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Downregulating Neuropilin-2 Triggers a Novel Mechanism Enabling EGFR-Dependent Resistance to Oncogene-Targeted Therapies

Sabrina Rizzolio, Chiara Battistini, Gabriella Cagnoni, Maria Apicella, Viviana Vella, Silvia Giordano and

Luca Tamagnoe

Abstract

Neuropilins are a class of cell surface proteins implicated in cell migration and angiogenesis, with aberrant expression in human tumors. Here, we show that the expression of Neuropilin-2 (NRP2) controls EGFR protein levels, thereby impinging on intracellular signaling, viability, and response to targeted therapies of oncogene-addicted cells. Notably, increased NRP2 expression in EGFR-addicted tumor cells led to downregulation of EGFR protein and tumor cell growth inhibition. NRP2 also blunted upregulation of an EGFR "rescue" pathway induced by targeted therapy in Met-addicted carcinoma cells. Cancer cells acquiring resistance to MET oncogene-targeted drugs invariably underwent NRP2 loss, a step required for EGFR upregulation. Mechanistic investigations revealed that NRP2 loss activated NFkB and upregulated the EGFR-associated protein KIAA1199/CEMIP, which is known to oppose the degradation of activated EGFR kinase. Notably, KIAA1199 silencing in oncogene-addicted tumor cells improved therapeutic responses and counteracted acquired drug resistance. Our findings define NRP2 as the pivotal switch of a novel broad-acting and actionable pathway controlling EGFR signaling, and driving resistance to therapies targeting oncogene-addiction.

Significance: These important findings identify the cell surface molecule Nrp2 as the pivotal switch of a novel, actionable pathway driving EGFR upregulation and resistance to oncogene- targeted therapies.

Introduction

Most oncogenes encode for proteins implicated in sustaining cell proliferation and/or protecting cell viability, and their deregulated activation can support uncontrolled neoplastic growth. It is commonly assumed that multiple genetic changes are required to drive cancer formation; however, certain constitutively activated oncogenes can rearrange cell signaling pathways to the point of becoming pivotal (and thereby essential) regulators of proliferation and survival of tumor cells, which makes them also perfect targets for therapeutic approaches, a status called “oncogenic addiction” (1, 2). On the other hand, accumulating studies over the last decade, have consistently demonstrated that tumor cells that are specifically targeted in their Achille's heel, tend to upregulate alternative pathways capable of rescuing cell viability. In fact, Darwinian selection of the fittest, favored by cell heterogeneity in eliciting these rescue mechanisms, eventually leads to the rise of drug-resistant cells, which account for disease progression. In this setting, experimental studies aim not only at the identification of novel potentially druggable addiction mechanisms of cancer cells, but also focus onto pathways that could maintain cell viability in the face of targeted therapies. In many cases, these adaptive mechanisms of drug resistance do not imply genetic changes (at least at start), but depend on gene expression or functional regulations, maintained under the selective pressure of the therapy. For example, we have previously shown that impaired response to MET oncogene-targeted therapy may be sustained by EGFR kinase activity, both in cell lines and in patient-derived gastric cancer xenografts grown in mice (3, 4). Indeed, MET and EGFR combined targeting achieved full tumor regression and prevented resistance onset in this setting; however, the molecular mechanism sustaining EGFR overexpression and resistance could not be elucidated.

Neuropilins (NRP1 and NRP2) are a small family of conserved and widely expressed transmembrane proteins, originally implicated in the regulation of axon guidance and vascular development (5, 6). Neuropilins are widely distributed in the adult tissues, and their levels are often altered in human tumors, compared with normal tissues (7). We and others have previously shown that NRP1 is crucially required for the viability and growth of a range of cancer cells (8), which is consistent with its abundant expression in advanced stage human tumors (9). We have also identified one molecular mechanism accounting for a selective advantage coupled with NRP1 overexpression in cancer cells. In fact, NRP1 can form a complex with EGFR on the surface of cancer cells, where it promotes ligand-induced EGFR clustering and endocytosis, leading to intracellular activation of AKT signaling cascade (8). The NRP1-homologus molecule Neuropilin-

2 (NRP2) is commonly found at low levels in carcinomas, despite its upregulated expression has been associated with metastatic progression (7). The implicated signaling mechanisms are still unclear, although in vitro experiments suggested that NRP2 can act, mainly in response to paracrine VEGF, by promoting TGF-β1 or beta-catenin/Wnt signaling pathways (10, 11). In another study, however, VEGF/NRP2 pathway was also found to repress IGF1R expression and oncogenic signaling in prostate carcinoma cells (12).

Thus, we decided to investigate further the role of NRP2 in cancer cells, and unexpectedly found it to be implicated in the negative regulation of EGFR levels. Consistently, we found that NRP2 could critically control the viability of EGFR-addicted cancer cells. Moreover, tumor cells dependent on the constitutive activity of the MET oncogene became less sensitive to targeted inhibitors upon NRP2-silencing, due to EGFR upregulation. Notably, we found that tumor cells developing secondary resistance to MET-targeted drugs had dramatically downregulated NRP2 expression, whereas restoring its expression suppressed the resistant phenotype and rescued drug-sensitivity. In fact, NRP2 does not impact on EGFR transcription, but rather controls an EGFR-regulatory protein, KIAA1199 (also known as CEMIP), which was previously shown to prevent ligand-induced EGFR degradation and to increase EGFR signaling in cervical carcinoma cells (13). We finally demonstrated that NRP2-dependent regulation of KIAA1199 expressions is instrumental to uphold EGFR signaling in cancer cells, and protect them from treatment by the onset of drug-resistance. Moreover, direct interference against KIAA1199 was effective in inducing viability loss in EGFR-addicted cancer cells, and counteracting secondary resistance mediated by adaptive EGFR signaling upregulation.

Materials and Methods

Cell lines

All immortalized cell lines used in our study were provided by the cell line biobank of the Candiolo Cancer Institute, under the supervision of Ms. Raffaella Albano. The bank had acquired original stocks of following human cell lines from the indicated sources: HS746T gastric carcinoma, and A375 melanoma cells were provided by ATCC (in 2013); SKMEL5 and SKMEL28 melanoma, PC3 prostate cancer and A549 lung adenocarcinoma cells were provided by NCI (in 2011); EBC1 human lung squamous cell carcinoma line was purchased from the Health Science Research Resources Bank (in 2012). GTL16 is a clonal cell line previously established in our laboratory (14), derived from the poorly differentiated gastric carcinoma cell line MKN45. Our internal batch of PC9 lung adenocarcinoma cells was re-authenticated soon before experimental application by short tandem repeat (STR) profiling (Cell-ID, Promega). SG16 primary gastric carcinoma cells have been previously described (4), and their genetic identity with the tumor of origin had been validated by short tandem repeat (STR) profiling (Cell-ID, Promega). Upon request of the scientists, the technical assistant of the bank thaws certificated vials of frozen cells, which are expanded and handed out for research. The cells used in this study were maintained in culture for up to 2 to 4 months after resuscitation, and then discarded. New cell batches put in culture are subjected to Mycoplasmatesting, an institutional service offered on a weekly base that applies a PCR Mycoplasma Detection kit (Applied Biological Materials Inc.). On a periodical base, all cell lines thawed from the biobank are re-authenticated at the genomic facility of the Candiolo Cancer Institute, by applying the PowerPlex16 Cell-ID assay (Promega), based on the analysis of 15 genomic STR markers (plus amelogenin). All cell lines used in our study have been authenticated no more than 3 years before their experimental application. RPMI culture medium was used for most cell lines applied in this study, except for HS746T, A375, and HEK-293T cells that were maintained in DMEM medium. The media were supplemented with 1% l-glutamine (2 mmol/L), 10% FBS (Sigma), penicillin (5,000 U/mL, Faber), and 0.1% streptomycin (5 mg/mL, Squibb) and incubated in a humidified incubator with 5% CO_2 at 37°C.

Antibodies and other reagents

Anti–Neuropilin-2 antibody used for Western blotting analysis was purchased from R&D Systems (AF2215). EGFR was detected by an antibody from Santa Cruz Biotechnology (clone 1005) and EnzoLifeSciences (ALX-804-064-C100) for Western blot detection, whereas EGFR was immunoprecipitated with an antibody purchased from Upstate (clone LA22). EGFR phosphorylation was detected by a phospho-specific antibody (directed against p-Tyr_{1068}) from Abcam (ab5644). ErbB2/Neu was detected with an antibody from Santa Cruz Biotechnology (clone C-18), whereas ErbB3 with an antibody from Millipore (clone 2F12). Total and phosphorylated forms of MAPK and AKT (against pAKT-S^{473} and pMAPK-Thr^{202}/Tyr^{204}) were detected with antibodies from Cell Signaling Technology. Total IkB was detected with an antibody purchased from Abcam (ab32518). Other antibodies applied in this study were: anti-vinculin (V4505, Sigma) and anti–β-actin from Santa Cruz Biotechnology (clone I-19) for loading control. Secondary antibodies were purchased from Promega or The Jackson Laboratory. EGF and TGFα were purchased from Abcam. The IKK inhibitor BMS-345541 was supplied by Sigma, the IKK-16 from Santa Cruz Biotechnology. JNJ38877605 MET kinase inhibitor was kindly provided by Janssen. Crizotinib was purchased from Carbosynth.

Cell proliferation analysis

Tumor cells were seeded in multiple 96-well plates at an initial density of $1.5–3 \times 10^3$ cells per well (depending on the cell line), and subsequently grown in complete medium. At each experimental time point, one multiwell dish was fixed with 11% glutheraldehyde, stained with crystal violet, and the absorbance was read using a standard colorimetric system at 595nm.

Cell viability assay

Tumor cells were seeded in 96-well plastic culture plates at an initial density of $1–2 \times 10^3$ cells per well (depending on the cell line) in the presence of the indicated drugs or vehicle (DMSO). Cell viability was assessed using a luminescence assay (CellTiter-Glo Luminescent Cell Viability Assay, Promega). After 72 hours from seeding, the medium was changed and the cells incubated with CellTiter-Glo reagent. The number of viable cells was directly proportional to the luminescent signal recorded.

Establishment of acquired resistance to targeted inhibitors in MET-addicted cancer cells

To establish cells resistant to JNJ38877605, we treated parental EBC1 cells with escalating concentrations of the drug, starting from 15 nmol/L (around IC_{50}), until they reacquired the ability to grow in presence of JNJ38877605 at the same rate of parental cells (in absence of the drug); then the inhibitor concentration was doubled until the cells accomplished resistance to 180 nmol/L JNJ38877605. Following a similar protocol, we treated parental SG16 cells with escalating concentrations of JNJ38877605, starting from 25 nmol/L, until establishing resistance to 250 nmol/L.

Gene expression knockdown by RNA-interference

Neuropilin-2 expression was silenced in tumor cells by transfecting targeted siRNA sequences (with Lipofectamine 2000, Invitrogen) or by transducing shRNA-expressing lentiviral constructs (to achieve stable knock-out). A pool of three different chemically synthesized siRNA sequences targeting Neuropilin-2 transcripts were used for transient gene silencing (#1: GCCGCAGCUUUGCCUGACU; #2: UUCCAAAGAUGCUGCCUAU; #3: GAGCCACCUUCUCCAAAAU). To achieve long-term expression, the best targeting sequence (#2) was inserted into the lentiviral transfer plasmid pCCLsin.PPT.hPGK.GFP.Wpre as previously reported (15), in the frame of a sequence driving the transcription of a short hairpin RNA under control of the H1 promoter (indicated as shNRP2). As control shRNAs (shC), we expressed a nontargeting sequence (GATAGGTCATGACTGTCCC). We furthermore silenced Neuropilin-2 with a puromycin-selectable construct purchased from Sigma-Aldrich (TRCN0000063312) targeting an independent sequence, and used pLKO empty vector as control. Three different sequences of chemically synthesized siRNAs targeting KIAA1199 transcripts were used, either alone or as a pool (#a: ACAUUGAAAUAUUCGCCAUGCUC; #b: GACAAGGAGGCCAAGACAGTGGT; #c: GGUAUUCAGCCGGAUCCUU). To achieve long-term silencing of KIAA1199, we applied two selected independent puromycin-selectable constructs provided by Sigma-Aldrich: TRCN0000118787 and TRCN0000118791 (indicated in the text as shKIAA1199 #87 and shKIAA1199 #91).

Statistical analysis

Statistical significance was performed by the two tailed Student t test. Error bars represent the SD, as indicated in each figure legend. All experiments were repeated at least three times (biological replicates) with consistent results, even if figures may show one representative experiment (with the average of technical replicates). Statistical significance is indicated by asterisks in the figures, as follows: *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$.

For additional information, see Supplementary Methods.

Results

Neuropilin-2 negatively controls EGFR levels and dampens the growth of EGFR-addicted tumor cells

Neuropilin-2 (NRP2) is differentially distributed in human tumors, but its functional relevance in this context is largely unclear. We therefore knocked down its expression by RNA interference in two human cancer cell models, that is, A549 lung and PC3 prostate carcinoma (Fig. 1A). Notably, neither the phenotype nor the growth rate of the two different cell lines appeared to be affected upon NRP2 downregulation (Fig. 1B; Supplementary Fig. S1A). Because we have previously shown that the homologous protein NRP1 promotes EGFR signaling in cancer cells (8), we asked whether NRP2 could also regulate this pathway. In fact, NRP2 silencing resulted instead in a strong increase of EGFR protein levels (Fig. 1C; Supplementary Fig. S1B), but not of other family members ERBB2 or ERBB3 (Supplementary Fig. S1C), underlying the specificity of this mechanism. Notably, EGFR regulation did not occur at transcriptional level, because mRNA abundance was not significantly affected (Fig. 1D). Moreover, in complementary experiments, we found that NRP2 overexpression downregulated EGFR levels in these cells (Supplementary Fig. S1D). These results strongly suggested that NRP2 can negatively regulate EGFR signaling.

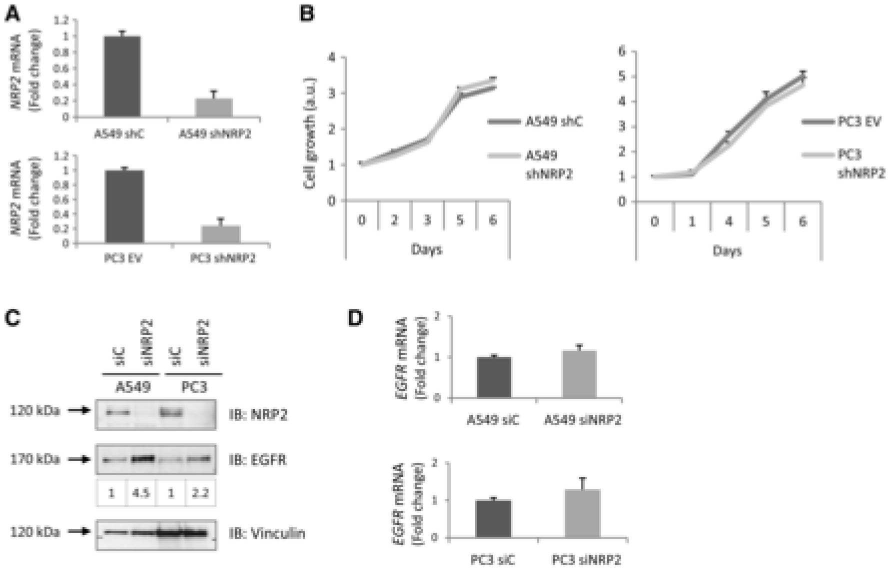

Figure 1. NRP2 negatively regulates EGFR expression in cancer cells. A, A549 and PC3 carcinoma cells were subjected to stable NRP2 silencing by shRNAs (shNRP2), as demonstrated by real-time qPCR analysis (performed in triplicate); controls were provided by cells transduced with a nontargeting construct (shC or empty vector, EV). B, The growth rate of the same cells shown in A was assayed in cell culture; cell numbers were estimated by crystal violet staining (of quadruplicate wells per each condition) and normalized to the first assessment after seeding (day 0). The experiment was repeated with two independent batches of transduced cells, showing similar gene knockdown as in A. C, Western blotting analysis of NRP2 and EGFR protein expression in the same cells transiently transfected with siRNAs directed against NRP2 (siNRP2), or control siRNAs (siC). EGFR band intensity quantification (vs. respective controls) is shown at the bottom. Vinculin staining provided a loading control. Images depict representative results of three independent experiments. D, EGFR mRNA levels were quantified by real-time qPCR analysis (in triplicate) in the same cells as in C.

Notably, most cancer cells growing in permissive conditions in vitro do not depend on the EGFR pathway for proliferation. Thus, to assess the functional relevance of this mechanism, we initially assayed the proliferation of A549 cells in serum-deprived medium solely containing EGF or TGFα growth factors. Notably, in these stringent conditions, EGFR ligands improved the viability of NRP2-depleted cells, whereas control cells were unaffected (Supplementary Fig. S2A). We then assessed NRP2 activity in PC9 lung carcinoma cells that are actually addicted to EGFR signaling, and can be targeted with inhibitor drugs, such as erlotinib, resulting in dramatic viability loss (16). In keeping with the mechanism described above, NRP2-overexpressing PC9 cells showed reduced proliferation rate (Fig. 2A), accompanied by partial downregulation of EGFR levels and intracellular signaling (Fig. 2B). Moreover, they acquired increased susceptibility to targeted therapy (Fig. 2C); for instance, at drug concentrations totally ineffective in control cells, erlotinib could achieve highly significant viability loss in cells overexpressing NRP2. Consistent with the observed growth disadvantage, NRP2 overexpression in stably transduced cells was progressively lost in culture in a few weeks period (Supplementary Fig. S2B). These data further validated the functional relevance of NRP2-dependent negative regulation of EGFR levels in tumor cells.

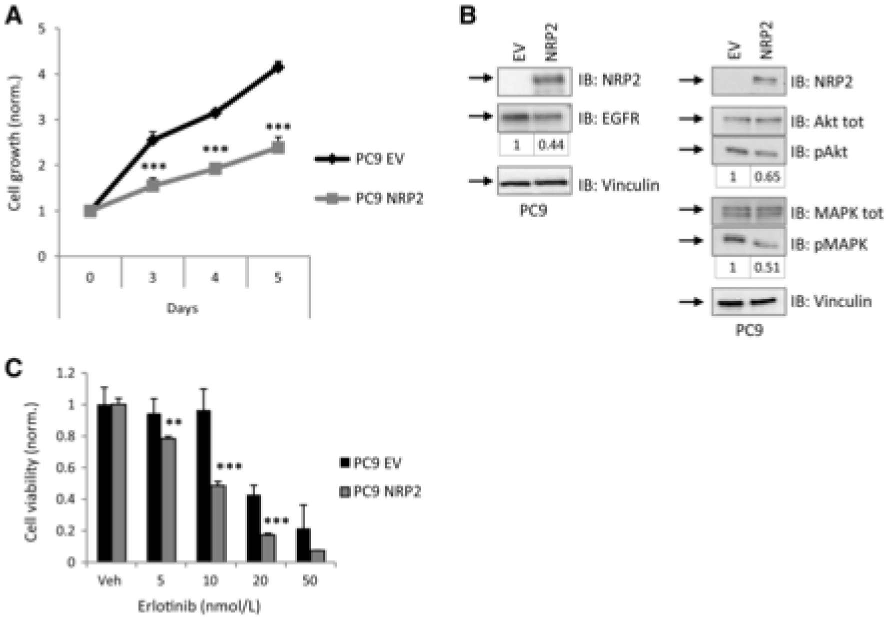

Figure 2. NRP2 inhibits growth and viability of EGFR-addicted PC9 cells. A, EGFR-addicted PC9 carcinoma cells were transduced to express NRP2 (or mock-transduced with an empty vector, EV) and the growth rate was assayed in cell culture; cell numbers were estimated by crystal violet staining (of quadruplicate wells per each condition) and normalized to the first assessment, shortly after seeding (day 0). Four independent batches of transduced cells were analyzed in independent experiments, with consistent results. B, Western blotting analysis of the same cells shown in A, with the indicated antibodies. The same cell lysates were loaded in two separate blots (left and right images). Three independent experiments yielded consistent results. Band intensities were quantified (vs. respective controls) and are shown at the bottom for EGFR, pAKT/totAKT ratio, and pMAPK/totMAPK ratio. C, The same cells as above were incubated for 72 hours with the indicated concentrations of EGFR kinase inhibitor erlotinib (or DMSO as vehicle, Veh), and cell viability was assessed by CellTiter Glo assay (each point in quadruplicate) in three independent experiments. Statistical analysis compared cells transduced with NRP2 vs. empty vector. Statistical significance: **, P < 0.005; ***, P< 0.0005.

NRP2 downregulation in MET-addicted cells unleashes EGFR expression and drives EGFR-dependent resistance to targeted therapy

We then analyzed the functional relevance of NRP2 in other tumor cell models of oncogene-addiction, in particular EBC1 lung cancer, and GTL16 and HS746T gastric cancers, carrying METgene amplification and susceptibility to MET kinase inhibitors, although to different extent and IC_{50}values (4, 17). Notably, in these cells, EGFR expression is basally low, but we confirmed a prominent EGFR upregulation upon NRP2 silencing, achieved by means of two independent shRNAs (Fig. 3A; Supplementary Fig. S3A). It was previously reported that NRP2 is produced in at least two splice variants, with putative functional differences (18); actually, upon either combined or selective knock down of the two NRP2 isoforms by independent siRNAs, we achieved EGFR upregulation in cancer cells (Supplementary Fig. S3BC). Importantly, the therapeutic response to two different MET kinase inhibitors (JNJ38877605 and crizotinib) was significantly reduced in NRP2-depleted cancer cells (Fig. 3B–E; Supplementary Fig. S3D). This suggested that NRP2 negatively regulates a mechanism that can prevent responsiveness to MET oncogene-targeted therapy, and based on our results EGFR featured a likely candidate for this. Indeed, tumor cell treatment with a combined regimen targeting both MET and EGFR signaling could blunt the drug refractoriness elicited by NRP2 downregulation (Fig. 3FG).

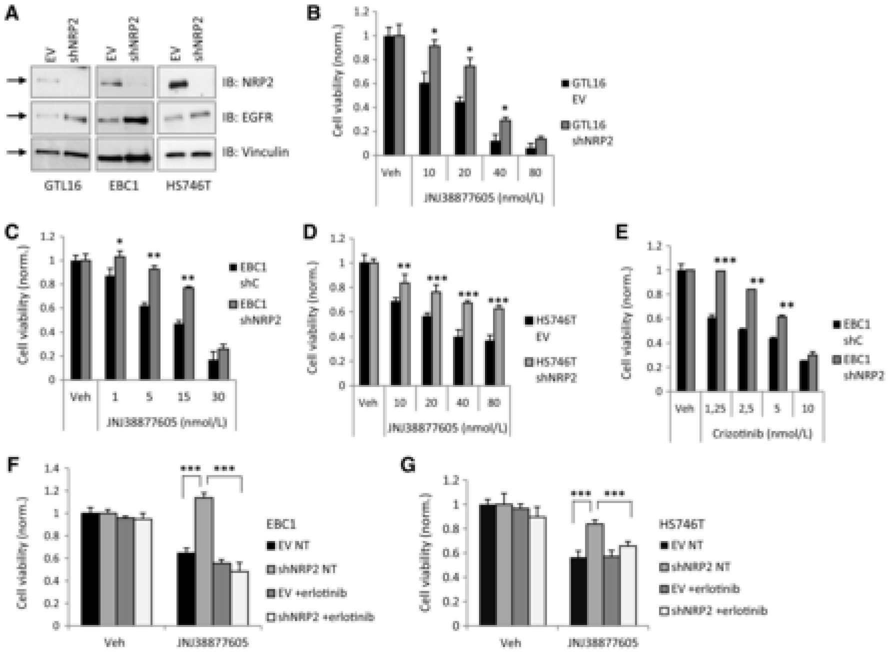

Figure 3. NRP2 knockdown results in EGFR upregulation and refractoriness to targeted inhibitors of MET-addicted cancer cells. A, MET-addicted GTL16, EBC, and HS7546T carcinoma cells were subjected to NRP2 knockdown, or transduced with a nontargeting construct (empty vector, EV, or shC in experiments shown in other panels), and analyzed by Western blotting with the indicated antibodies. At least three independent batches of transduced cells were analyzed in independent experiments with consistent results; images show representative data. B–D, The indicated cells (described in A) were incubated for 72 hours with the indicated concentrations of the specific MET kinase inhibitor JNJ38877605 (or DMSO as vehicle, Veh); the cellular viability was assessed by CellTiter Glo assay (each point in quadruplicate) in at least three independent experiments. Statistical analysis compared NRP2-silenced cells with controls treated with the same drug concentration. E, Same as in the previous panels, for cells treated with an alternative MET-inhibitor drug crizotinib. F and G, NRP2-depleted and mock-transduced EBC1 or HS746T cells (as indicated) were exposed (or not) to MET-inhibitor JNJ38877605 (respectively, 15 nmol/L and 20 nmol/L) in presence or absence of the EGFR inhibitor erlotinib (1 μmol/L). The cell viability was assessed as in previous panels. Three independent experiments were performed. Statistical significance: $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$.

To validate the relevance of NRP2 in controlling the onset of resistance to targeted drugs, we derived cells stably refractory to the MET kinase inhibitor JNJ38877605 from two distinct oncogene-addicted models, that is, the immortalized lung carcinoma cells EBC1 and the primary gastric cancer cells SG16 (4), by sustained treatment with escalating concentrations of the drug (Fig. 4A). Of note, in the course of this process, EBC1 cells incidentally became dependent on the presence of the inhibitor (Supplementary Fig. S4A), a status also known as “drug-addiction” that we and others have previously described (19, 20). Importantly, although the IC_{50} and activity of the MET inhibitor were different in the two cellular models, in both cases we observed a dramatic downregulation of endogenous NRP2 expression in cells that became insensitive to the treatment, accompanied by a striking increase in EGFR levels (Fig. 4B). In fact, NRP2 downregulation was more profound in cells resistant to progressively higher drug concentrations (Supplementary Fig. S4B). This mechanism appeared to be due to epigenetic downregulation of NRP2 gene expression, as it could be largely reversed by treatment with the HDAC inhibitor TSA (Fig. 4C). Moreover, analysis of NRP2 gene copy number at gDNA level did not reveal any significant change in resistant versus parental cells (Supplementary Fig. S4C). In addition, by means of a validated NRP2 gene promoter reporter construct (12), we confirmed that NRP2 gene transcription was significantly reduced in drug-resistant cells (Fig. 4D).

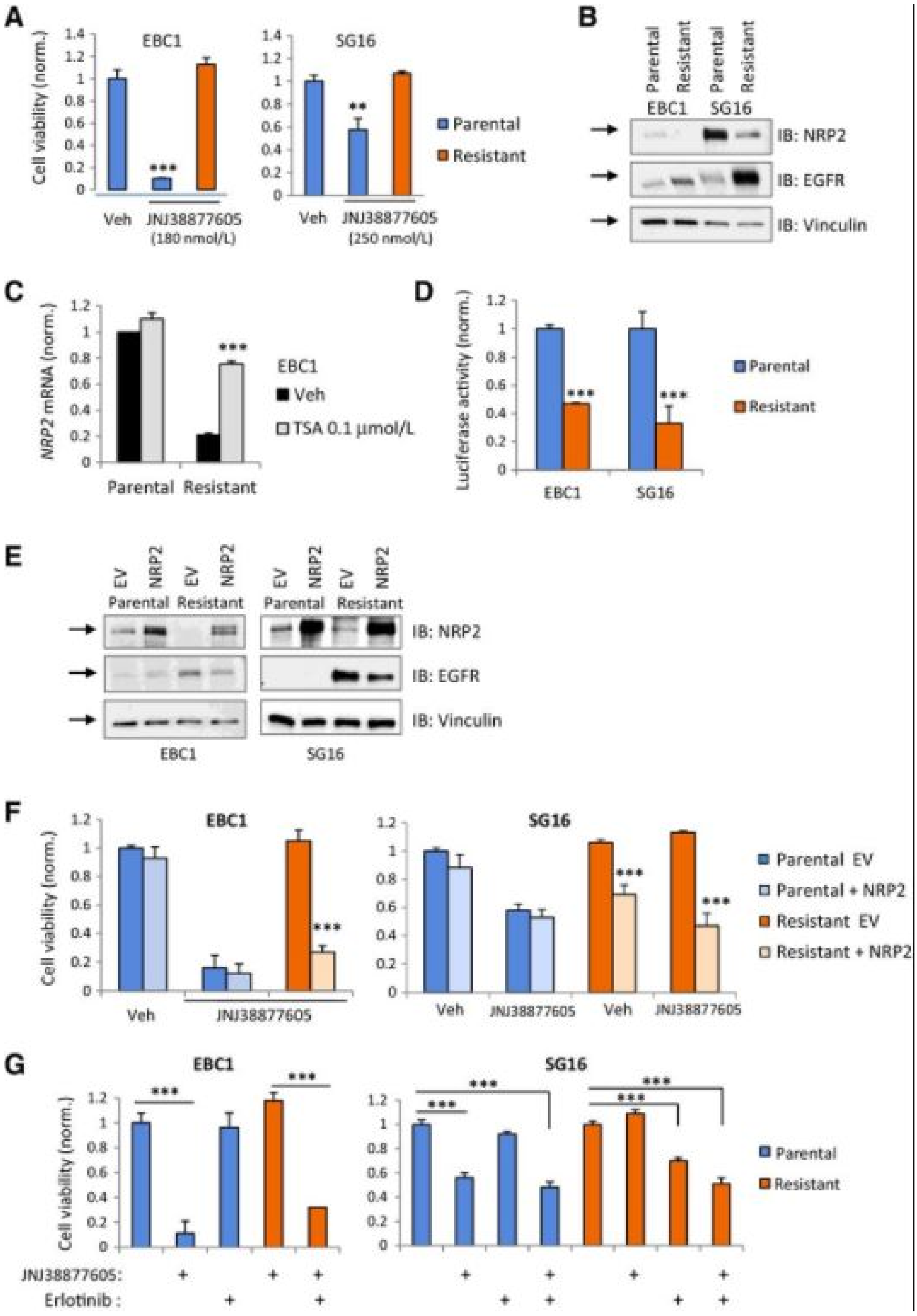

Figure 4. The onset of acquired resistance to targeted therapy in MET-addicted cells depends on adaptive NRP2 loss of expression, leading to EGFR upregulation. A, MET-addicted EBC1 or SG16 cells were treated with the escalating concentrations of the targeted inhibitor JNJ38877605, until the onset of drug resistance. Cell viability was assessed as in Fig. 3. Resistant cells were equally viable at the tolerated drug concentrations (indicated), as parental cells in the absence of the drug. Due to drug-addiction (see Supplementary Fig. S4A), resistant EBC1 cells were always maintained in the presence of the MET-inhibitor. B, Western blotting analysis of NRP2 and EGFR protein expression in the same cells described in A. Three independent experiments were performed. C, NRP2 mRNA levels in the above shown drug-resistant (and parental) EBC1 cells, either treated with the histone deacetylase inhibitor trichostatin A (TSA) or DMSO (Veh), were assessed by real-time qPCR. Statistical analysis compared resistant cells treated with TSA versus vehicle. Three different experiments were performed. D, NRP2 gene promoter activity was assessed in

parental and drug-resistant cells by transfection of a luciferase reporter construct. At least two experiments were performed per each cell line, yielding consistent results. E, EBC1 and SG16 cells, either parental or drug-resistant (the same as described in A), were subject to reverse transfection (in quadruplicate points) with NRP2 construct or EV as control. Western blotting analysis revealed changes in EGFR expression. The results were confirmed in three independent experiments. F, The same cells described in E were treated with JNJ38877605 (180 nmol/L for EBC1 and 250 nmol/L for SG16) or incubated with vehicle only, and cell viability was assessed after 96 hours (with CellTiter Glo assay). At least three independent experiments were performed. Statistical analysis compared cells transduced with NRP2 versus empty vector (EV). G, Cell viability assays of the same cells described in A, upon single or combined treatment of parental or drug-resistant cells with the MET-inhibitor JNJ38877605 (180 nmol/L for EBC1 and 250 nmol/L for SG16) and/or the EGFR-inhibitor erlotinib (1 μmol/L). The experiments were repeated at least three times. Statistical significance: **, $P < 0.005$; ***, $P < 0.0005$.

The causal role of NRP2 downregulation in drug resistance was demonstrated by forcing its re-expression in the two drug-resistant tumor models. Consistent with the described mechanism, this approach curbed EGFR expression in resistant cells (Fig. 4E), and basally impaired their viability (Fig. 4F); most importantly, NRP2 re-expression in resistant cells recovered full sensitivity to the targeted therapy (Fig. 4F). We confirmed that drug resistance associated with NRP2 downregulation was due to EGFR signaling by assaying combined treatments with the specific inhibitor erlotinib (Fig. 4G); in fact, the latter proved effective (alone or in combination with JNJ38877605) in drug-resistant, but not in parental cells (which are not dependent on EGFR signaling). Interestingly, by a complementary approach, we found that drug-resistant cells were enabled to proliferate in serum-deprived medium containing EGF or TGFα, whereas parental cells were insensitive to these factors and virtually growth arrested in these conditions (Supplementary Fig. S4DE).

NRP2 controls the expression of the EGFR-associated protein KIAA1199, in NFkB-dependent manner, impinging on EGFR protein stability

EGFR signaling upregulation sustaining tumor cell growth is often associated with gene overexpression; however, we had observed that NRP2 did not impact on EGFR mRNA levels. Actually, EGFR is furthermore posttranslationally regulated, by endocytosis and intracellular proteasomal and lysosomal-mediated degradation (21, 22). Thus, we initially assessed EGFR ligand-induced internalization rate in control and NRP2-silenced A549 cells (Supplementary Fig. S5A), as well as in parental and drug-resistant EBC1 cells (Supplementary Fig. S5B), but did not observe any significant difference in EGFR endocytosis. Conversely, we found that, upon NRP2 overexpression, ligand-induced EGFR degradation was occurring with faster kinetics (Supplementary Fig. S5C). Thus, we investigated the potential relevance of NRP2 in controlling mechanisms held responsible for the regulation of intracellular EGFR protein stability. In particular, we found that NRP2 silencing strongly upregulated the expression of the EGFR-associated protein KIAA1199 (also known as CEMIP) in both oncogene-addicted and non-oncogene addicted cells (Fig. 5A; Supplementary Fig. S3C). As shown in Fig. 5B, KIAA1199 levels were clearly upregulated in drug-resistant carcinoma cells described in the previous paragraph, consistent with NRP2 loss; moreover, this mechanism was fully reverted by re-installing NRP2 expression (Fig. 5C).

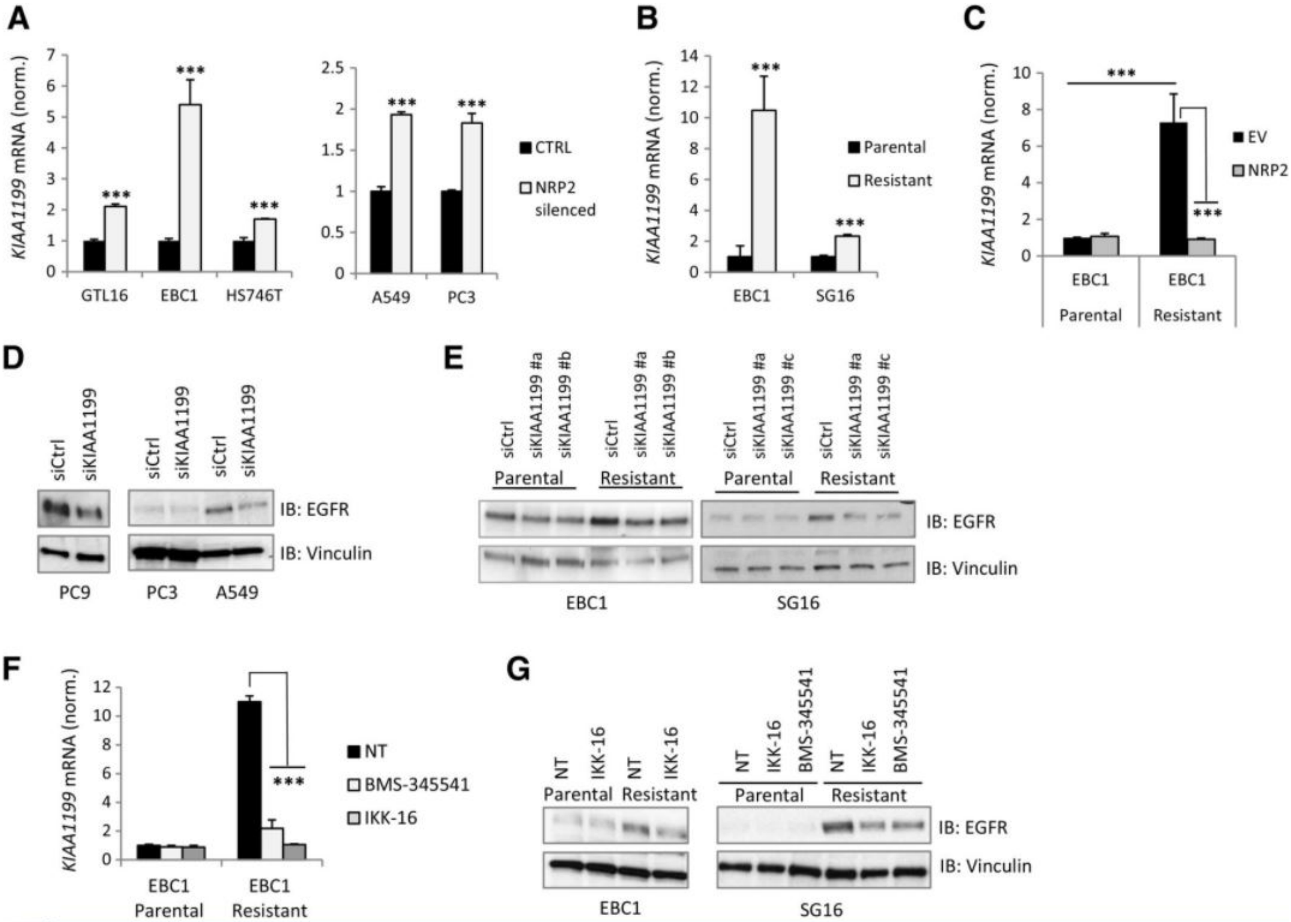

Figure 5. NRP2 knockdown upregulates the expression of KIAA1199, which is responsible for EGFR stabilization in a range of cancer cells. A, Real-time qPCR analysis (in triplicate) demonstrating the upregulation of KIAA1199 in various different cancer cells upon NRP2 silencing by shRNAs. B, KIAA1199 expression was analyzed (as in previous panel) in parental or drug-resistant EBC1 and SG16 cells (described in Fig. 4). C, KIAA1199 expression (analyzed as above) in parental or drug-resistant EBC1, either basally or upon NRP2 overexpression. D, Western blotting analysis of EGFR levels in PC9, PC3 and A549 carcinoma cells (as indicated), subjected to KIAA1199 silencing by transfection with a pool of siRNAs (siKIAA1199) or treated with control siRNAs (siC). Validation of KIAA1199 expression knockdown in the various conditions is shown in Supplementary Fig. S4A. Three independent experiments were performed, yielding consistent results. E, Western blotting analysis of EGFR levels in EBC1 cells (parental and JNJ38877605-resistant) on the left, and SG16 cells (parental and JNJ38877605-resistant) on the right, subjected to KIAA1199 silencing by two independent siRNAs or treated with control siRNAs. Validation of KIAA1199 expression knockdown in the various conditions is shown in Supplementary Fig. S4BC. Three independent experiments were performed, yielding consistent results. F, KIAA1199 expression in EBC1 cells (parental or JNJ38877605-resistant) treated with two inhibitors of the NFkB pathway (i.e. BMS-345541 1μmol/L or IKK-16 1μmol/L, or DMSO as vehicle) was analyzed by real-time qPCR. Three independent experiments confirmed these results. G, EGFR expression was analyzed by Western blotting in (parental or JNJ38877605-resistant) EBC1 cells on the left and SG16 cells on the right, upon treatment with the same NFkB inhibitors as in F. Three independent experiments confirmed these results. Statistical significance: $^{**}P < 0.005$; $^{***}P < 0.0005$. EV, empty vector.

KIAA1199 has been reported to increase EGFR protein stability and signaling in cervical and breast carcinoma cells, acting as a viability-protecting mechanism elicited by the NFkB pathway (13). Consistent with that, KIAA1199 knockdown curtailed EGFR protein levels in both oncogene-addicted and non-oncogene-addicted cells (Fig. 5D; Supplementary Fig. S5D). Moreover, the observed upregulation of EGFR levels in drug-resistant EBC1 and SG16 cells was impaired by KIAA1199 silencing (Fig. 5E; Supplementary Fig. S5E). Notably, we found that both KIAA1199 and EGFR upregulations in drug-resistant cells were dependent on the activation of the NFkB signaling pathway, as demonstrated by blockade with two distinct specific inhibitors (Fig. 5FG). To assess the hypothesis of a negative regulation of NFkB by NRP2, we analyzed the upstream inhibitor of NFkB cascade, IkB. Indeed, IkB levels were decreased (and thereby NFkB signaling increased) in both EBC1 and SG16 drug-resistant cells, characterized by reduced NRP2 expression and KIAA1199 and EGFR upregulation (Supplementary Fig. S6A), notably, NRP2 re-expression in resistant cells restored IkB levels (Supplementary Fig. S6A), leading to NFkB pathway inhibition. Conversely, NRP2 silencing resulted in IkB-loss in PC3 carcinoma cells (Supplementary Fig. S6B), thereby suggesting a general relevance of this regulatory pathway.

KIAA1199 targeting downregulates EGFR levels, thus counteracting resistance to targeted drugs and promoting viability loss in oncogene-addicted cells

KIAA1199 is widely expressed in human tumors (23); thus, we asked whether impairing KIAA1199 expression in cancer cells could inhibit EGFR-dependent mechanisms sustaining proliferation, such as in EGFR-addicted cells or in cells resistant to therapy due to NRP2 downregulation. To this end, we knocked down KIAA1199 expression in EGFR-addicted PC9 lung carcinoma cells, which resulted in an early antiproliferative effect and dramatic viability loss in culture (Fig. 6A; Supplementary Fig. S6C), consistent with a blockade of the addition pathway in these cells. Similarly, KIAA1199 knock-down in MET-inhibitor–resistant cells largely rescued drug sensitivity (Fig. 6BC; Supplementary Fig. S6DE), consistent with the consequent loss of the EGFR-dependent mechanism sustaining drug resistance.

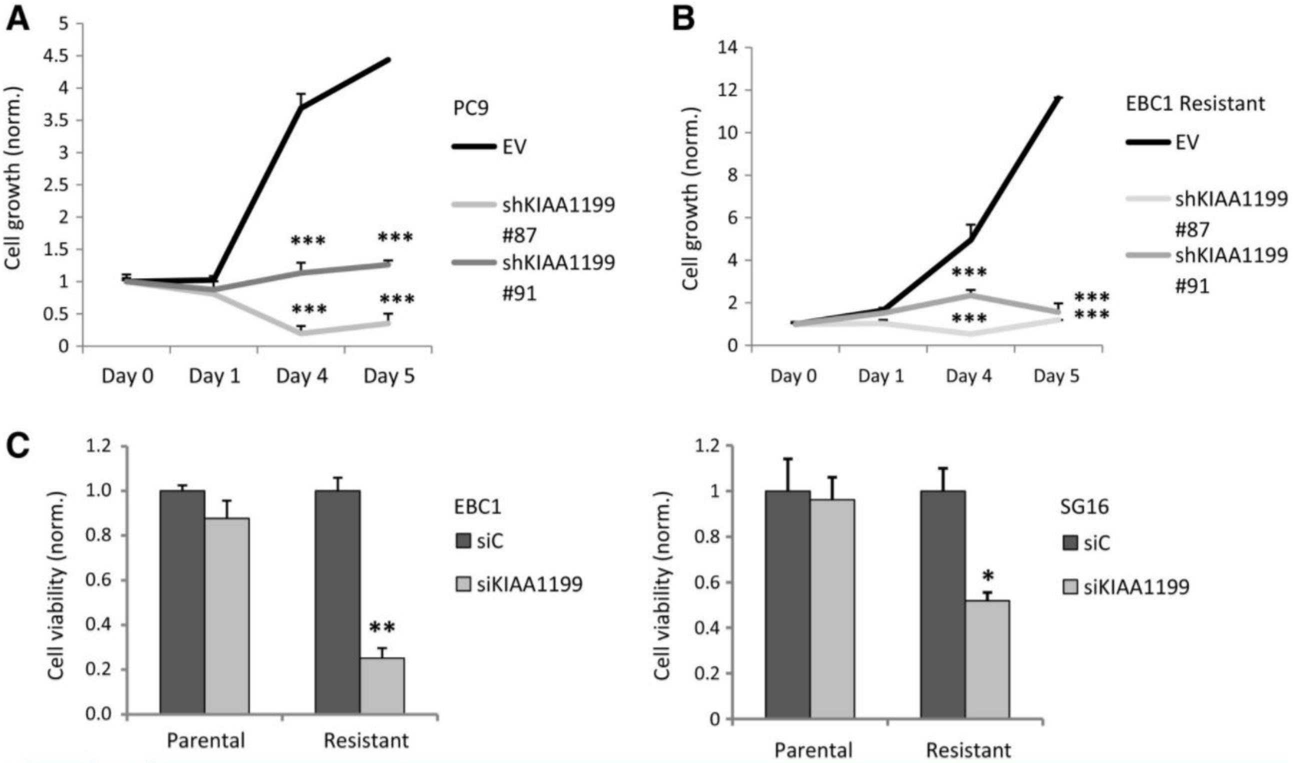

Figure 6. KIAA1199 targeting counteracts resistance to targeted drugs and elicits viability loss in oncogene-addicted cells. A,EGFR-addicted PC9 carcinoma cells were subjected to KIAA1199 silencing with two different shRNA constructs or mock-transduced with an empty vector (EV). The growth rate was assayed in cell culture; cell numbers were estimated by crystal violet staining (of quadruplicate wells per each condition) and normalized to the first assessment after seeding (day 0). Three independent experiments were performed, yielding consistent results. B, The same experimental setting as in A was applied to EBC1 JNJ38877605-resistant cells (described in Fig. 4); the experiment was repeated at least three times. C, EBC1 cells (parental and JNJ38877605-resistant) on the left and SG16 cells (parental and JNJ38877605-resistant) on the right were subjected to reverse transfection (in quadruplicate wells), with a pool of three siRNAs against KIAA1199 or control siRNAs. Parental cells were left untreated, whereas resistant cells were kept in the presence of the drug. Cell viability was assessed after 96 hours with CellTiter Glo assay. Statistical analysis compared KIAA1199-silenced cells with controls in the same experimental conditions. Validation of KIAA1199 expression knockdown in the various conditions is shown in Supplementary Fig. S6. At least three independent experiments were performed. Statistical significance: *P < 0.05; **P < 0.005; ***P < 0.0005.

These data not only underscore the relevance of KIAA1199 in NRP2-dependent EGFR regulation, but identify a novel potential target for dwindling EGFR function in tumor cells and promoting the efficacy of targeted therapies by counteracting a mechanism of drug resistance.

Discussion

The so-called “addiction” of certain tumors to activated oncogenes is currently exploited therapeutically by the treatment with specific targeted drugs, which can achieve dramatic tumor shrinkage. Yet, this therapeutic effect is often lost upon the upregulation of alternative tyrosine kinase signaling pathways sustaining cell survival, and eventually leading to acquired drug resistance. For instance, we have previously shown that loss of responsiveness to MET oncogene-targeted inhibitors may be driven by the upregulation of EGFR kinase (3, 4), although the implicated mechanisms were not elucidated. In the present study, we describe a previously unknown function of NRP2 in the regulation of EGFR expression in cancer cells, identify the implicated effectors of this pathway, and unveil its role in a mechanism of acquired resistance to drugs targeting oncogene-addiction.

NRP2 expression is typically found: in neural crest derived cells (24); in a subset of endothelial cells (25), whereby it was found to regulate tumor lymphangiogenesis; as well as in tumor cells (26), where its functional role is still unclear. In fact, although we and others have demonstrated that the homologous receptor NRP1 is often required for cancer cell viability (8), NRP2 knockdown experiments rather suggest a regulatory function in epithelial to mesenchymal transition, cell migration, and integrin function (10, 27). Notably, a recent study reported that a NRP2 splice isoform (NRP2b) promotes TGFβ-R signaling (18), which is an important pathway controlling cell differentiation and the acquisition of a mesenchymal phenotype. It was also reported that NRP2 silencing can lead to cancer cell death in response to active EGFR entrapment in intracellular vesicles (28), but we have not observed this effect in our cellular models. Actually, here we show for the first time that NRP2 is an important negative regulator of EGFR expression in a range of tumor cells.

EGFR tyrosine kinase elicits a major signaling pathway sustaining cancer cell proliferation. In fact, EGFR ligands are abundantly present in the tumor microenvironment, due to autocrine circuits in cancer cells and paracrine release by stromal cells (29). Moreover, EGFR protein levels may be upregulated in tumor cells, as the gene becomes amplified or mutated, leading to ligand-independent constitutive activation and oncogenic addiction. However, in many other cases, EGFR expression is induced in cancer cells as expedience mechanism, for taking advantage of its ligand-activated pathway sustaining survival and growth. It could therefore be speculated that NRP2 expression in tumor cells is leveled depending on the selective advantage conferred by the downstream regulated signaling cascades. For instance, in cells that are addicted to EGFR signaling, like PC9 lung cancer, NRP2 is almost undetectable, and we showed that its forced expression is hardly compatible with cell survival and growth. In MET-addicted cells, NRP2 is basally expressed at intermediate levels, but it is adaptively downregulated to allow the onset of EGFR-dependent acquired resistance to MET-targeted inhibitors. Moreover, in non oncogene-addicted cells, this NRP2-gated mechanism is relevant to enable responsiveness to environmental EGFR ligands, under nutrient-deprivation conditions.

Intriguingly, although NRP2 levels are suppressed in conditions earning from EGFR upregulation, its expression might be valuable in cells exploiting specific pathways promoted by NRP2, such as TGFβ-R (18) or Hedgehog/Gli-1 (10). For instance, Gli-1 expression was consistently reduced in all batches of MET-inhibitor resistant tumor cells, in association with NRP2 downregulation (Supplementary Fig. S7A). It was demonstrated that Hedgehog/Gli-1 signaling is required for melanoma cell growth (30). Notably, melanoma cells are originally derived from the neural crest and indeed express high levels of NRP2, whereas NRP1 is almost undetectable. Interestingly, we found that - unlike in most carcinoma cell lines - the silencing of NRP2 caused growth arrest and viability loss in melanoma cells (Supplementary Fig. S7BC), indicating dependence on a NRP2-driven signaling cascade, to be elucidated. On a different note, it was recently shown that mesenchymal-type MDAMB231 cells are characterized by low levels of miRNA-196a-3p, which hinders both NRP2 expression and TGFβ-R signaling in other breast cancer cells (31). This is consistent with the reported association between NRP2 and TGFβ-R (18), as well as with the known dependence of MDAMB231 cells on the latter signaling pathway (32). Indeed, we observed that—unlike other carcinoma cells—MDAMB231 cells were dependent on NRP2 for viability and growth (Supplementary Fig. S7D).

In addition to showing previously unknown functions of NRP2 in diverse cancer cells, in this study we unveiled a novel signaling cascade controlled by NRP2 and responsible for EGFR regulation at posttranslational level, through the effector molecule KIAA1199/CEMIP. KIAA1199 was previously found in association with EGFR and capable of preventing its ligand-induced intracellular degradation (13). Here, we found that NRP2 is a negative regulator of KIAA1199 mRNA expression, via NFkB pathway inactivation, thus controlling EGFR protein levels and signaling cascade. Notably, the intracellular signaling mechanisms downstream to NRP2 are poorly understood, and this is the first time that its role in the negative regulation of NFkB pathway is unveiled.

Importantly, our data suggest that KIAA1199 targeting in tumor cells that are dependent on EGFR signaling (either primarily oncogene addicted or exploiting this pathway to acquire drug resistance) is strikingly effective in hindering growth, promoting response to targeted therapy, and counteracting resistance. For instance, KIAA1199 knockdown in EGFR-addicted PC9 carcinoma cells led to growth arrest, consistent with what we observed upon NRP2 overexpression. Indeed, we could not establish long-standing KIAA1199 silencing or NRP2 overexpression in PC9 cells, likely due to negative selection of cells undergoing EGFR downregulation. Moreover, interfering with KIAA1199 was effective in sensitizing to targeted therapy cancer cells, which were not primarily addicted to EGFR, but upregulated its expression as an adaptation and a secondary drug-resistance mechanism. Thus, KIAA1199 may be considered a promising target for future combined therapeutic regimens attacking human tumors that could use EGFR as adaptive mechanism of resistance.

In summary, here we describe a novel signaling cascade mediated by NRP2 in diverse tumor cells, hindering the NFkB-dependent induction of the EGFR-stabilizing protein KIAA1199/CEMIP. In fact, NRP2 downregulation proves to be a mechanism of adaptive secondary resistance to oncogene-targeted therapy in tumor cells. Conversely, NRP2 overexpression or blockade of the downstream effector KIAA1199 results in EGFR depletion and therapeutic targeting of cancer cells, either primarily EGFR-addicted, or developing drug resistance due to adaptive EGFR upregulation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: S. Rizzolio, L. Tamagnone

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Rizzolio, C. Battistini, G. Cagnoni, M. Apicella, V. Vella

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Rizzolio, S. Giordano, L. Tamagnone

Writing, review, and/or revision of the manuscript: S. Rizzolio, S. Giordano, L. Tamagnone

Study supervision: S. Giordano, L. Tamagnone

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References

1. Chong CR, Jänne PA. The quest to overcome resistance to EGFR-targeted therapies in cancer. Nat Med2013;19:1389–400.

2. Stommel JM, Kimmelman AC, Ying H, Nabioullin R, Ponugoti AH, Wiedemeyer R, et al. Coactivation of receptor tyrosine kinases affects the response of tumor cells to targeted therapies. Science 2007;318:287–90.

3. Corso S, Ghiso E, Cepero V, Sierra JR, Migliore C, Bertotti A, et al. Activation of HER family members in gastric carcinoma cells mediates resistance to MET inhibition. MolCancer 2010;9:121.

4. Apicella M, Migliore C, Capelôa T, Menegon S, Cargnelutti M, Degiuli M, et al. Dual MET/EGFR therapy leads to complete response and resistance prevention in a MET-amplified gastroesophageal xenopatient cohort. Oncogene 2017;36:1200–10.

5. Guttmann-Raviv N, Kessler O, Shraga-Heled N, Lange T, Herzog Y, Neufeld G. The neuropilins and their role in tumorigenesis and tumor progression. Cancer Lett 2006;231:1–11.

6. Parker MW, Guo HF, Li X, Linkugel AD, Vander Kooi CW. Function of members of the neuropilin family as essential pleiotropic cell surface receptors. Biochemistry 2012;51:9437–46.

7. Rizzolio S, Tamagnone L. Multifaceted role of neuropilins in cancer. CurrMedChem 2011;18:3563–75.

8. Rizzolio S, Rabinowicz N, Rainero E, Lanzetti L, Serini G, Norman J, et al. Neuropilin-1-dependent regulation of EGF-receptor signaling. Cancer Res 2012;72:5801–11.

9. Wild JR, Staton CA, Chapple K, Corfe BM. Neuropilins: expression and roles in the epithelium. Int J Exp Pathol 2012;93:81–103.

10. Goel HL, Pursell B, Chang C, Shaw LM, Mao J, Simin K, et al. GLI1 regulates a novel neuropilin-2/α6β1 integrin based autocrine pathway that contributes to breast cancer initiation. EMBO Mol Med 2013;5:488–508.

11. Grandclement C, Pallandre JR, Valmary Degano S, Viel E, Bouard A, Balland J, et al. Neuropilin-2 expression promotes TGF-β1-mediated epithelial to mesenchymal transition in colorectal cancer cells. PLoS ONE 2011;6:e20444.

12. Goel HL, Chang C, Pursell B, Leav I, Lyle S, Xi HS, et al. VEGF/neuropilin-2 regulation of Bmi-1 and consequent repression of IGF-IR define a novel mechanism of aggressive prostate cancer. Cancer Discov2012;2:906–21.

13. Shostak K, Zhang X, Hubert P, Göktuna SI, Jiang Z, Klevernic I, et al. NF-κB-induced KIAA1199 promotes survival through EGFR signalling. Nat Commun 2014;5:5232.

14. Smolen GA, Sordella R, Muir B, Mohapatra G, Barmettler A, Archibald H, et al. Amplification of MET may identify a subset of cancers with extreme sensitivity to the selective tyrosine kinase inhibitor PHA-665752. Proc Natl Acad Sci U S A 2006;103:2316–21.

15. Corso S, Migliore C, Ghiso E, De RG, Comoglio PM, Giordano S. Silencing the MET oncogene leads to regression of experimental tumors and metastases. Oncogene 2008;27:684–93.

16. Yeo WL, Riely GJ, Yeap BY, Lau MW, Warner JL, Bodio K, et al. Erlotinib at a dose of 25 mg daily for non-small cell lung cancers with EGFR mutations. J Thorac Oncol 2010;5:1048–53.

17. Cepero V, Sierra JR, Corso S, Ghiso E, Casorzo L, Perera T, et al. MET and KRAS gene amplification mediates acquired resistance to MET tyrosine kinase inhibitors. Cancer Res 2010;70:7580–90.

18. Gemmill RM, Nasarre P, Nair-Menon J, Cappuzzo F, Landi L, D'Incecco A, et al. The neuropilin 2 isoform NRP2b uniquely supports TGFβ-mediated progression in lung cancer. Sci Signal 2017;10.

19. Martin V, Corso S, Comoglio PM, Giordano S. Increase of MET gene copy number confers resistance to a monovalent MET antibody and establishes drug dependence. Mol Oncol 2014;8:1561–74.

20. Das Thakur M, Salangsang F, Landman AS, Sellers WR, Pryer NK, Levesque MP, et al. Modelling vemurafenib resistance in melanoma reveals a strategy to forestall drug resistance. Nature 2013;494:251–5.

21. Sigismund S, Argenzio E, Tosoni D, Cavallaro E, Polo S, Di Fiore PP. Clathrin-mediated internalization is essential for sustained EGFR signaling but dispensable for degradation. DevCell 2008;15:209–19.

22. Longva KE, Blystad FD, Stang E, Larsen AM, Johannessen LE, Madshus IH. Ubiquitination and proteasomal activity is required for transport of the EGF receptor to inner membranes of multivesicular bodies.J Cell Biol 2002;156:843–54.

23. Zhang Y, Jia S, Jiang WG. KIAA1199 and its biological role in human cancer and cancer cells (review).Oncol Rep 2014;31:1503–8.

24. Gammill LS, Gonzalez C, Gu C, Bronner-Fraser M. Guidance of trunk neural crest migration requires neuropilin 2/semaphorin 3F signaling. Development 2006;133:99–106.
25. Karpanen T, Heckman CA, Keskitalo S, Jeltsch M, Ollila H, Neufeld G, et al. Functional interaction of VEGF-C and VEGF-D with neuropilin receptors. FASEB J 2006;20:1462–72.
26. Jubb AM, Sa SM, Ratti N, Strickland LA, Schmidt M, Callahan CA, et al. Neuropilin-2 expression in cancer.Histopathology 2012;61:340–9.
27. Wittmann P, Grubinger M, Gröger C, Huber H, Sieghart W, Peck-Radosavljevic M, et al. Neuropilin-2 induced by transforming growth factor-β augments migration of hepatocellular carcinoma cells. BMC Cancer2015;15:909.
28. Dutta S, Roy S, Polavaram NS, Stanton MJ, Zhang H, Bhola T, et al. Neuropilin-2 regulates endosome maturation and EGFR trafficking to support cancer cell pathobiology. Cancer Res 2016;76:418–28.
29. Singh AB, Harris RC. Autocrine, paracrine and juxtacrine signaling by EGFR ligands. Cell Signal2005;17:1183–93.
30. Stecca B, Mas C, Clement V, Zbinden M, Correa R, Piguet V, et al. Melanomas require HEDGEHOG-GLI signaling regulated by interactions between GLI1 and the RAS-MEK/AKT pathways. Proc Natl Acad Sci U S A2007;104:5895–900.
31. Chen Y, Huang S, Wu B, Fang J, Zhu M, Sun L, et al. Transforming growth factor-β1 promotes breast cancer metastasis by downregulating miR-196a-3p expression. Oncotarget 2017;8:49110–22.
32. Lei X, Bandyopadhyay A, Le T, Sun L. Autocrine TGFbeta supports growth and survival of human breast cancer MDA-MB-231 cells. Oncogene 2002;21:7514–23