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## **ProNGF increases breast tumor aggressiveness through functional association of TrkA with EphA2**

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1 **ProNGF increases breast tumor aggressiveness through functional**  
2 **association of TrkA with EphA2**

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39

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43 **Highlights**

- 44 • EphA2 is a key element of proNGF signaling in breast cancer cells
- 45 • ProNGF-induced Src activation through EphA2 is independent of TrkA  
46 phosphorylation
- 47 • TrkA/EphA2 PLA signal is associated with decrease of overall survival in  
48 breast cancer

49

50 **Abstract**

51 ProNGF expression has been linked to several types of cancers including breast  
52 cancer, and we have previously shown that proNGF stimulates breast cancer  
53 invasion in an autocrine manner through membrane receptors sortilin and TrkA.  
54 However, little is known regarding TrkA-associated protein partners upon proNGF  
55 stimulation. By proteomic analysis and proximity ligation assays, we found that  
56 proNGF binding to sortilin induced sequential formation of the functional  
57 sortilin/TrkA/EphA2 complex, leading to TrkA-phosphorylation dependent Akt  
58 activation and EphA2-dependent Src activation. EphA2 inhibition using siRNA  
59 approach abolished proNGF-stimulated clonogenic growth of breast cancer cell  
60 lines. Combinatorial targeting of TrkA and EphA2 dramatically reduced colony  
61 formation *in vitro*, primary tumor growth and metastatic dissemination towards the  
62 brain *in vivo*. Finally, proximity ligation assay in breast tumor samples revealed that  
63 increased TrkA/EphA2 proximity ligation assay signals were correlated with a  
64 decrease of overall survival in patients.

65 All together, these data point out the importance of TrkA/EphA2 functional  
66 association in proNGF-induced tumor promoting effects, and provide a rationale to  
67 target proNGF/TrkA/EphA2 axis by alternative methods other than the simple use  
68 of tyrosine kinase inhibitors in breast cancer.

## 69 **1. Introduction**

70 Elevated levels of pleiotropic growth factors and their cognate receptor tyrosine  
71 kinases (RTK) as well as mutated receptors actively participates to cancer  
72 progression and resistance to targeted therapies using tyrosine kinase inhibitors  
73 [1,2]. Tumor resistance to specific tyrosine kinase inhibitors may be due to complex  
74 cross-talk in terms of receptor interactions and their redundant or diversified  
75 downstream signaling partners [1,2]. This underlies the need to better characterize  
76 growth factor signaling and RTK cooperation in order to optimize such therapies.

77 In the case of TrkA signaling networks, elicited by nerve growth factor (NGF),  
78 several co-receptors such as p75<sup>NTR</sup>, ErbB2, and Ret-5 [3–5] have been reported to  
79 modulate TrkA-induced biological effects. Moreover, sortilin is known to be involved  
80 in TrkA signaling networks under proNGF (precursor of NGF) stimulation. Sortilin, a  
81 member of the Vps10p-domain proteins, is mainly known to be involved in vesicular  
82 trafficking but it also acts as a membrane receptor for neurotensin and  
83 proneurotrophins [6,7]. Although recombinant proNGF has been described to  
84 directly bind to sortilin, p75<sup>NTR</sup> and TrkA, it has been found that, in neuron cells,  
85 proNGF induces sortilin/p75<sup>NTR</sup> complex formation, leading to apoptotic cell death  
86 [8]. Further study showed that high ratio between p75<sup>NTR</sup> and TrkA stimulates  
87 proNGF-induced neuron apoptosis, while low ratio between p75<sup>NTR</sup> and TrkA  
88 stimulates proNGF-induced survival [9]. ProNGF is also associated with non-  
89 neuronal malignancies and its expression has been reported in melanoma, prostate,  
90 breast and thyroid cancers [10–13]. In melanoma, proNGF can stimulate cancer cell  
91 invasion through p75<sup>NTR</sup> [12] while in prostate cancer, its expression correlates with  
92 aggressiveness and nerve infiltration into the tumor site [11]. In breast cancer, we  
93 previously reported high levels of proNGF are correlated with lymph node invasion

94 [10]. In addition, proNGF stimulates breast cancer cell invasion through sortilin and  
95 TrkA receptors, independently of p75<sup>NTR</sup>, leading to the subsequent activation of Src  
96 and Akt signaling pathways [10].

97 Despite the tumor-promoting effects of proNGF, associated signaling pathways still  
98 remain fragmentary. Here, we identified EphA2, a membrane receptor tyrosine  
99 kinase, as a key element of the proNGF signaling in breast cancer cells. Moreover,  
100 increased TrkA/EphA2 proximity ligation assay signals were correlated with a  
101 decrease of overall survival in breast cancer patients, further pointing out the  
102 importance of TrkA/EphA2 functional association in proNGF-mediated tumor  
103 progression. Thus, our results provide a rationale to target proNGF/TrkA/EphA2 axis  
104 as a promising therapeutic strategy in breast cancer.

105

## 106 **2. Materials and Methods**

### 107 2.1. Cell culture

108 All breast cancer cell lines were acquired from the American Type Culture Collection  
109 (ATCC) except for SUM159-PT, which is from Asterand Bioscience. MDA-MB-231  
110 breast cancer cells stably overexpressing HA-tagged native TrkA (MDA-MB-231  
111 HA-TrkA) or a kinase-dead TrkA were established as previously described [10].  
112 Cells were maintained in Eagle's Minimal Essential Medium (Life Technologies)  
113 (MDA-MB-231 and MCF-7 cells), or RPMI 1640 medium (Life Technologies) (HCC-  
114 1954, T-47D) supplemented with 10 % inactivated FBS (Fetal Bovine Serum)  
115 (Hyclone), 2 mM L-glutamine, 1 % non-essential amino acids, 40 UI/ml penicillin,  
116 40 µg/ml streptomycin, 50 µg/ml gentamycin and ZellShield™ (Biovalley) at 37°C in  
117 5 % CO<sub>2</sub>-humidified atmosphere. SUM159-PT were grown in Ham's F12 nutrient  
118 mix supplemented with 5 % FBS, 10 mM HEPES, 0.1 % insulin, 1 mg/ml  
119 hydrocortisone, 40 UI/ml penicillin, 40 µg/ml streptomycin, 50 µg/ml gentamycin and  
120 ZellShield™ (Biovalley) at 37°C in 5 % CO<sub>2</sub>-humidified atmosphere. Before  
121 treatment, cells were rinsed twice with PBS, left for 24h in culture medium  
122 supplemented with 0.1 % FBS, and then treated with recombinant human non-  
123 cleavable proNGF (denoted as proNGF and used at 0.5 nM in all the experiments)  
124 (Alomone Labs) or recombinant human mature NGF at 16 nM (Alomone labs). For  
125 some experiments, cells were pre-incubated for 1h with the TrkA pharmacological  
126 inhibitor K252a (10 nM, Calbiochem) or neurotensin (1 µM, R&D systems).

### 127 2.2. Transfection

128 Tumor cells were transfected with 2 nM siRNA using INTERFERin™ transfection  
129 reagent (Polyplus transfection) following the manufacturer's instructions. The siRNA  
130 sequences used against EphA2 were: GCAAGGAAGUGGUACUGCUGGACUU

131 (from Eurogentec) or GCGUAUCUUCAUUGAGCUCAA [14] compared to a control  
132 siRNA sequence (siGFP) GAUGAACUUCAGGGUCAGCTT. For TrkA, a pool of  
133 three siRNA sequences (Eurogentec) was used: GAACCUGACUGAGCUCUAC,  
134 UGGAGUCUCUCUCCUGGAA and GCUGCAGUGUCAUGGGCAA.

### 135 2.3. Immunoprecipitation and Western blot analysis

136 Immunoprecipitation and Western blotting experiments were carried out as  
137 previously reported [15]. The primary antibodies used for Western blotting were:  
138 anti-sortilin (#612101, BD Biosciences), anti-TrkA (ANT-018, Alomone Labs), anti-  
139 EphA2 (clone 1E3, Abnova), anti-HA (Covance), anti-phospho-Akt (Ser-473)  
140 (#9271), anti-pan-Akt (#4691), anti-phospho-Src (Tyr-416) (#2105), anti-Src  
141 (#2109) and anti-phospho-TrkA (Tyr-674/675) (#4621) (Cell Signaling Technology).  
142 For immunoprecipitation studies, anti-HA (12CA5, Roche), anti-sortilin (BAF2934,  
143 R&D Systems) and anti-EphA2 (clone C-20, Santa Cruz Biotechnologies) were  
144 used.

### 145 2.4. Nano-LC-MS/MS Q-Star analysis

146 MDA-MB-231 HA-TrkA cells were treated with proNGF for 5 or 30 min. Total cell  
147 lysates were subjected to immunoprecipitation using anti-HA (Covance).  
148 Immunoprecipitated proteins were then separated by 10 % SDS-PAGE. After  
149 colloidal Coomassie blue staining, bands, which intensities were increased under  
150 proNGF stimulation, were cut and peptide digests were extracted from the 1-D gel  
151 band and nanoLC-nanoESI-MS/MS analyses were performed on a hybrid  
152 quadrupole time-of-flight mass spectrometer (Q-Star, Applied Biosystems)  
153 equipped with a nano-electrospray ion source coupled with a nano high pressure



154 liquid chromatography system (LC Packings Dionex) as previously described [16].  
155 Identified proteins were classified by Panther software (<http://www.pantherdb.org>).

### 156 2.5. *In situ* proximity ligation assay (PLA)

157 Cells grown on acid-washed eight-well glass slides ( $10^4$  cells per well) (Thermo  
158 Scientific) in appropriate medium with 5 or 10 % FBS for 24h. After treatment,  
159 paraformaldehyde-fixed cells were incubated with 4 % BSA (1h, 20°C) followed by  
160 overnight incubation with primary antibodies [mouse anti-HA, 1:50 (Covance); goat  
161 anti-sortilin, 1:50 (R&D systems); rabbit anti-EphA2, 1:100 (Cell Signaling  
162 Technology), mouse anti-TrkA, 1:50 (Alomone Labs)]. PLA was performed as  
163 recommended by manufacturer's instructions. Briefly, slides were incubated with  
164 secondary antibodies complexed with complementary nucleotide sequences for 2h.  
165 The formed DNA circle, resulting from complementary nucleotides was then  
166 amplified using fluorescent oligonucleotides. Nuclei were counterstained with  
167 Hoechst 33258 (Sigma-Aldrich) and samples were mounted with fluorescence  
168 mounting medium (Dako). PLA images (fluorescent red dots) were acquired using  
169 a fluorescence microscope (100X oil immersion objective,  $\lambda_{\text{excitation}}$ : 562 nm,  $\lambda_{\text{emission}}$ :  
170 624 nm, microscope Eclipse Ti; Nikon) and analyzed with NIS-Elements BR  
171 software (Nikon) and Image J.

172 Tissue microarrays were from SuperBioChips (CBA4) and US BioMax (HBre-  
173 Duc150Sur01) allowing analysis in 189 individual tumor samples. For PLA in  
174 paraffin-embedded patient tumor samples, primary antibodies anti-TrkA (ANT-018,  
175 Alomone Labs) and anti-EphA2 (AF3035, R&D systems) were incubated overnight  
176 at 4°C. Subsequent steps were done according to the manufacturer's instructions.  
177 Briefly, slides were incubated with secondary antibodies complexed with  
178 complementary nucleotide sequences for 2h. The formed DNA circle, resulting from

179 complementary nucleotides was then amplified using oligonucleotides labeled with  
180 horseradish peroxidase. PLA signal was evaluated according to the number of dots  
181 per cell and the number of stained cells in a double-blind analysis (by TRA and DJ).  
182 PLA signal <2 dots/cell was considered as low, PLA signal between 2-10 dots/cell  
183 was considered as medium, PLA signal >10 dots/cell was considered as high.  
184 Kaplan-Meier curves were obtained by using GraphPad Prism software.

## 185 2.6. Clonogenic cell growth

186 Clonogenic assays were performed as previously described [15]. After siRNA  
187 transfection, 2000 cells were seeded in 35 mm Petri dishes and treated with proNGF  
188 for 10 days. Colonies were then stained with crystal violet before counting.

## 189 2.7. *In vivo* experiments

190 All the experiments involving mice received the approval of the local ethic committee  
191 and were carried out according to French national animal care regulations. MDA-  
192 MB-231 HA-TrkA cells ( $3 \times 10^6$ ) were subcutaneously injected into six-week old  
193 female SCID mice. Two weeks after cell injection, mice were randomized into four  
194 groups (n=7), and were treated a total of three times at 3-day intervals. Lestaurtinib  
195 (CEP-701; Calbiochem) was suspended in vehicle (40% polyethylene glycol 1000,  
196 10% povidone C30 and 2% benzyl alcohol in distilled water) and injected  
197 intraperitoneally (10 mg/kg). siEphA2 (Eurogentec; 7.5  $\mu$ g/mouse) was delivered  
198 using *in vivo* jetPEI<sup>®</sup> according to the manufacturer's instructions (Polyplus  
199 transfection) and injected subcutaneously near the tumor mass. Tumor volume was  
200 determined throughout the experiment by measuring the length (l) and width (w) and  
201 then calculated as  $\pi/6 \times l \times w \times (l+w)/2$ .

202 For analyses of breast cancer cell dissemination in mice, xenograft experiments  
203 were conducted using MDA-MB-231 HA-TrkA cells. The tumors were allowed to

204 develop for 14 days and the mice were then submitted to 5 injections (every 3 days)  
205 of either scrambled siRNA, or TrkA- and EphA2-targeting siRNAs alone or in  
206 combination (7.5 µg siRNA/mouse). Tumor volume was determined throughout the  
207 experiment by measuring the length (l) and width (w) and tumors were allowed to  
208 grow up to 2 cm<sup>3</sup> to allow metastasis of cancer cells. After animal sacrifice, lungs,  
209 liver and brain were collected and detection of cancer cells in those organs was  
210 carried out by evaluating human microglobulin mRNA expression by RT-PCR as  
211 previously described [17].

## 212 2.8. Statistical analysis

213 Results are expressed as mean ± SEM of at least three independent experiments.  
214 Two-tailed unpaired Student t-tests, one-way or two-way ANOVA tests (Bonferroni's  
215 post-hoc test) and Mann-Whitney tests were used where appropriate.

216

217 **3. Results**

218 3.1. TrkA is associated with sortilin and EphA2 in proNGF-treated MDA-MB-231  
219 breast cancer cells

220 In order to decipher TrkA signaling partners under proNGF stimulation, we first  
221 performed proteomic analysis in MDA-MB-231 breast cancer cells stably expressing  
222 HA-tagged TrkA [10]. Proteins co-immunoprecipitated with HA-TrkA were identified  
223 by mass spectrometry analysis (Figure 1). Individual proteins (985) were identified  
224 in selected bands from cells treated with proNGF (5 and 30 min) (Figure 1A). Using  
225 Panther classification software, we observed that most of the identified proteins  
226 were implicated in maturation and vesicular trafficking (binding and catalytic  
227 activities). Seven percent were related to membrane receptor and signaling (Figure  
228 1B). As expected, sortilin, the known receptor of proNGF was found to be co-  
229 immunoprecipitated with HA-TrkA (Figure 1C). Interestingly, we found that EphA2,  
230 a membrane receptor tyrosine kinase, was also co-immunoprecipitated with HA-  
231 TrkA. EphA2 is of particular interest, as it is known to be expressed in breast cancer  
232 in which it favors aggressive behavior and metastases formation [18]. We also  
233 identified Src, cortactin and p130 Cas (BCAR1, *Breast cancer anti-estrogen*  
234 *resistance protein 1*), which are reported to act as early effectors in EphA2  
235 downstream signaling pathways [19]. Other proteins such as SHEP1 (SH2D3C,  
236 *SH2 domain-containing protein 3C*), PTP-PEST (PTPN12, *Tyrosine-protein*  
237 *phosphatase non-receptor type 12*) and RIL (PDLI4, *PDZ and LIM domain protein*  
238 *4*) were also found to be pulled-down with TrkA; these proteins are well known to  
239 regulate Src and/or p130 Cas-mediated signaling pathways [20–22]. The other  
240 identified proteins including Lasp1 (*LIM and SH3 domain protein 1*), SNAP23  
241 (*Synaptosomal-associated protein 23*), FHL2 (*Four and a half LIM domains protein*  
242 *2*), HAX1 (*HCLS1-associated protein X-1*), adducin, MARCKS (*Myristoylated*

243 *alanine-rich C-kinase substrate*) gelsolin, and integrins  $\alpha_3\beta_1$  are downstream targets  
244 of Src and p130 Cas, which are associated with cytoskeleton remodeling and cell  
245 migration [23–28].

246 To confirm proNGF-induced TrkA association with sortilin and EphA2 in breast  
247 cancer cells, we further performed immunoprecipitation assays by using antibodies  
248 against HA (-TrkA), sortilin and EphA2, followed by Western blot analysis (Figure  
249 2A). In the absence of proNGF, none of the three receptors was found to be co-  
250 immunoprecipitated. Upon proNGF treatment, sortilin and EphA2 were co-  
251 immunoprecipitated with TrkA. These results were confirmed by reverse co-  
252 immunoprecipitation of sortilin and EphA2 (Figure 2A). Of note, in the presence of  
253 NGF, sortilin binding to TrkA was detected after 30 min of treatment but EphA2  
254 binding was not observed after neither 5 min nor 30 min of treatment (Figure 2A).  
255 Thus, NGF seemed to induce a late TrkA/sortilin association, suggesting that sortilin  
256 acts as an endocytic receptor, as previously reported in neuronal cell models [8].

257 Proximity ligation assays (PLA) were then carried out to confirm any receptor  
258 association (distance <40 nm) at the plasma membrane. In the absence of  
259 exogenous proNGF stimulation, a basal PLA signal (red dots) was observed for  
260 sortilin/TrkA (Figure 2B-C) and TrkA/EphA2 (Figure 2D-E). ProNGF stimulation (5  
261 min) enhanced PLA signals of sortilin/TrkA and EphA2/TrkA (Figure 2C and 2E);  
262 this increase was transient as membrane PLA signals decreased to basal levels  
263 after 30 min of proNGF treatment.

264 Together, these data indicated that proNGF specifically induces the association of  
265 TrkA with sortilin and EphA2, even if we cannot exclude the existence of other  
266 intermediary partners.

267 3.2. Sequential sortilin/TrkA/EphA2 association induces TrkA-dependent Akt  
268 phosphorylation and EphA2-dependent Src phosphorylation

269 In order to put insight into the dynamics of the receptors association, we first treated  
270 cells with neurotensin that inhibits, by competition, proNGF binding to sortilin [8]. As  
271 shown in Figure 3A, in the presence of neurotensin, TrkA was not  
272 immunoprecipitated with either sortilin or EphA2, indicating that proNGF binding to  
273 sortilin was necessary for sortilin/TrkA/EphA2 association. When TrkA expression  
274 was silenced by siRNA (Figure 3B, S1), sortilin did not pull-down EphA2, indicating  
275 that sortilin could not associate with EphA2 in the absence of TrkA; this was further  
276 confirmed by PLA, as no signal was detected for sortilin/EphA2.

277 Interestingly, in cells stably expressing a kinase-dead TrkA that prevents receptor  
278 phosphorylation (Figure 3D), proNGF still induced TrkA association with sortilin and  
279 EphA2, suggesting that TrkA phosphorylation was not necessary for  
280 sortilin/TrkA/EphA2 association. Finally, when EphA2 expression was silenced by  
281 siRNA (Figure 3E), TrkA and sortilin were co-immunoprecipitated in proNGF-treated  
282 cells, indicating that EphA2 was not required for TrkA and sortilin association.

283 Together, these results suggested that proNGF binding to sortilin elicits sortilin  
284 association with TrkA, which in turn recruits EphA2 at cell surface.

285 As we have previously shown that proNGF stimulates breast cancer cell invasion  
286 through TrkA activation, we asked if EphA2 could be involved in TrkA-mediated cell  
287 invasion. When EphA2 expression was inhibited by siRNA approach, proNGF was  
288 no longer able to stimulate cell invasion both in native and HA-TrkA over-expressing  
289 MDA-MB-231 cells (Figure S2), implying that EphA2 was necessary for proNGF-  
290 stimulated and TrkA-mediated cell invasion. ProNGF-stimulated invasion implicates  
291 the activation of downstream signaling pathways including Akt and Src [10]. On the

292 other hand, Src is also a downstream effector of EphA2 [19], we then determined  
293 the respective role of TrkA and EphA2 in Akt and Src activation by using a kinase-  
294 dead mutant of TrkA (Figure 3F) or siEphA2 approach (Figure 3G). Following  
295 proNGF treatment, Akt was not phosphorylated in cells expressing a kinase-dead  
296 mutant of TrkA while Src phosphorylation was maintained (Figure 3F), indicating  
297 that Akt phosphorylation depended upon TrkA phosphorylation while Src  
298 phosphorylation did not. By contrary, in cells transfected with siEphA2, proNGF still  
299 induced Akt phosphorylation but not that of Src, indicating that Akt phosphorylation  
300 did not depend on EphA2 while Src activation did (Figure 3G).

301 Collectively, our data showed that, in MDA-MB-231 breast cancer cells, proNGF  
302 binding to sortilin induced functional sortilin/TrkA/EphA2 association, leading to  
303 TrkA-dependent Akt phosphorylation and EphA2-dependent Src phosphorylation  
304 (Figure 3H).

305

### 306 3.3. ProNGF increases breast cell clonogenic growth through functional association 307 of TrkA with EphA2

308 In order to determine the relevance of TrkA/EphA2 functional association in breast  
309 cancer cells, we further extended our study on a panel of representative breast  
310 cancer cell lines including basal-like (wild type MDA-MB-231, SUM159-PT), luminal-  
311 like (MCF-7, T-47D), and HER2-overexpressing basal-like cell line HCC-1954. PLA  
312 signals of TrkA/EphA2 were increased after 5 min of stimulation with proNGF in all  
313 cell lines tested, except for MCF-7 (Figures 4A and S3). Clonogenic assays were  
314 then carried out to evaluate the impact of EphA2 invalidation on proNGF-stimulated  
315 cell growth. As shown in Figure 4B, proNGF stimulated clonogenic cell growth in all  
316 the cell lines tested; siEphA2 totally abolished proNGF-induced cell growth except  
317 in MCF-7, this was consistent with the results of PLA assay revealing that proNGF

318 did not increase TrkA/EphA2 complex in these cells and with the fact that MCF-7  
319 cells express low levels of both TrkA and EphA2 compared to the other cell lines  
320 (Figure S4). Use of another siEphA2 sequence [14] confirmed also the implication  
321 of EphA2 in pro-NGF induced clonogenic growth of MDA-MB-231 cells (Figure S5).  
322 These results indicated that proNGF-induced cell growth involved TrkA/EphA2  
323 association in different cell lines whatever their molecular classification (*i.e.* basal,  
324 luminal or HER2-like). We then examined the impact of TrkA and/or EphA2 inhibition  
325 on colony formation in MDA-MB-231 cells. As shown in Figure 4C, the TrkA inhibitor  
326 K252a inhibited colony formation to about 85 % of control, siEphA2 to 60 % of  
327 control, while combinatory treatment with K252a and siEphA2 inhibited colony  
328 formation to less than 30 % of control.

329

330 3.4 Simultaneous targeting of TrkA and EphA2 reduces tumor growth and brain  
331 metastasis *in vivo*

332 Given the above results indicating the importance of proNGF-induced TrkA/EphA2  
333 association in breast cancer cells, we determined the potential benefit of a  
334 combinatorial targeting of TrkA and EphA2 in xenograft mouse model. As shown in  
335 Figure 5A and B, CEP-701 (clinical derivative of K252a) alone did not significantly  
336 reduce tumor growth, while siEphA2 alone delayed tumor growth when compared  
337 to the control group (scrambled siRNA). Combined treatment of CEP-701 and  
338 siEphA2 resulted in a dramatic reduction of tumor burden when compared to CEP-  
339 701 or siEphA2 treatment alone. We then evaluated the impact of TrkA and/or  
340 EphA2 invalidation on breast cancer cell dissemination in different organs including  
341 lungs, liver and brain (Figure 5C). In these conditions, we first confirmed that  
342 combined inhibition of TrkA and EphA2 inhibited tumor growth as the median



343 survival of the mice was increased: Median of survival were 40 days in control group,  
344 53 days in siTrkA group, 49 days in siEphA2 group and 57.5 days in siTrkA and  
345 siEphA2 mice (Figure S6). We found that tumor cells disseminated readily in these  
346 three distant organs. Lung metastasis was not modified under any invalidation  
347 condition. Interestingly, TrkA invalidation alone was sufficient to reduce liver  
348 metastasis while combined inhibition of TrkA and EphA2 was required to decrease  
349 brain metastasis. These results indicated that simultaneous inhibition of TrkA and  
350 EphA2 was not only efficient in inhibiting primary tumor growth, but also in reducing  
351 brain metastasis formation.

352

353 3.5. ProNGF-induced TrkA/EphA2 association is correlated with poor prognosis in  
354 breast cancer

355 To go further on the significance of TrkA/EphA2 functional complex in breast cancer,  
356 we performed immunostainings of TrkA, EphA2, and PLA labeling to colocalize  
357 TrkA/EphA2 in breast tumor samples in a tissue microarray (TMA) cohort of 189  
358 patients (Figure 6, Table 1 and S1). We found that TrkA expression was associated  
359 with PR-negative status (Table 1). However, neither TrkA nor EphA2 alone  
360 correlated with overall survival of patients (Figure 6A-H and Table 1). By PLA, we  
361 distinguished the differential PLA signals of TrkA/EphA2 in the samples, and found  
362 that high level of TrkA/EphA2 PLA signals in tumors was correlated with a significant  
363 decrease of overall survival of patients (Figure 6I-L).

364

#### 365 4. Discussion

366 Compelling evidences showed that proNGF is more than just a metabolic precursor  
367 of NGF, as it exhibits biological activities in a wide range of normal and neoplastic  
368 tissues, including breast cancer [6,8–13,17]. Nevertheless, proNGF functions in  
369 cells are still in debate due to its pro-survival and pro-apoptotic activities, according  
370 to cell types. A recent study reconciled these findings by showing that these  
371 opposite biological effects depend on TrkA levels [29]. Indeed, the authors showed  
372 that proNGF elicits apoptotic signaling in PC-12 cells expressing low levels of TrkA  
373 while it favors survival of cells expressing high levels of TrkA. In agreement with  
374 these findings, increased levels of TrkA are associated with tumor growth and  
375 metastasis in breast cancer [30] and melanoma [31].

376 In breast cancer cells, we previously observed that uncleavable proNGF induces  
377 sortilin recruitment at plasma membrane and TrkA activation, leading to increased  
378 cell invasion, independently of p75<sup>NTR</sup> [10]. Herein, by studying TrkA-interacting  
379 proteins upon proNGF stimulation, we identified EphA2, a membrane receptor  
380 tyrosine kinase, as a key element of proNGF signaling in breast cancer cells.  
381 ProNGF signaling through sortilin and TrkA allowed for EphA2 recruitment, which in  
382 turn activated Src in a TrkA phosphorylation-independent manner.

383 Implications of EphA2 in proNGF-induced signaling is of particular interest. Indeed,  
384 EphA2 binds to its ligand ephrin-A1 to maintain cell adhesion and tissue  
385 homeostasis in normal breast epithelial cells [32], ephrin-A1 downregulation favors  
386 ligand-independent activation of EphA2 and associated downstream signaling  
387 pathways (*e.g.* MAP-kinase, RhoGTPase and Src) in several types of cancer cells,  
388 leading to cell invasion and metastasis [33–35]. Although the mechanisms of ligand-  
389 independent activation of EphA2 remain fragmentary, it has been shown that EphA2

390 phosphorylation by Akt or Rsk can lead to its activation [35,36]. Here, we observed  
391 that in the context of proNGF stimulation, EphA2 activated Src *via* an Akt-  
392 phosphorylation independent mechanism. Moreover, our proteomic analysis  
393 revealed that several Src-associated signaling proteins like cortactin and p130 Cas  
394 were pulled-down with TrkA upon proNGF treatment, suggesting that proNGF could  
395 activate a signaling cascade involving Src/p130 Cas/cortactin complex. Although  
396 further study should be done to confirm this hypothesis, the Src/p130 Cas/cortactin  
397 complex is already reported to induce cell invasion [37].

398 Song *et al.* [14] have recently reported that EphA2 is overexpressed in the basal-  
399 like breast cancer molecular subtype and this overexpression is correlated with poor  
400 recurrence-free survival in triple-negative breast cancers. Loss of EphA2 function in  
401 both human and genetically engineered mouse models of triple negative breast  
402 cancers reduced tumor growth. Herein, we observed that EphA2 silencing inhibited  
403 proNGF-stimulated clonogenic cell growth of not only triple negative, but also  
404 luminal ER-positive and HER2-positive breast cancer cell lines. This implies that  
405 EphA2 is involved in proNGF-stimulated clonogenic cell growth, independently of  
406 molecular subtypes. Consistently, we could not find any significant correlation  
407 between TrkA/EphA2 complex or co-localization and different subtypes of breast  
408 cancer. Of importance, the level of TrkA/EphA2 co-localization is significantly  
409 correlated with poor overall survival of patients suffering from breast cancer  
410 regardless the cancer subtype, suggesting the potential involvement of  
411 proNGF/TrkA/EphA2 axis in breast cancer progression whatever the cancer  
412 subtype. These findings reinforced our previous results showing a significant  
413 correlation between the expression of proNGF and lymph node invasion [10].

414 Cross-talk in growth factor-induced signaling pathway is a leading cause of  
415 resistance to targeted therapies [38,39]. EphA2 was identified to mediate resistance  
416 to multiple targeted therapies including trastuzumab (HER-2 inhibitor) *via* Src  
417 activation in breast cancer cells [40], as well as erlotinib (EGFR inhibitor) in lung  
418 cancer models [41]. We postulate that the existence of a proNGF-induced EphA2-  
419 Src pathway, independently of TrkA phosphorylation and Akt activation, may  
420 contribute to tumor resistance to therapies targeting TrkA kinase domain  
421 (lestaurtinib, larotrectinib, entrectinib...). Here, we showed that simultaneous  
422 targeting of TrkA and EphA2 receptors, dramatically reduced colony formation *in*  
423 *vitro* and tumor development *in vivo*, suggesting that inhibiting both TrkA- and  
424 EphA2-dependent signaling pathways may improve the therapeutic benefit in  
425 patients (over)expressing TrkA, EphA2 and proNGF.

426 In conclusion, our data demonstrated that functional interactions between sortilin,  
427 TrkA and EphA2 are essential for the tumor-promoting effect of proNGF in breast  
428 cancer. Although further translational work is required, our results suggest that  
429 proNGF/TrkA/EphA2 axis could be used as both a prognostic marker and a potential  
430 therapeutic target in breast cancer.

431

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437

438 **Conflicts of interest**

439 All authors declare no conflict of interest

440

441 **Reference**

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**TABLE**

**Table 1:** Correlation between expression levels of TrkA and EphA2, TrkA/EphA2 association (PLA signal) and clinical parameters of patient samples

		TrkA	EphA2	TrkA/EphA2 PLA
<b>Estrogen receptor</b>	<b>Positive (n=109)</b>	1.725±0.09478 p= 0.1408	1.817±0.1011 p=0.2155	2.193±0.1169 p=0.1608
	<b>Negative (n=71)</b>	1.944±0.1115	2.014±0.1212	1.915±0.1615
<b>Progesterone receptor</b>	<b>Positive (n=95)</b>	<b>1.663±0.09996</b> <b>p=0.0318</b>	1.758±0.1097 p=0.0536	2.053±0.1201 p=0.5557
	<b>Negative (n=84)</b>	<b>1.976±0.1044</b>	2.060±0.1089	2.141±0.1541
<b>HER-2</b>	<b>Positive (n=69)</b>	1.783±0.1254 p=0.8799	1.884±0.1248 p=0.9606	2.217±0.1650 p=0.2717
	<b>Negative (n=113)</b>	1.805±0.08876	1.876±0.09988	2.000±0.1166
<b>Triple negative</b>	<b>Positive (n=34)</b>	2.088±0.1544 p=0.0578	2.176±0.1661 p=0.0715	1.765±0.2315 p=0.1053
	<b>Negative (n=147)</b>	1.735±0.08153	1.816±0.08743	2.163±0.1049
<b>Lymph node invasion</b>	<b>Positive (n=116)</b>	1.724±0.09372 p=0.1166	1.897±0.09976 p=0.9171	2.138±0.1193 p=0.2127
	<b>Negative (n=69)</b>	1.957±0.1081	1.913±0.1181	1.899±0.1193

611

612 **FIGURE LEGENDS**

613

614 **Figure 1: Proteomic analysis of HA-TrkA partners revealed EphA2 association**

615 **and downstream signaling pathways proteins. (A)** MDA-MB-231 HA-TrkA cells

616 were treated in absence or presence of non-cleavable proNGF (0.5nM) for 5 and 30

617 min. Total cell lysates were subjected to HA immunoprecipitation and separated in

618 10% SDS-PAGE. Proteins were revealed by colloidal blue Coomassie staining.

619 Using transilluminator, intensity of bands was appreciated by CC and R-A T and

620 bands with stronger intensity than that of control were cut (red square). Protein

621 peptide digests were then subjected to mass spectrometry analysis. **(B)** Identified

622 proteins were analyzed for Biological pathways using Panther software. **(C)** Mass

623 spectrometry identification of selected putative interacting partners of TrkA under

624 proNGF stimulation (5 or 30 min). For each identified protein, the Uniprot ID, the

625 number and sequence of the different peptides allowing protein identification and

626 the individual peptide Mascot score are summarized. Underlined amino-acids (C

627 and M) are oxidized residues. MW: Molecular Weight. GO: Gene Ontology.

628

629 **Figure 2: ProNGF induced association of sortilin, TrkA and EphA2. (A)**

630 Representative immunoblotting for sortilin, EphA2 and TrkA following IP anti-HA

631 (TrkA), sortilin or EphA2 in HA-TrkA MDA-MB-231 cells after non-cleavable proNGF

632 treatment. **(B-E)** Representative pictures (B and D) and quantification (C and E) for

633 sortilin/TrkA (B and C) and TrkA/EphA2 (D and E) PLA signals in HA-TrkA MDA-

634 MB-231 cells after non-cleavable proNGF treatment. PLA signals were quantified

635 on three independent experiments. In (C) and (E), data are expressed as scatter

636 plots. \*\*\*p<0.001; ns, not significant.

637

638 **Figure 3: ProNGF-induced association of TrkA with sortilin and EphA2 was**  
639 **sequential.**

640 **(A-B)** Representative immunoblotting for sortilin, EphA2 and TrkA after IP anti-HA  
641 (TrkA) following treatment with 1  $\mu$ M neurotensin (A) or after IP anti-sortilin in cells  
642 transfected with TrkA-targeting siRNA (B). **(C)** Representative pictures of  
643 sortilin/EphA2 PLA. **(D-E)** Representative immunoblotting for sortilin, EphA2 and  
644 TrkA after IP anti-HA in HA-TrkA MDA-MB-231 cells expressing a kinase-dead TrkA  
645 mutant (D) or transfected with EphA2-targeting siRNA (E). In (D), TrkA  
646 phosphorylation in the kinase domain (Y674/675 residues) was also evaluated by  
647 immunoblotting. **(F-G)** Representative immunoblotting for the phosphorylated and  
648 total forms of Akt and Src in non-cleavable proNGF-stimulated HA-TrkA MDA-MB-  
649 231 cells expressing a kinase-dead TrkA mutant (F) or cells transfected with EphA2-  
650 targeting siRNA (G). Immunoprecipitations and immunoblots were carried out at  
651 least 2 times with similar results. **(H)** Putative dynamic of proNGF-induced  
652 sortilin/TrkA/EphA2 signaling in breast cancer cells. After proNGF binding to sortilin  
653 (1), TrkA is phosphorylated allowing Akt activation (2). Additionally, EphA2 is also  
654 recruited to the sortilin/TrkA complex in a TrkA kinase-independent manner, leading  
655 to Src activation (3).

656

657 **Figure 4: TrkA/EphA2 complex was involved in proNGF-stimulated clonogenic**  
658 **cell growth. (A)** PLA of TrkA/EphA2 complex in wild-type MDA-MB-231, SUM159-  
659 PT, MCF-7, T-47D and HCC-1954 breast cancer cells following proNGF treatment  
660 (5 and 30 min). **(B)** Clonogenic cell growth following proNGF treatment (5 and 30  
661 min) and transfection of EphA2-targeting siRNA. **(C)** Clonogenic cell growth of MDA-  
662 MB-231 cells following treatment with 10 nM K252a and/or transfection with EphA2-

663 targeting siRNA. Data are expressed as scatter plots (A) or means  $\pm$  SEM (B-C).  
664 \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; ns, not significant.

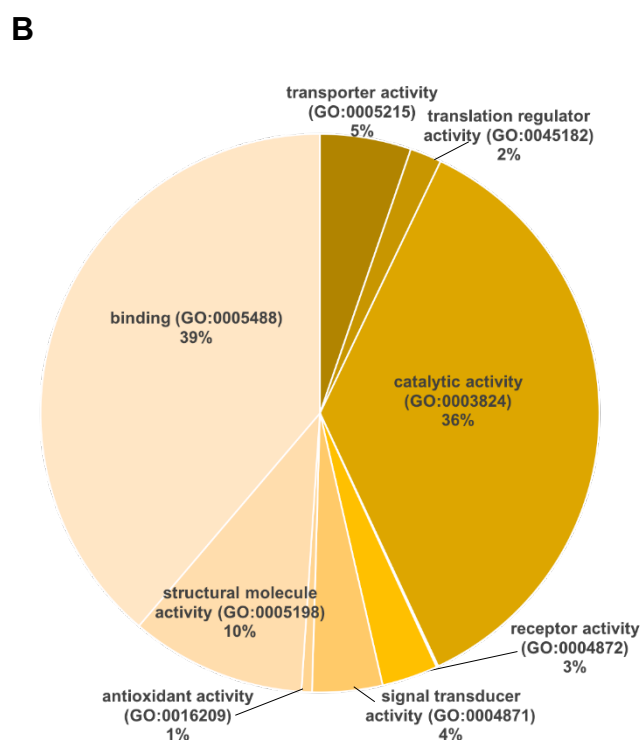
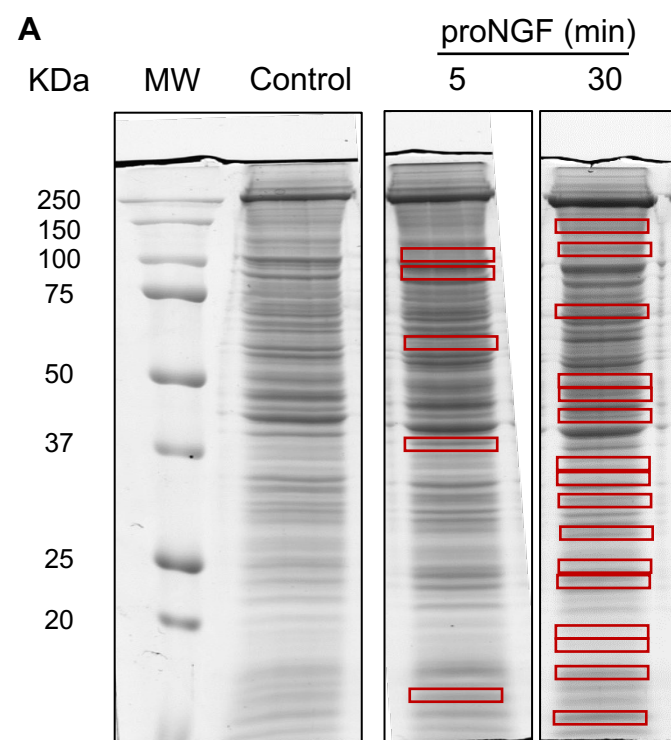
665

666 **Figure 5: Combinatorial treatment with TrkA- and EphA2-targeting modalities**  
667 **delays primary tumor growth and metastasis formation. (A-B)** Tumor growth of  
668 MDA-MB-231 xenografts in SCID mice submitted to 3 injections (every 3 days; black  
669 arrows) of either *in vivo* JetPEI + control or EphA2-targeting siRNA (7.5  $\mu$ g/mouse),  
670 or CEP-701 (10 mg/kg) alone or in combination. Tumor volumes were measured at  
671 different intervals (A) and represented as scatter plots at the end of the experiment  
672 (B). **(C)** Detection of metastatic human breast cancer cells (as determined by RT-  
673 PCR for the expression of the human microglobulin) in different organs (lungs, liver  
674 and brain) of MDA-MB-231 xenograft-bearing mice submitted to 5 injections (every  
675 3 days; black arrows) of either *in vivo* JetPEI + control or EphA2-targeting siRNA  
676 (7.5  $\mu$ g/mouse), or CEP-701 (10 mg/kg) alone or in combination. \* $p < 0.05$ ; \*\* $p < 0.01$ ;  
677 \*\*\* $p < 0.001$ ; ns: not significant. Data are expressed as means  $\pm$  SEM (A) or scatter  
678 plots (B).

679

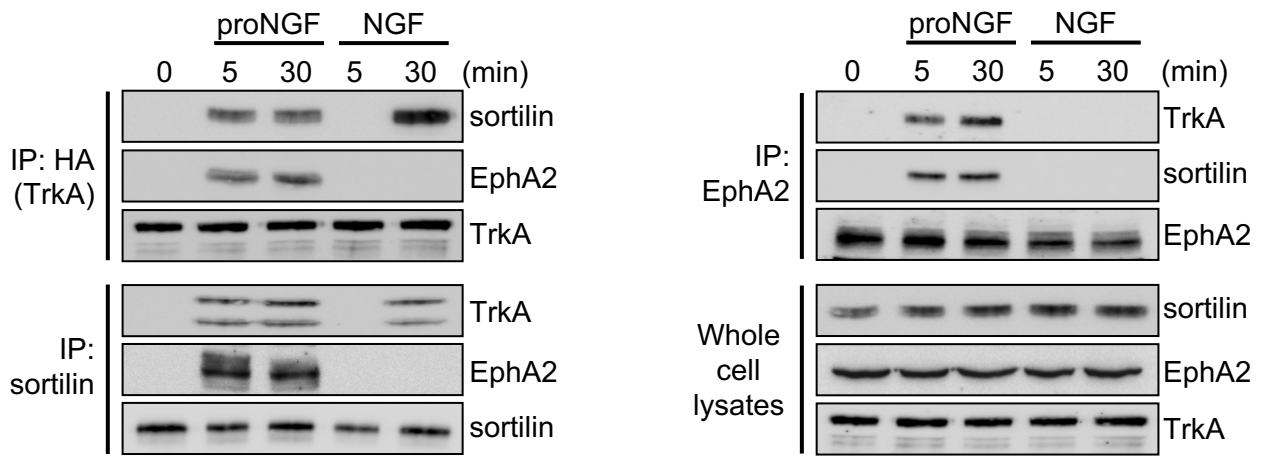
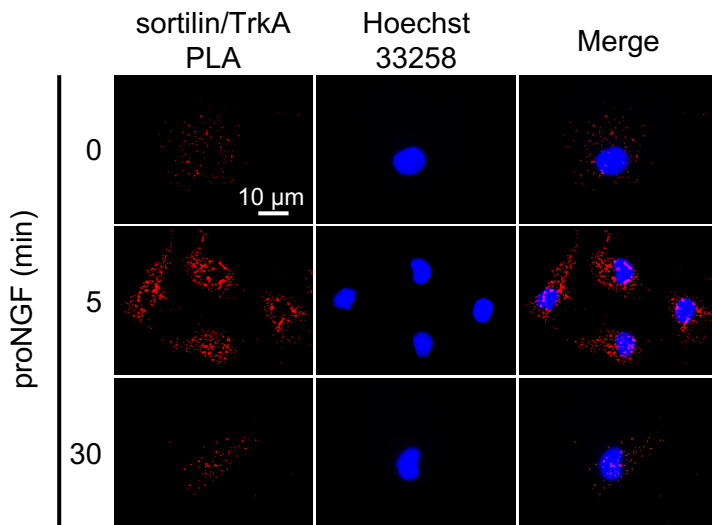
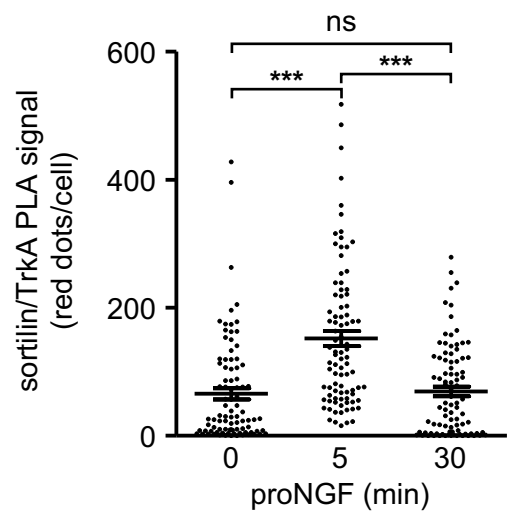
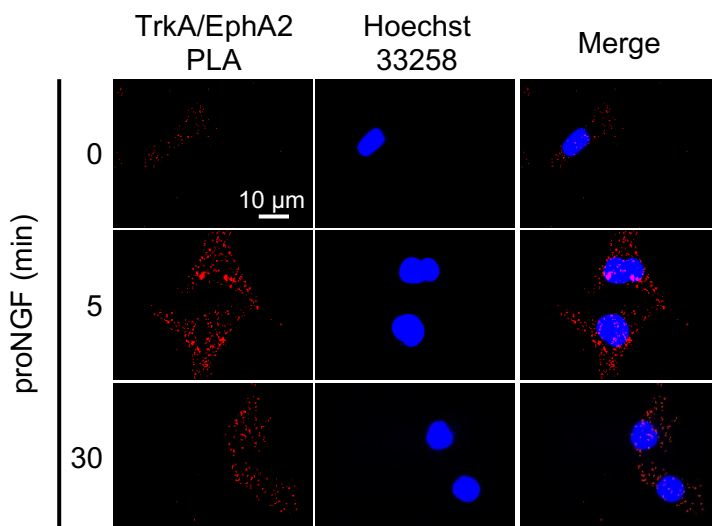
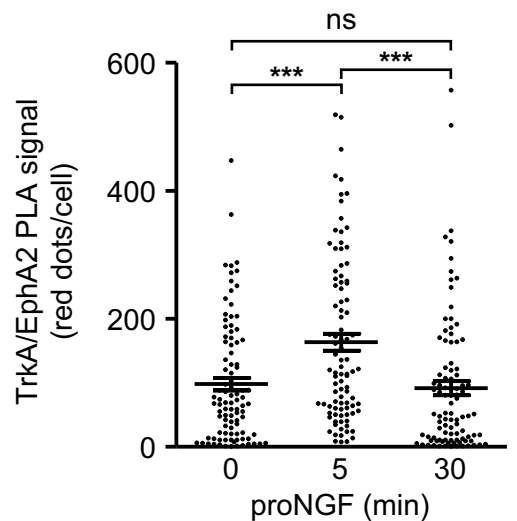
680 **Figure 6: TrkA/EphA2 complex expression is associated with overall survival**  
681 **(OS) decrease for breast cancer patients. (A-C)** Representative pictures for TrkA  
682 immunostaining in breast tumors, defined as low (A), medium (B) or high (C). **(D)**  
683 Kaplan-Meier OS curves in breast cancer patients according to TrkA staining. **(E-G)**  
684 Representative pictures for EphA2 immunostaining in breast tumors, defined as low  
685 (E), medium (F) or high (G). **(H)** Kaplan-Meier OS curves in breast cancer patients  
686 according to EphA2 staining. **(I-K)** Representative PLA images depicting low (I),  
687 medium (J) and high staining (K) for TrkA/EphA2 complex on patient breast tumor

688 samples. **(L)** Kaplan-Meier OS curves in breast cancer patients according to  
689 TrkA/EphA2 complex abundance.

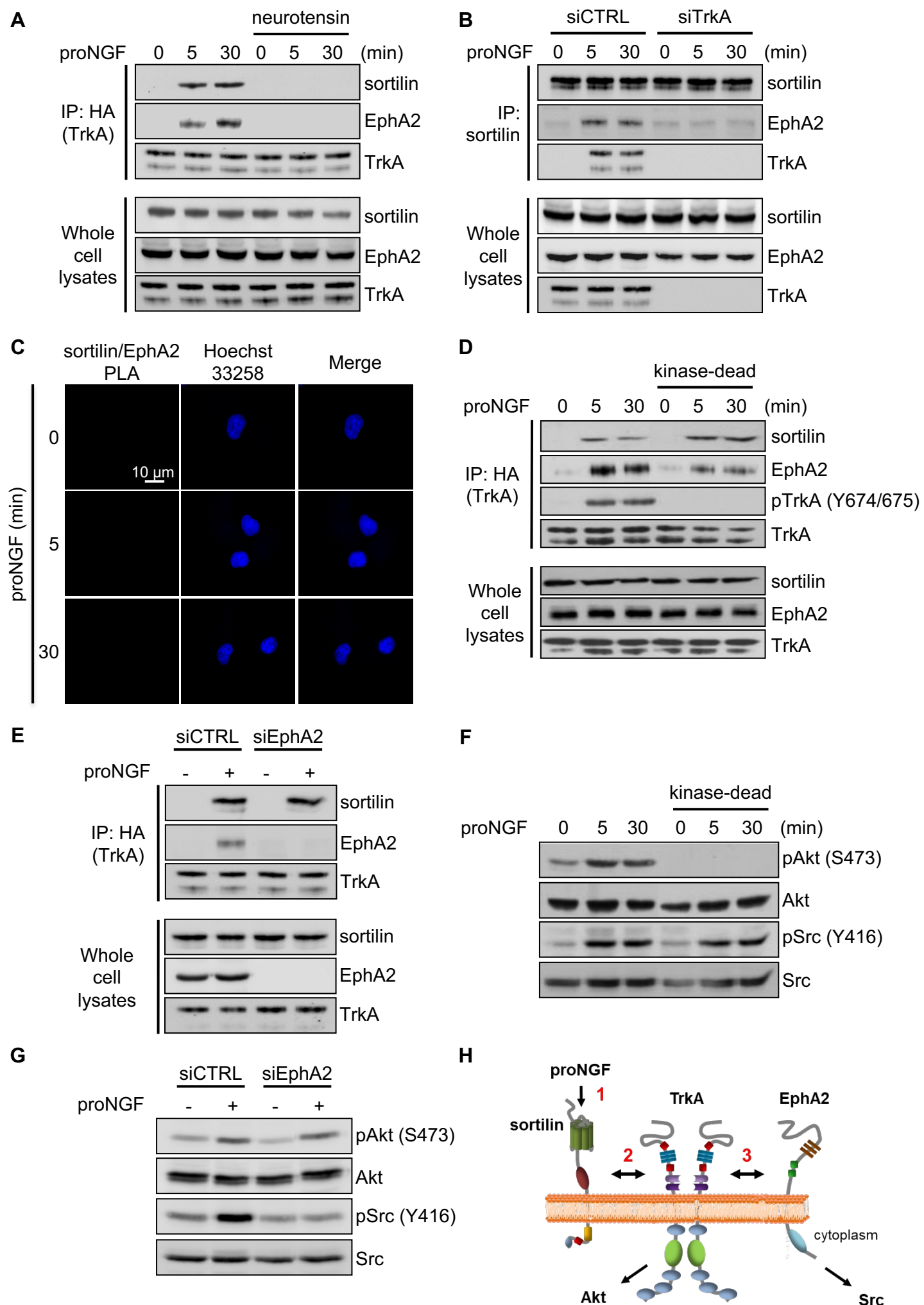


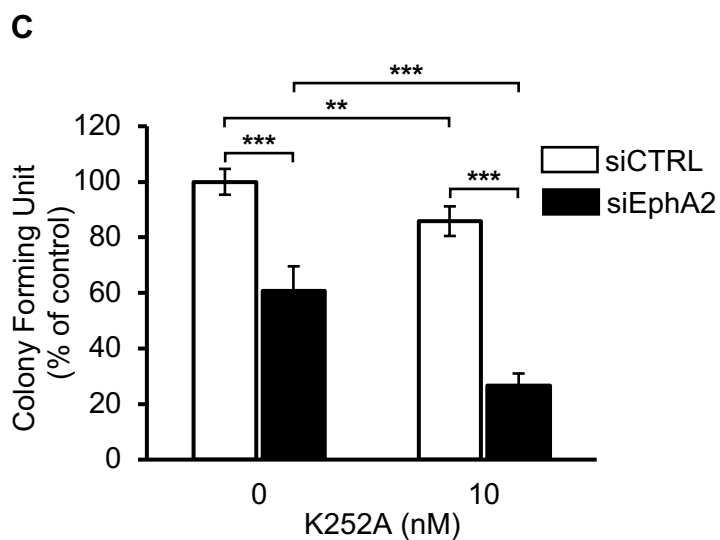
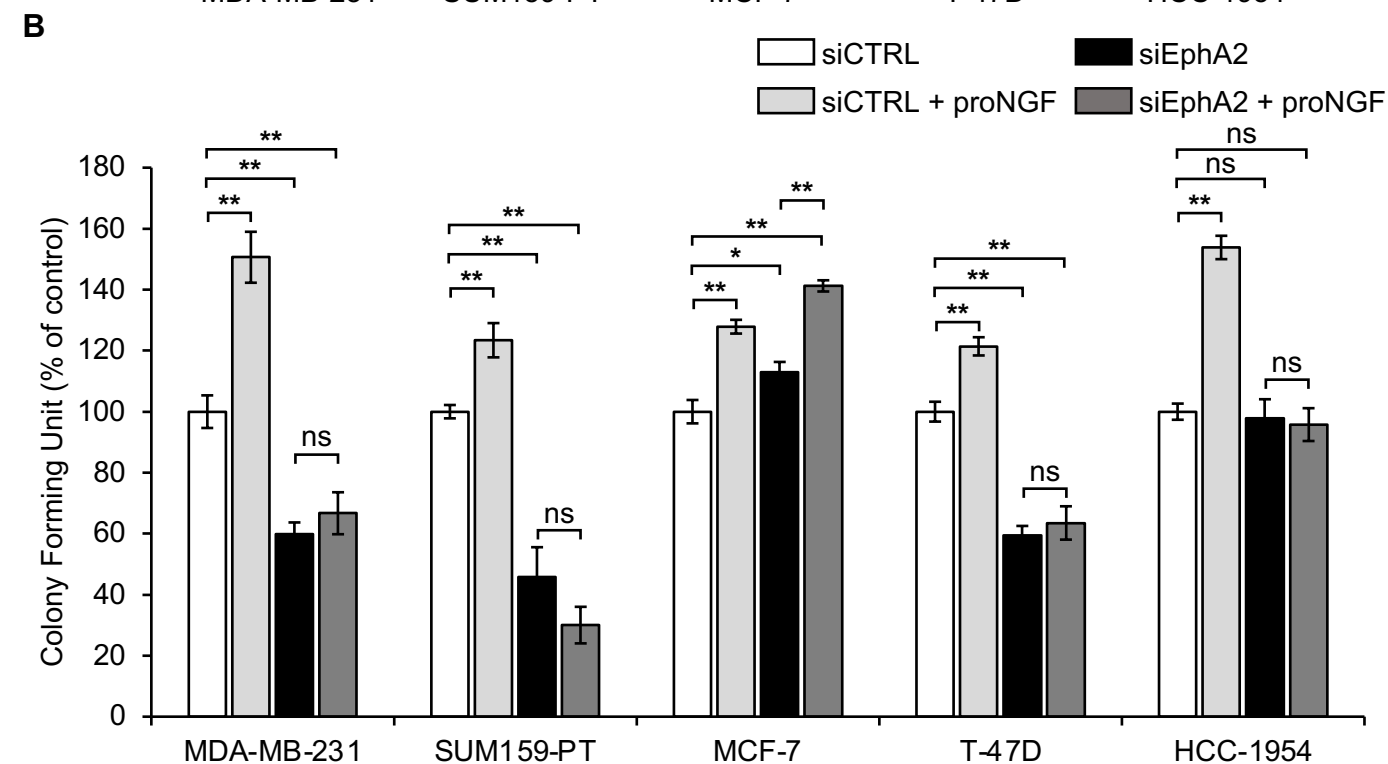
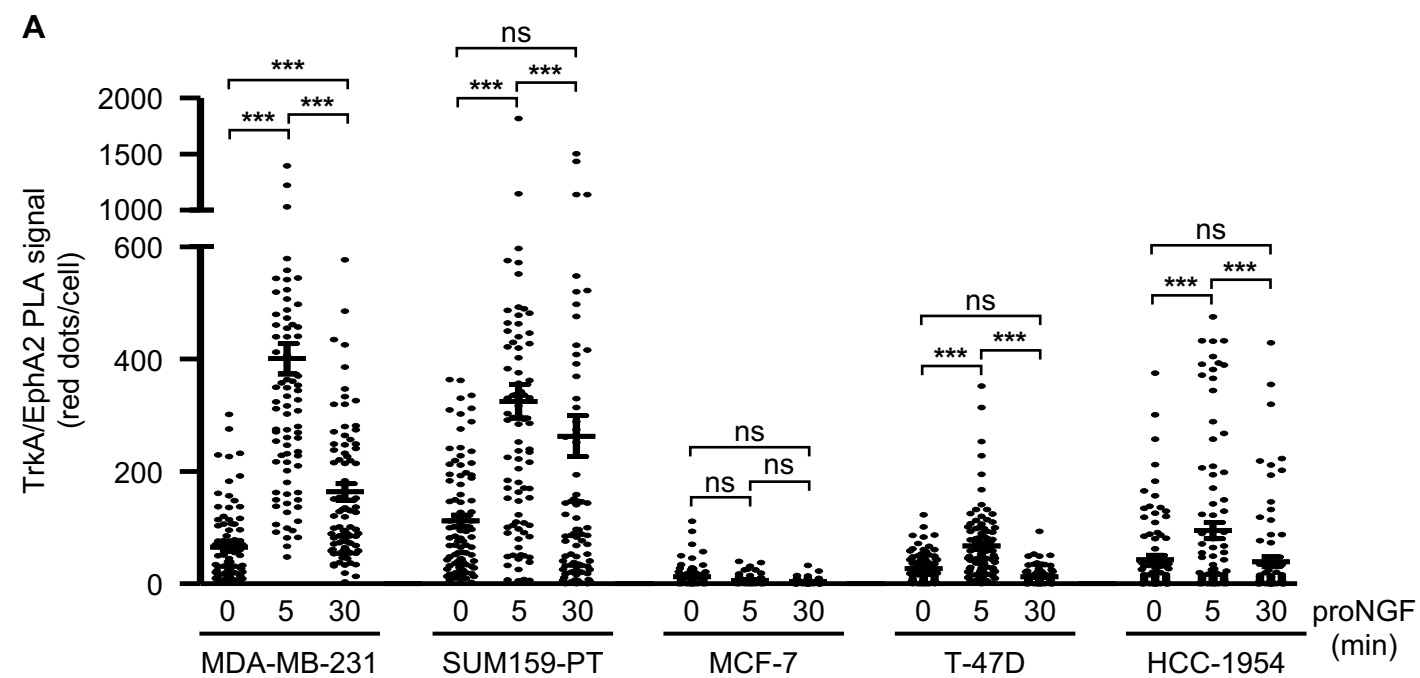
**C**

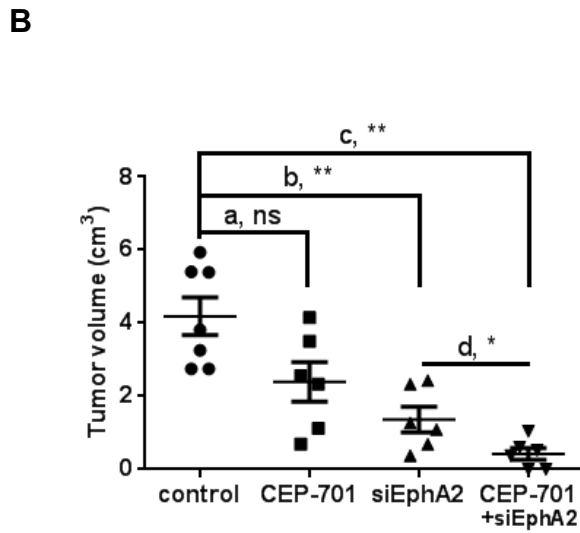
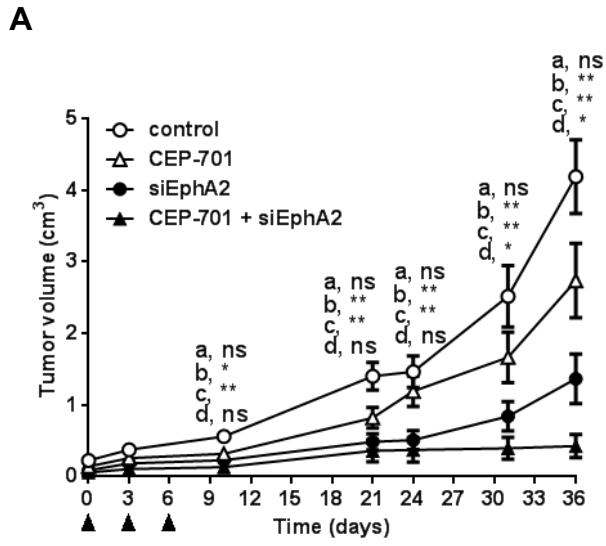
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Src	P12931	AANILVGENLVCK	54
Cortactin	Q14247	VDQSAVGFEYQGK	44
		YGLFPANYVELR	84
p130 Cas	P56945	LVFIGDTLSR	33
		ATAPGPEGGTLHPNPTDK	20
SHEP1	Q8N5H7	TEGTK	30
		LDLLER	39
PTP-PEST	Q05209	TLLLEFQNESR	55
RIL	P50479	GYFFLDER	46
		DFSAPLTISR	25
		VKPPEGYDVVAVYVYNAK	17
Lasp1	Q14847	GFSVVADTPELQR	60
SNAP23	O00161	ILGLAIESQDAGIK	76
FHL2	Q14192	CSLSLVGR	59
		YISFEER	43
		NSLVDKPFAAK	24
		CAGCTNPISGLGGTK	26
		GSSWHETCFIHR	54
		DDFAYCLNCFCDLYAK	61
		EDQLLCTDCYSEYSSK	29
HAX1	O00165	IFGGVLESDAR	38
adducin	P35611	INLQGDIVDR	49
MARCKS	P29966	LSGFSEFK	31
		GEPAAAAAPEAGASPVK	26
		EAPAEGEAAEPGSPTAAEGEA ASAASSTSSPK	86
Gelsolin	P06396	AGALNSNDAFVLK	41
Integrin alpha-3	P26006	YLLLAGAPR	23
		TVEDVGSPLK	34
		LELLMMDNLR	26
		LELLMMDNLR	41
		EAGNPGSLFGYSVALHR	27
Integrin beta-1	P05556	IGFGSFVEK	64
		LLVFSTDAGFHFAGDGK	63

**A****B****C****D****E**



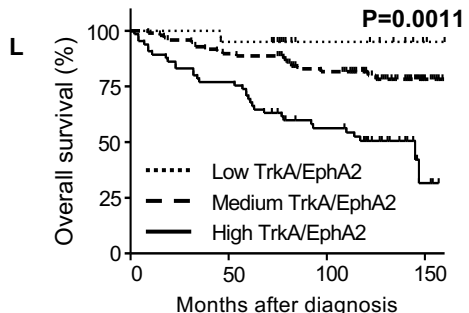
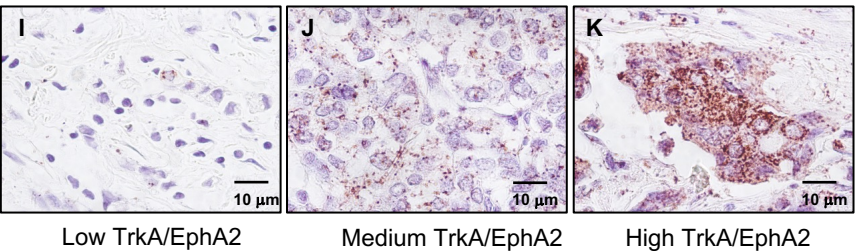
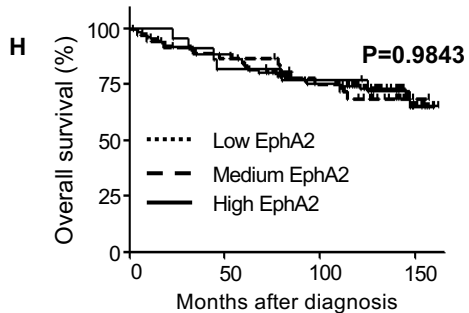
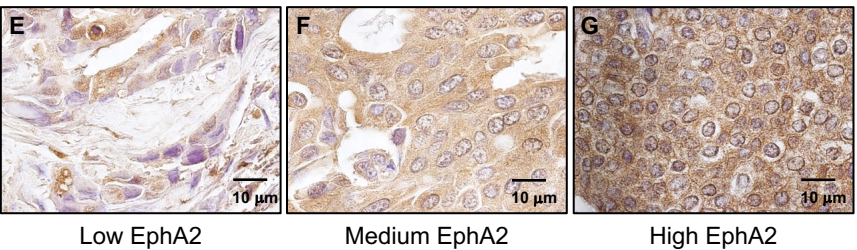
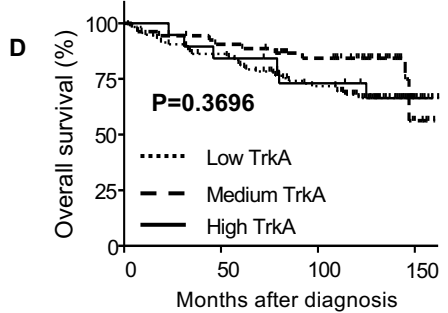
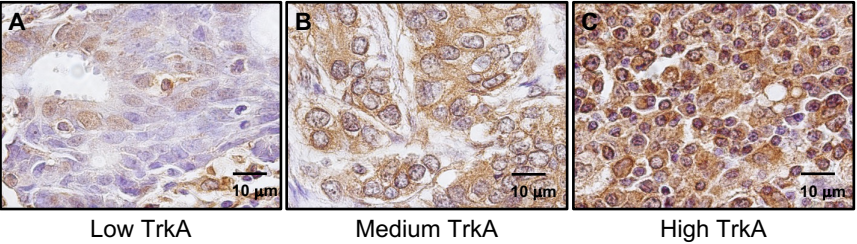






**C**

Metastasis in mice	siCTRL	siTrkA	siEphA2	siTrkA+siEphA2
Lung	10/10	10/10	10/10	10/10
Liver	7/10	4/10	7/10	4/10
Brain	8/10	8/10	7/10	4/10



Lévêque *et al.*, Figure 6

1 **ProNGF increases breast tumor aggressiveness through functional**  
2 **association of TrkA with Ephrin type-A receptor 2**

3  
4  
5 Lévêque Romain, Corbet Cyril, Aubert Léo, Guilbert Matthieu, Lagadec Chann,  
6 Adriaenssens Eric, Duval Jérémy, Finetti Pascal, Birnbaum Daniel, Magné Nicolas,  
7 Chopin Valérie, Bertucci François, Le Bourhis Xuefen & Toillon Robert-Alain

8  
9  
10  
11 **SUPPLEMENTARY FIGURE LEGENDS**

12  
13  
14 **Supplementary Figure S1: Efficacy of siTrkA.**

15 Representative Immunoblotting for HA (TrkA) in HA-TrkA MDA-MB-231 cells  
16 transfected with control, three different TrkA-targeting siRNAs or a pool of the three  
17 TrkA-targeting siRNAs.

18  
19 **Supplementary Figure S2: ProNGF-induced invasion is dependent of EphA2.**

20 **Native and HA-TrkA MDA-MB-231 cells** were transfected EphA2-targeting siRNA  
21 were seeded in collagen coated Boyden chambers and treated with non-cleavable  
22 proNGF (0.5nM) or NGF (16nM) for 16 hours. Invading cells were then counted and  
23 results were reported as a percentage of invading cells compared to control. Data  
24 are expressed as means  $\pm$  SEM. \*\*p<0.01; ns, not significant.

25  
26 **Supplementary Figure S3:** Representative pictures for sortilin/EphA2 PLA in wild-  
27 type MDA-MB-231 (A), SUM159-PT (B), MCF-7 (C), T-47D (D) and HCC1954 (E)  
28 cells. Cells were treated with non-cleavable proNGF (0.5 nM) for 5 or 30 min and  
29 then subjected to TrkA/EpHA2 PLA staining.

30

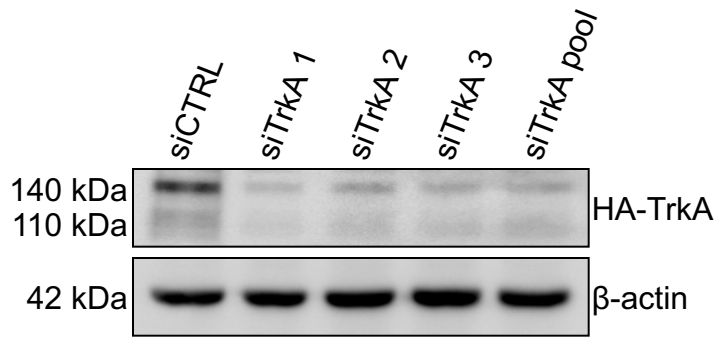
31 **Supplementary Figure S4:** Representative western blots for TrkA and EphA2  
32 expression in wild-type MDA-MB-231, SUM159-PT, MCF-7, T-47D and HCC1954  
33 cells.

34

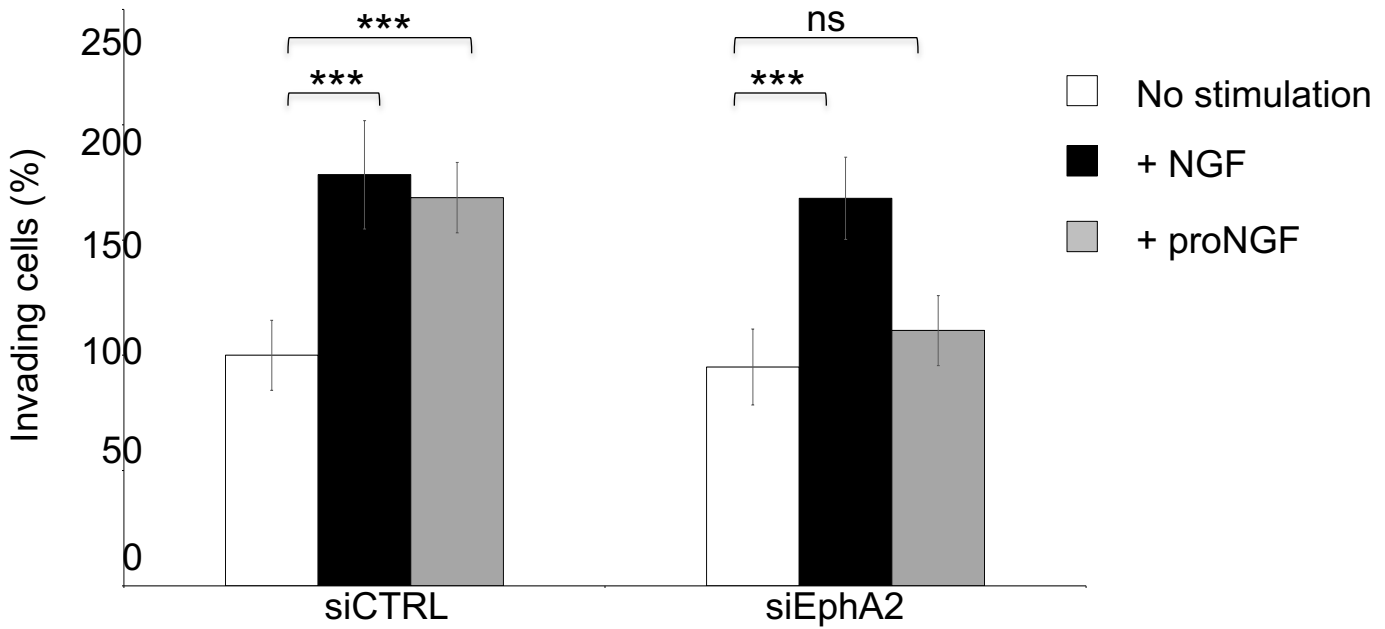
35 **Supplementary Figure S5: Efficacy of another siEphA2.** (A) Clonogenic cell  
36 growth of MDA-MB-231 cells following non-cleavable proNGF treatment and  
37 transfection of EphA2-targeting siRNA. Data are expressed as means  $\pm$  SEM.  
38 \*\* $p < 0.01$ ; ns, not significant. (B) Representative pictures of clonogenic growth.

39

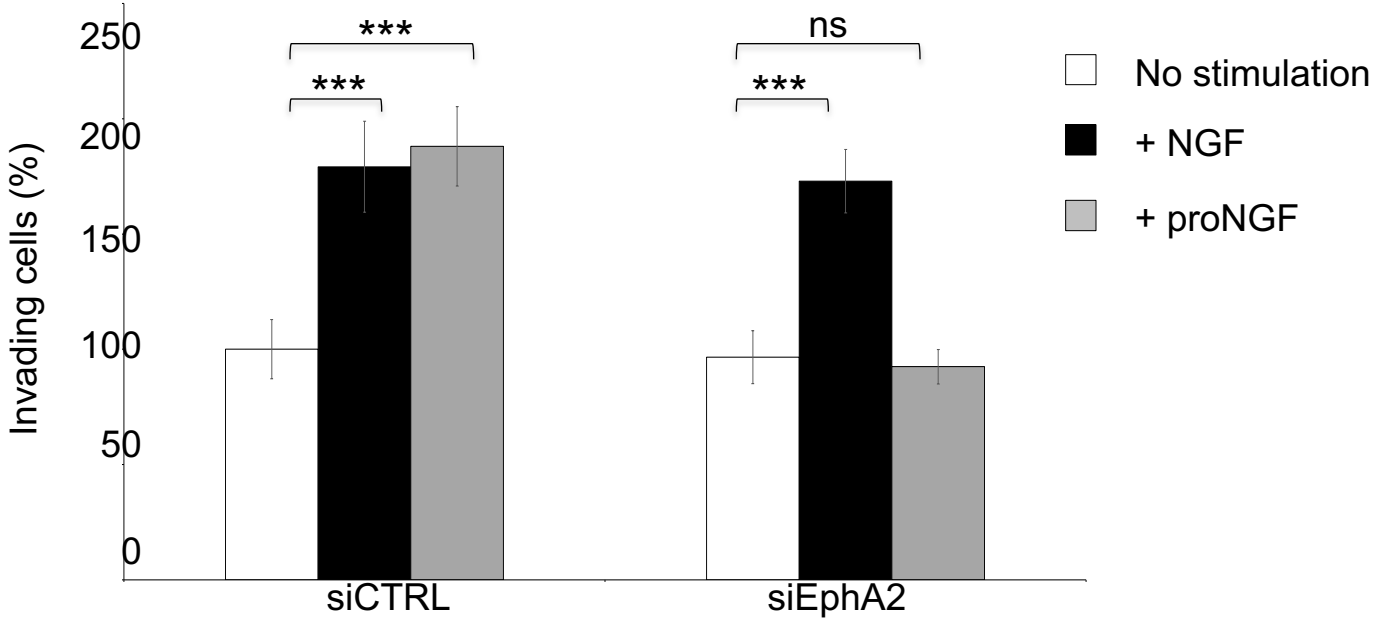
40 **Supplementary Figure S6:** Kaplan-Meier OS curves in mice according to siCTRL,  
41 siTrkA, Si EphA2 and combinatory siTrkA and siEphA2 treatments. Xenograft  
42 experiments were conducted using MDA-MB-231 HA-TrkA cells. The tumors were  
43 allowed to develop for 14 days and the mice were then submitted to 5 injections  
44 (every 3 days) of either scrambled siRNA, or TrkA- and EphA2-targeting siRNAs  
45 alone or in combination (7.5  $\mu\text{g}$  siRNA/mouse). Tumor volume was determined  
46 throughout the experiment by measuring the length (l) and width (w) and tumors  
47 were allowed to grow up to 2  $\text{cm}^3$  to allow metastasis of cancer cells and mice were  
48 sacrificed in accordance with ethical practices.



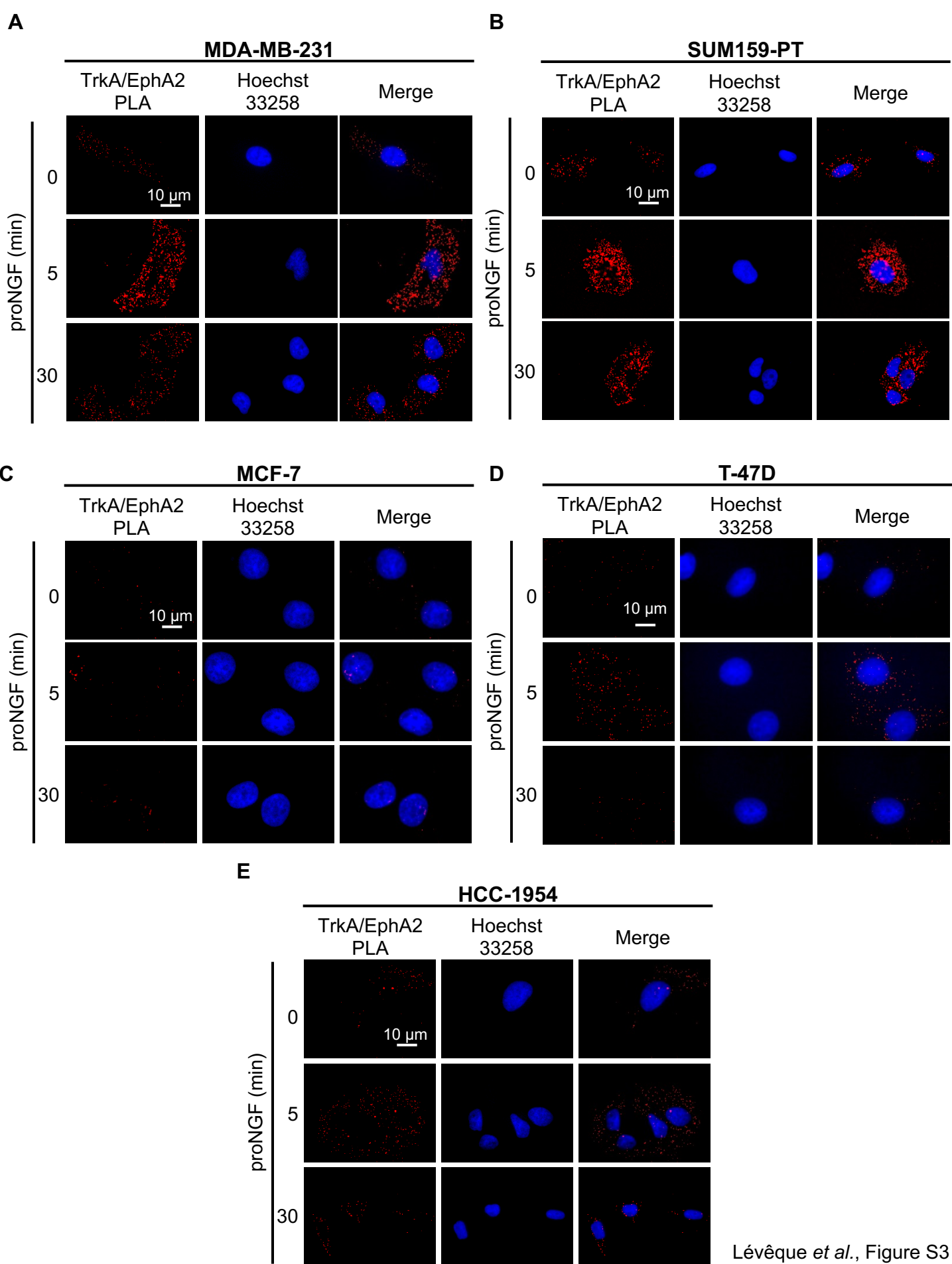
### HA-TrkA MDA-MB-231 cells



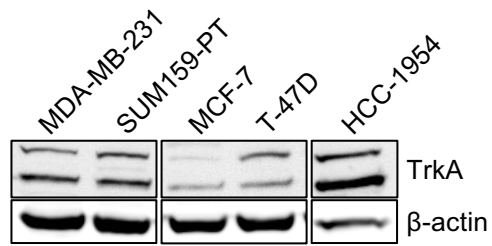
### native MDA-MB-231 cells



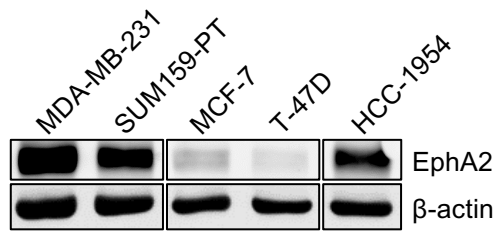


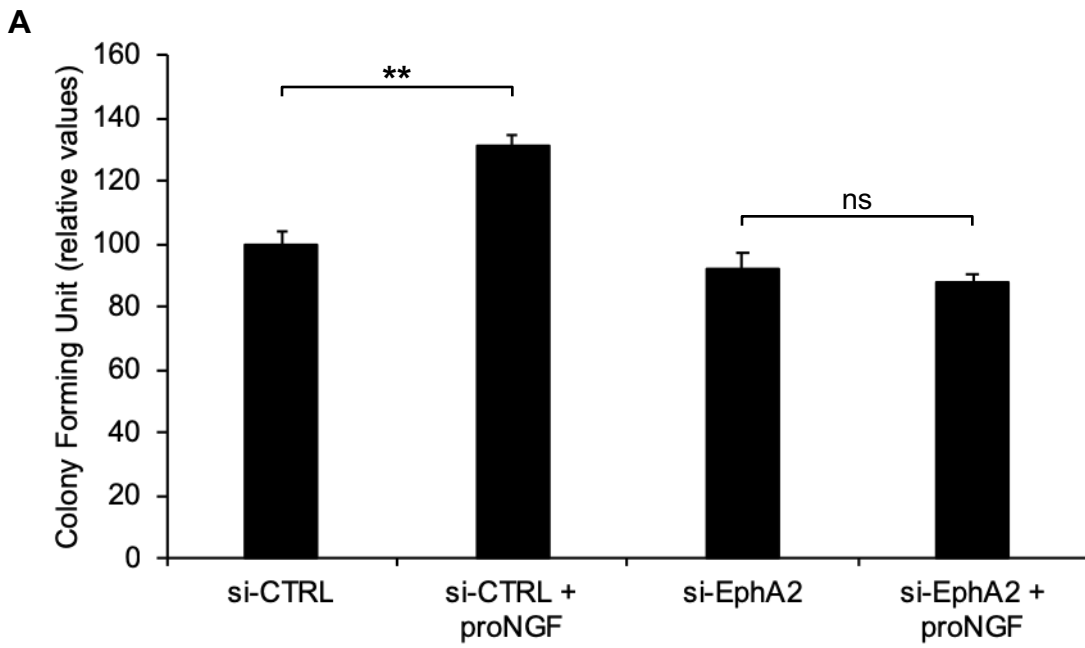


**A**



**B**





**B**

