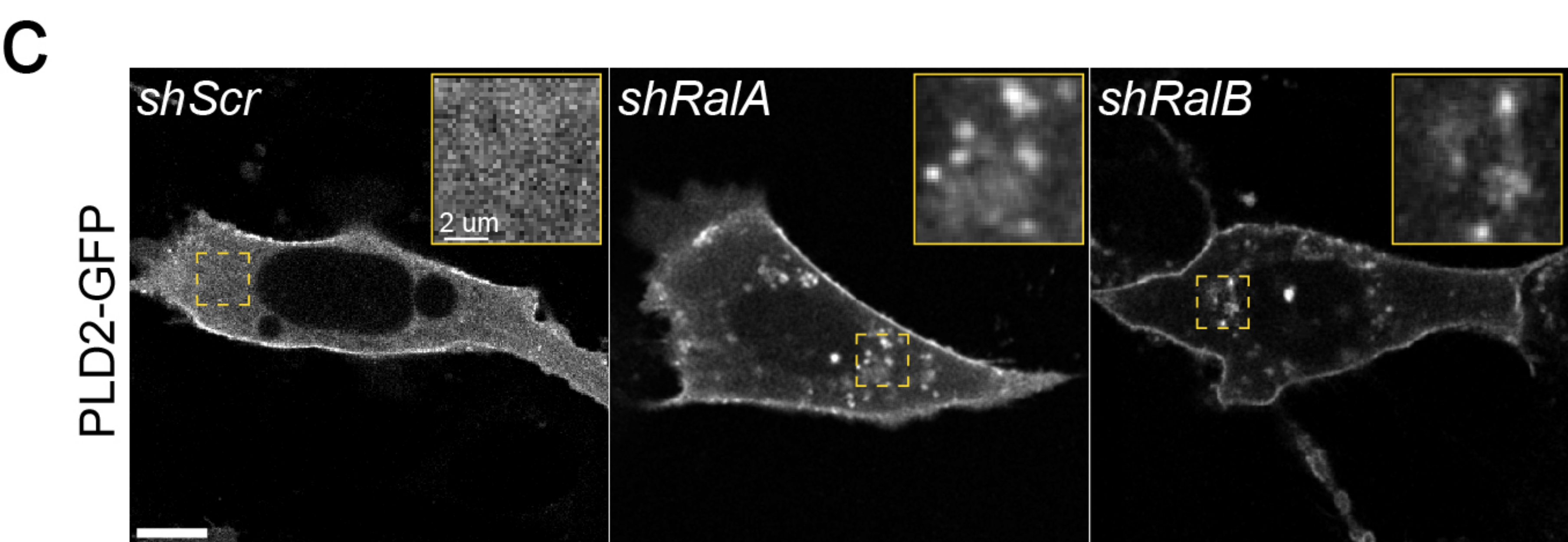
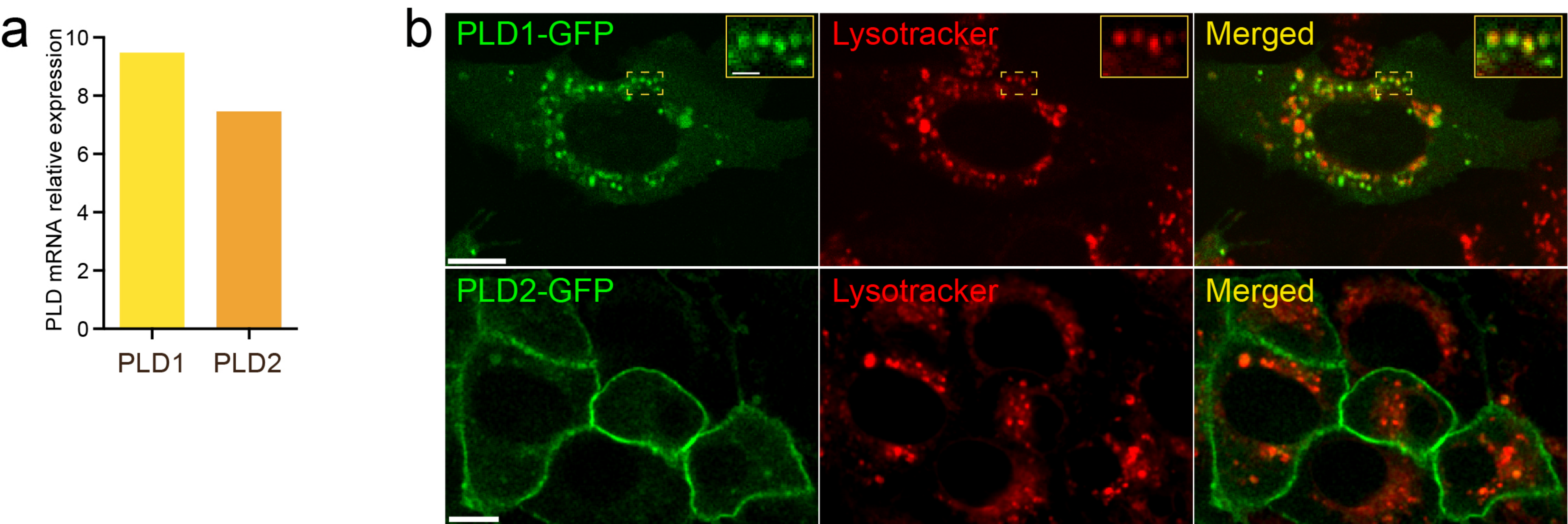


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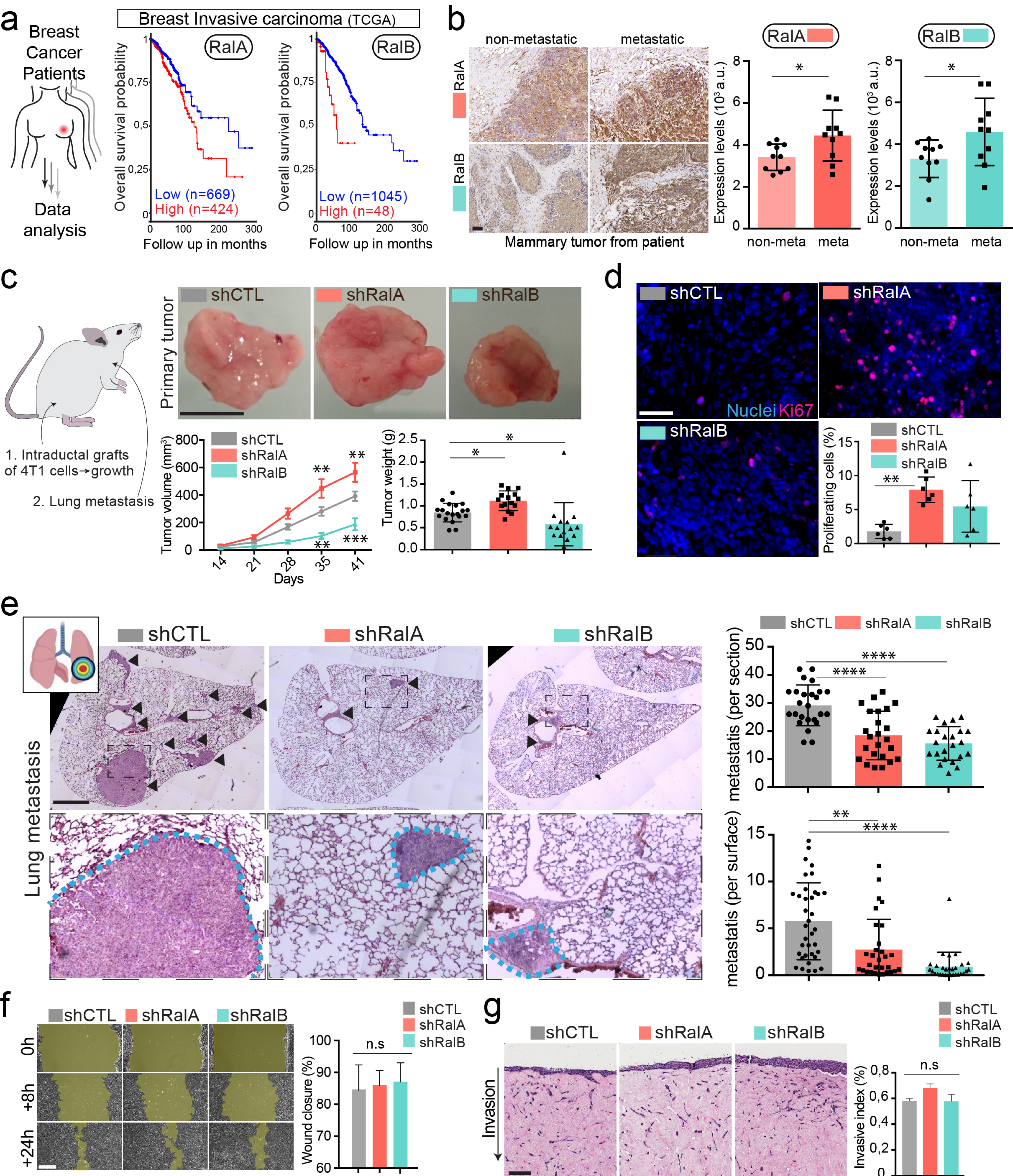


Figure 3\_Ghoroghi *et al.*



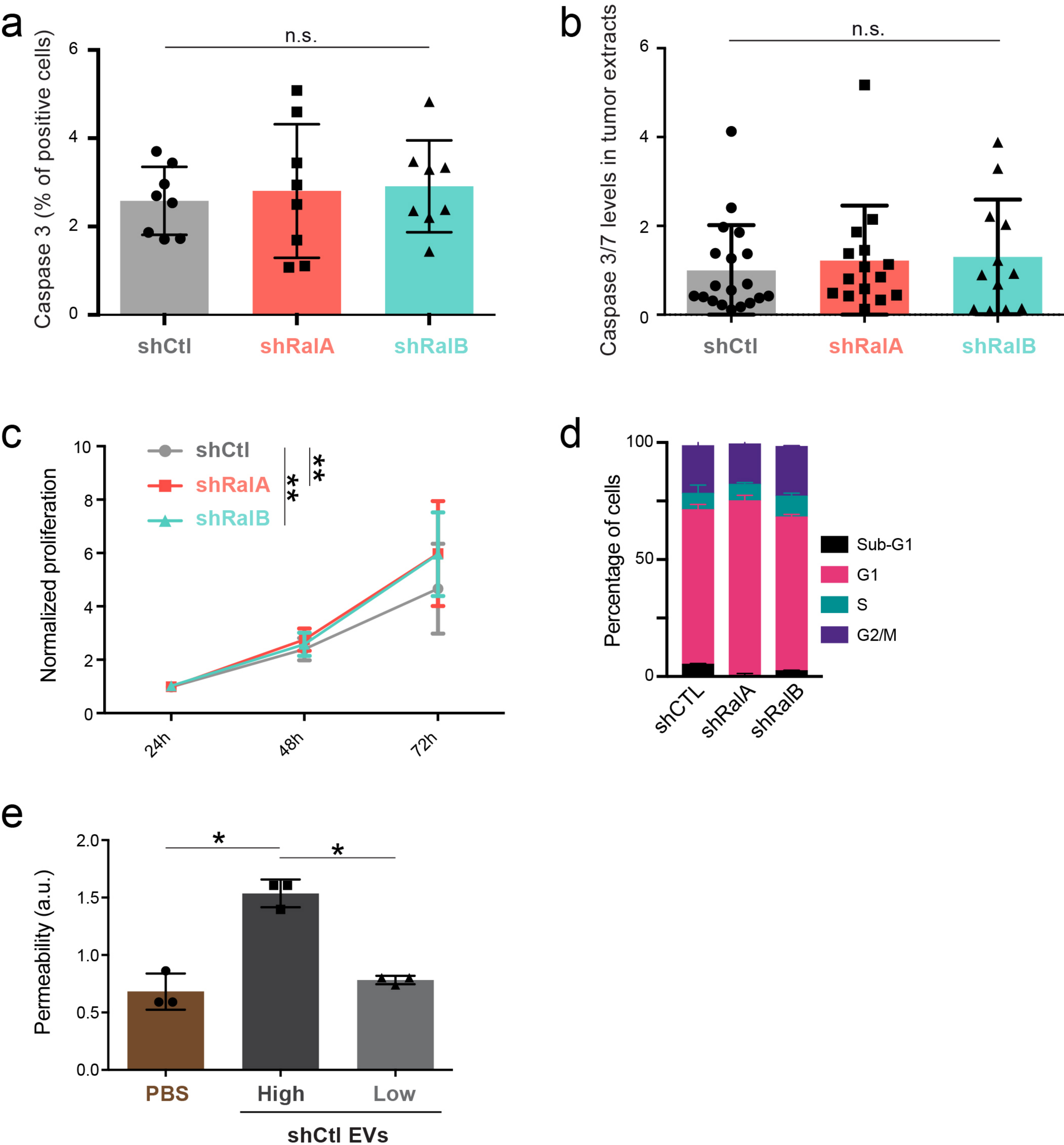


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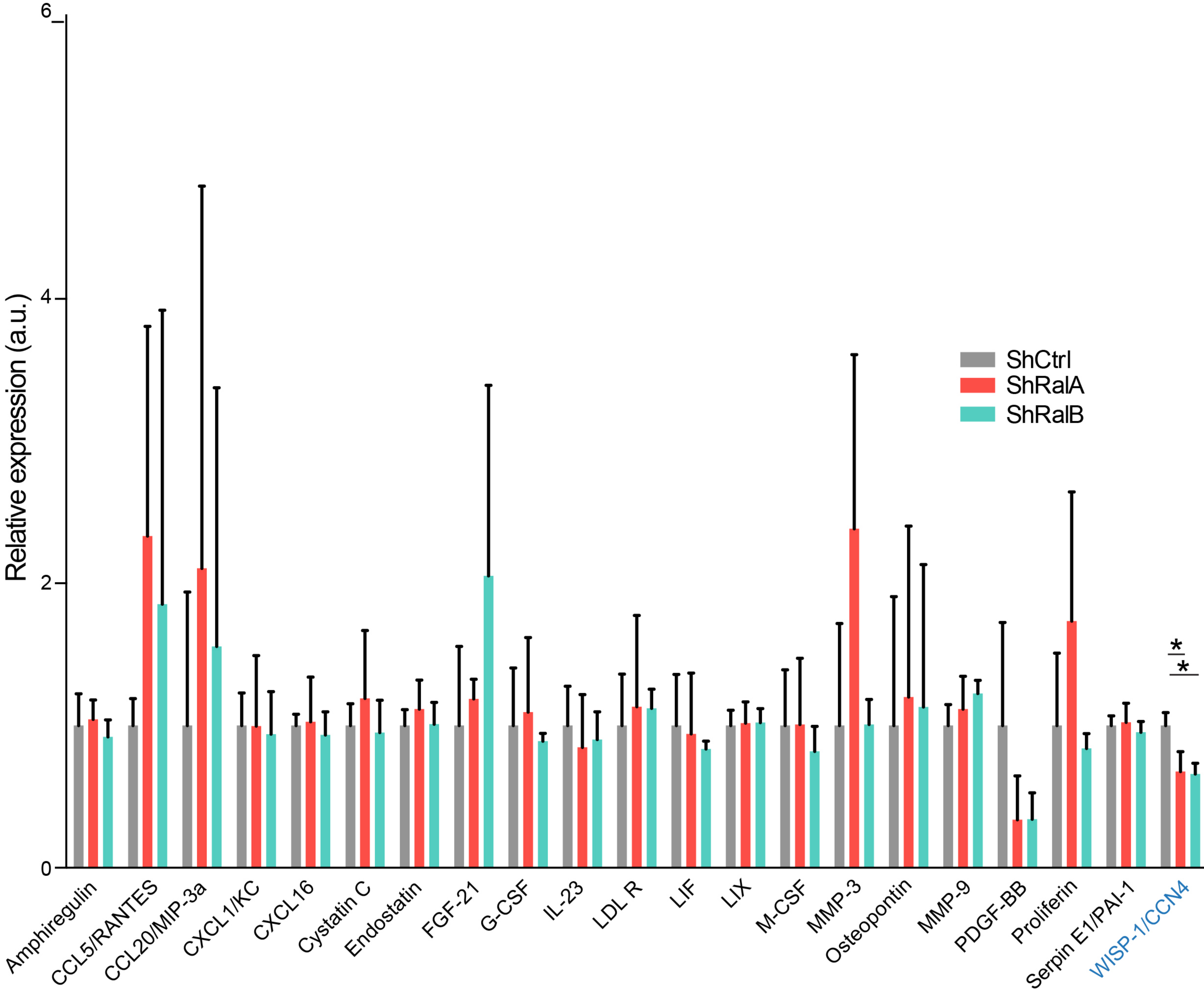


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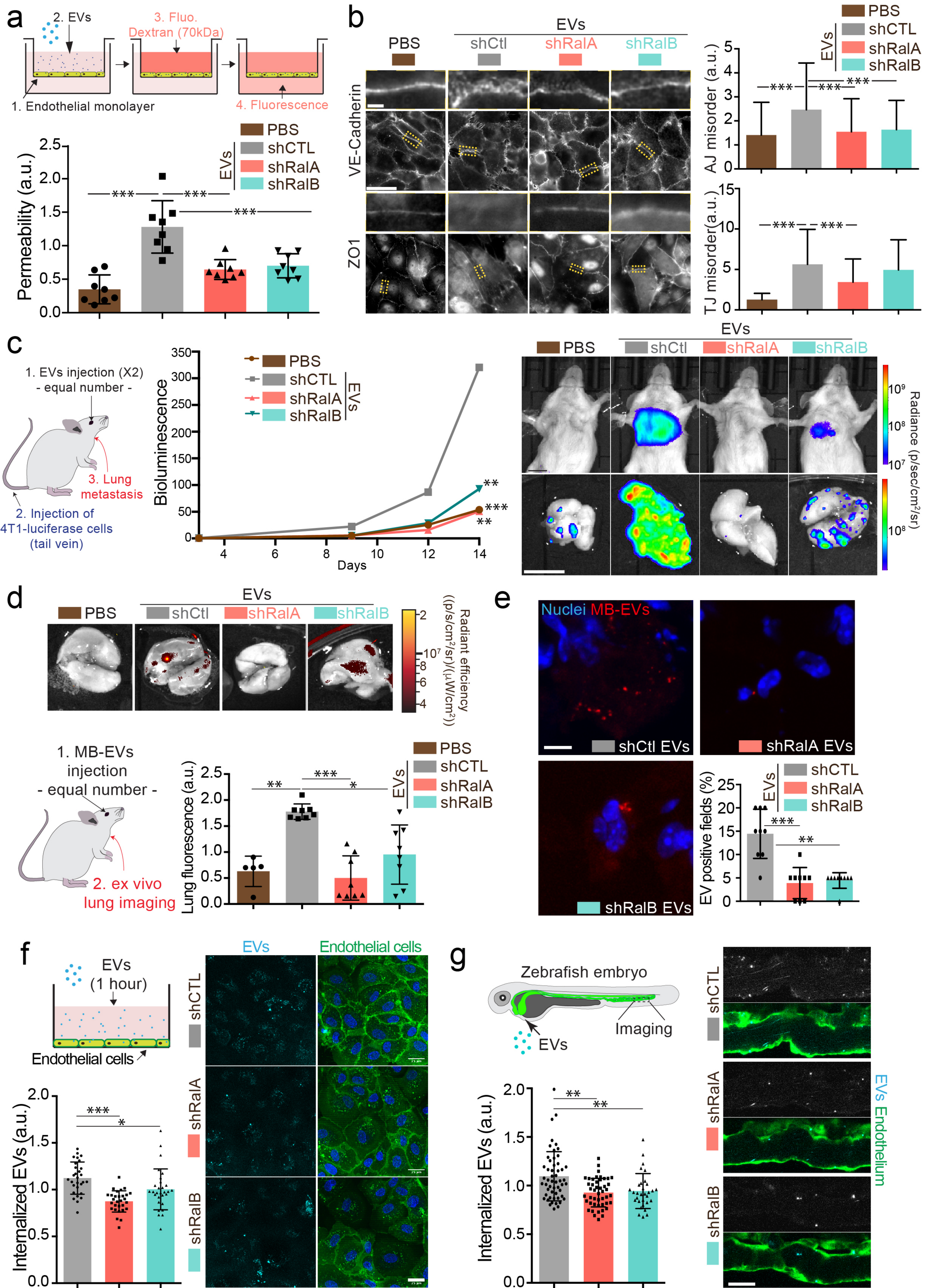


Figure 4\_Ghoroghi et al.



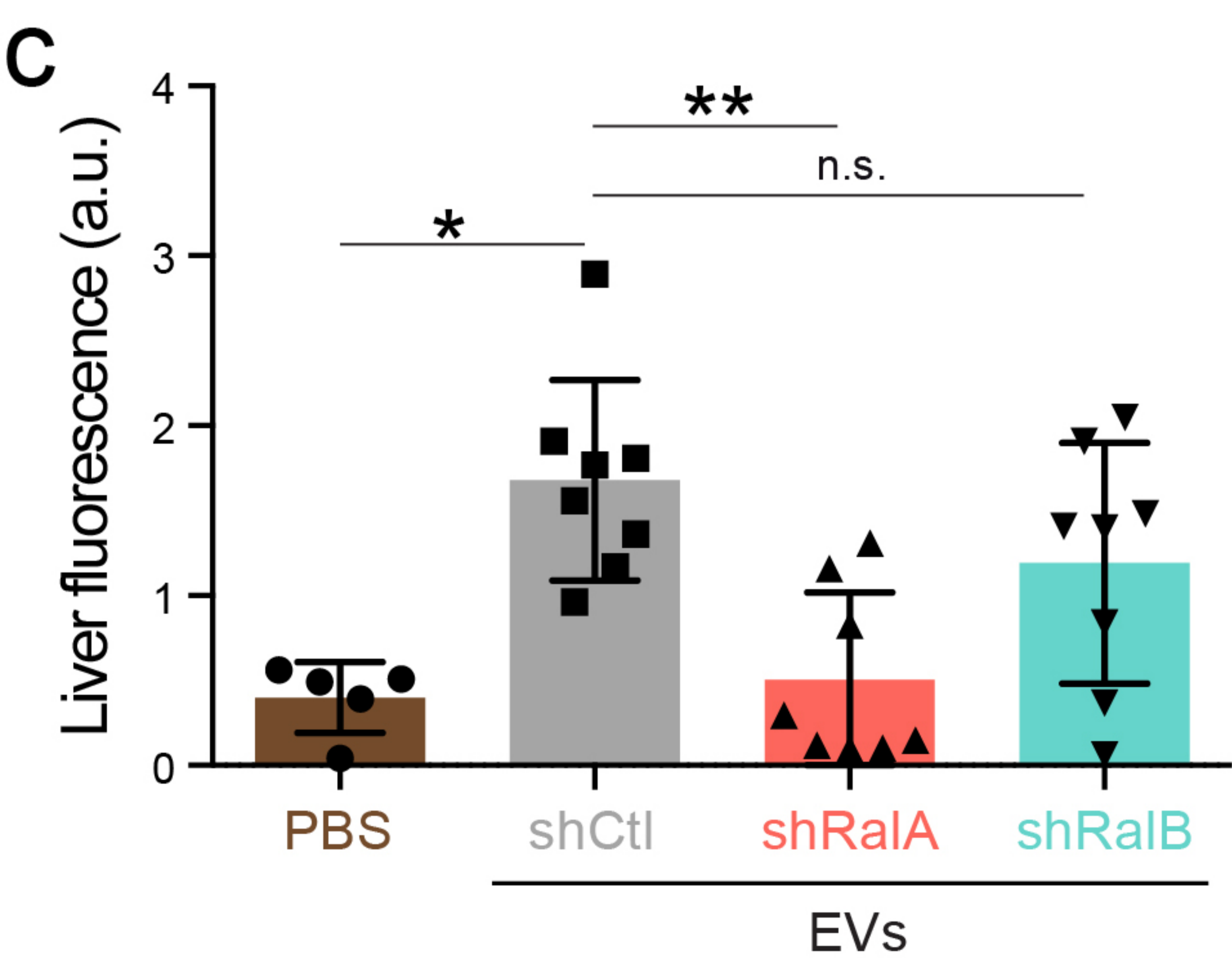
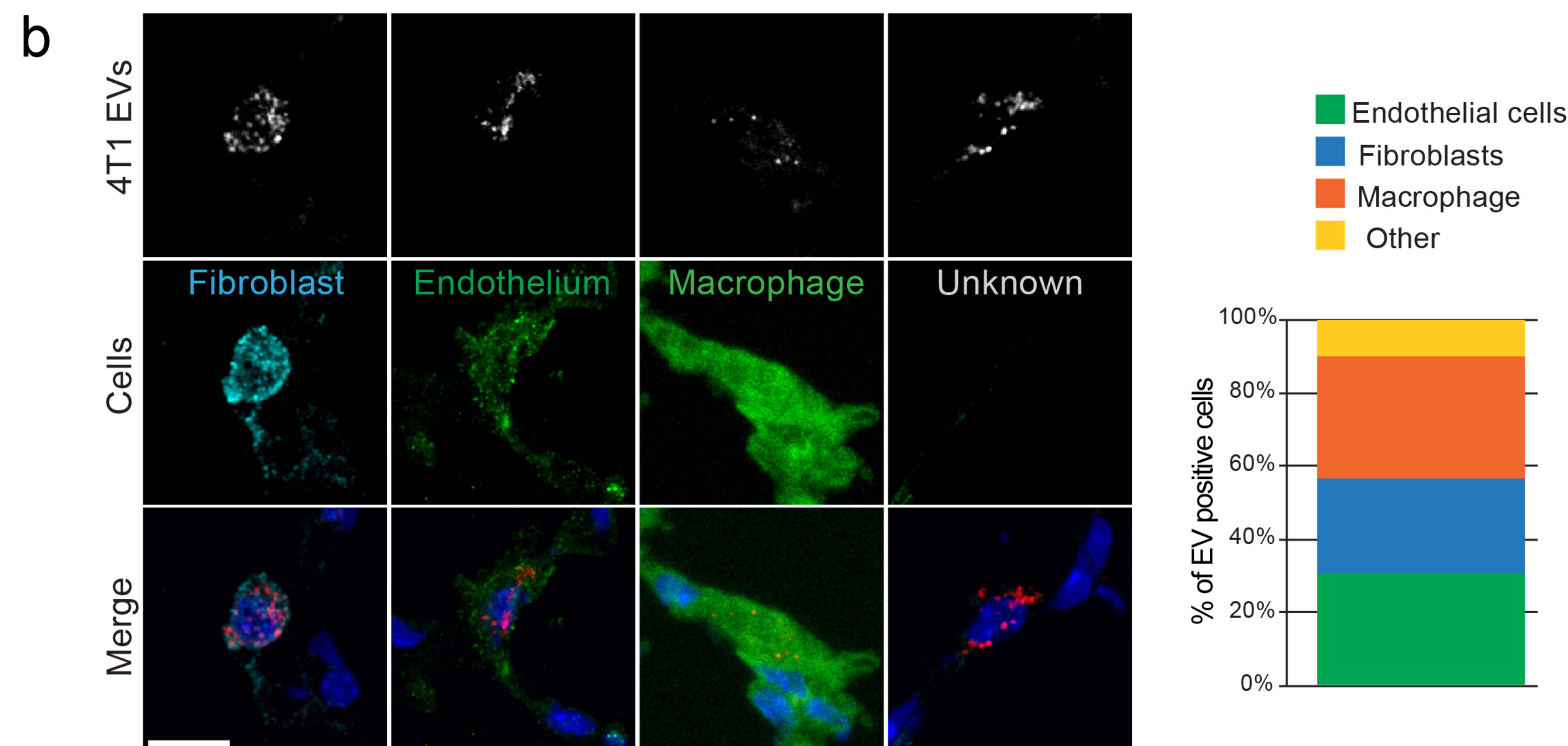
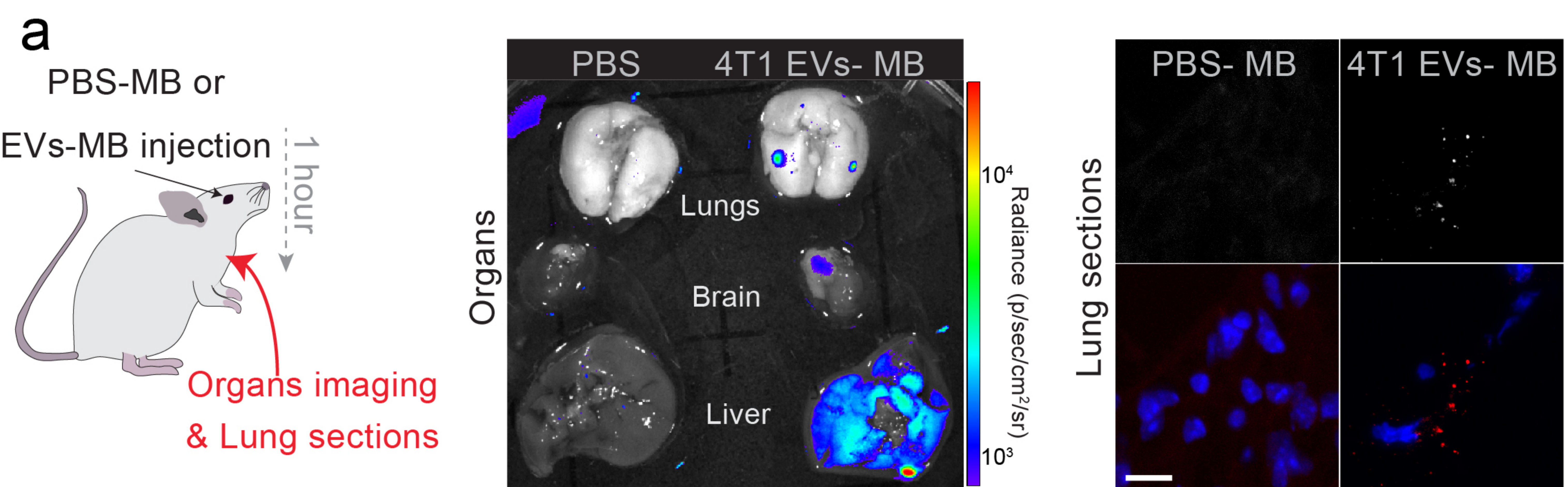


Figure 4 - Figure Supplement 1\_Ghoroghi *et al.*



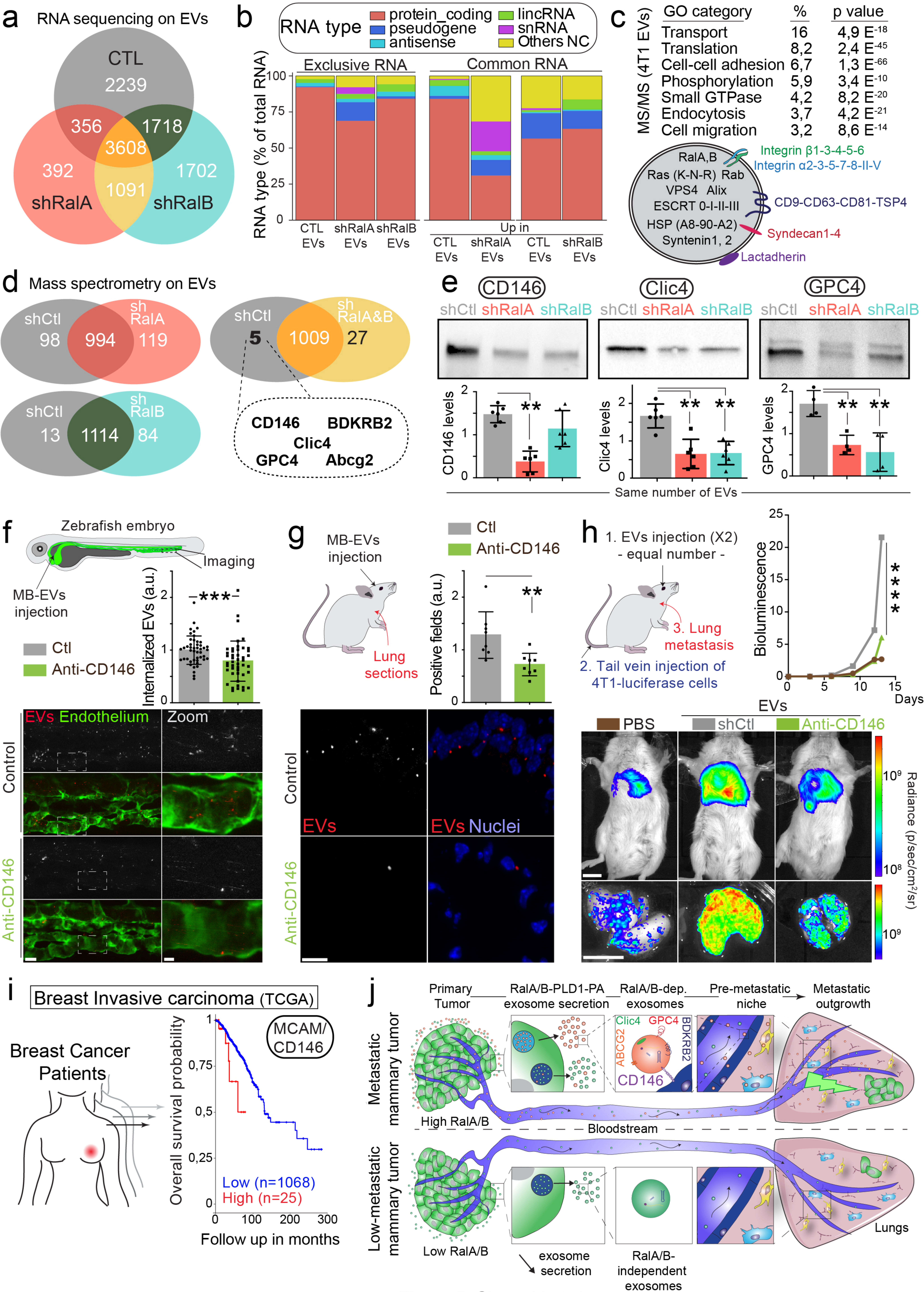
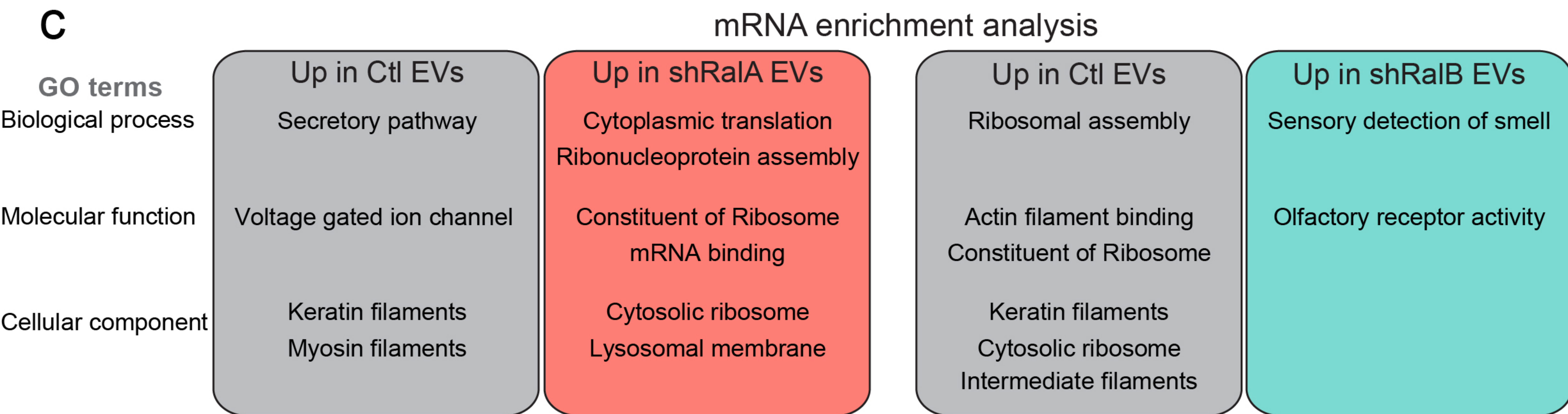
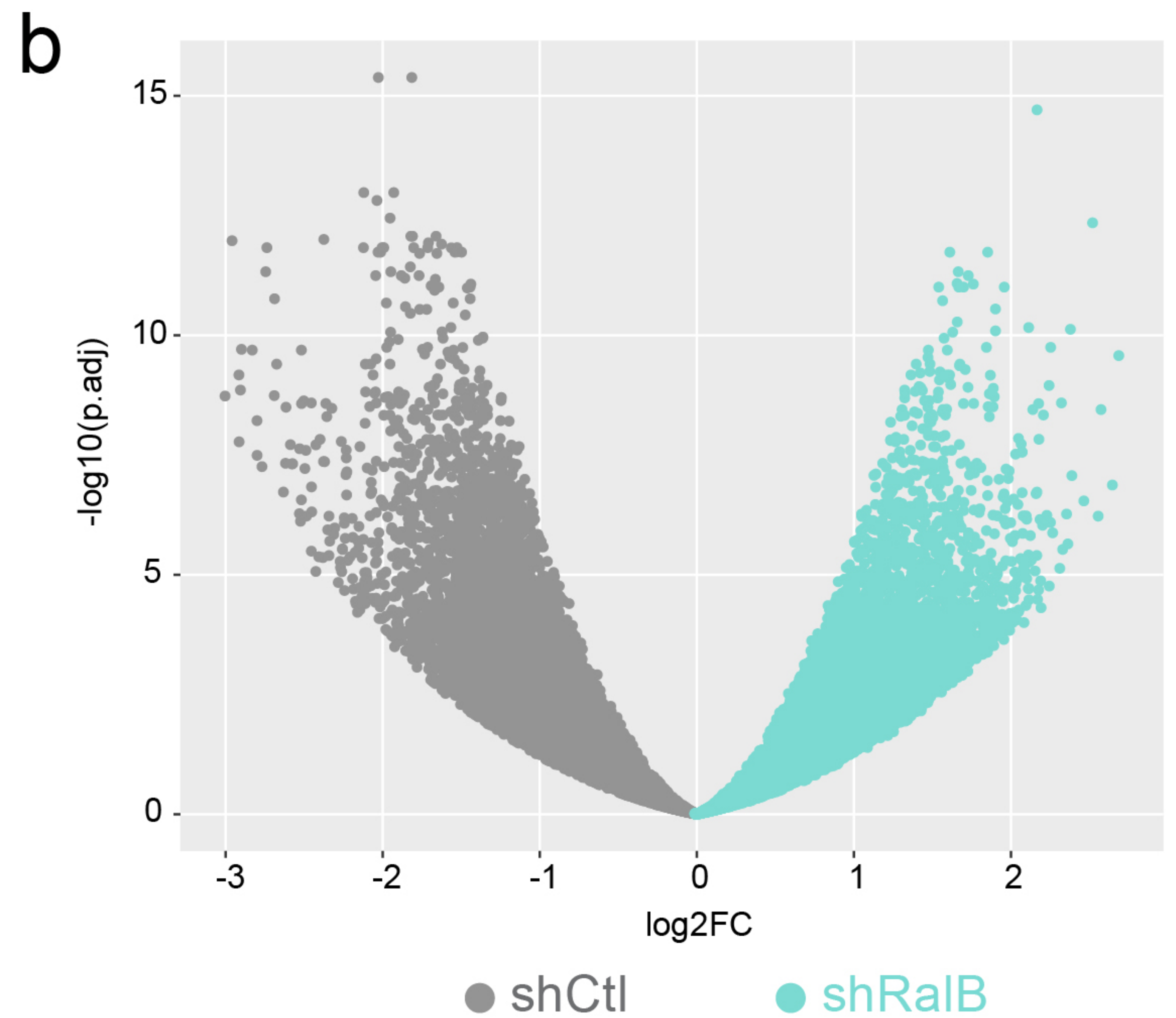
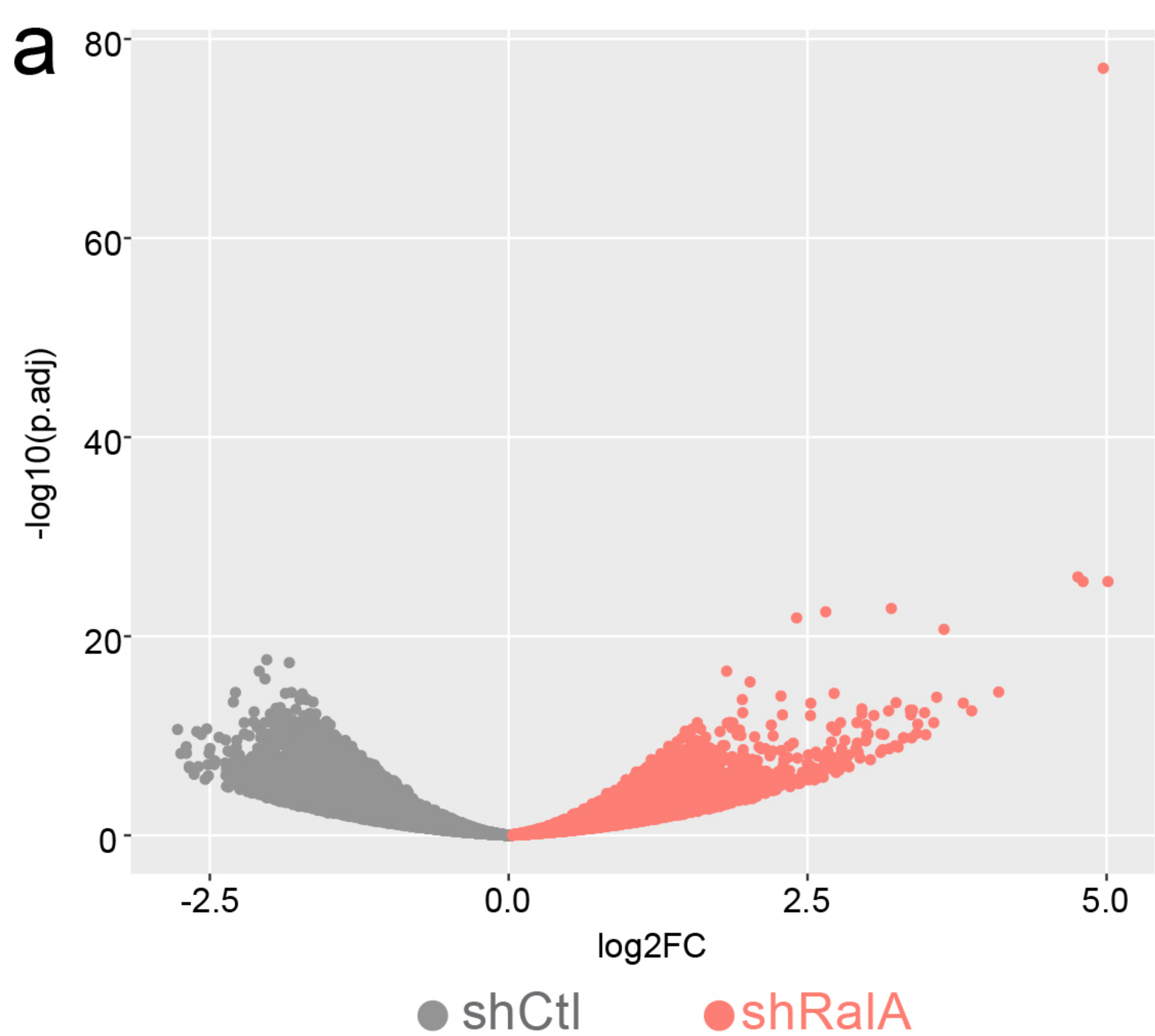


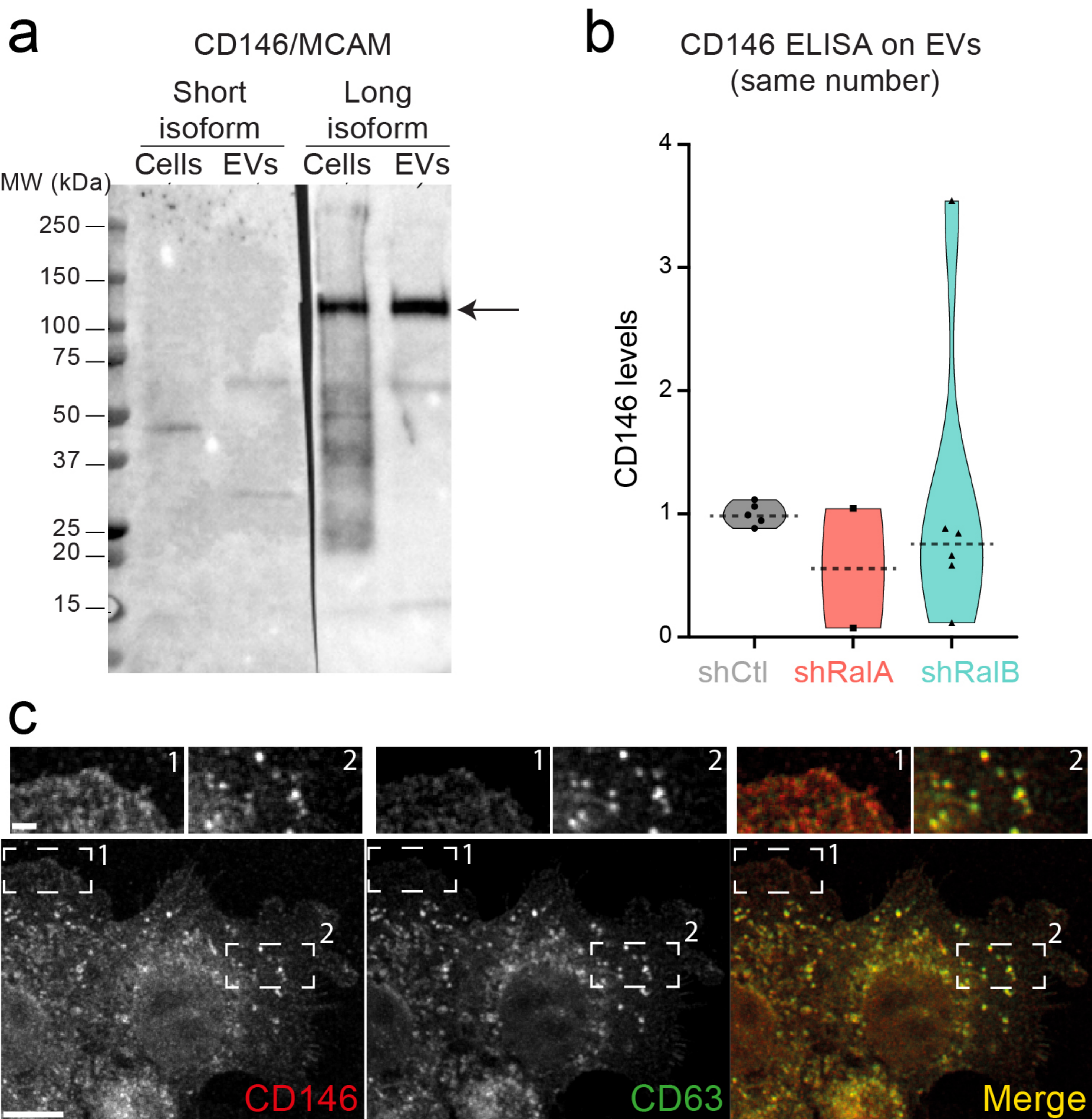
Figure 5\_Ghoroghi et al.





**Figure 5 - Figure Supplement 1\_Ghoroghi *et al.***





**Figure 5 - Figure Supplement 2\_Ghoroghi *et al.***