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ABSTRACT

Neuropilin-1 (NRP1) is a co-receptor for multiple extracellular ligands. NRP1 is widely expressed in cancer cells and in advanced human tumors; however, its functional relevance and signaling mechanisms are unclear. Here we show that NRP1 expression controls viability and proliferation of different cancer cells, independent of its short intracellular tail. We found that the extracellular domain of NRP1 interacts with the Epidermal Growth Factor Receptor (EGFR) and promotes its signaling cascade elicited upon EGF or TGF alpha stimulation. Upon NRP1 silencing, the ability of ligand-bound EGFR to cluster on the cell surface, internalize and activate the downstream AKT pathway is severely impaired. EGFR is frequently activated in human tumors due to overexpression, mutation, or sustained autocrine/paracrine stimulation. Here we show that NRP1-blocking antibodies and NRP1 silencing can counteract ligand-induced EGFR activation in cancer cells. Thus our findings unveil a novel molecular mechanism by which NRP1 can control EGFR signaling and tumor growth.

INTRODUCTION

Neuropilin-1 (Nrp1) and Neuropilin-2 (Nrp2) form a small family of conserved and widely-expressed transmembrane proteins, originally implicated in the regulation of axon guidance and vascular development (1,2). The extracellular portion of neuropilins mediates protein-protein interactions; in particular, "a" and "b" domains are known to interact with secreted class 3 semaphorins and vascular endothelial growth factors (VEGF) (3,4), while the "c" (MAM) domain mediates neuropilin homo- and hetero-dimerization (5) (see Figure 1A). The role of the short cytoplasmic tail of neuropilins is poorly understood, and its relevance is still controversial (6-8).

Beside embryonal development, neuropilins are widely distributed in the adult tissues, and their levels are often significantly increased in cancer cells and tumor biopsies of various origin, compared to normal counterparts ((9,10); reviewed in (11). In addition, high levels of Nrp1 were significantly associated with poor outcome in patients with colon cancer (9), breast cancer (12) and non-small lung cancer (13), and correlated with invasive behavior and metastatic potential in gastrointestinal carcinoma, glioma and prostate carcinoma (11). Notably, EGF stimulation has been found to induce Nrp1 expression in tumor cells (9,14,15). Moreover, Nrp1 expression was upregulated in epithelial cells upon neoplastic transformation driven by constitutive activation of the Ras pathway (16). Nrp1 overexpression in advanced tumors may suggest a link with the acquisition of a functional advantage at the cellular level. Yet, experimental data on the role of Nrp1 in cancer cells are contradictory, and the implicated molecular mechanisms have not been elucidated. For example, knocking down Nrp1 expression in carcinoma cells inhibits proliferation, cell survival, and extracellular matrix invasion *in vitro* (13,17);

consistently, other studies indicated that Nrp1 overexpression can inhibit cancer cell apoptosis (18,19). These effects have been often explained by the role of Nrp1 in supporting VEGF signaling (17,19,20). In contrast to these findings, in other studies, an elevated expression of Nrp1 was associated with more favorable prognosis for colon cancer patients (21) and reduced tumor growth in experimental models in mice (22); moreover, a VEGF/Nrp1-dependent pathway suppressing cell viability has been proposed in one specific case (23). These discrepancies are currently unresolved and they might reflect cell-specific responses and/or the involvement of different signaling pathways.

Antibodies and short peptides interfering with Nrp1 function have been shown to inhibit tumor angiogenesis and tumor growth *in vivo* in mice (24-26); these data have validated Nrp1 as a significant target for anti-angiogenic and antitumor agents. Intriguingly, by applying a computational model, it was predicted that a more potent VEGF inhibition may be achieved by using an anti-Nrp1 antibody that does not block ligand binding, but rather interferes with Nrp1 oligomerization (27). This is consistent with previous evidence that deleting the "c"/MAM oligomerization domain impairs neuropilin function (5). Moreover, a synthetic peptide derived from the transmembrane segment of Nrp1 prevents Nrp1 dimerization and oligomerization and blocks glioma growth in mice (28).

In this study, we identified a novel molecular mechanism to account for the acquisition of selective advantage coupled with Neuropilin-1 overexpression in cancer cells. We found that Nrp1 is in complex with EGFR on the cell surface, where it mediates ligand-induced EGFR clustering and endocytosis, leading to intracellular activation of AKT signaling cascade. Notably, upon Nrp1 depletion, EGFR signaling is significantly affected in cancer cells. Our work identifies a new function of Nrp1 in

association with a growth factor receptor, and envisages a putative role of Nrp1 in sustaining EGFR activation in a large fraction of human tumors.

MATERIALS and METHODS

(also see Supplemental material)

Cell lines. American Type Culture Collection (ATCC) provided tested and authenticated cell lines used in our study, which were passaged in our laboratory for fewer than 6 months after resuscitation. Cells were grown in standard medium supplemented with 1% L-glutamine (2mM) and 10% FBS (Sigma).

Gene expression knock down by RNA-interference. Neuropilin expression was silenced by transfecting the following targeted siRNA sequences (chemically synthesized) with Lipofectamine 2000 (Invitrogen) or by using the Amaxa nucleofector kit T (program X-01, Lonza); #1: AGATCGACGTTAGCTCCAA; #2: AACACCTAGTGGAGTGATA; #3: CAATCACGTGCAGGCTCAA (where not specified, siRNA #2 was used). In order to achieve stable gene knock-down for long-term experiments, we transduced cells with lentiviral constructs expressing targeted shRNA. The sequence targeting Nrp1 was previously published (17). Control shRNAs (shC) were generated by introducing four base substitutions in Nrp1 targeting sequence (GATAGGTCATGACTGCCC). These shRNA sequences were inserted in

the lentiviral transfer plasmid pCCLsin.PPT.hPGK.GFP.Wpre under control of the H1 promoter, as previously reported (29). We similarly designed shRNA-expressing lentiviral constructs targeting the transcript of Nrp2 (TTCCAAAGATGCTGCCTAT), and EGFR (GCAGTCTTATCTAACTATGAT; kindly provided by L. Trusolino, IRCC, Candiolo, Italy). PTEN expression was silenced by using Sigma Mission shRNA-expressing lentiviral vector TRCN0000002749. Control empty vector pLKO was from Sigma.

RESULTS

Neuropilin-1 expression regulates tumor cell viability and proliferation

We knocked down the expression of either Nrp-1 or Nrp-2 by RNA interference in multiple different human cancer cells (validation data shown in Suppl. Fig. 1A-B). Proliferation and viability of Nrp2-depleted tumor cells were comparable to control cells treated with a non-targeting shRNA (see Suppl. Fig. 1C-D). In contrast, Nrp1 knock-down resulted in a significant impairment of viability and proliferation in a variety of cancer cell models (Fig. 1B and Suppl. Fig. 1E). The specificity of this effect was validated by applying three distinct siRNA sequences directed against Nrp1 (Suppl. Fig. 1F). Flow cytometry-based detection of the early apoptotic marker Annexin-V revealed a significantly increased number of apoptotic

cells following Nrp1 knock-down (Suppl. Fig. 2A); moreover, the pro-survival PI3K-AKT signaling pathway was strikingly attenuated in these cells compared to controls (Suppl. Fig. 2B). Notably, upon forcing the constitutive activation of AKT by means of PTEN silencing we could partly rescue cell viability in Nrp1-depeleted cells (Suppl. Fig. 2C-D); this implicated AKT signaling in a putative Nrp1-regulated pathway supporting cancer cell survival.

Complementary to gene knock-down experiments, we found that Nrp1 overexpression was sufficient to confer a proliferative advantage to tumor cells and to enhance their viability (Fig. 1C and Suppl. Fig. 3A). Notably, Nrp1 is known to bind VEGF-A, a growth factor especially active in endothelial cells via the tyrosine kinase receptor KDR/VEGF-R2. Although VEGF-R2 is not expressed in most cancer cells (including those used in our experiments), a previous report proposed that VEGF binding to Nrp1 could promote tumor cell survival via poorly defined KDR-independent pathways (19). We have therefore generated and expressed in cancer cells a mutated Nrp1 construct unable to bind VEGF-A_{165} (Nrp1_3mut, see Fig. 1A and Suppl. Fig. 3B). In analogy to the wild type counterpart, Nrp1_3mut promoted cancer cell growth and viability (Fig. 1D and Suppl. Fig. 3C), indicating that VEGF-binding is not implicated in this function. Moreover, cancer cells overexpressing either wild type Nrp1 or its Nrp1-3mut variant formed larger tumors in mice compared to controls (Fig. 1E). Unlike what reported in a previous study (20), Nrp1-overexpressing tumors (either wt or mutant forms) were not characterized by increased vessel density (Suppl. Fig. 3D), indicating that Nrp1 can sustain cancer cell growth in vivo independently of tumor angiogenesis.

Notably, the cytoplasmic domain of Nrp1 is very small, and its functional relevance in cancer cells is unclear. In order to experimentally address this issue, we

overexpressed in tumor cells a truncated secretable form of Nrp1, lacking both transmembrane and cytoplasmic domains (Nrp1ec; Fig. 2A). The isolated extracellular domain of Nrp1 was sufficient to promote tumor cell proliferation and viability (Fig. 2B-C), consistent with the effects mediated by full-length Nrp1. Moreover, Np1ec expression was sufficient to rescue the effect of Nrp1 depletion in cancer cells (Fig. 2D). In keeping with our findings in vitro, we observed a growth advantage of cancer cells overexpressing Nrp1ec transplanted in mice (Suppl. Fig.4A), and this was not accompanied by an increased tumor vessel density (Suppl. Fig. 4B). Altogether, these data suggested that Nrp1 activity promoting cancer cell survival and proliferation is not due to a signaling cascade elicited by its intracellular tail, but rather implicate a function of the extracellular domain of Nrp1, independent from VEGF binding.

Neuropilin-1 and EGFR form a signaling complex on the cell surface

Since Nrp1ec expression was sufficient to induce proliferation and rescue cell viability, we asked about the implicated mechanisms. Interestingly, we found that treating cancer cells with affinity-purified Nrp1ec activated AKT and MAPK signaling cascades (Fig. 3A-B). These effectors have a crucial role in controlling tumor cell survival and proliferation, and are often activated downstream to Receptor Tyrosine Kinases (RTK) expressed on the cell surface. By applying an unbiased screening approach, we found that the extracellular domain of Nrp1 could elicit the phosphorylation of Epidermal Growth Factor Receptor (EGFR)(Fig. 3C). This was further confirmed by western blotting using specific antibodies directed against the major EGFR auto-phosphorylation site P-Tyr_{1068} (Fig. 3D). Pre-treating cells with

Cetuximab (an EGFR-blocking monoclonal antibody) impaired Nrp1ec-induced MAPK and AKT activation (Fig. 3E), indicating pathway specificity; similar results were obtained by applying the small molecule EGFR inhibitor Gefitinib (not shown). These data suggested that the Nrp1-dependent signaling cascade controlling cell growth and survival is largely mediated by EGFR activation. Moreover, upon analysing tissue sections of Nrp1-overexpressing tumors grown in mice (described in Fig. 1E) we found that EGFR tyrosine phosphorylation was significantly increased compared to controls (Suppl. Fig. 5).

Co-immunoprecipitation experiments indicated that the extracellular domain of Nrp1 interacts with EGFR upon overexpression (Fig. 4A). Furthermore, endogenous Nrp1 and EGFR were basally associated in A549 non-small lung cancer cells, and the complex was induced upon stimulation with either EGF or TGFα, two major EGFR ligands found in tumors (Fig. 4B). By confocal microscopy analysis, we found that endogenous Nrp1 and EGFR largely co-localized on the surface of A549 cells (Suppl. Fig. 6A). Moreover, in response to EGF stimulation, a large fraction of Nrp1 was found in intracellular vesicles co-localizing with EGFR (Suppl. Fig. 6A). Based on the co-localization with the Early Endosome Antigen-1 (EEA1; (30) (Suppl. Fig. 6B), these vesicles were identified as early endosomes, a vesicular compartment that typically traffics internalized EGFR (31).

Neuropilin-1 ectodomain regulates EGFR clustering and endocytosis

In order to understand the functional relevance of Nrp1 in EGFR signaling, we first checked EGFR levels on the plasma membrane in control and Nrp1-depleted cells by cell surface biotinylation experiments, and found them to be comparable

(Suppl. Fig. 7A). Moreover, cell surface incubation with fluorescently-labeled EGF revealed that receptor binding was not affected upon Nrp1 knock down (Suppl. Fig. 7B). Ligand-induced activation of EGFR is thought to elicit receptor oligomerization and clustering on the cell surface, followed by internalization by endocytosis (32,33). We therefore applied Total Internal Reflection Fluorescence (TIRF) microscopy to study EGFR distribution at the cell surface. This revealed that, at the steady state, EGFR-signal was dispersed on the plasma membrane and rarely aggregated in small spots (Fig. 4C). In contrast, upon stimulation with physiological concentrations of EGF (2 ng/ml) the receptors clustered in many distinct aggregates at the cell surface (Fig. 4C). Importantly, this ligand-induced effect was barely detectable in Nrp1-depleted cells, which behaved similarly to non-stimulated cells (Fig. 4C), strongly suggesting that Nrp1 plays a role in ligand-engaged EGFR clustering on the cell surface. Consistent results were furthermore obtained assessing EGF- or TGFα-induced EGFR clustering by conventional confocal microscopy (Fig. 4D). Notably, the treatment with soluble extracellular domain of Nrp1 (Nrp1ec) promoted EGFR clustering in tumor cells and was sufficient to rescue the functional defect resulting from endogenous Nrp1 knock down (Fig. 4D). Altogether, these data indicate that the extracellular domain of Nrp1 is required and sufficient to induce EGFR oligomerization and clustering on the cell surface.

Ligand-induced EGFR clustering is followed by its internalization in endocytic vesicles, from where EGFR can sustain prolonged intracellular signals (34-36). Intriguingly, we noticed that ligand-induced EGFR trafficking into EEA1-positive endosomes was impaired in Nrp1-silenced cells, potentially suggesting the requirement of Nrp1 for EGFR endocytosis (Suppl. Fig. 8). In order to track the internalization of activated EGFR, A549 carcinoma cells were ligated with EGF at

+4°C (to put on hold the endocytosis of activated receptors), and then surface biotin-labeled either before or after allowing ligand-induced endocytosis to occur (by shifting the cells at 37°C). Whereas in control cells activated EGFR was efficiently internalized and its levels on the cell surface dramatically decreased, in Nrp1-deficient cells ligand-engaged EGFR largely remained at the cell surface indicating that it was inefficiently internalized (Fig. 5A). By immunofluorescence experiments, we tracked EGFR trafficking in response to EGF or TGFα; unlike in control cells, in Nrp1-depleted cells ligated receptors remain on the cell surface and their internalization is impaired (Fig. 5B and Suppl. Fig. 9A-C). Further experiments applying fluorescent-labeled EGF to track the internalization of receptor complexes (Fig. 5C) demonstrated the requirement of Nrp1 expression for this function. Notably, Nrp1-deficient cells did not show any defect in the uptake mediated by Transferrin receptor (TfR), a cargo receptor which is continuously internalized from the cell surface (Suppl. Fig. 10); this indicated that Nrp1 is not part of the basic molecular machinery mediating receptor endocytosis, but it specifically affects the endocytosis of selected molecules, such as EGFR.

We found that the differential internalization of EGF-EGFR complexes in control and Nrp1-depleted cells was unchanged upon treatment with the catalytic inhibitor Erlotinib (selectively blocking EGFR autophosphorylation) (Suppl. Fig. 11A), or with the tyrosine phosphatase inhibitor sodium orthovanadate (preventing phosphorylation turnover)(Suppl. Fig. 11B), strongly suggesting that Nrp1 can control ligand-induced EGFR internalization even independently from its tyrosine phosphorylation. Thus, to elucidate the functional role of specific Nrp1 domains for EGFR internalization, we reconstituted its expression in silenced cells by ectopically transfecting either non-targetable full-length Nrp1 or two different mutants of the

intracellular domain, lacking either the C-terminal -SEA_{cooh} sequence (Nrp1_ΔSEA) or the entire cytoplasmic domain (Nrp1_Δcyto). Re-expression of full-length Nrp1, as well as of either of the above mutants (shown in Suppl. Fig. 12A), restored ligand-induced EGFR internalization to normal rates, indicating that the cytoplasmic domain of Nrp1 is not implicated for this function.

We then focused our attention on the extracellular domain of Nrp1, based on our data supporting its crucial role in eliciting EGFR clustering on the cell surface. A Nrp1 mutant lacking the extracellular MAM domain, required for receptor oligomerization (ΔMAM) (5) (expression shown in Suppl. Fig. 12B), could not rescue EGFR endocytosis (Fig. 5E). Unlike wild type Nrp1, the monomeric ΔMAM mutant was furthermore unable to reinstall ligand-induced EGFR clustering in Nrp1-depleted cells (Suppl. Fig. 13A), consistent with that Nrp1 oligomerization is required to mediate the clustering of associated EGFR. We then tested a complementary truncated mutant of Nrp1 including the oligomerization MAM domain only, anchored to the plasma membrane (Nrp1-MAM-TM). This protein strongly associated with endogenous Nrp1 (Suppl. Fig. 13B) but was unable to interact with EGFR (not shown). Notably, not only Nrp1-MAM-TM could not rescue the effect of Nrp1 knock down, but it even acted as dominant negative molecule, by blocking the function of endogenous Nrp1 and leading to impaired EGFR clustering on the cell surface (Suppl. Fig. 13C), as well as defective AKT activation (Suppl. Fig. 13D) and reduced tumor cell viability (Suppl. Fig. 13E). Taken together, these data strongly suggest that the oligomerization of the extracellular domain of Nrp1 associated with EGFR is responsible for ligand-induced EGFR clustering on the cell surface and subsequent internalization.

Nrp1 expression controls ligand-induced EGFR activation and signaling in tumor cells

In keeping with its impact on EGFR oligomerization, Nrp1 knock-down also inhibited ligand-induced EGFR phosphorylation and the activation of intracellular AKT effector pathway in cancer cells (Fig. 6A-B). Notably, AKT activation in response to the ErbB3/ErbB4-ligand Heregulin-β1 or the unrelated growth factor Insulin was unaffected by Nrp1 knock down in the same cells (Fig. 6C). We further assayed the functional relevance of Nrp1 in EGFR signaling by treating two different cancer cell lines with a Nrp1-blocking antibody (3). In treated cells, ligand-induced phosphorylation of both EGFR and the downstream effector AKT was impaired (Fig. 6D). These data suggest that Nrp1 is specifically required for EGFR signaling in cancer cells.

EGFR pathway is frequently activated in human tumors, and pivotally implicated in sustaining cell proliferation (37). This may be associated with receptor gene amplification and overexpression (38), or ligand over-expression and autocrine signaling in cancer cells (39,40). Interestingly, upon forced EGFR overexpression and constitutive activation in A549, Nrp1 expression became dispensable for cell viability (Fig. 7A). On the other hand, constitutive EGFR activation sustained by autocrine TGFα signaling was strikingly dependent on Nrp1 expression in cancer cells (Fig. 7B, left panel). Consistently, cells carrying TGFα overexpression displayed a modest but significant increment of cell viability in culture, which was completely lost upon Nrp1 silencing (Fig. 7B, right panel).

DISCUSSION

Accumulating evidence seems to associate neuropilins expression in cancer cells with tumor progression. In particular, Nrp1 overexpression is often found in human cancers of different origin, and correlates with tumor aggressiveness and poor clinical outcome. Tumor models, in vivo and in vitro, have been used to try elucidating the functional role of Nrp1 in cancer progression; however experimental data were partly contradictory and did not identify the implicated molecular mechanisms. The results of the present study indicate that Nrp1 expression plays a pivotal role in cancer cell survival and proliferation. In fact, while knocking down Nrp1 expression severely impaired growth and viability of different cancer cells, its overexpression conversely promoted cell proliferation in vitro and tumor growth in mouse models.

By investigating the molecular mechanisms underlying this activity, we found that the extracellular domain of Nrp1 (Nrp1ec) could elicit the phosphorylation of both AKT and MAPK intracellular effectors and promote cell proliferation, recapitulating the effects of overexpressing the full-length molecule; moreover Nrp1ec was sufficient to restore viability of cancer cells deprived of the endogenous transmembrane molecule. These results suggested that the small cytoplasmic domain of Nrp1 is not specifically required for the regulation of cancer cell viability and proliferation. Moreover, by experiments in vitro and in vivo, we demonstrated that the VEGF-binding site in the extracellular domain of Nrp1 is not required to mediate these effects, thereby implicating a major alternative signaling pathway.

Initially, we ruled out two candidate effectors previously associated with Nrp1: VEGF-R2 and c-Met (data not shown). Then, by applying an unbiased screening for RTK activation in cancer cells, we found out that Nrp1ec selectively triggered the phosphorylation of EGFR (but not other ErbB family members). Notably, Nrp1ec did not interfere with EGF-induced receptor activation, or even slightly improved it in co-stimulation experiments (data not shown), thus suggesting that Nrp1ec does not engage EGFR ligand-binding site or EGF itself. We then demonstrated the physical association between endogenous Nrp1 and EGFR in cancer cells and found it to be induced by EGF and TGFα. Moreover, while ligand stimulation induced EGFR clustering in large complexes on the cell surface, this was impaired upon Nrp1 silencing. We found that this process is mediated by the oligomerization (MAM) domain of Nrp1.

Ligand-induced EGFR internalization into endocytic vescicles was furthermore impaired in the absence of Nrp1. Importantly, EGFR internalization was recovered in Nrp1-silenced cells upon re-expression of mutated Nrp1 isoforms lacking the cytoplasmic domain, or by the treatment with Nrp1ec alone. On the other hand, EGFR internalization could not be recovered upon re-expression of mutated Nrp1 lacking the oligomerization MAM domain, responsible for EGFR clustering on the cell surface. It is known from literature that EGFR endocytic traffic is intimately connected to receptor activation (41). However, whether the kinase activity is required for EGFR internalization is debated (33,42,43), and it cannot be ruled out that tyrosine phosphorylation and receptor internalization may be two partly independent consequences of receptor ligation on the cell surface. This issue started to be mechanistically addressed, and receptor oligomerization *per se* is emerging as a driving force for EGFR internalization independent of kinase activity (44). In this

framework, our study points at Nrp1 as an additional player in the regulation of EGFR trafficking. Indeed, in our experimental model we found that Nrp1 controls EGFR endocytosis even independently from tyrosine autophosphorylation, by mediating receptor oligomerization and clustering on the cell surface.

It is well known that receptor endocytosis is a major regulatory mechanism controlling receptor signaling in space and time (45). This is particularly important for EGFR, because the signaling cascade elicited by receptor activation is not limited to the plasma membrane, and crucially continues during receptor trafficking through endosomal compartments, especially for mediating AKT activation (36). Because ligand-induced EGFR internalization in tumor cells was severely reduced in the absence of Nrp1, EGF-bound receptors remained at the cell surface, where they are hardly capable to activate AKT (46). This mechanism is potentially implicated in the pathway leading to tumor cell growth and survival mediated by EGF. Indeed, we found that the extracellular domain of Nrp1 is sufficient to promote AKT activation, cancer cell viability and tumor growth in vivo, independent from VEGF binding. Moreover, our data suggest that Nrp1-dependent AKT activation is mediated by EGFR signaling, thereby suggesting a molecular mechanism to account for increased Nrp1 expression in human tumors. Complementary to this, and relevant for cancer treatment, the treatment with a Nrp1-blocking antibody (or knocking down Nrp1 expression by RNAi) abated EGFR auto-phosphorylation and downstream AKT signaling induced by EGF or TGFalpha, which sustains tumor cell survival and proliferation.

EGFR is in fact a major driver of progression in several human cancers. Even tumors carrying wild type non-amplified EGFR have been found to depend on the signaling activity of this proto-oncogene for progression, and they can be successfully

targeted with EGFR-blocking drugs (47-49). Intriguingly, our data suggest that Nrp1 function is dispensable in EGFR-overexpressing cancer cells. Instead, we found that constitutive EGFR activation and pro-survival signaling due to an autocrine loop of TGFα, which is also frequently seen in human tumors, is dependent on Nrp1 expression.

Preclinical trials in mice with two different anti-Nrp1 antibodies demonstrated a strong tumor suppressor effect that could not be explained by interference with VEGF binding or VEGFR2 signaling (24), although the implicated molecular mechanisms remained unclear. On the other hand, one anti-Nrp1 MoAb, selected for targeting the VEGF-binding domain in order to block tumor angiogenesis (MNRP1685A), was tested in the clinics revealing moderate toxicity. Therefore, our data could prompt future studies focusing on antibodies or therapeutic active molecules targeting Nrp1-EGFR interaction in tumor cells, independent of its function as co-receptor for VEGF in endothelium.

In conclusion, Nrp1, thanks to its ability to regulate EGFR activation, internalization and signaling, could represent a new and relevant target for interfering with the EGF/TGFα-dependent survival and proliferation of cancer cells.

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LEGENDS to FIGURES

Figure 1. Neuropilin-1 expression levels regulate tumor cell growth and viability.

A. Schematic drawing depicting Nrp1 structure and the conserved domains comprised in the different constructs analyzed in this study (including point mutations in Nrp1_3mut).

B. The viability of multiple different Nrp1-depleted tumor cells (expression data shown in Suppl. Fig. 1A) was assessed by MTT assay (see Suppl. Methods). Values shown are the mean ±SD of three independent experiments performed in triplicate, normalized to controls for each cell line; *p<0.01; **p<0.001; ***p<0.0001.

C. The growth of control-EV, Nrp1-depleted (shNrp1), and Nrp1-overexpressing A549 carcinoma cells was compared, in cell proliferation assays (see Suppl. Methods). Plotted values represent the mean ±SD of three independent experiments performed in triplicate; *p<0.01; **p<0.001; ***p<0.0001.

D. Wild type or point-mutated Nrp1 unable to bind VEGF (Nrp1_3mut, see Fig. 1A and Suppl. Fig. 3B) were overexpressed in U87MG tumor cells, and cell growth was assessed in culture (compared to controls) as in panel C. Data shown are the mean ±SD of three independent experiments performed in triplicate; **p<0.001; ***p<0.0001. Immuno-blotting analysis of protein expression is shown on the right.

E. U87MG cells engineered as described above were transplanted s.c. in immunodeficient mice. Tumor growth was assessed by periodical volumetric measurements (left panel), and tumor weight was eventually measured after excision

(right panel). Values reported in graphs represent the average ±SD of 6 mice per each experimental condition; *p<0.01; ***p<0.0001.

Figure 2. The extracellular domain of Nrp1 is sufficient to promote tumor cell viability/proliferation.

A. Immuno-blotting analysis of expression of the isolated extracellular domain of Nrp1 (Nrp1ec) in MDAMB435 cells, and its secretion in the conditioned medium.

B. The growth of tumor cells expressing Nrp1ec or mock (illustrated above) was assessed as in Fig. 1C. The graph shows mean values ±SD of three independent experiments performed in triplicate; *p<0.01.

C. The viability of MDAMB45 and A549 tumor cells overexpressing Nrp1ec was assessed by MTT conversion assays (in 1% FBS-containing medium). Data shown are the mean ±SD of three independent experiments performed in triplicate; *p<0.01; **p<0.001.

D. MDAMB435 cells expressing recombinant Nrp1ec, or transfected with an empty vector (EV), were furthermore subjected to knock down of endogenous Nrp1 by treatment with three independent siRNA sequences (siNrp1) or a control (siC). On the left, are shown the results of MTT assays; average values (from two independent experiments performed in triplicate) were normalized to the respective siC-treated

conditions. Unlike EV-control cells, Nrp1ec-overexpressing cells were unaffected by silencing endogenous Nrp1; *p<0.01; **p<0.001. On the right: immunoblotting analysis of Nrp1 expression in the different conditions.

Figure 3. Nrp1ec elicits EGFR activation and downstream signaling.

A-B. Western blotting analysis of different tumor cell lines incubated with affinity purified soluble Nrp1ec (or mock) for 15 minutes, revealing AKT (A) and MAPK (B) activation by means of phospho-specific antibodies. Detection of total AKT and MAPK levels in the same samples (shown at the bottom) provided protein loading controls.

C. By applying a Proteome Profiler antibody array (ARY001, by R&D systems), we assessed the tyrosine phosphoryation of 42 different RTKs in protein lysates of T47D tumor cells incubated with mock, 300 ng/ml Nrp1ec, or Heregulin (0.2 nM, as internal positive control). Nrp1ec selectively induced EGFR phosphorylation.

D. EGFR phosphorylated in tyrosine 1068 was detected by immunoblotting with anti-phosphospecific antibodies in total protein lysates of different tumor cells incubated with 2 ng/ml EGF or 300 ng/ml Nrp1ec. Vinculin was also detected in the same lysates to provide a reference of protein loading.

E. A549 cells were treated with EGF or Nrp1ec (as above), in absence or presence of the EGFR-blocking antibody Cetuximab (100 μg/ml). Western blotting analysis (as in panels A-B) showed that EGFR blockade prevented AKT and MAPK phosphorylation induced by both Nrp1ec and EGF.

Figure 4. Nrp1 associates with EGFR and regulates ligand-induced EGFR clustering on the cell surface.

A. Nrp1ec and EGFR (transfected in HEK-293T cells) were co-immunopurified from cell lysates, as demonstrated by Western blotting.

B. Endogenous EGFR and Nrp1 co-purified from protein lysates of A549 cells, especially upon stimulation with EGFR cognate ligands 2 ng/ml EGF and 20 ng/ml TGFα for 15 minutes.

C. A549 cells were transfected with GFP-EGFR in presence of a non-targeting siRNA (siC) or a siRNA targeting Nrp1 (siNrp1) and plated onto glass-bottomed dishes. Cells were treated with 2ng/ml EGF, and images were acquired with a TIRF microscope (Nikon, objective 100x). Scale bar: 5μm. Image-J software was used to score the number of EGFR-positive clusters per cell (n= 65, per condition); and measure the length (μm) of their major axis (shown in left and right graph, respectively). Data represent mean value ± SEM from three independent experiments; ***$p<0.001$ (Mann-Whitney test).

D. A549 were transfected with GFP-EGFR and siNrp1 (or siC), as above. The cells were then incubated with 2 ng/ml EGF or 20 ng/ml TGFα, in the presence of the dynamin inhibitor Dynasore (to prevent receptor internalization; see (50). Confocal fluorescence microscopy analysis in non-permeabilized conditions, revealed the presence of large EGFR clusters on the cell surface, induced upon ligand stimulation in Nrp1-dependent manner. Cell treatment with 400 ng/ml Nrp1ec basally induced EGFR clusters and promoted EGF-mediated effects. The fraction of EGFR clusters-containing cells (out of a count of 100 cells in different fields) was normalized to that observed in non-treated siC control condition. Representative microscopic fields and

magnified insets are shown on the left (scale bars: 20 μm; insets are magnified 2-fold).

Figure 5. Nrp1 regulates ligand-induced EGFR internalization.

A. A549 cells, either control or Nrp1-depleted by siRNA, were incubated with 2 ng/ml EGF at 4°C, and subsequently surface biotinylated, either directly (on the left) or after allowing protein internalization to occur by shifting cells at 37°C for 25 minutes (on the right).

B. EGFR internalization was tracked by "antibody feeding" experiments (see Suppl. Methods for a detailed description, and Suppl. Fig. 9A for a schematic protocol and microscopic images of representative fields). The graphs show a quantification of the ratio between internalized EGFR and total EGFR staining in control and Nrp1-silenced cells, as the mean ±SD of 20 independent fields acquired in three independent experiments; ***$p < 0.0001$.

C. EGF-EGFR endocytosis was assayed in A549 cells, either control or Nrp1-depleted, upon incubation with 2 ng/ml fluorescent-labeled EGF_{555} (red) (see Suppl. Methods for details). Representative images are shown on the right: Nrp1 staining in green allows to identify few non-silenced cells in siRNA-Nrp1 treated samples that provide a convenient internal control for EGF555 binding and internalization. Scale bars: 25 μm. The graph on the left shows a normalized signal quantification of internalized EGF-EGFR complexes, as the mean ±SD of 50 fields derived from four independent experiments; **$p < 0.001$; ***$p < 0.0001$.

D. Nrp1 wild type or the indicated mutant constructs (non-targetable by siRNAs) were re-expressed in Nrp1-depleted (siNrp1) or control (siC) A549 cells. Fluorescent EGF internalization experiments were performed as above. The graph shows mean values

±SD of at least 20 fields derived from three independent experiments (normalized to siC controls); **p< 0.001. Nrp1 constructs expression was verified by immuno-blotting analysis (shown in Suppl. Fig. 12A).

E. Nrp1 wild type or the indicated mutant constructs (non-targetable by siRNAs) were re-expressed in Nrp1-depleted (siNrp1) or control (siC) A549 cells. Fluorescent EGF internalization experiments were performed and quantified as above (panels C-D) **p< 0.001. Protein expression was verified by immuno-blotting (see Suppl. Fig. 12A). Co-treatment of Nrp1-deficient cells with 400 ng/ml purified soluble Nrp1ec (rightmost bars) reconstituted the functional response to EGF.

Figure 6. Nrp1 regulates EGFR signaling in cancer cells.

A. Western blotting analysis of EGFR and AKT phosphorylation in control and Nrp1-depleted A549 cells, upon stimulation with 2 ng/ml EGF. In the upper rows, EGFR was immunopurified and then analyzed by anti-phosphotyrosine antibodies. In lower rows, total protein extracts were analyzed with anti-phospho-AKT antibodies.

B. Analysis of EGF-induced EGFR and AKT phosphorylation in total lysates of control and Nrp1-depleted Hela cells. Data shown are representative of three independent experiments.

C. Nrp1-depleted A549 cells were stimulated with 2 ng/ml EGF, 0.2 nM Heregulin-β1 or 10nM Insulin and AKT phosphorylation was assessed as above.

D. Immunoblotting analysis of EGFR and AKT phosphorylation induced by 2 ng/ml EGF in tumor cells pre-treated for 1hour with a Nrp1-blocking antibody (3) (or an unrelated antibody). Data shown are representative of four independent experiments.

Figure 7. Constitutive EGFR activation by autocrine ligand stimulation in cancer cells is dependent on Nrp1 expression.

A. Western blotting analysis of EGFR tyrosine phosphorylation in control and EGFR-transfected A549 cells, recapitulating constitutive receptor activation in tumors due to receptor overexpression. Constitutive EGFR tyrosine phosphorylation (left panel) was associated with increased cell viability (right panel), independent of Nrp1 expression (knocked down by targeted shRNA expression). Values are the mean ±SD of three independent experiments performed in triplicate; **$p < 0.001$; ***$p < 0.0001$.

B. Immunoblotting analysis of EGFR tyrosine phosphorylation in control and TGFα-transfected A549 cells. In presence of constitutive TGFα loop, EGFR phosphorylation is strongly induced at the steady state, but this effect is lost upon Nrp1 knock down. Consistent results were observed by analyzing cell viability (right panel). Results are the mean ±SD of three independent experiments performed in triplicate; *$p < 0.01$; ***$p < 0.0001$.

Figure 1

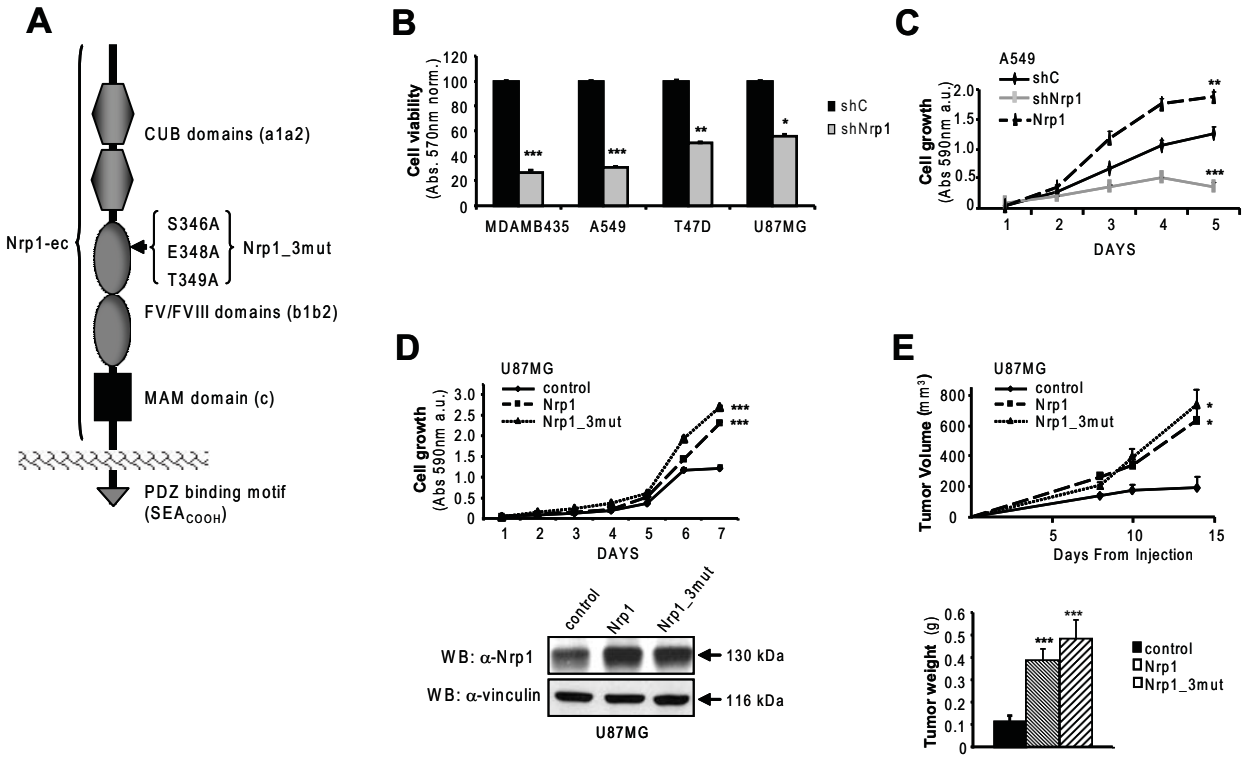
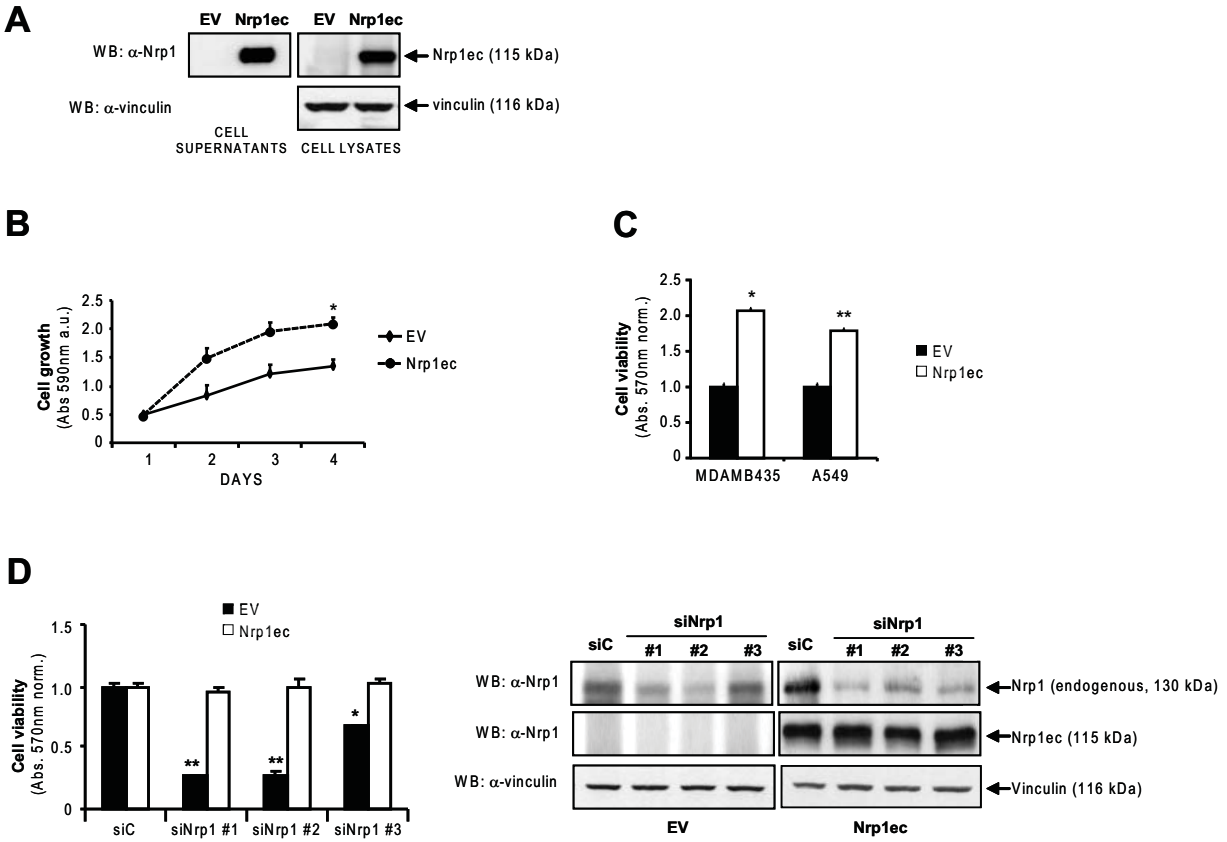


Figure 2



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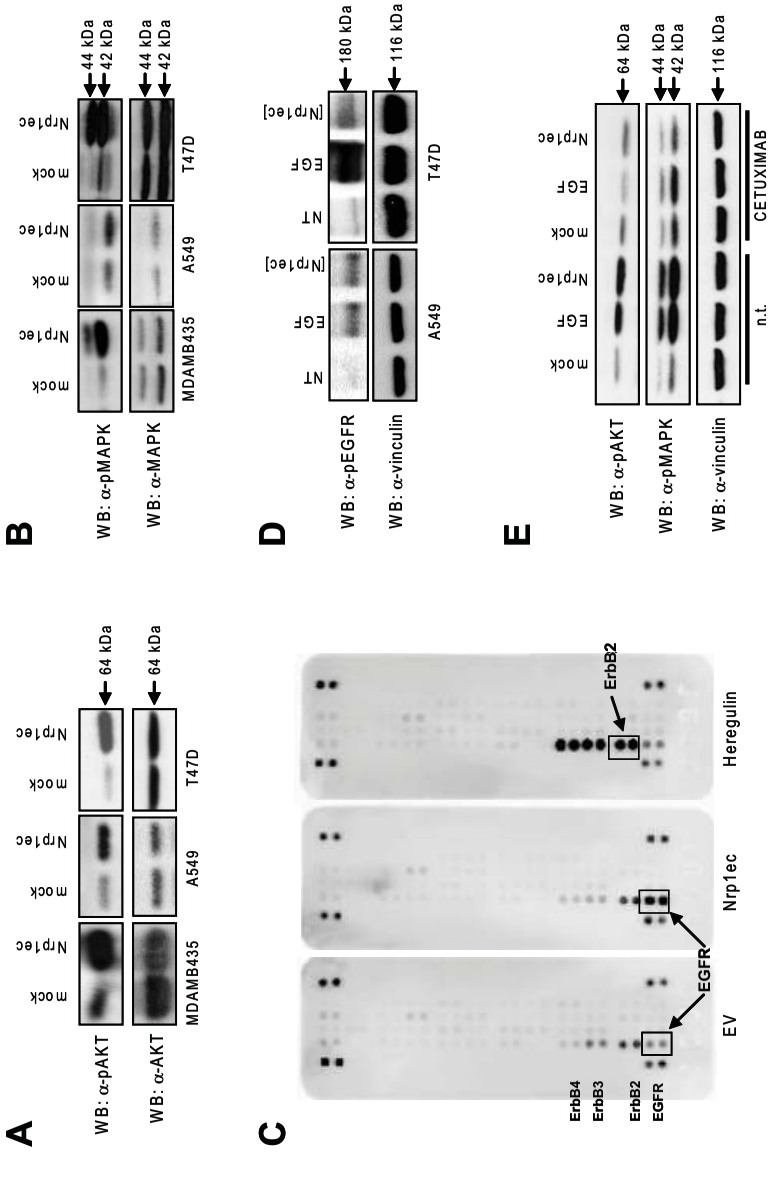

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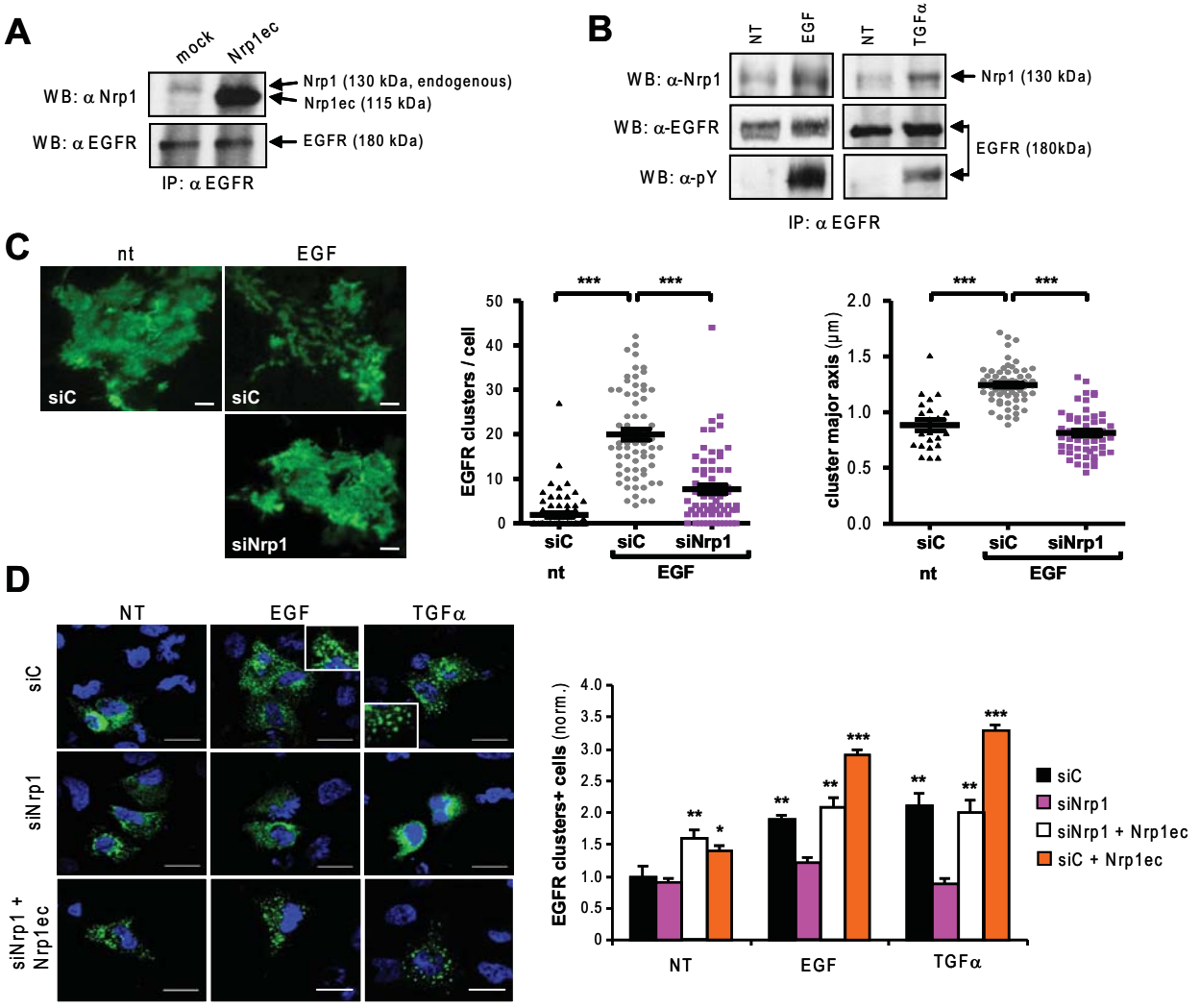

Figure 5

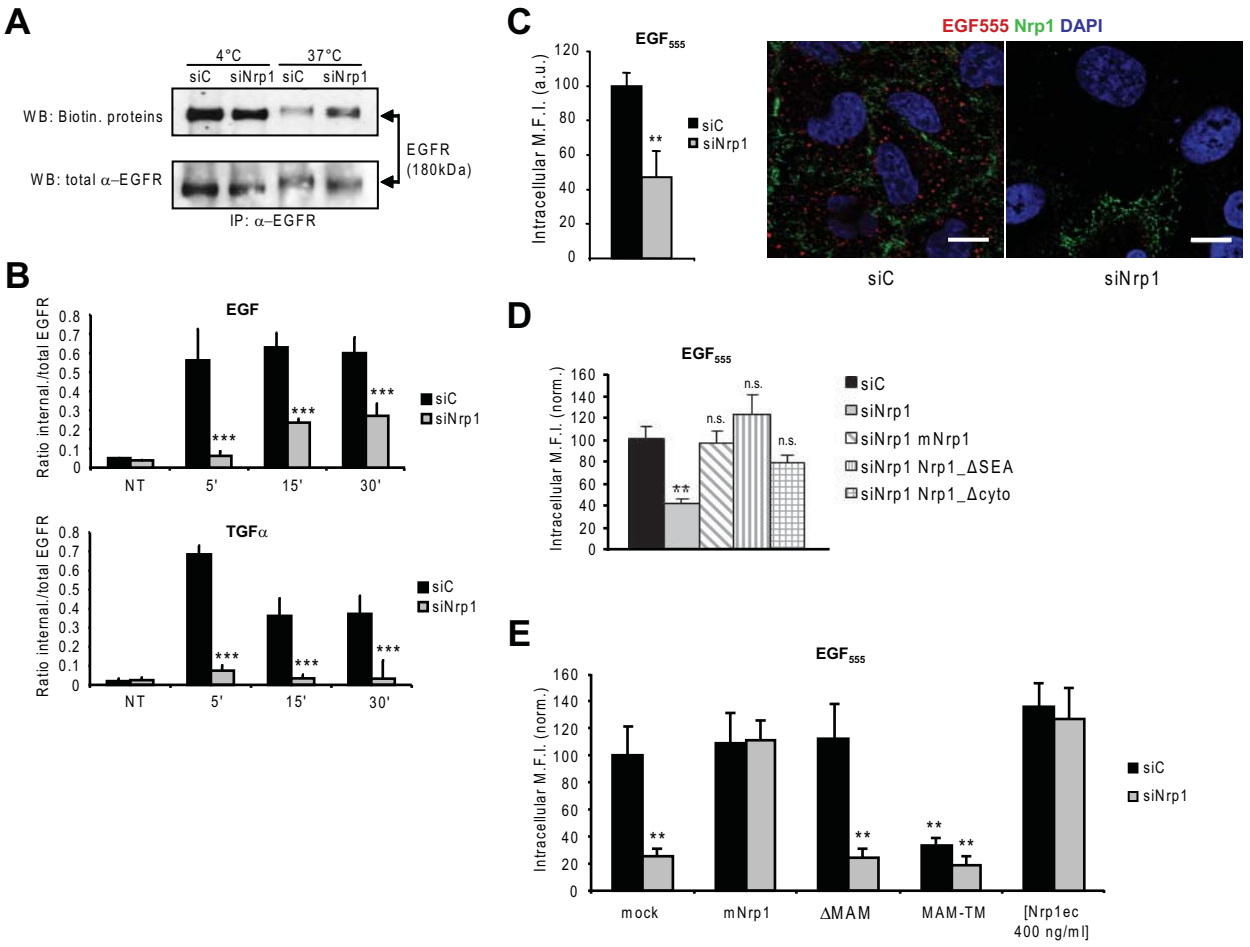

Figure 6

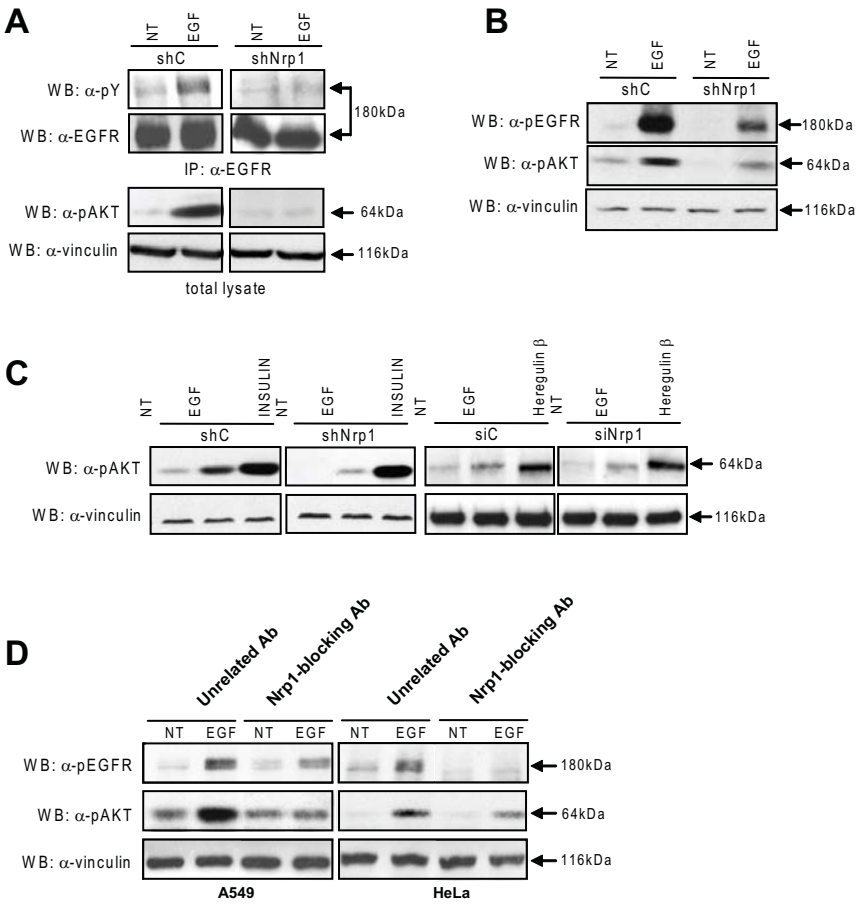

Figure 7