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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 IC₅₀) 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% CO₂ 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 GAPDH 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 (8.50x10⁸ 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), α -
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 (7×10^8 - 9.5×10^9) 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% OsO₄ (Electron Microscopy Sciences) and 0.8%
485 K₃Fe(CN)₆ (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% OsO₄ 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 AccuriTM C6 cell analyzer with BD CSamplerTM Analysis Software.
506 Results were analyzed with FlowJo software version 10 (TreeStar).

507 For lysosomal analysis, confluent cells were incubated with 1 μ 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 μ 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 8.4×10^4 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 μ g/ml) for 72 hours before 8×10^4 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 μ m, 12 mm diameter, polyester membrane, Corning, New York, USA)
535 were coated with fibronectin (10 μ g/ml; Sigma). Then, HUVECs were seeded (0.3×10^6 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 μ g) of 4T1-EVs, PBS (as a negative control) or with 100ng/ml TNF- α (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 (F2, F4, F6, and F8) 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 (F4).

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 μ L of H₂O 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 μ m particle size; Waters) over 3 min in 1% solvent B at a flow rate of 5 μ L/min
591 followed by reverse-phase separation (ACQUITY UPLC BEH130 C18, 200 mm x 75 μ m id, 1.7 μ 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 ≥ 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 software¹ 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 repository⁵
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 log₂FC 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/CH₃OH/H₂O (5/1/4) +0.2 %
650 formic acid+0.028 % NH₃ and eluent B was isopropanol+0.2 % formic acid+0.028 % NH₃. 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 μ 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 1.5×10^8 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 1.4×10^8 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 Axiolmager
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% CO₂. 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 2x10⁸ 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% CO₂.

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

799

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 μm ; zoom: 2 μm . d) Representative electron micrographs of 4T1 shCtl, shRalA and shRalB
811 cells, with zoom on MVBs; Scale bar: 1 μm ; 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 μm ; zoom: 2 μm . b) Electron microscopy analysis of 4T1 cells treated
818 with PLD1 or PLD2 inhibitor. Scale bar: 1 μm . 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 μm ; zoom: 2 μm . 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 μm 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 μm . 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 μm . 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 μm .

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 μm ; zoom: 2 μm . 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 (3×10^8 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 μm . **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 μm . 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 μ 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 μ m; Zoom scale bar: 5 μ 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 μ 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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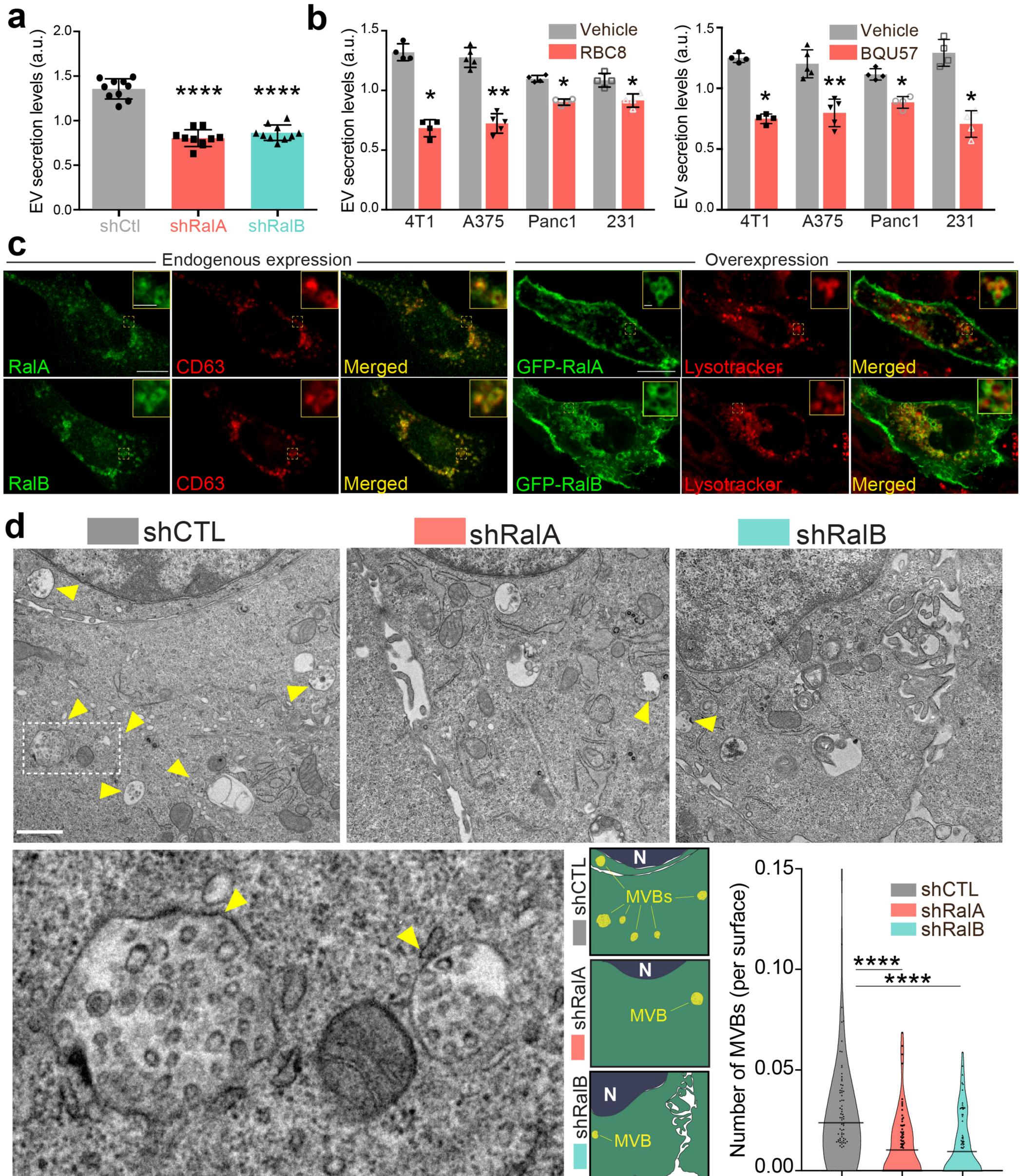


Figure 1_Ghoroghi *et al.*

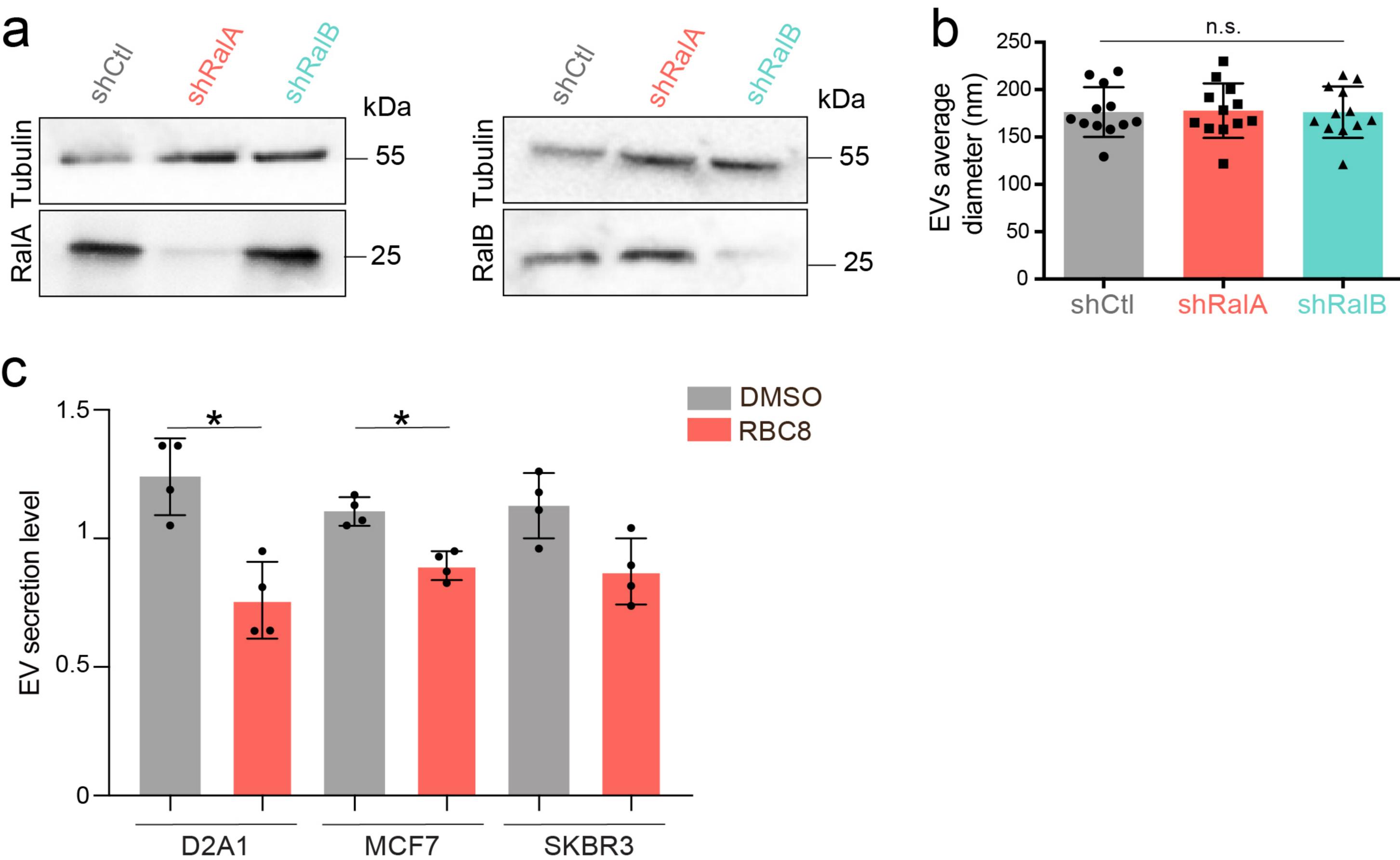


Figure 1 - Figure Supplement 1_Ghoroghi *et al.*

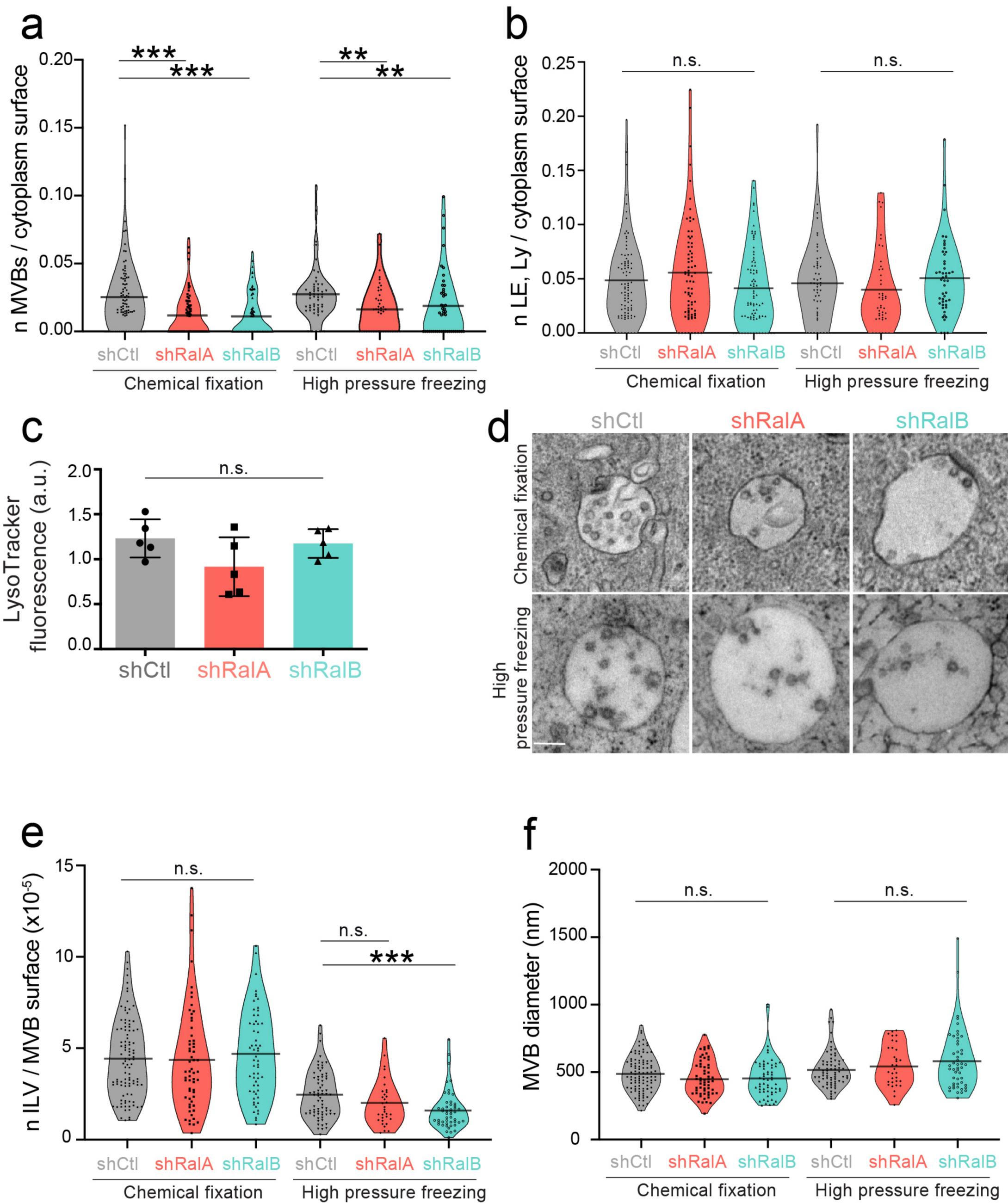


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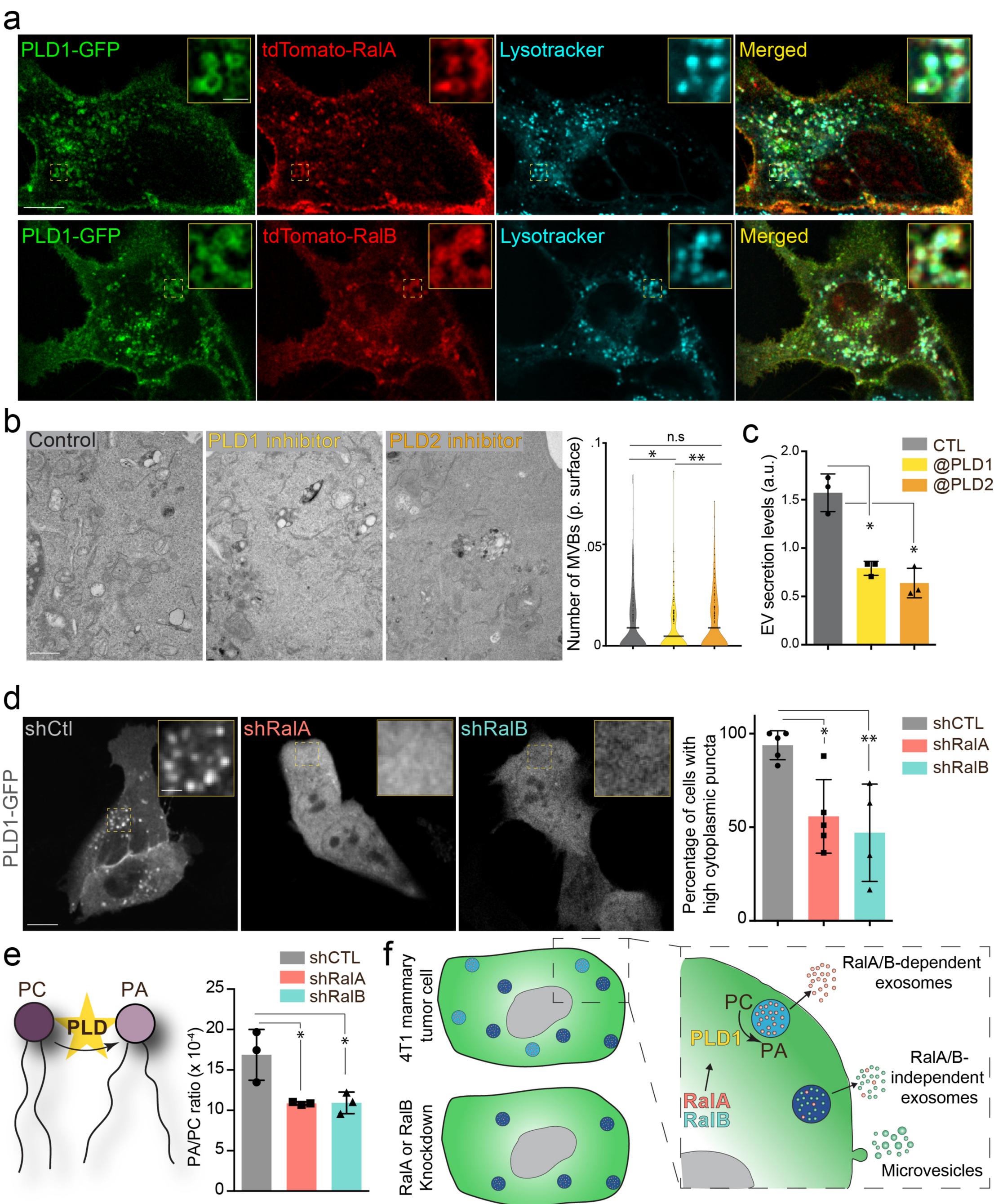


Figure 2_Ghoroghi *et al.*

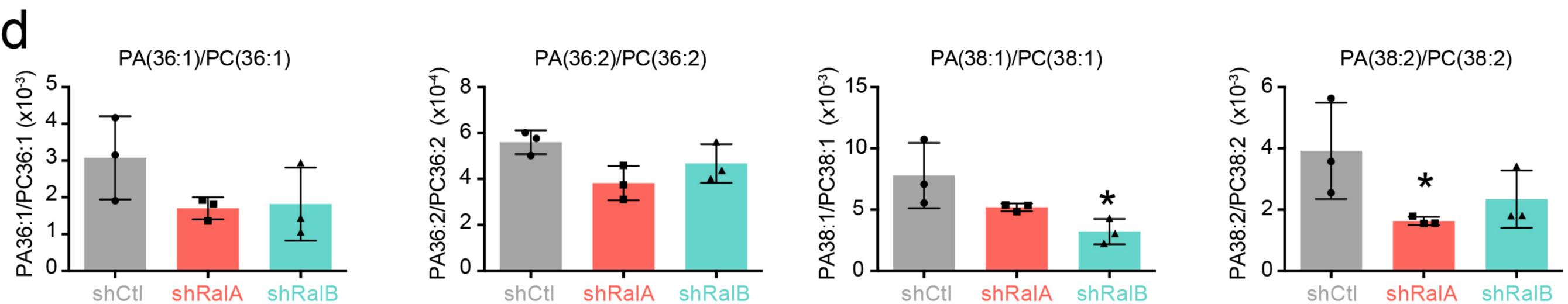
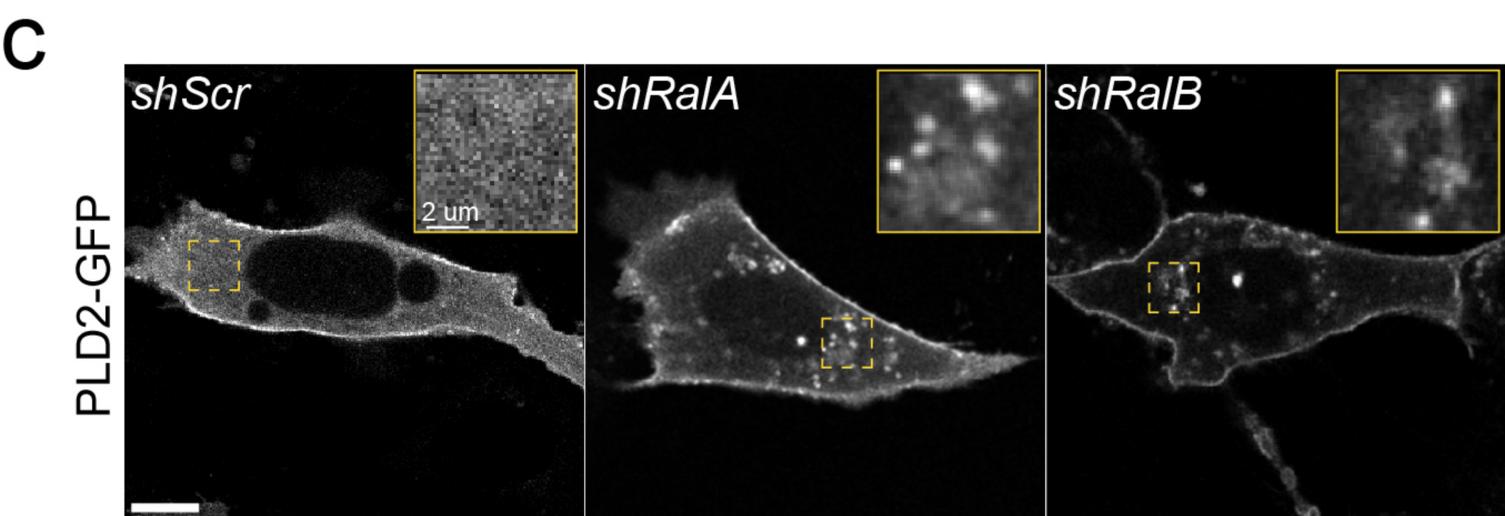
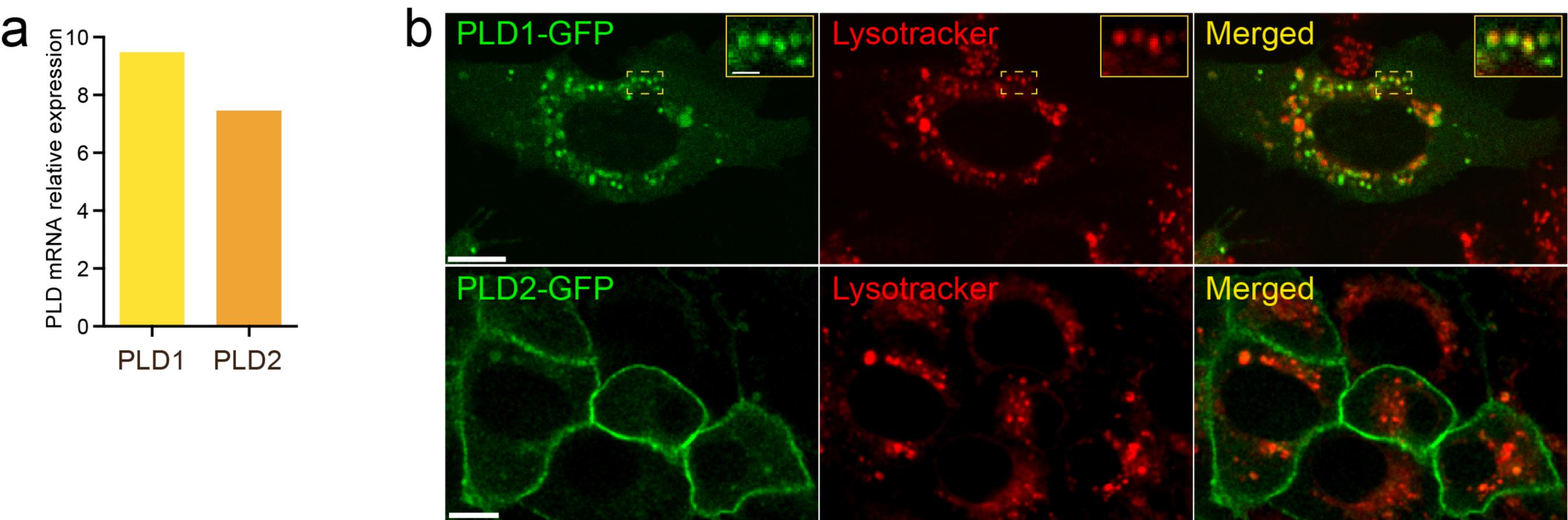


Figure 2 - Figure Supplement 1_Ghoroghi *et al.*

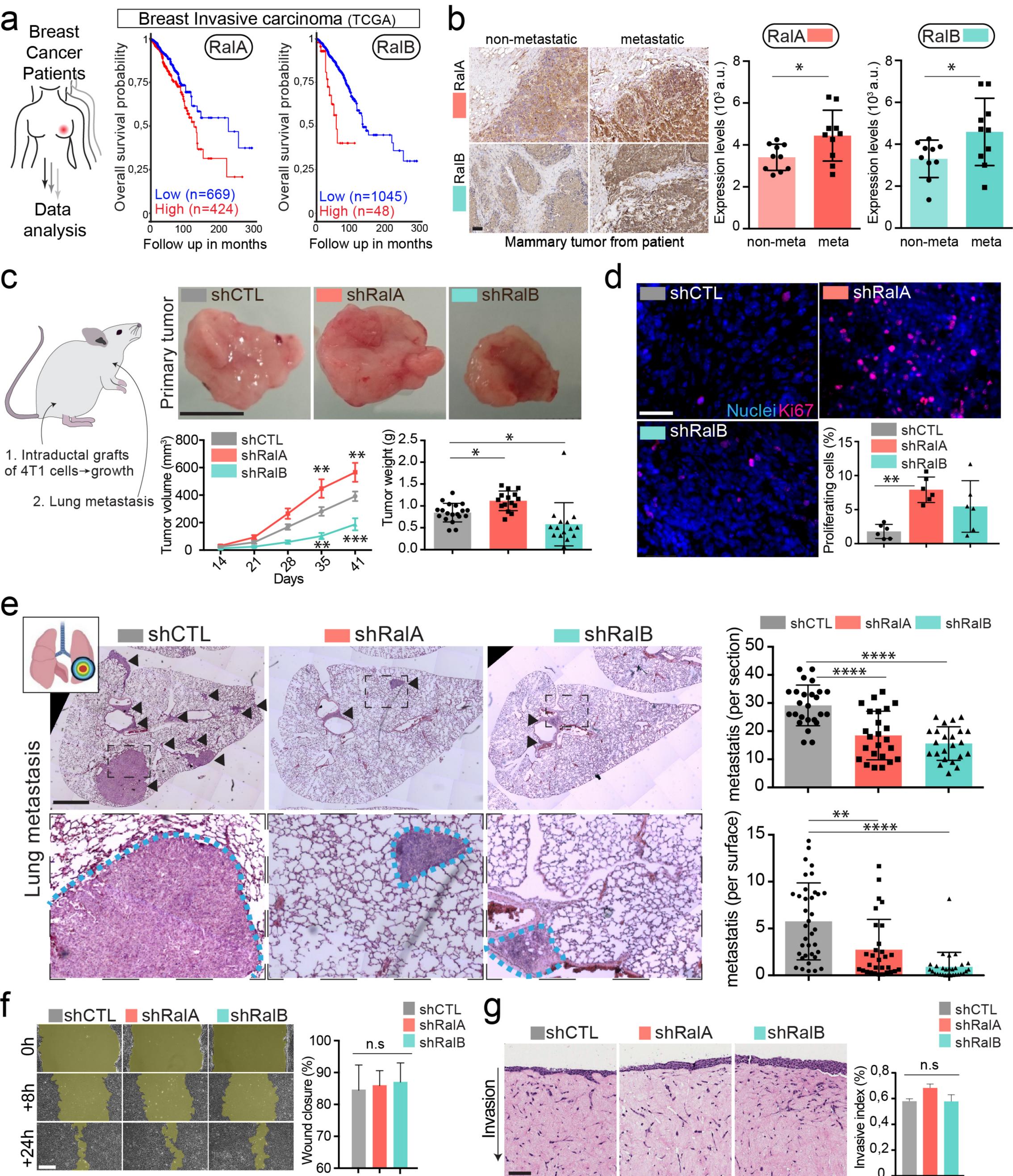


Figure 3_Ghoroghi *et al.*

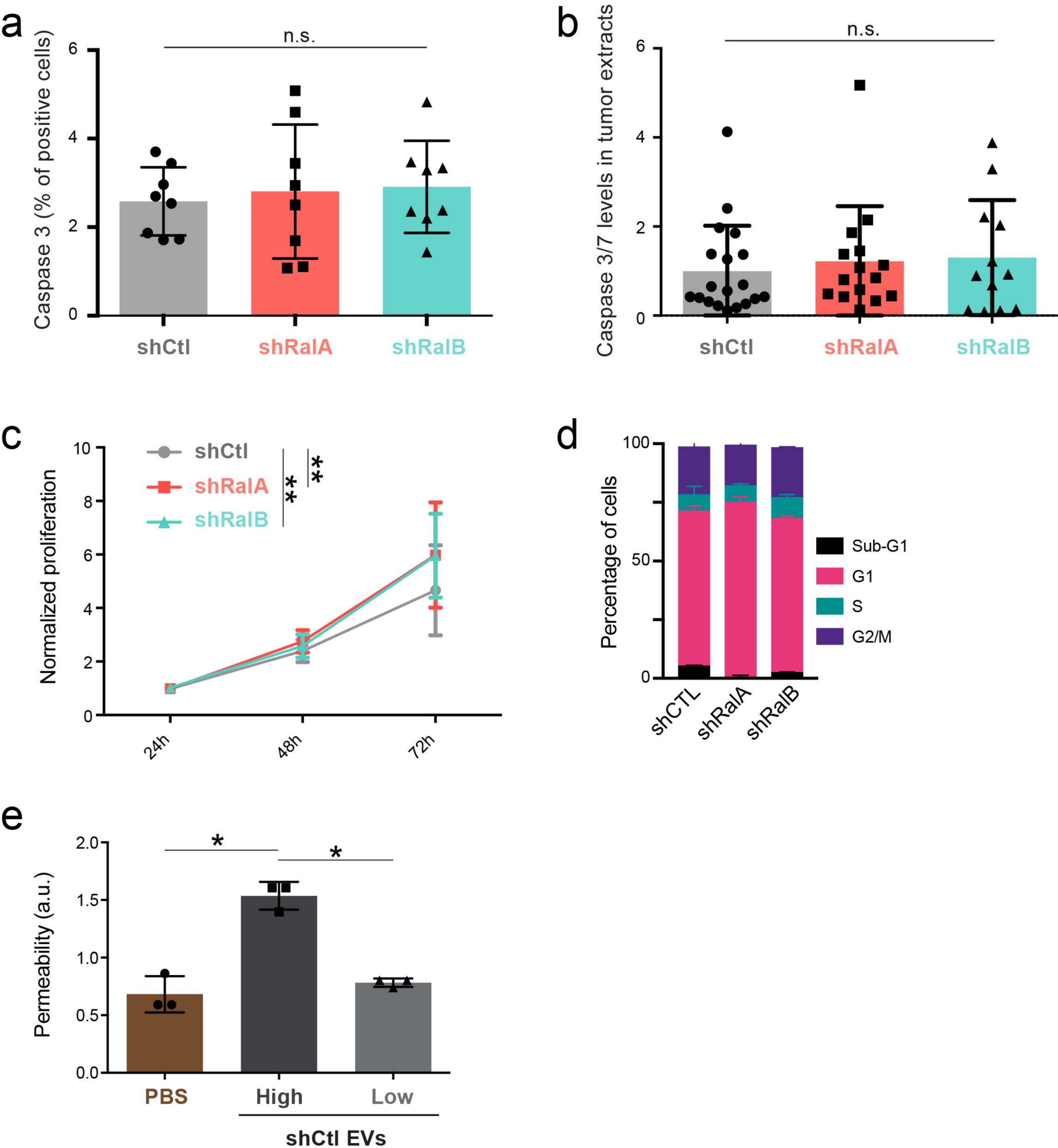


Figure 3 - Figure Supplement 1_Ghoroghi *et al.*

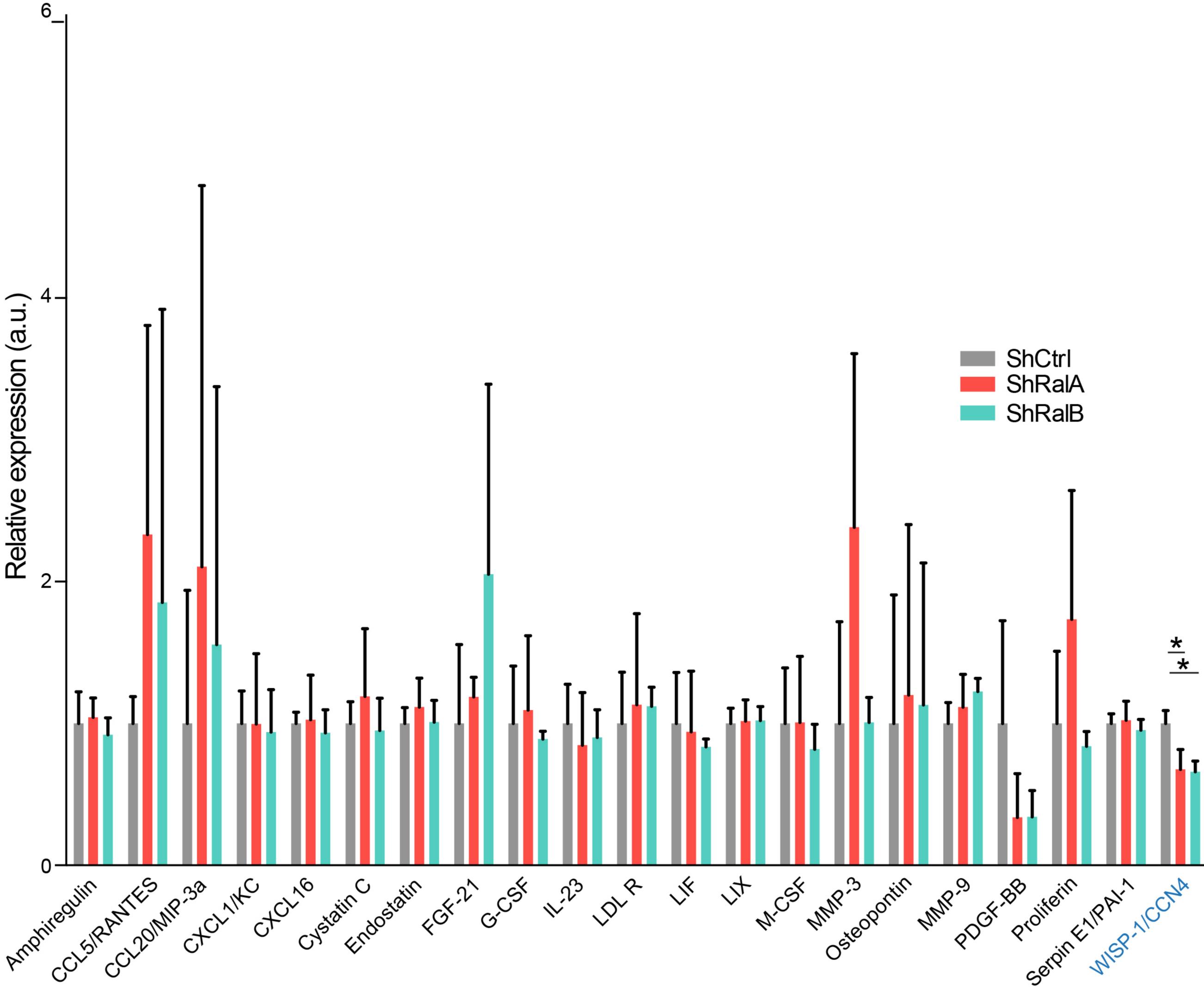


Figure 3 - Figure Supplement 2_Ghoroghi *et al.*

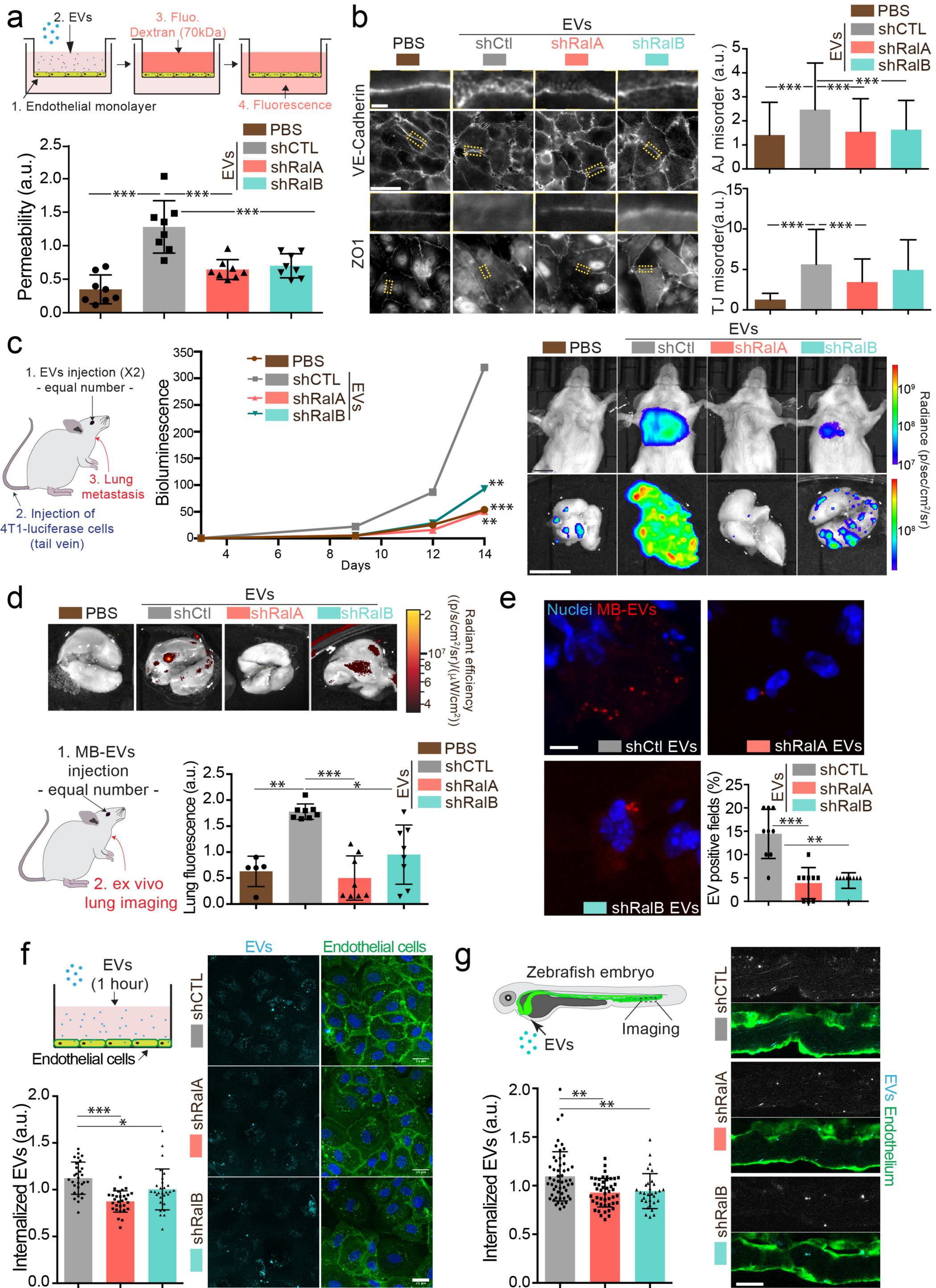


Figure 4_Ghoroghi et al.

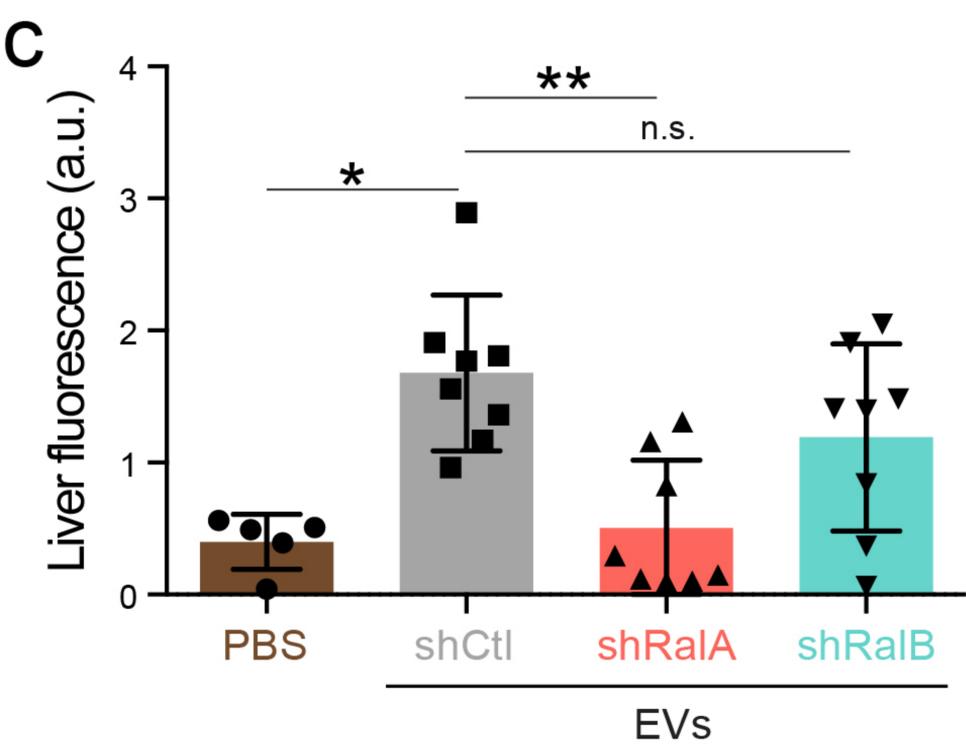
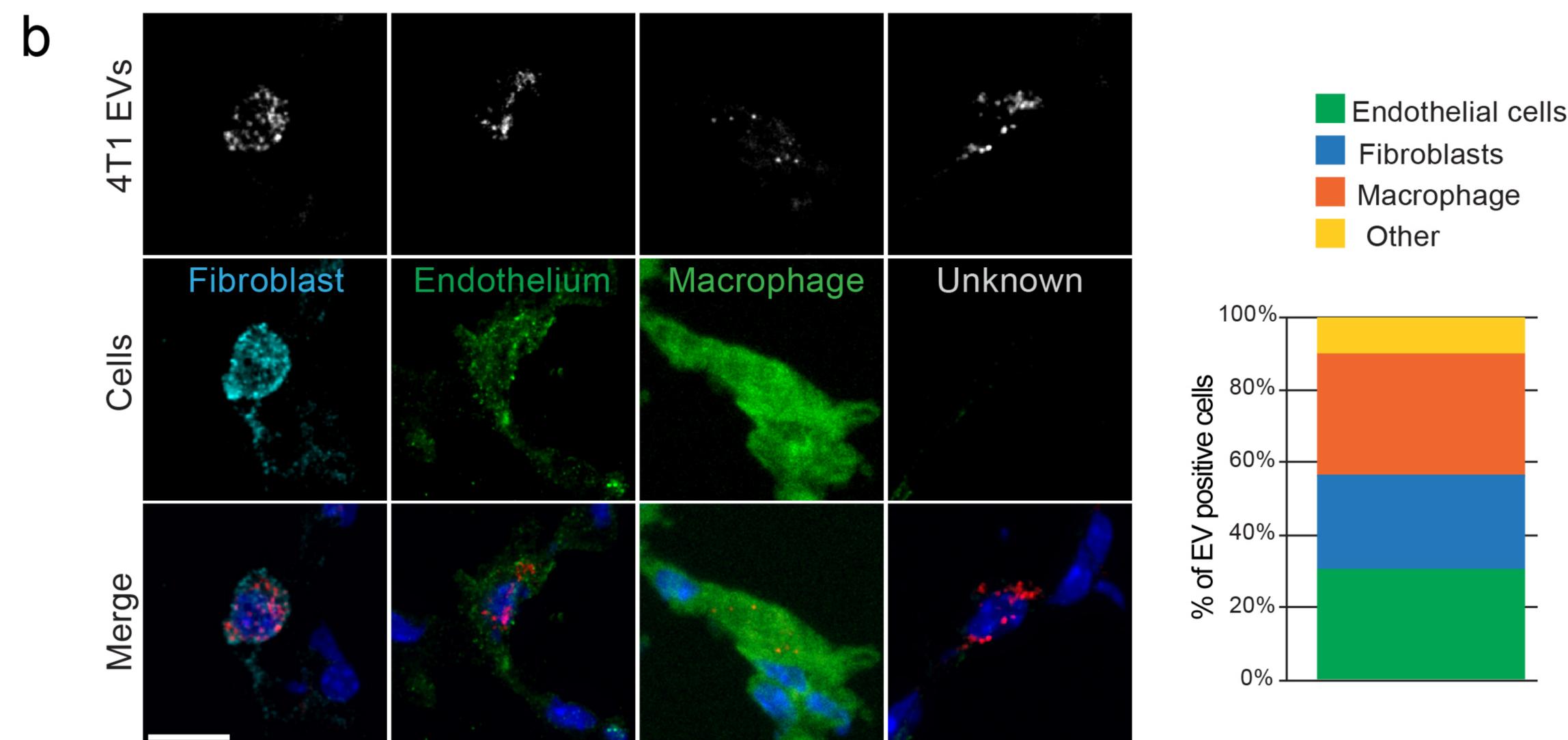
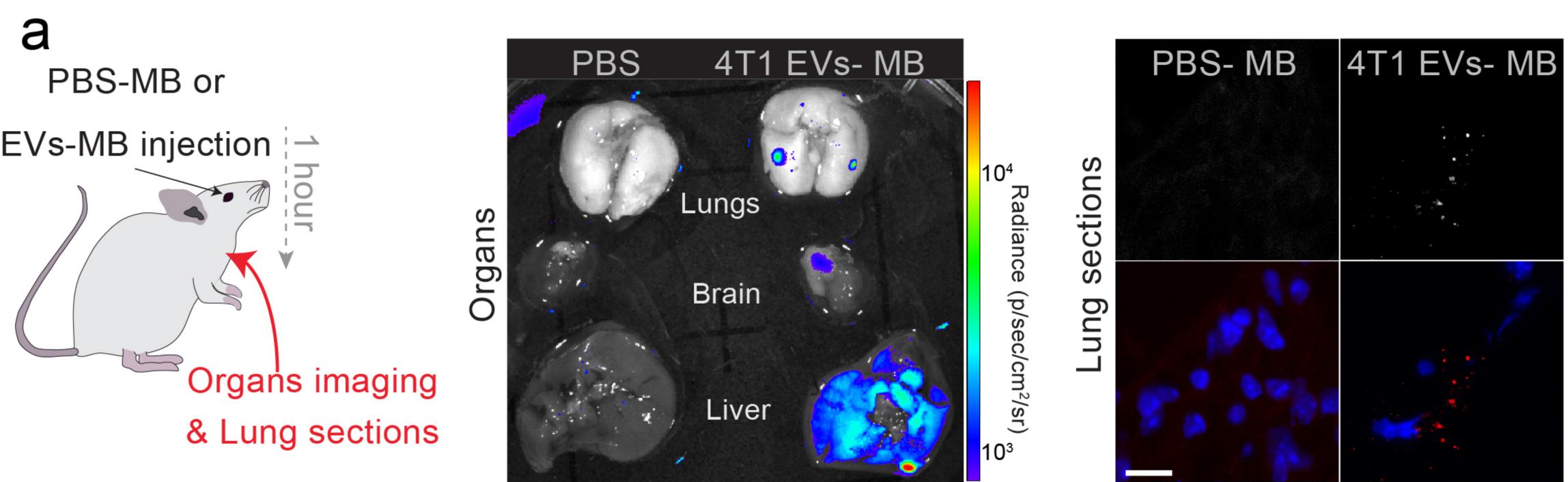


Figure 4 - Figure Supplement 1_Ghoroghi *et al.*

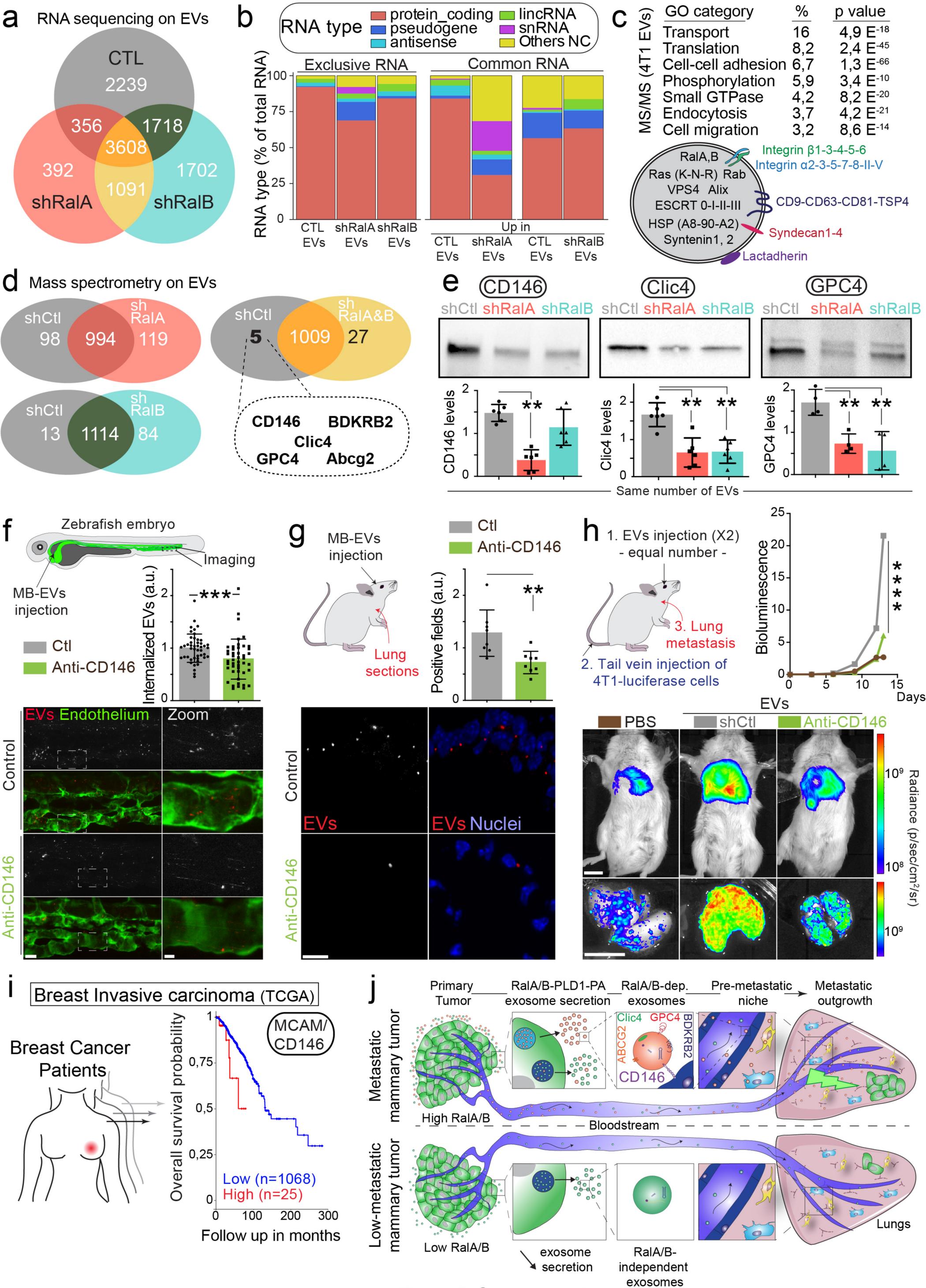


Figure 5_Ghoroghi et al.

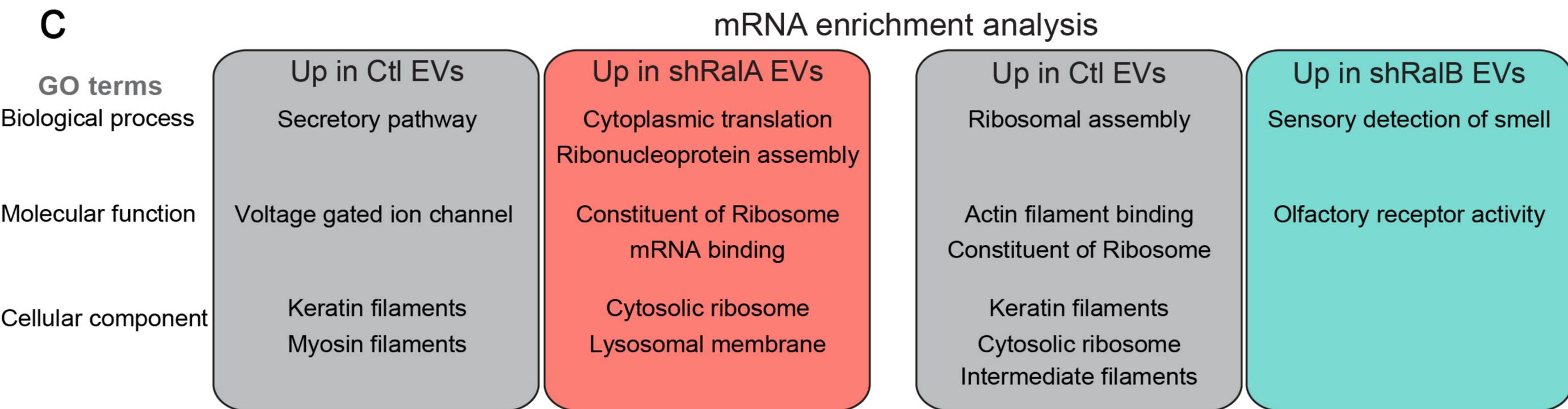
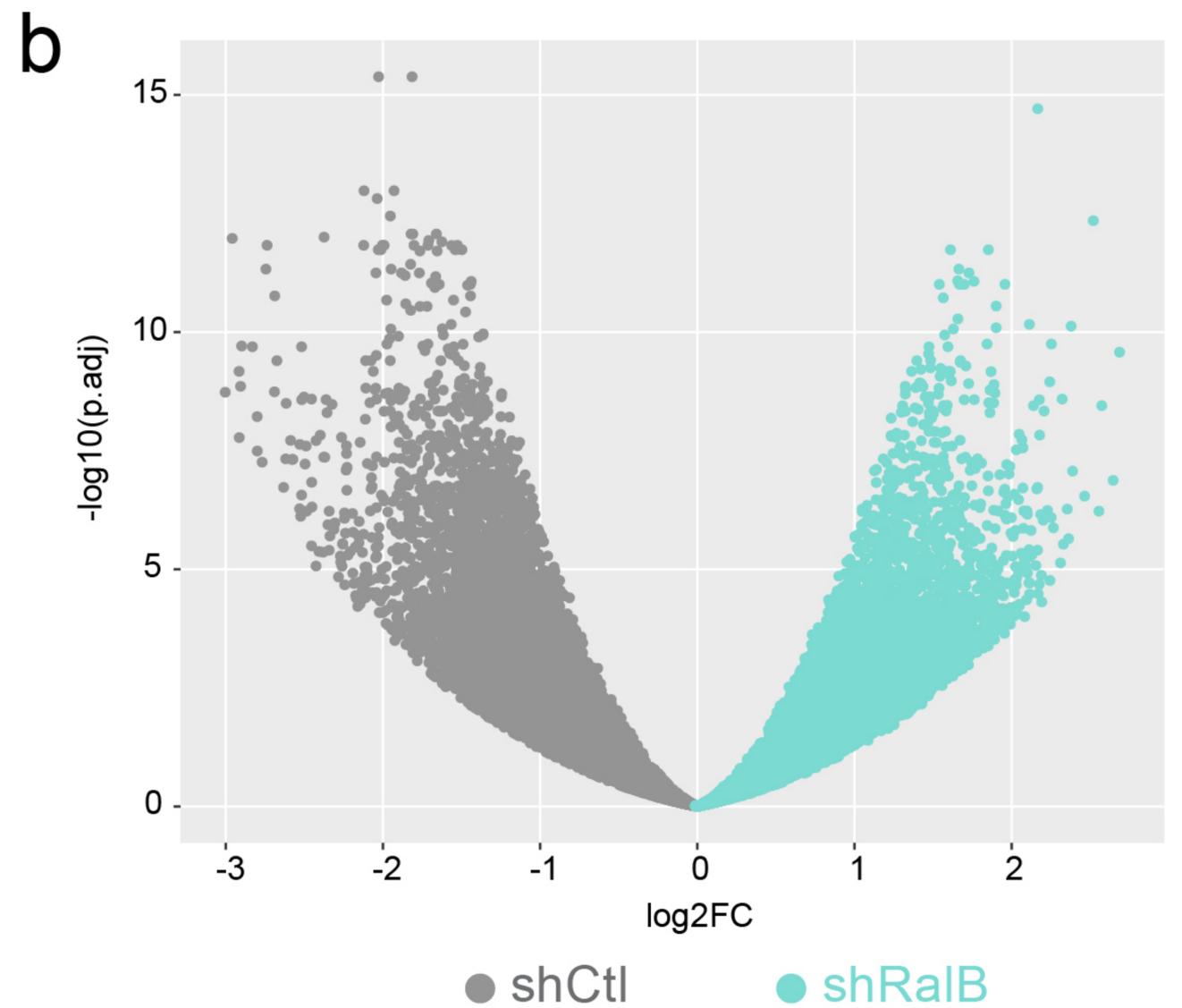
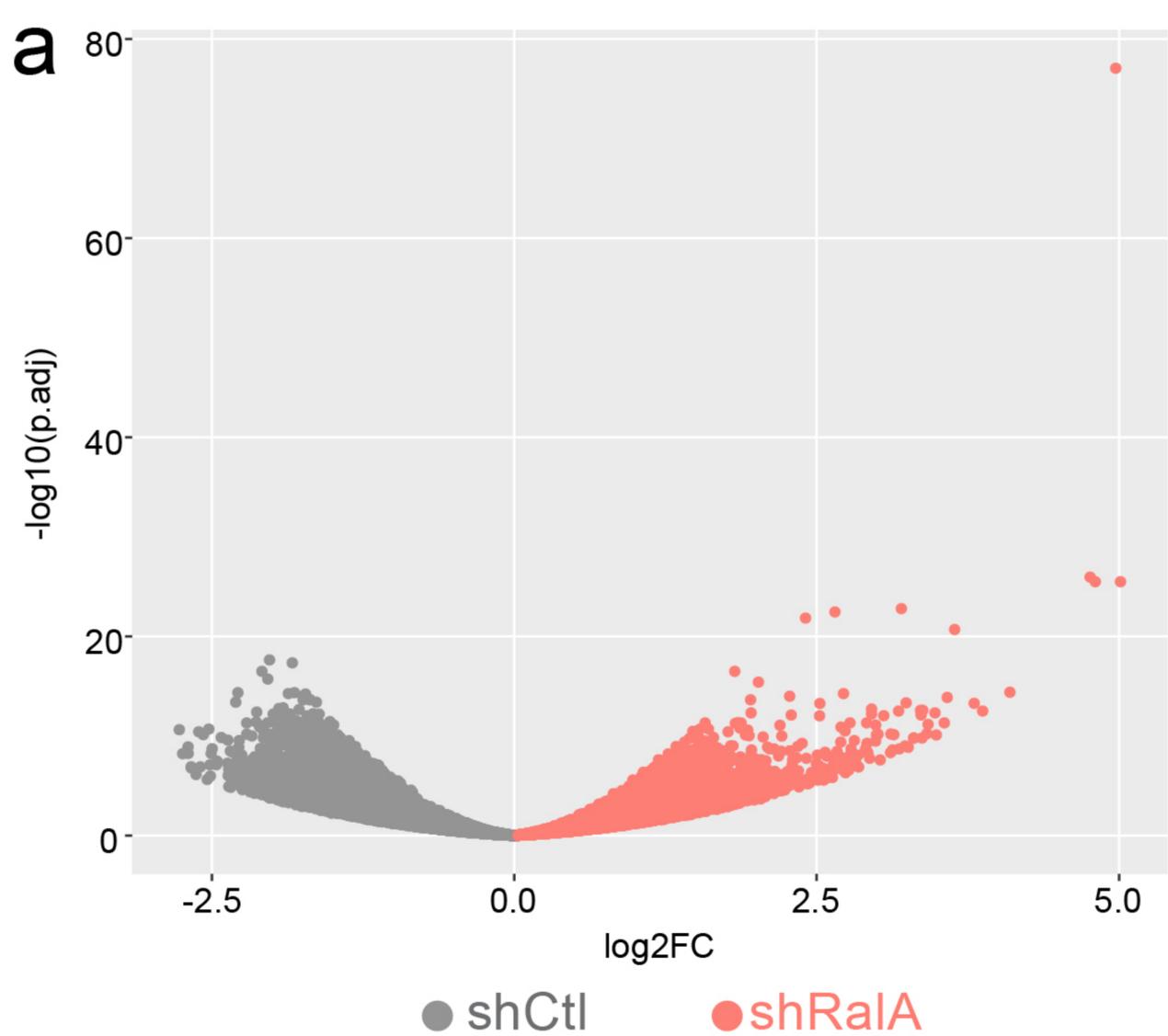


Figure 5 - Figure Supplement 1_Ghoroghi *et al.*

