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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
63 that 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
74 complex cross-talk in terms of receptor interactions and their redundant or
75 diversified downstream signaling partners [1,2]. This underlies the need to better
76 characterize growth factor signaling and RTK cooperation in order to optimize
77 such therapies.

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

94 breast cancer, we previously reported high levels of proNGF are correlated with
95 lymph node invasion [10]. In addition, proNGF stimulates breast cancer cell
96 invasion through sortilin and TrkA receptors, independently of p75^{NTR}, leading to
97 the subsequent activation of Src and Akt signaling pathways [10].
98 Despite the tumor-promoting effects of proNGF, associated signaling pathways
99 still remain fragmentary. Here, we identified EphA2, a membrane receptor tyrosine
100 kinase, as a key element of the proNGF signaling in breast cancer cells. Moreover,
101 increased TrkA/EphA2 proximity ligation assay signals were correlated with a
102 decrease of overall survival in breast cancer patients, further pointing out the
103 importance of TrkA/EphA2 functional association in proNGF-mediated tumor
104 progression. Thus, our results provide a rationale to target proNGF/TrkA/EphA2
105 axis as a promising therapeutic strategy in breast cancer.

106

107 **2. Materials and Methods**

108 2.1. Cell culture

109 All breast cancer cell lines were acquired from the American Type Culture
110 Collection (ATCC) except for SUM159-PT, which is from Asterand Bioscience.
111 MDA-MB-231 breast cancer cells stably overexpressing HA-tagged native TrkA
112 (MDA-MB-231 HA-TrkA) or a kinase-dead TrkA were established as previously
113 described [10]. Cells were maintained in Eagle's Minimal Essential Medium (Life
114 Technologies) (MDA-MB-231 and MCF-7 cells), or RPMI 1640 medium (Life
115 Technologies) (HCC-1954, T-47D) supplemented with 10 % inactivated FBS (Fetal
116 Bovine Serum) (Hyclone), 2 mM L-glutamine, 1 % non-essential amino acids,
117 40 UI/ml penicillin, 40 µg/ml streptomycin, 50 µg/ml gentamycin and ZellShield™
118 (Biovalley) at 37°C in 5 % CO₂-humidified atmosphere. SUM159-PT were grown in
119 Ham's F12 nutrient mix supplemented with 5 % FBS, 10 mM HEPES, 0.1 %
120 insulin, 1 mg/ml hydrocortisone, 40 UI/ml penicillin, 40 µg/ml streptomycin,
121 50 µg/ml gentamycin and ZellShield™ (Biovalley) at 37°C in 5 % CO₂-humidified
122 atmosphere. Before treatment, cells were rinsed twice with PBS, left for 24h in
123 culture medium supplemented with 0.1 % FBS, and then treated with recombinant
124 human non-cleavable proNGF (denoted as proNGF and used at 0.5 nM in all the
125 experiments) (Alomone Labs) or recombinant human mature NGF at 16 nM
126 (Alomone labs). For some experiments, cells were pre-incubated for 1h with the
127 TrkA pharmacological inhibitor K252a (10 nM, Calbiochem) or neurotensin (1 µM,
128 R&D systems).

129 2.2. Transfection

130 Tumor cells were transfected with 2 nM siRNA using INTERFERin™ transfection
131 reagent (Polyplus transfection) following the manufacturer's instructions. The

132 siRNA sequences used against EphA2 were:
133 GCAAGGAAGUGGUACUGCUGGACUU (from Eurogentec) or
134 GCGUAUCUUCAUUGAGCUCAA [14] compared to a control siRNA sequence
135 (siGFP) GAUGAACUUCAGGGUCAGCTT. For TrkA, a pool of three siRNA
136 sequences (Eurogentec) was used: GAACCUGACUGAGCUCUAC,
137 UGGAGUCUCUCUCCUGGAA and GCUGCAGUGUCAUGGGCAA.

138 2.3. Immunoprecipitation and Western blot analysis

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

148 2.4. Nano-LC-MS/MS Q-Star analysis

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

156 equipped with a nano-electrospray ion source coupled with a nano high pressure
157 liquid chromatography system (LC Packings Dionex) as previously described [16].
158 Identified proteins were classified by Panther software (<http://www.pantherdb.org>).

159 2.5. *In situ* proximity ligation assay (PLA)

160 Cells grown on acid-washed eight-well glass slides (10^4 cells per well) (Thermo
161 Scientific) in appropriate medium with 5 or 10 % FBS for 24h. After treatment,
162 paraformaldehyde-fixed cells were incubated with 4 % BSA (1h, 20°C) followed by
163 overnight incubation with primary antibodies [mouse anti-HA, 1:50 (Covance); goat
164 anti-sortilin, 1:50 (R&D systems); rabbit anti-EphA2, 1:100 (Cell Signaling
165 Technology), mouse anti-TrkA, 1:50 (Alomone Labs)]. PLA was performed as
166 recommended by manufacturer's instructions. Briefly, slides were incubated with
167 secondary antibodies complexed with complementary nucleotide sequences for
168 2h. The formed DNA circle, resulting from complementary nucleotides was then
169 amplified using fluorescent oligonucleotides. Nuclei were counterstained with
170 Hoechst 33258 (Sigma-Aldrich) and samples were mounted with fluorescence
171 mounting medium (Dako). PLA images (fluorescent red dots) were acquired using
172 a fluorescence microscope (100X oil immersion objective, $\lambda_{excitation}$: 562 nm,
173 $\lambda_{emission}$: 624 nm, microscope Eclipse Ti; Nikon) and analyzed with NIS-Elements
174 BR software (Nikon) and Image J.

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

181 complementary nucleotide sequences for 2h. The formed DNA circle, resulting
182 from complementary nucleotides was then amplified using oligonucleotides
183 labeled with horseradish peroxidase. PLA signal was evaluated according to the
184 number of dots per cell and the number of stained cells in a double-blind analysis
185 (by TRA and DJ). PLA signal <2 dots/cell was considered as low, PLA signal
186 between 2-10 dots/cell was considered as medium, PLA signal >10 dots/cell was
187 considered as high. Kaplan-Meier curves were obtained by using GraphPad Prism
188 software.

189 2.6. Clonogenic cell growth

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

194 2.7. *In vivo* experiments

195 All the experiments involving mice received the approval of the local ethic
196 committee and were carried out according to French national animal care
197 regulations. MDA-MB-231 HA-TrkA cells (3×10^6) were subcutaneously injected
198 into six-week old female SCID mice. Two weeks after cell injection, mice were
199 randomized into four groups (n=7), and were treated a total of three times at 3-day
200 intervals. Lestaurtinib (CEP-701; Calbiochem) was suspended in vehicle (40%
201 polyethylene glycol 1000, 10% povidone C30 and 2% benzyl alcohol in distilled
202 water) and injected intraperitoneally (10 mg/kg). siEphA2 (Eurogentec; 7.5
203 $\mu\text{g}/\text{mouse}$) was delivered using *in vivo* jetPEI[®] according to the manufacturer's
204 instructions (Polyplus transfection) and injected subcutaneously near the tumor

205 mass. Tumor volume was determined throughout the experiment by measuring the
206 length (l) and width (w) and then calculated as $\pi/6 \times l \times w \times (l+w)/2$.

207 For analyses of breast cancer cell dissemination in mice, xenograft experiments
208 were conducted using MDA-MB-231 HA-TrkA cells. The tumors were allowed to
209 develop for 14 days and the mice were then submitted to 5 injections (every 3
210 days) of either scrambled siRNA, or TrkA- and EphA2-targeting siRNAs alone or in
211 combination (7.5 μ g siRNA/mouse). Tumor volume was determined throughout the
212 experiment by measuring the length (l) and width (w) and tumors were allowed to
213 grow up to 2 cm³ to allow metastasis of cancer cells. After animal sacrifice, lungs,
214 liver and brain were collected and detection of cancer cells in those organs was
215 carried out by evaluating human microglobulin mRNA expression by RT-PCR as
216 previously described [17].

217 2.8. Statistical analysis

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

221

222 **3. Results**

223 3.1. TrkA is associated with sortilin and EphA2 in proNGF-treated MDA-MB-231 224 breast cancer cells

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

248 adducin, MARCKS (*Myristoylated alanine-rich C-kinase substrate*) gelsolin, and
249 integrins $\alpha_3\beta_1$ are downstream targets of Src and p130 Cas, which are associated
250 with cytoskeleton remodeling and cell migration [23–28].

251 To confirm proNGF-induced TrkA association with sortilin and EphA2 in breast
252 cancer cells, we further performed immunoprecipitation assays by using antibodies
253 against HA (-TrkA), sortilin and EphA2, followed by Western blot analysis (Figure
254 2A). In the absence of proNGF, none of the three receptors was found to be co-
255 immunoprecipitated. Upon proNGF treatment, sortilin and EphA2 were co-
256 immunoprecipitated with TrkA. These results were confirmed by reverse co-
257 immunoprecipitation of sortilin and EphA2 (Figure 2A). Of note, in the presence of
258 NGF, sortilin binding to TrkA was detected after 30 min of treatment but EphA2
259 binding was not observed after neither 5 min nor 30 min of treatment (Figure 2A).
260 Thus, NGF seemed to induce a late TrkA/sortilin association, suggesting that
261 sortilin acts as an endocytic receptor, as previously reported in neuronal cell
262 models [8]. Proximity ligation assays (PLA) were then carried out to confirm any
263 receptor association (distance <40 nm) at the plasma membrane. In the absence
264 of exogenous proNGF stimulation, a basal PLA signal (red dots) was observed for
265 sortilin/TrkA (Figure 2B-C) and TrkA/EphA2 (Figure 2D-E). ProNGF stimulation (5
266 min) enhanced PLA signals of sortilin/TrkA and EphA2/TrkA (Figure 2C and 2E);
267 this increase was transient as membrane PLA signals decreased to basal levels
268 after 30 min of proNGF treatment.

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

272 3.2. Sequential sortilin/TrkA/EphA2 association induces TrkA-dependent Akt
273 phosphorylation and EphA2-dependent Src phosphorylation

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

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

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

291 As we have previously shown that proNGF stimulates breast cancer cell invasion
292 through TrkA activation, we asked if EphA2 could be involved in TrkA-mediated
293 cell invasion. When EphA2 expression was inhibited by siRNA approach, proNGF
294 was no longer able to stimulate cell invasion both in native and HA-TrkA over-
295 expressing MDA-MB-231 cells (Figure S2), implying that EphA2 was necessary for
296 proNGF-stimulated and TrkA-mediated cell invasion. ProNGF-stimulated invasion

297 implicates the activation of downstream signaling pathways including Akt and Src
298 [10]. On the other hand, Src is also a downstream effector of EphA2 [19], we then
299 determined the respective role of TrkA and EphA2 in Akt and Src activation by
300 using a kinase-dead mutant of TrkA (Figure 3F) or siEphA2 approach (Figure 3G).
301 Following proNGF treatment, Akt was not phosphorylated in cells expressing a
302 kinase-dead mutant of TrkA while Src phosphorylation was maintained (Figure
303 3F), indicating that Akt phosphorylation depended upon TrkA phosphorylation
304 while Src phosphorylation did not. By contrary, in cells transfected with siEphA2,
305 proNGF still induced Akt phosphorylation but not that of Src, indicating that Akt
306 phosphorylation did not depend on EphA2 while Src activation did (Figure 3G).
307 Collectively, our data showed that, in MDA-MB-231 breast cancer cells, proNGF
308 binding to sortilin induced functional sortilin/TrkA/EphA2 association, leading to
309 TrkA-dependent Akt phosphorylation and EphA2-dependent Src phosphorylation
310 (Figure 3H).

311

312 3.3. ProNGF increases breast cell clonogenic growth through functional
313 association of TrkA with EphA2

314 In order to determine the relevance of TrkA/EphA2 functional association in breast
315 cancer cells, we further extended our study on a panel of representative breast
316 cancer cell lines including basal-like (wild type MDA-MB-231, SUM159-PT),
317 luminal-like (MCF-7, T-47D), and HER2-overexpressing basal-like cell line HCC-
318 1954. PLA signals of TrkA/EphA2 were increased after 5 min of stimulation with
319 proNGF in all cell lines tested, except for MCF-7 (Figures 4A and S3). Clonogenic
320 assays were then carried out to evaluate the impact of EphA2 invalidation on
321 proNGF-stimulated cell growth. As shown in Figure 4B, proNGF stimulated
322 clonogenic cell growth in all the cell lines tested; siEphA2 totally abolished

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

336

337 3.4 Simultaneous targeting of TrkA and EphA2 reduces tumor growth and brain 338 metastasis *in vivo*

339 Given the above results indicating the importance of proNGF-induced TrkA/EphA2
340 association in breast cancer cells, we determined the potential benefit of a
341 combinatorial targeting of TrkA and EphA2 in xenograft mouse model. As shown in
342 Figure 5A and B, CEP-701 (clinical derivative of K252a) alone did not significantly
343 reduce tumor growth, while siEphA2 alone delayed tumor growth when compared
344 to the control group (scrambled siRNA). Combined treatment of CEP-701 and
345 siEphA2 resulted in a dramatic reduction of tumor burden when compared to CEP-
346 701 or siEphA2 treatment alone. We then evaluated the impact of TrkA and/or
347 EphA2 invalidation on breast cancer cell dissemination in different organs

348 including lungs, liver and brain (Figure 5C). In these conditions, we first confirmed
349 that combined inhibition of TrkA and EphA2 inhibited tumor growth as the median
350 survival of the mice was increased: Median of survival were 40 days in control
351 group, 53 days in siTrkA group, 49 days in siEphA2 group and 57.5 days in siTrkA
352 and siEphA2 mice (Figure S6). We found that tumor cells disseminated readily in
353 these three distant organs. Lung metastasis was not modified under any
354 invalidation condition. Interestingly, TrkA invalidation alone was sufficient to
355 reduce liver metastasis while combined inhibition of TrkA and EphA2 was required
356 to decrease brain metastasis. These results indicated that simultaneous inhibition
357 of TrkA and EphA2 was not only efficient in inhibiting primary tumor growth, but
358 also in reducing brain metastasis formation.

359

360 3.5. ProNGF-induced TrkA/EphA2 association is correlated with poor prognosis in
361 breast cancer

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

371

372 **4. Discussion**

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

383 In breast cancer cells, we previously observed that uncleavable proNGF induces
384 sortilin recruitment at plasma membrane and TrkA activation, leading to increased
385 cell invasion, independently of p75^{NTR} [10]. Herein, by studying TrkA-interacting
386 proteins upon proNGF stimulation, we identified EphA2, a membrane receptor
387 tyrosine kinase, as a key element of proNGF signaling in breast cancer cells.
388 ProNGF signaling through sortilin and TrkA allowed for EphA2 recruitment, which
389 in turn activated Src in a TrkA phosphorylation-independent manner.

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

397 that EphA2 phosphorylation by Akt or Rsk can lead to its activation [35,36]. Here,
398 we observed that in the context of proNGF stimulation, EphA2 activated Src *via* an
399 Akt-phosphorylation independent mechanism. Moreover, our proteomic analysis
400 revealed that several Src-associated signaling proteins like cortactin and p130 Cas
401 were pulled-down with TrkA upon proNGF treatment, suggesting that proNGF
402 could activate a signaling cascade involving Src/p130 Cas/cortactin complex.
403 Although further study should be done to confirm this hypothesis, the
404 Src/p130 Cas/cortactin complex is already reported to induce cell invasion [37].
405 Song *et al.* [14] have recently reported that EphA2 is overexpressed in the basal-
406 like breast cancer molecular subtype and this overexpression is correlated with
407 poor recurrence-free survival in triple-negative breast cancers. Loss of EphA2
408 function in both human and genetically engineered mouse models of triple
409 negative breast cancers reduced tumor growth. Herein, we observed that EphA2
410 silencing inhibited proNGF-stimulated clonogenic cell growth of not only triple
411 negative, but also luminal ER-positive and HER2-positive breast cancer cell lines.
412 This implies that EphA2 is involved in proNGF-stimulated clonogenic cell growth,
413 independently of molecular subtypes. Consistently, we could not find any
414 significant correlation between TrkA/EphA2 complex or co-localization and
415 different subtypes of breast cancer. Of importance, the level of TrkA/EphA2 co-
416 localization is significantly correlated with poor overall survival of patients suffering
417 from breast cancer regardless the cancer subtype, suggesting the potential
418 involvement of proNGF/TrkA/EphA2 axis in breast cancer progression whatever
419 the cancer subtype. These findings reinforced our previous results showing a
420 significant correlation between the expression of proNGF and lymph node invasion
421 [10].

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

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

439

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444 de Cavel, M T Chassat).

445

446 **Conflicts of interest**

447 All authors declare no conflict of interest

448

449 **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
Estrogen receptor	Positive (n=109)	1.725±0.09478 p= 0.1408	1.817±0.1011 p=0.2155	2.193±0.1169 p=0.1608
	Negative (n=71)	1.944±0.1115	2.014±0.1212	1.915±0.1615
Progesterone receptor	Positive (n=95)	1.663±0.09996 p=0.0318	1.758±0.1097 p=0.0536	2.053±0.1201 p=0.5557
	Negative (n=84)	1.976±0.1044	2.060±0.1089	2.141±0.1541
HER-2	Positive (n=69)	1.783±0.1254 p=0.8799	1.884±0.1248 p=0.9606	2.217±0.1650 p=0.2717
	Negative (n=113)	1.805±0.08876	1.876±0.09988	2.000±0.1166
Triple negative	Positive (n=34)	2.088±0.1544 p=0.0578	2.176±0.1661 p=0.0715	1.765±0.2315 p=0.1053
	Negative (n=147)	1.735±0.08153	1.816±0.08743	2.163±0.1049
Lymph node invasion	Positive (n=116)	1.724±0.09372 p=0.1166	1.897±0.09976 p=0.9171	2.138±0.1193 p=0.2127
	Negative (n=69)	1.957±0.1081	1.913±0.1181	1.899±0.1193

624

625 **FIGURE LEGENDS**

626

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

628 **association and downstream signaling pathways proteins. (A)** MDA-MB-231

629 HA-TrkA cells were treated in absence or presence of non-cleavable proNGF

630 (0.5nM) for 5 and 30 min. Total cell lysates were subjected to HA

631 immunoprecipitation and separated in 10% SDS-PAGE. Proteins were revealed by

632 colloidal blue Coomassie staining. Using transilluminator, intensity of bands was

633 appreciated by CC and R-A T and bands with stronger intensity than that of control

634 were cut (**red square**). Protein peptide digests were then subjected to mass

635 spectrometry analysis. **(B)** Identified proteins were analyzed for Biological

636 pathways using Panther software. **(C)** Mass spectrometry identification of

637 selected putative interacting partners of TrkA under proNGF stimulation (5 or 30

638 min). For each identified protein, the Uniprot ID, the number and sequence of the

639 different peptides allowing protein identification and the individual peptide Mascot

640 score are summarized. Underlined amino-acids (C and M) are oxidized residues.

641 MW: Molecular Weight. GO: Gene Ontology.

642

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

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

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

646 proNGF treatment. **(B-E)** Representative pictures (B and D) and quantification (C

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

648 TrkA MDA-MB-231 cells after non-cleavable proNGF treatment. PLA signals were

649 quantified on three independent experiments. In (C) and (E), data are expressed

650 as scatter plots. ***p<0.001; ns, not significant.

651

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

654 **(A-B)** Representative immunoblotting for sortilin, EphA2 and TrkA after IP anti-HA
655 (TrkA) following treatment with 1 μ M neurotensin (A) or after IP anti-sortilin in cells
656 transfected with TrkA-targeting siRNA (B). **(C)** Representative pictures of
657 sortilin/EphA2 PLA. **(D-E)** Representative immunoblotting for sortilin, EphA2 and
658 TrkA after IP anti-HA in HA-TrkA MDA-MB-231 cells expressing a kinase-dead
659 TrkA mutant (D) or transfected with EphA2-targeting siRNA (E). In (D), TrkA
660 phosphorylation in the kinase domain (Y674/675 residues) was also evaluated by
661 immunoblotting. **(F-G)** Representative immunoblotting for the phosphorylated and
662 total forms of Akt and Src in non-cleavable proNGF-stimulated HA-TrkA MDA-MB-
663 231 cells expressing a kinase-dead TrkA mutant (F) or cells transfected with
664 EphA2-targeting siRNA (G). Immunoprecipitations and immunoblots were carried
665 out at least 2 times with similar results. **(H)** Putative dynamic of proNGF-induced
666 sortilin/TrkA/EphA2 signaling in breast cancer cells. After proNGF binding to
667 sortilin (1), TrkA is phosphorylated allowing Akt activation (2). Additionally, EphA2
668 is also recruited to the sortilin/TrkA complex in a TrkA kinase-independent manner,
669 leading to Src activation (3).

670

671 **Figure 4: TrkA/EphA2 complex was involved in proNGF-stimulated**
672 **clonogenic cell growth. (A)** PLA of TrkA/EphA2 complex in wild-type MDA-MB-
673 231, SUM159-PT, MCF-7, T-47D and HCC-1954 breast cancer cells following
674 proNGF treatment (5 and 30 min). **(B)** Clonogenic cell growth following proNGF
675 treatment (5 and 30 min) and transfection of EphA2-targeting siRNA. **(C)**

676 Clonogenic cell growth of MDA-MB-231 cells following treatment with 10 nM
677 K252a and/or transfection with EphA2-targeting siRNA. Data are expressed as
678 scatter plots (A) or means \pm SEM (B-C). ** $p < 0.01$; *** $p < 0.001$; ns, not significant.

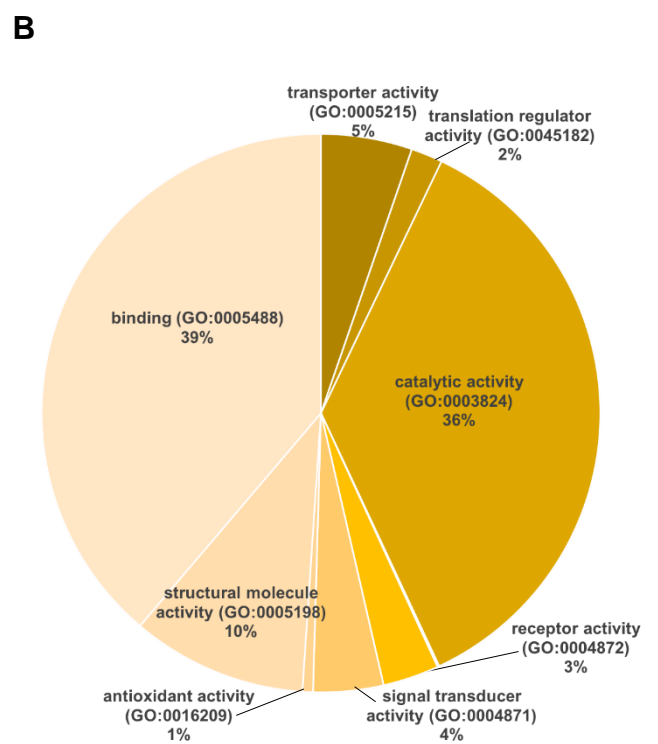
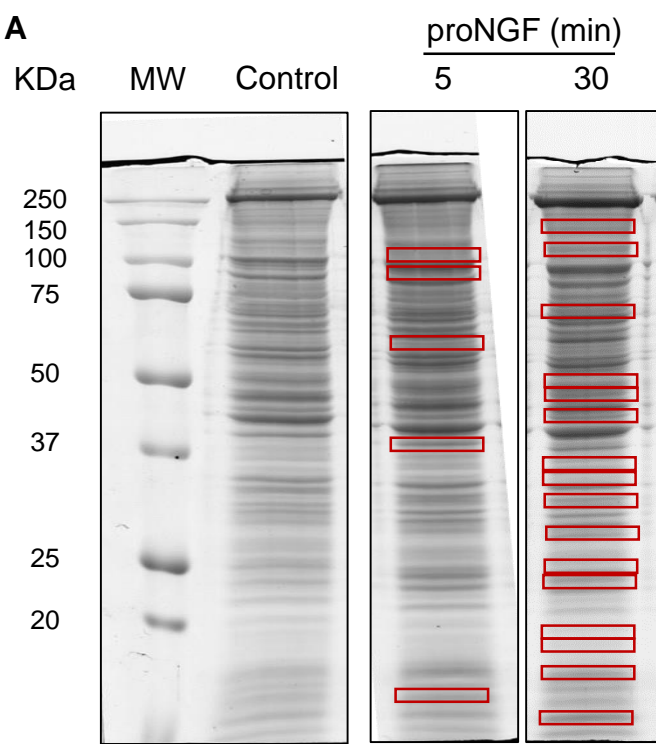
679

680 **Figure 5: Combinatorial treatment with TrkA- and EphA2-targeting modalities**
681 **delays primary tumor growth and metastasis formation. (A-B)** Tumor growth
682 of MDA-MB-231 xenografts in SCID mice submitted to 3 injections (every 3 days;
683 black arrows) of either *in vivo* JetPEI + control or EphA2-targeting siRNA (7.5
684 $\mu\text{g}/\text{mouse}$), or CEP-701 (10 mg/kg) alone or in combination. Tumor volumes were
685 measured at different intervals (A) and represented as scatter plots at the end of
686 the experiment (B). **(C)** Detection of metastatic human breast cancer cells (as
687 determined by RT-PCR for the expression of the human microglobulin) in different
688 organs (lungs, liver and brain) of MDA-MB-231 xenograft-bearing mice submitted
689 to 5 injections (every 3 days; black arrows) of either *in vivo* JetPEI + control or
690 EphA2-targeting siRNA (7.5 $\mu\text{g}/\text{mouse}$), or CEP-701 (10 mg/kg) alone or in
691 combination. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns: not significant. Data are
692 expressed as means \pm SEM (A) or scatter plots (B).

693

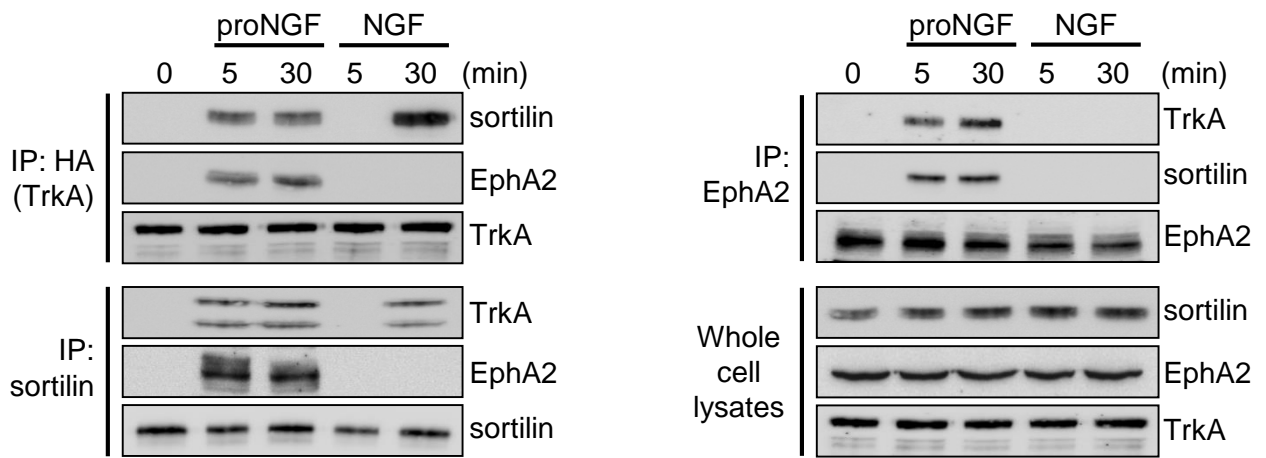
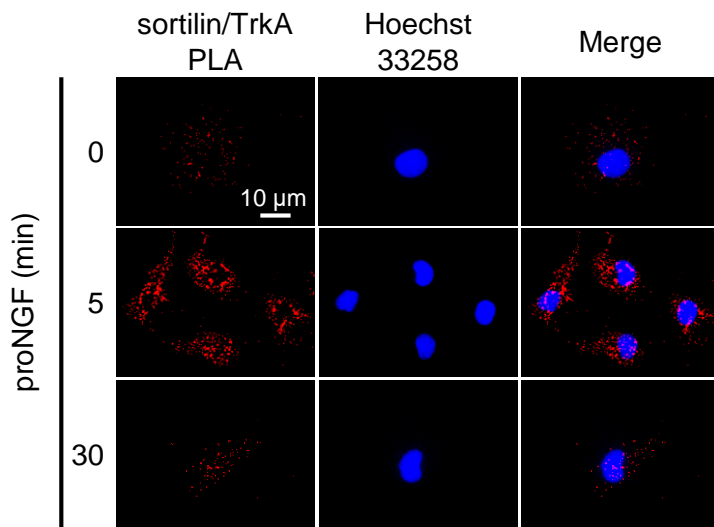
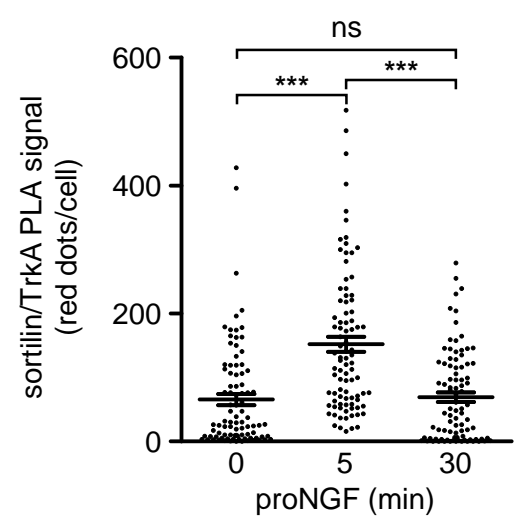
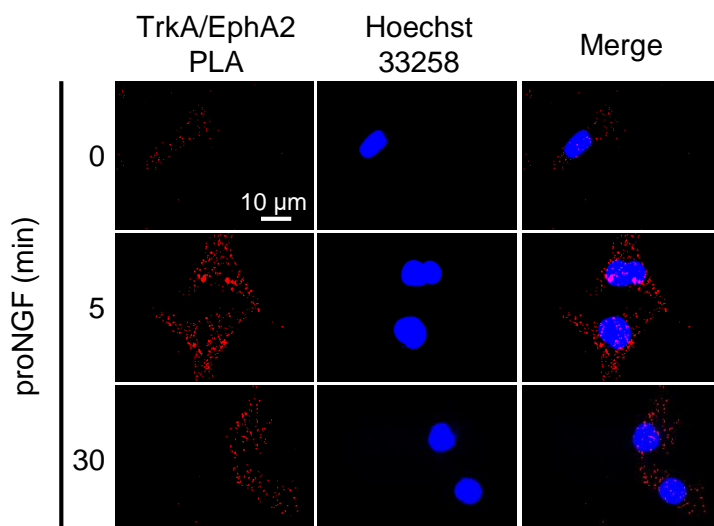
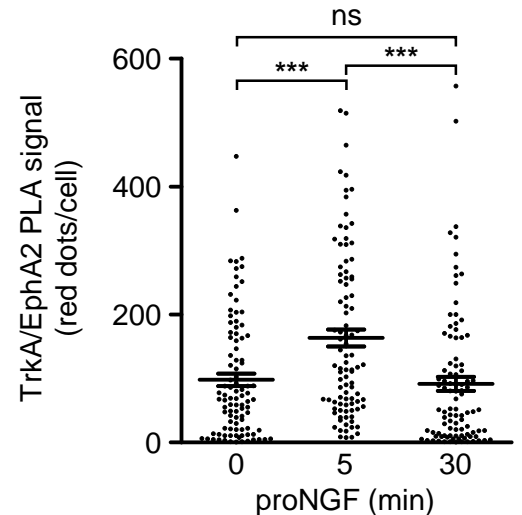
694 **Figure 6: TrkA/EphA2 complex expression is associated with overall survival**
695 **(OS) decrease for breast cancer patients. (A-C)** Representative pictures for
696 TrkA immunostaining in breast tumors, defined as low (A), medium (B) or high (C).
697 **(D)** Kaplan-Meier OS curves in breast cancer patients according to TrkA staining.
698 **(E-G)** Representative pictures for EphA2 immunostaining in breast tumors, defined
699 as low (E), medium (F) or high (G). **(H)** Kaplan-Meier OS curves in breast cancer
700 patients according to EphA2 staining. **(I-K)** Representative PLA images depicting

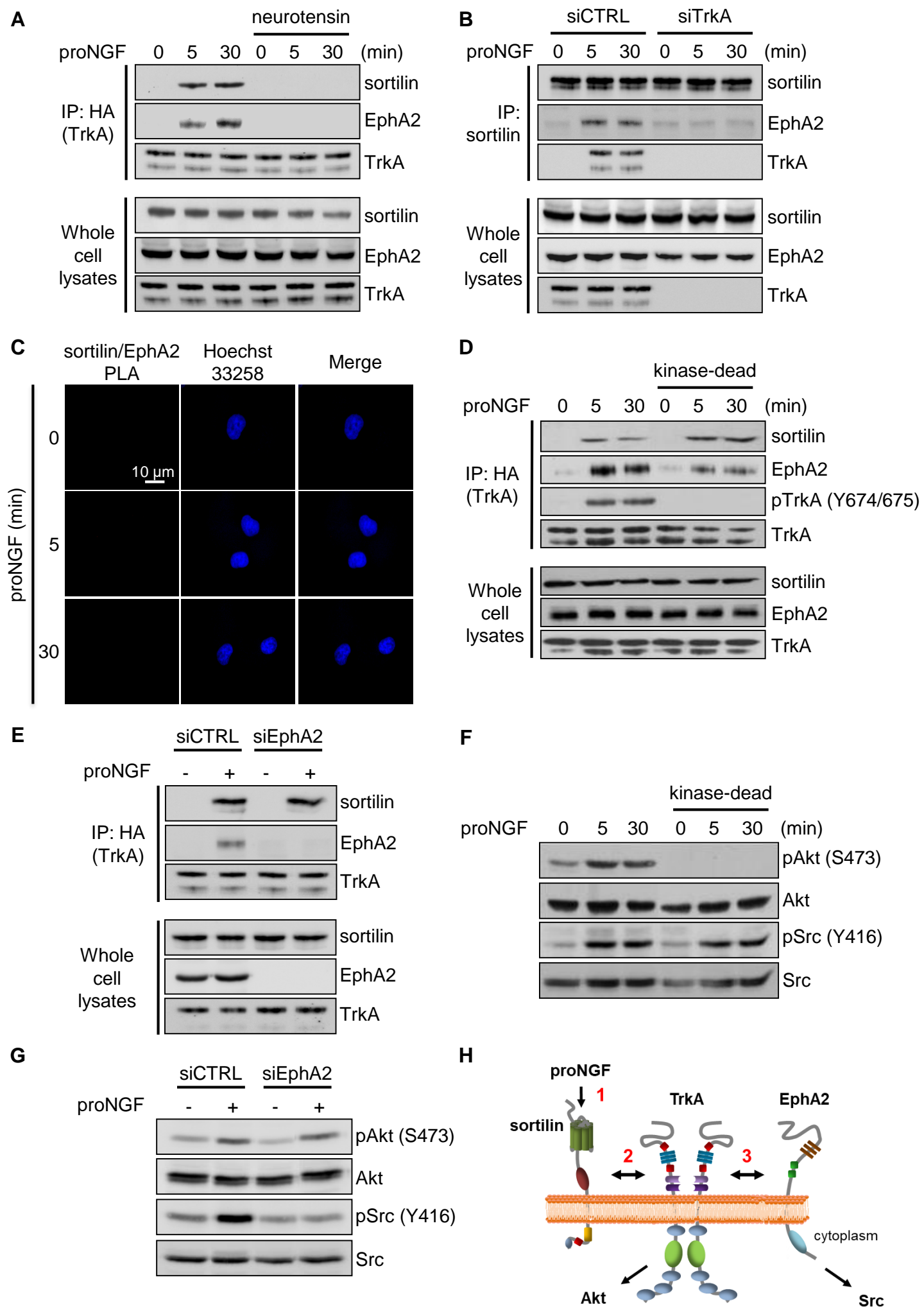
701 low (I), medium (J) and high staining (K) for TrkA/EphA2 complex on patient breast
702 tumor samples. (L) Kaplan-Meier OS curves in breast cancer patients according to
703 TrkA/EphA2 complex abundance.

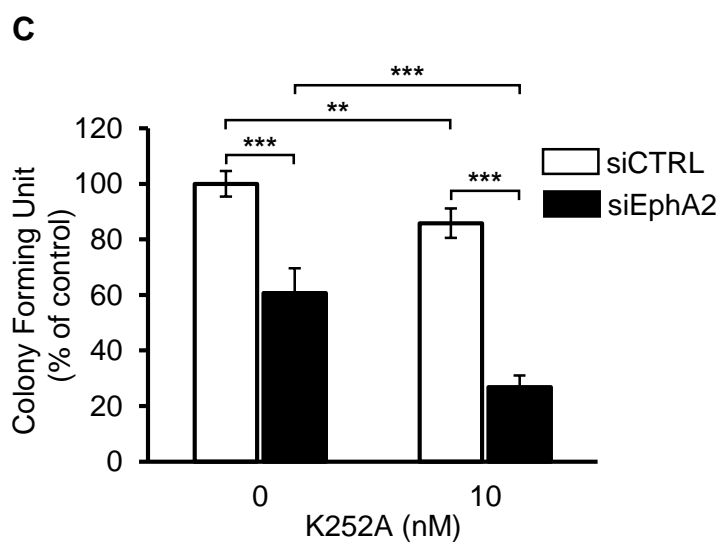
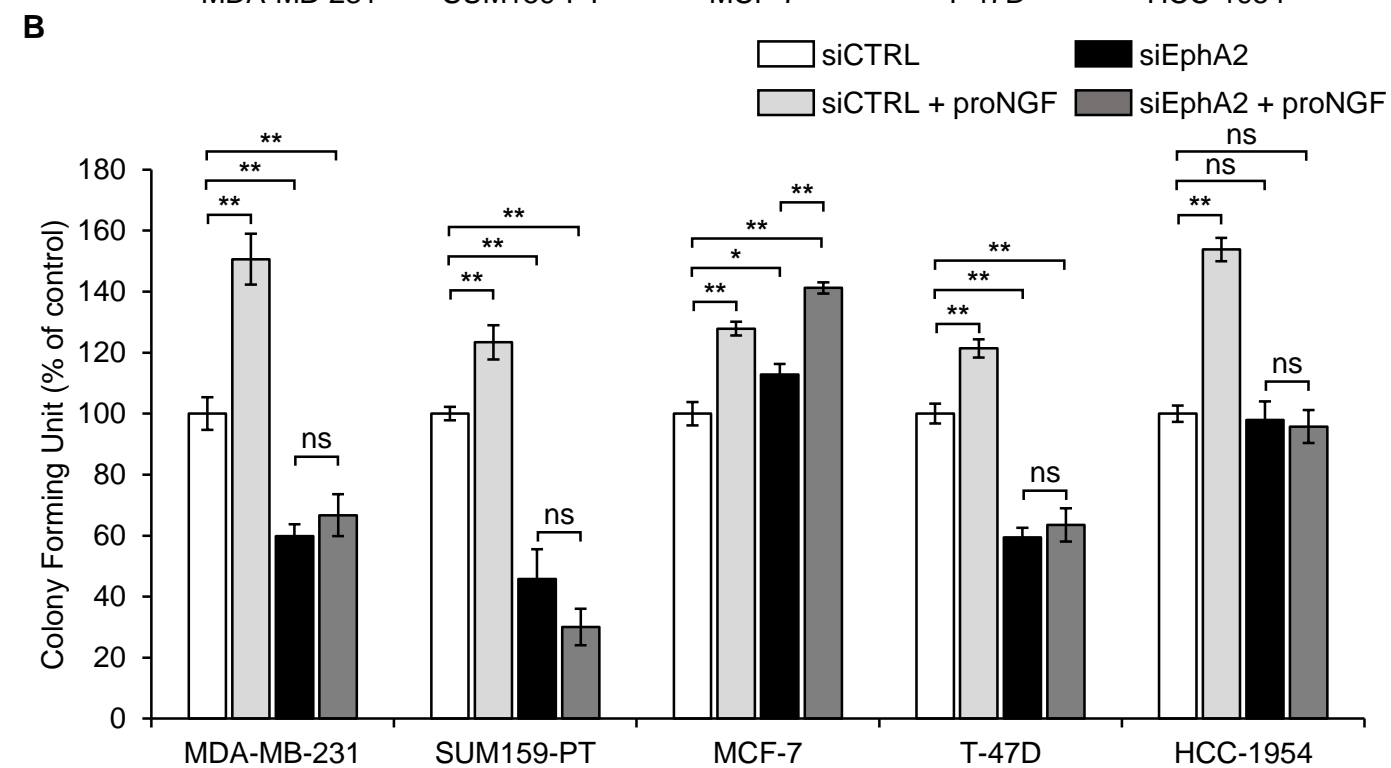
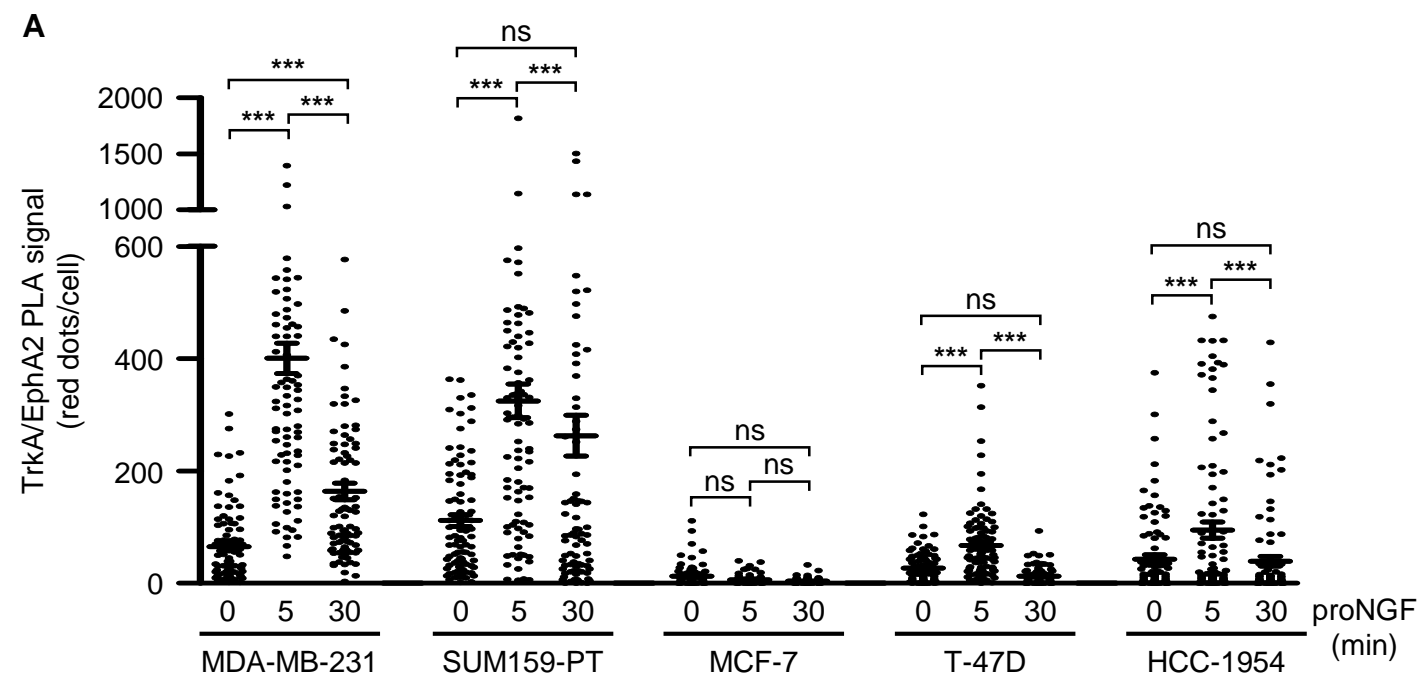


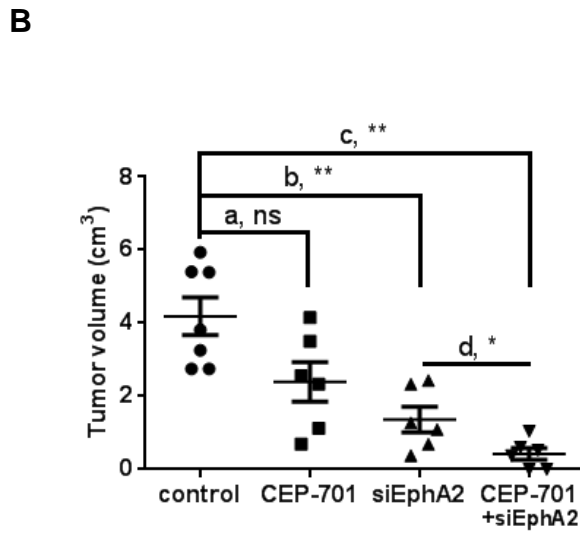
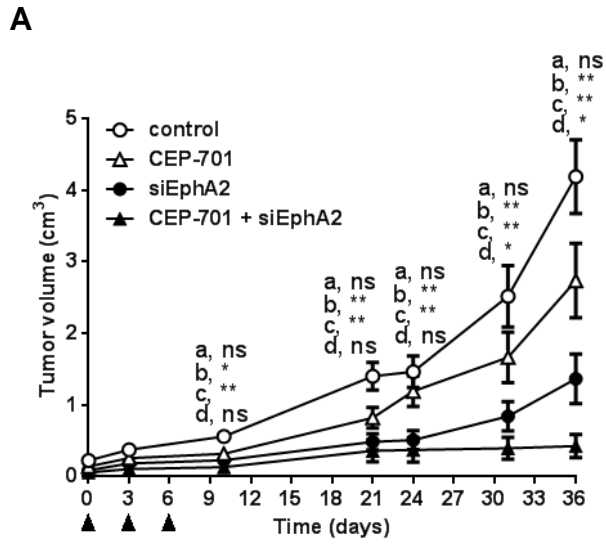
C

Protein name	Uniprot ID	Peptide sequences	Mascot score
Sortilin	Q99523	IYSFGLGGR	31
EphA2	P29317	FADIVSILDK	45
		TVSEWLESIK	25
Src	P12931	AANILVGENLVCK	54
Cortactin	Q14247	VDQSAVGFEYQGGK	44
		YGLFPANYVELR	84
p130 Cas	P56945	LVFIGDTLSR	33
		ATAPGPEGGGTLHPNPTDK	20
SHEP1	Q8N5H7	TEGTK	30
		LDLLER	39
PTP-PEST	Q05209	TLLLEFQNESR	55
RIL	P50479	GYFFLDER	46
		DFSAPLTISR	25
		VKPPEGYDVVAVYRNAK	17
Lasp1	Q14847	GFSVVADTPELQR	60
SNAP23	O00161	ILGLAIESQDAGIK	76
FHL2	Q14192	CSLSLVGR	59
		YISFEER	43
		NSLVDKPFNAK	24
		CAGCTNPISGLGGTK	26
		GSSWHETCFIHR	54
		DDFAYCLNCFDLYAK	61
		EDQLLCTDCYSNEYSSK	29
HAX1	O00165	IFGGVLESDAR	38
adducin	P35611	INLQGDIVDR	49
MARCKS	P29966	LSGFSEK	31
		GEPAAAAAPEAGASPVK	26
		EAPAEGEAAEPGSPTAAEGEA ASAASSTSSPK	86
Gelsolin	P06396	AGALNSNDAFVLK	41
Integrin alpha-3	P26006	YLLLAGAPR	23
		TVEDVGSPLK	34
		LELLMDNLR	26
		LELLMDNLR	41
		LELLMDNLR	27
		EAGNPGSLFGYSVALHR	27
Integrin beta-1	P05556	IGFGSFVEK	64
		LLVFSTDAGHFHAGDGK	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

