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VEGF–Neuropilin-2 signaling promotes stem-like traits in breast cancer cells by TAZ-mediated repression of the Rac GAP β 2-chimaerin

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Abstract

The role of vascular endothelial growth factor (VEGF) signaling in cancer is well-known in the context of angiogenesis but is also important in the functional regulation of tumor cells themselves. Notably, autocrine VEGF signaling mediated by its co-receptors called neuropilins (NRPs) appears to be essential for sustaining the proliferation and survival of cancer stem cells (CSCs), which are implicated in mediating tumor growth, progression and drug resistance. Therefore, understanding the mechanisms involved in VEGF-mediated support of CSCs is critical to successfully treating cancer patients. The expression of the Hippo effector TAZ is associated with breast CSCs and has been shown to confer stem cell-like properties. We found that VEGF-NRP2 signaling contributes to the activation of TAZ in various breast cancer cells, which mediates a positive feedback loop that promotes mammosphere formation. VEGF-NRP2 signaling activated the GTPase Rac1, which inhibited the Hippo kinase LATS, which enabled the activity of TAZ. In

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Author Contributions

A.L.E. and A.M.M. designed experiments and wrote the manuscript. A.L.E. performed experiments. S.G. performed immunofluorescence confocal microscopy. C.C. cloned wild-type and S89A TAZ plasmids, and generated puromycin-sensitive EPTB and MES cells. J.O. and J.Z. performed the ENCODE, cBioPortal and microarray analyses. J.J.A. provided technical support. H.L.G. contributed to ChIP experiments, provided feedback on data and helped write the manuscript. All authors read and approved the final manuscript.

Competing Interests

The authors of this manuscript do not have any competing interests.

Data and materials availability

All data needed to evaluate the conclusions in the paper are present in the paper or the Supplementary Materials.

complex with the transcription factor TEAD, TAZ then bound and repressed the promoter of the gene encoding the Rac GTPase-activating protein (Rac GAP) β 2-chimaerin. By activating GTP hydrolysis, Rac GAPs effectively turn off Rac signaling; hence, TAZ-mediated repression of β 2-chimaerin sustained Rac1 activity in CSCs. Depletion of β 2-chimaerin in non-CSCs increased Rac1 activity, TAZ abundance and mammosphere formation. Analysis of a breast cancer patient database revealed an inverse correlation between β 2-chimaerin and TAZ expression in tumors. Our findings highlight an unexpected role for β 2-chimaerin in a feedforward loop of TAZ activation and the acquisition of CSC properties.

Introduction

Vascular endothelial growth factor (VEGF) was originally characterized as a protein that promotes endothelial growth (1) and increases vascular permeability (2). For these and other reasons, it was presumed that the role of VEGF in cancer was limited to angiogenesis (1, 3–5). It is evident now, however, that there are angiogenesis-independent functions of VEGF in cancer that are mediated by specific receptors. Tumor cells express VEGF receptor tyrosine kinases (VEGFR1 and VEGFR2) and neuropilins (NRPs), another family of VEGF receptors. NRP1 and NRP2 were identified initially as neuronal receptors for semaphorins, which are axon guidance factors that function primarily in the developing nervous system (6). The finding that NRPs can also function as VEGF receptors and that they are expressed on endothelial and tumor cells launched studies aimed at understanding their contribution to angiogenesis and tumor biology (7). NRPs have the ability to interact with and modulate the function of VEGFR1 and VEGFR2, as well as other receptors (8–10). There is also evidence that NRPs are valid targets for therapeutic inhibition of angiogenesis and cancer (11–14).

A surge of evidence has implicated autocrine VEGF signaling mediated by NRPs in the function of cancer stem cells (CSCs), a sub-population of cells that function in tumor initiation, the differentiation of multi-lineage cancer cell hierarchies, therapy resistance and metastasis (12, 15–21). These observations have led to intense investigation into the mechanisms by which VEGF sustains CSCs and how these processes can be exploited therapeutically. Previously, we reported that NRP2 is highly expressed in breast CSCs and that VEGF-NRP2 signaling contributes to breast tumor initiation (22). A key issue that emerges from these findings is the mechanism by which VEGF-NRP2 signaling contributes to the function of CSCs. In pursuit of this issue, we were intrigued by reports that the Hippo pathway transducer TAZ confers stem cell properties and contributes to breast tumorigenesis, especially in high-grade tumors, which are distinguished by high NRP2 expression and VEGF-NRP2 signaling activity (22). Moreover, TAZ expression in breast cancer correlates with tumor grade (23) and high-grade tumors harbor a higher frequency of CSCs than do lower grade tumors (24). Mechanistic studies have shown that TAZ can induce an epithelial to mesenchymal transition (EMT) in mammary epithelial cells (25), a process that can increase stem cell properties (26). Moreover, TAZ is necessary for the self-renewal of CSCs (23). In contrast, the role of YAP in breast cancer is less clear and its expression does not correlate with clinical outcome (27).

The Hippo pathway consists of core kinases and regulatory molecules that facilitate TAZ phosphorylation, cytoplasmic retention and inactivation (28–30). For this reason, identifying upstream receptors that disrupt Hippo signaling and, consequently, enhance TAZ activity is critical for understanding how these effectors contribute to the function of CSCs. In this study, we discovered that VEGF-NRP2 signaling contributes to increased TAZ activity by a Rac1-dependent, feed-forward mechanism.

Results

VEGF-NRP2 Signaling Contributes to TAZ Activation:

Initially, we assessed the contribution of VEGF-NRP2 signaling to TAZ activation. For this purpose, we used an inducible system to transform MCF10A cells with Src, which generates a population of CD44^{high}/CD24^{low} cells with CSC properties (31). This population is actually comprised of distinct epithelial (EPH) and mesenchymal (MES) populations that differ in TAZ activity and tumor initiating potential. The MES population has enhanced TAZ activity, self-renewal potential and tumor initiating capability compared to the EPH population (32). Importantly, MES cells express increased levels of VEGF and NRP2 compared to EPH cells and are dependent on VEGF-NRP2 signaling for self-renewal (33). Expression of either NRP2 (Figure 1A) or VEGF (Figure 1B) was diminished in MES cells using shRNAs, which resulted in decreased TAZ abundance compared to control cells as assessed by immunoblotting (Figures 1, A and B). As reported previously, TAZ abundance is an indicator of its activation status (34, 35), as well as its nuclear localization. For this latter reason, we compared TAZ localization in control cells to VEGF- and NRP2-depleted cells by immunofluorescence (Figure 1C; fig. S1A). TAZ is localized primarily in the nucleus in control MES cells (Figure 1C; fig. S1A). In contrast, little, if any, TAZ was detected in VEGF- and NRP2-depleted cells, which is consistent with our immunoblotting data (Figures 1, A and B). The TAZ that was detected in VEGF-depleted cells is localized in the cytoplasm (Figure 1C; fig. S1A).

Depletion of NRP2 in MES cells also reduced the mRNA expression of the TAZ target genes *CTGF* and *CYR61* (Figure 1D). To substantiate the data obtained with shRNAs, we treated MES cells with a function-blocking NRP2 antibody (11) and observed a concentration - dependent decrease in TAZ abundance (Figure 1E). Although similar results were obtained with YAP (fig. S1B–C), we focused subsequent experiments on TAZ because convincing data correlating YAP expression and clinical parameters in breast cancer are lacking (27).

We extended this analysis to MDA-MB-231 cells because they exhibit mesenchymal properties and highly express VEGF and NRP2 (33). Similar to MES cells, NRP2 depletion reduced the abundance of TAZ, as well as TAZ target genes, (Figures 1, F and G) suggesting that NRP2 affects TAZ-mediated transcription. TAZ regulates gene expression by associating with the TEAD family of transcription factors (36), which infers that NRP2 should affect TEAD transcriptional activity. Indeed, the activity of a TEAD luciferase reporter was reduced significantly in MDA-MB-231 cells with diminished NRP2 expression compared to control cells (Figure 1H).

Rac1 Facilitates VEGF-NRP2-mediated Activation of TAZ:

To investigate the mechanism by which VEGF-NRP2 signaling activates TAZ, we focused on Rac1 for several reasons. This GTPase is a major effector of NRP/plexin signaling in neurons (37, 38) and it has been implicated in VEGF signaling in endothelial cells (39, 40). Moreover, Rac1 has also been implicated in TAZ activation (35, 41–43). We observed that depleting VEGF expression or treating MES cells with the NRP2 function-blocking antibody resulted in a substantial decrease in Rac1 activity (Figures 2, A and B). Similar results were obtained with MDA-MB-231 cells (Figure 2C). Conversely, stimulating MDA-MB-231 cells with VEGF resulted in an increase in Rac1 activity (Figure 2D).

An important issue is whether VEGFRs contribute to Rac1 activation in breast cancer cells. Treatment of MDA-MB-231 cells with the VEGFR inhibitors pazopanib and sunitinib in the presence of VEGF did not decrease Rac1 activity compared to VEGF alone (fig. S2), suggesting that VEGF-NRP2 activation of Rac1 is VEGFR-independent. This result is not surprising because we reported previously that MDA-MB-231 and other breast cancer cell lines express very low levels of VEGFRs (44). In contrast, VEGF-NRP2-mediated Rac1 activation appears to be focal adhesion kinase (FAK)-dependent, because the FAK inhibitor FAK14 significantly decreased Rac1 activity in MES cells (Figure 2E). This result is consistent with previous findings that FAK is a downstream effector of VEGF-NRP2 signaling (22), and it has been implicated in Rac1 activation (45–47), as well as Hippo pathway regulation (48, 49).

The results described above prompted us to evaluate whether VEGF-NRP2 signals through Rac1 to promote TAZ activation. Based on our finding that depletion of NRP2 in MDA-MB-231 cells decreased the expression of TAZ target genes (Figure 1G), we found that expression of a constitutively active V12 Rac1 in these cells rescued their expression (Figure 2F). This result provides evidence that the VEGF-NRP2-Rac1 axis contributes to TAZ activation. Subsequently, we assessed the role of Rac1 inhibition on mammosphere formation in MES cells and observed decreased mammosphere formation upon treatment with the Rac inhibitors EHT1864 and NSC23766 (Figure 2G).

We next sought to investigate the mechanism by which VEGF-NRP2-mediated regulation of Rac1 contributes to TAZ activation. We focused on the LATS tumor suppressor kinases because they phosphorylate TAZ directly at the Ser⁸⁹ position and promote its cytoplasmic retention and degradation when phosphorylated and activated on their hydrophobic motifs (Thr¹⁰⁷⁹ in LATS1, Thr¹⁰⁴¹ in LATS2) (25, 50, 51). Moreover, LATS can be regulated by Rac1 (35, 41, 42). Treatment of MES cells with the Rac inhibitor EHT1864 resulted in a concentration-dependent increase in the abundance of phosphorylated Ser⁸⁹ (pSer⁸⁹) TAZ and a concomitant decrease in TAZ abundance (Figure 3A). It also increased the abundance of pThr¹⁰⁷⁹ LATS1 and decreased TAZ abundance as early as 15 minutes after treatment, and this pattern persisted for up to 6 hours (Figure 3B). Expression of dominant-negative N17 Rac1 also decreased TAZ abundance in MES cells (Figure 3C). Similar results were obtained with the NRP2 function-blocking antibody (Figure 3D). NRP2 knockdown in MDA-MB-231 cells also increased the abundance of pSer⁸⁹ TAZ and pThr¹⁰⁷⁹ LATS1 and decreased the abundance of TAZ (Figures 3, E and F). NRP2 depletion also increased LATS-mediated YAP phosphorylation (pSer¹²⁷) (fig. S1B). LATS knockdown rescued TAZ

abundance in NRP2 depleted MDA-MB-231 cells, which shows that VEGF-NRP2-Rac1 regulation of TAZ is LATS-dependent (fig. S3A).

Given the importance of TAZ in promoting CSC properties (23), we hypothesized that VEGF-NRP2-Rac1-mediated LATS inhibition is a critical upstream regulator of TAZ-mediated mammosphere formation. Depletion of NRP2 in MDA-MB-231 cells significantly reduced mammosphere formation, which was partially rescued by expression of wild-type TAZ (Figure 3G; fig. S4). Expression of S89A TAZ, which is resistant to LATS-mediated phosphorylation at that site, rescued mammosphere formation significantly more than did expression of wild-type TAZ (Figure 3G; fig. S4). Similar results were obtained by VEGF depletion in MES cells (Figure 3H). These results indicate that the wild-type TAZ ectopically expressed is subject to regulation by upstream VEGF-NRP2 signaling, but that the S89A TAZ mutant is not. Together, these data provide functional evidence that VEGF-NRP2-Rac1 promotes a TAZ-dependent stem-like phenotype through inhibition of LATS-mediated phosphorylation of TAZ at Ser⁸⁹.

To gain insight into the mechanism by which VEGF-NRP2-Rac1 signaling inhibits LATS activity, we postulated that this signaling regulates Merlin, the protein product of the Neurofibromatosis type 2 (NF2) gene, because phosphorylation of Merlin on Ser⁵¹⁸ by p21-activated kinase (PAK) is inhibitory (52–54). Moreover, Merlin phosphorylation inhibits LATS phosphorylation (55). These findings are relevant because PAK is a Rac-activated kinase (56). Following these observations, we found that either Rac inhibition or NRP2 depletion in MES cells reduced the abundance of Ser⁵¹⁸-phosphorylated Merlin (fig. S3, B and C).

VEGF-NRP2 Signaling Represses the Rac GAP β 2-chimaerin:

Rac1 cycles from GTP-bound active states to GDP-bound inactive states, which, in large part, is regulated by the expression of guanine nucleotide exchange factors (GEFs) and GAPs. Therefore, we profiled the expression of known Rac GEFs and GAPs in EPTB and MES cells (Table 1). Notably, we observed that the expression of the Rac GAP β 2-chimaerin was markedly reduced in MES cells compared to EPTB cells (Table 1), which we verified by immunoblotting (Figure 4A). β 2-chimaerin is a Rac-specific GAP that has been implicated as a tumor suppressor in breast cancer (57–61). Given that β 2-chimaerin abundance is reduced in MES cells and these cells highly express VEGF and NRP2 (33), we assessed whether VEGF-NRP2 signaling repressed β 2-chimaerin expression. Indeed, we observed that NRP2 depletion increased β 2-chimaerin mRNA and protein expression in MES cells (Figures 4, B and C). Treatment of MES cells with the NRP2 function-blocking antibody also increased β 2-chimaerin abundance (Figure 4D). These observations provide evidence that VEGF-NRP2 signaling represses β 2-chimaerin expression.

TAZ Activates Rac1 by Repressing β 2-chimaerin through a TEAD-Dependent Mechanism:

Based on our observation that VEGF-NRP2 signaling activates TAZ and represses β 2-chimaerin, we assessed the possibility that TAZ represses β 2-chimaerin. This possibility is supported by studies demonstrating that TAZ can function in transcriptional repression (62, 63). Depletion of TAZ in MES cells caused an increase in β 2-chimaerin mRNA and protein

expression and a consequent decrease in Rac1 activity (Figures 5, A to C). TAZ knockdown in MDA-MB-435 cells (Figure 5D) and MDA-MB-231 cells (Figure 5E) also increased $\beta 2$ -chimaerin mRNA expression. Conversely, TAZ overexpression repressed $\beta 2$ -chimaerin mRNA expression in MDA-MB-231 cells (Figure 5F). Given our observations that Rac1 activates TAZ (Figure 3) and that TAZ represses $\beta 2$ -chimaerin expression, we inhibited Rac1 in MES cells using EHT1864 and observed an increase in the expression of $\beta 2$ -chimaerin mRNA (Figure 5G). These results provide evidence that VEGF-NRP2-Rac1-mediated TAZ activation maintains elevated Rac1 activity by repressing $\beta 2$ -chimaerin in a positive feedback loop.

TAZ-mediated transcriptional repression is dependent on the TEAD1–4 family of transcription factors (62, 63). TEAD4, in particular, is expressed at relatively high levels in breast cancer, especially triple negative breast cancer (64, 65). Given this information, we initially searched the encyclopedia of DNA elements (ENCODE) for TEAD4 ChIP-seq datasets and found 4 cell types (h1-human embryonic stem cells, HCT116 colon cancer cells, Ishikawa endometrial adenocarcinoma cells and SK-N-SH neuroblastoma cells) where TEAD4 bound to the promoter region of the $\beta 2$ -chimaerin gene (Figure 6A). Specifically, a conserved peak was observed near position 29229000 (chr7) in all of the cell types. These findings are significant because they demonstrate direct binding of TEAD4 to the $\beta 2$ -chimaerin promoter. Also, h1-hESCs, HCT116, Ishikawa and SK-N-SH cells have enhanced TAZ/TEAD activity (66, 67). To validate the ENCODE data in our model system, we performed ChIP in MES cells using antibodies specific for TEAD4 and TAZ. The results verify that TEAD4 and TAZ are recruited to the genomic region in the $\beta 2$ -chimaerin promoter identified in ENCODE (Figure 6B). Based on these data, we depleted TEAD1/3/4 expression in MES cells and observed an increase in $\beta 2$ -chimaerin mRNA and protein expression (Figures 6, C and D) and a decrease in Rac1 activity (Figure 6E), consistent with our TAZ knockdown results (Figures 5, A to C). Similarly, expression of dominant-negative TEAD4 in MDA-MB-231 cells increased $\beta 2$ -chimaerin mRNA expression (Figure 6F).

$\beta 2$ -chimaerin Repression Contributes to Enhanced TAZ Activity:

An important issue that arises from the data thus far is whether $\beta 2$ -chimaerin repression has a causal role in TAZ activation. Expression of $\beta 2$ -chimaerin in MDA-MB-231 cells decreased Rac1 activity, as well as the abundance of TAZ itself (Figure 7A) and TAZ target genes (Figure 7B). Conversely, $\beta 2$ -chimaerin knockdown in EPTH cells increased Rac1 activity, TAZ abundance (Figure 7C) and TAZ target genes (Figure 7D). Notably, $\beta 2$ -chimaerin-depleted EPTH cells exhibited increased mammosphere formation compared to control EPTH cells (Figure 7E). Expression of $\beta 2$ -chimaerin also reduced TAZ-mediated, but not S89A TAZ-mediated, mammosphere formation in MDA-MB-231 cells (Figure 7F), providing further evidence that Rac1 inhibition of LATS contributes to TAZ activation and CSC properties.

These in vitro data indicating an inverse causal relationship between $\beta 2$ -chimaerin and TAZ and a positive causal relationship between VEGF-NRP2 and TAZ were substantiated by analysis of their expression in invasive breast carcinomas in The Cancer Genome Atlas (TCGA) database obtained from cBioPortal (68, 69) (Figure 7G). Indeed, the expression of

TAZ and $\beta 2$ -chimaerin were inversely correlated. In contrast, the expression of TAZ correlated with that of VEGF and NRP2. An inverse correlation between TAZ and $\beta 2$ -chimaerin was also detected in glioblastoma and colorectal adenocarcinoma samples in the TCGA. These findings are significant because both glioblastoma and colon cancer exhibit enhanced TAZ activity (66, 70, 71). Lastly, TAZ and $\beta 2$ -chimaerin exhibited an inverse correlation in the cancer cell line encyclopedia obtained from cBioPortal (967 cell lines) (Figure 7G), which provides further evidence of a repressive role (68, 69).

Our data indicate that TAZ-mediated repression of $\beta 2$ -chimaerin is associated with a mesenchymal phenotype. To substantiate this conclusion, we analyzed a microarray (GSE48204) that utilized TGF- β -treated NMuMG mammary epithelial cells to induce an EMT (72). In support of our conclusion, we found that the EMT reduced $\beta 2$ -chimaerin expression and increased expression of VEGF and NRP2, as well as TAZ target genes (fig. S5). We also used cBioPortal to stratify breast cancer patients in the TCGA database based on their expression of the estrogen receptor (ER), which is associated with an epithelial phenotype. Comparison of $\beta 2$ -chimaerin expression in the estrogen receptor-positive (ER⁺) and ER⁻ subgroups revealed that ER⁻ patients have lower expression of $\beta 2$ -chimaerin compared to ER⁺ patients (Figure 7H).

Discussion

The results of this study establish a causal role for VEGF-NRP2 signaling in sustaining the activation of TAZ, a critical effector molecule of the Hippo pathway that contributes to breast tumorigenesis and is associated with aggressive, high-grade tumors. An essential component of this mechanism is the repression of $\beta 2$ -chimaerin, a Rac GAP, by TAZ and the consequent activation of Rac1 resulting in a positive feedback loop driven by VEGF-NRP2 signaling that sustains TAZ activation (Figure 8). These findings increase our understanding of autocrine VEGF signaling in tumor cells and they substantiate the importance of Rac in the biology of CSCs and TAZ regulation.

Our conclusion that repression of $\beta 2$ -chimaerin contributes to TAZ activation and self-renewal indicates that this Rac GAP is an important gatekeeper that impedes the acquisition of stem cell properties. Based on the hypothesis that breast CSCs are de-differentiated and exhibit features of an EMT (26), this conclusion infers that repression of $\beta 2$ -chimaerin is a consequence of the EMT and that its expression is associated with an epithelial phenotype. Indeed, we uncovered that $\beta 2$ -chimaerin is repressed by the TGF- β -induced EMT of mammary epithelial cells. Importantly, we also demonstrated that ER⁺ patients have higher $\beta 2$ -chimaerin expression compared to ER⁻ patients. These observations contrast with the report that $\beta 2$ -chimaerin reduces E-cadherin levels in an *in vitro* overexpression system (60). Interestingly, however, this report also demonstrated that low expression of $\beta 2$ -chimaerin is associated with reduced relapse-free survival of breast cancer patients, which supports our findings.

Our results need to be discussed in the context of the report that NRP2 binds $\beta 2$ -chimaerin directly, and that Semaphorin3F-NRP2 signaling reduces this association to activate $\beta 2$ -chimaerin and regulate axonal pruning in the hippocampus (38). Although $\beta 2$ -chimaerin and

NRP2 exhibit an inverse expression pattern in breast cancer, we tested the hypothesis that residual β 2-chimaerin may be sequestered and inactivated by NRP2 as a mechanism of Rac1 regulation. However, we were unable to co-immunopurify NRP2 with β 2-chimaerin. This is not definitive, but it does suggest NRP2 regulation of β 2-chimaerin differs in breast cancer cells and neurons. This difference may reflect the fact that different ligands (Semaphorin3F and VEGF) engage NRP2 in these cells types. It is also worth mentioning that we previously demonstrated that Semaphorin3A and VEGF compete for NRP binding in breast cancer cells, and that these two ligands have opposite effects on the behavior of these cells (73). Nonetheless, the existing data highlight an important causal effect of NRP2 on β 2-chimaerin that is executed by distinct mechanisms.

A major conclusion of this study is that VEGF-NRP2 signaling contributes to TAZ activation by a Rac1-dependent mechanism. This role for Rac1 differs from its more established role in regulating cell invasion and migration in cancer, but it is consistent with other reports implicating Rac1 in the function of CSCs (74–76), as well as in YAP/TAZ activation (35, 41–43). Our results on the ability of VEGF-NRP2 signaling to inhibit LATS by a Rac1-dependent mechanism support these observations and they identify a novel ligand-receptor interaction that can mediate this regulation. Furthermore, our data suggest that the ability of Rac1 to inhibit LATS is mediated by its regulation of Merlin, which is an important organizer of the membrane-cytoskeleton interface (52–55, 77–79). Although we are not ruling out the possibility that VEGF-NRP2 signaling may activate RhoA, which has also been implicated in YAP/TAZ activation (35, 41, 79–81), we focused our attention on Rac1 because β 2-chimaerin is Rac-specific and does not have GAP activity against Rho (57, 58).

Although many studies have implicated autocrine VEGF signaling in the function of CSCs (20), its ability to contribute to Hippo-TAZ regulation provides a new dimension to our understanding of VEGF biology. While this manuscript was in review, however, it was reported that VEGF-VEGFR2 signaling contributes to YAP/TAZ activation during developmental angiogenesis (82). Our data support the role of VEGF in promoting YAP/TAZ activation, but the mechanism used by breast cancer cells is distinct because it appears to be dependent on VEGF-NRP2 activation of FAK-Rac1 but independent of VEGFR. Moreover, our findings reveal a pivotal role for β 2-chimaerin as a repressive intermediary between VEGF-NRP2 and TAZ activation. They also reinforce the hypothesis that targeting VEGF-NRP signaling is a viable therapeutic strategy for tumor cells that are dependent on TAZ activation.

Materials and Methods

Reagents and Antibodies

EHT1864 was purchased from Tocris, NSC23766 was purchased from Selleckchem, FAK14 was purchased from Sigma, Sunitinib and Pazopanib were purchased from LC Laboratories, human VEGFA165 was purchased from R&D Systems and the function-blocking Neuropilin-2 antibody was provided by Genentech (11). Immunoblotting antibodies were acquired as follows: Actin (A2066, Sigma), TAZ (560235, BD Biosciences), YAP/TAZ (8418S, Cell Signaling Technologies), pS89 TAZ (sc-17610, Santa Cruz Biotechnology),

pS127 YAP (4911A, Cell Signaling Technologies), Neuropilin-2 (sc-7242, Santa Cruz Biotechnology), β 2-chimaerin (CHN2) (HPA018989, Sigma), pT1079 LATS1 (8654S, Cell Signaling Technologies), LATS1 (9153S, Cell Signaling Technologies), VEGF (sc-152, Santa Cruz Biotechnology), Rac1 (610650, BD Biosciences), Pan-TEAD (13295S, Cell Signaling Technologies), pS518 Merlin (9163S, Cell Signaling Technologies), Merlin (6995S, Cell Signaling Technologies), HA-Tag (3724S, Cell Signaling Technologies), GST (sc-138, Santa Cruz Biotechnology) and Myc-Tag (2278S, Cell Signaling Technologies).

Constructs

The following lentiviral shRNA vectors were used: VEGF (TRCN0000003343, TRCN0000003344, TRCN0000003345), Neuropilin-2 (TRCN0000063309, TRCN0000063312, TRCN0000063310), β 2-chimaerin (provided by Dr. Alex Kolodkin, Johns Hopkins Medical Institute (38)) and TEAD 1/3/4 (provided by Dr. Junhao Mao, University of Massachusetts Medical School (66)). Retroviral shTAZ vectors were used as previously described (32). Stable shRNA expression was accomplished by selecting cells in 2 μ g/mL puromycin for 2 to 4 days. Myc-tagged (6X) β 2-chimaerin was provided by Dr. Alex Kolodkin, Johns Hopkins Medical Institute (38), Myc-tagged dominant negative TEAD4 was provided by Dr. Junhao Mao, University of Massachusetts Medical School, dominant negative Rac1 (N17Rac1) and constitutively active Rac1 (V12Rac1) were described previously (21). Human TAZ was cloned into pcDNA3.1 vector and site-directed mutagenesis was performed to generate TAZ S89A.

Cell Culture

ER-SRC transformed MCF10A cells were provided by Dr. Kevin Struhl (Harvard Medical School). To generate puromycin sensitive MCF10A ER-SRC cells, v-SRC was cloned from the cDNA pool of the original MCF10A ER-SRC cell line. Subsequently, pWZL Blast Twist ER plasmid (Addgene Plasmid #18799) was digested by BamHI to remove the Snai1 cDNA, and replaced with v-SRC cDNA, resulting in the expression of the fusion protein, v-SRC-ER, by the new recombinant plasmid. This plasmid was subsequently used to produce retrovirus for infecting MCF10A cells. Stable clones were selected by blasticidin. Isolation of the EPTH and MES populations of CD44⁺CD24^{-/low} MCF10A ER-SRC transformed cells using flow cytometry has been previously described (33). EPTH and MES cells were cultured as subclones for 2–3 passages and used for experiments. MDA-MB-231 and MDA-MB-435 cells were obtained from the American Type Culture Collection. All experiments were performed at a cell density of 25–35%.

Transfection and siRNA Knockdown

For overexpression, plasmids were transfected using Lipofectamine 2000 (Thermo Fisher Scientific). Cells were processed for immunoblotting, qPCR or mammosphere formation approximately 24 hours following transfection. For LATS 1/2 siRNA knockdown, MDA-MB-231 cells were transfected using DharmaFect 4 (Dharmacon). Cells were processed for immunoblotting 48 hours following transfection. LATS 1/2 siRNA has been previously described (32).

Mammosphere Assay

Cells were plated in UltraLow attachment 6 well plates in DMEM/F12 medium supplemented with B27, EGF and fibroblast growth factor as previously described (33). For serial passaging, mammospheres were pelleted and dissociated with 0.05% Trypsin for 15 minutes at 37 degrees Celsius to obtain single cells. These cells were washed in 1X PBS, counted and re-plated in UltraLow attachment 6 well plates.

Immunolotting

Cells were washed in 1X PBS and scraped on ice in RIPA buffer with EDTA and EGTA (BP-115DG, Boston Bioproducts) supplemented with protease and phosphatase inhibitors (Pierce, #88669). Laemmli 6X SDS sample buffer (BP-111R, Boston Bioproducts) was added to each sample and the protein lysate was boiled for 10 minutes and separated using SDS-PAGE. Rac activity was assessed using a GST fusion protein containing the Rac/cdc42 binding domain of PAK (PBD) as previously described (21, 83).

Luciferase Reporter Assay

TEAD transcriptional activity was assessed using a luciferase reporter construct (8XGTIIC-Addgene #34615) with the Dual-Luciferase Reporter Assay System (#E2940, Promega). Approximately 24 hours following transfection, luciferase activity was measured as the average ratio of firefly to Renilla luciferase.

Real time quantitative PCR

RNA extraction was accomplished using an RNA isolation kit (BS88133, Bio Basic Inc) and cDNAs were produced using qScript cDNA synthesis kit (#95047, Quantabio). SYBR green (Applied Biosystems) was used as the qPCR master mix. Experiments were performed in triplicate and normalized to GAPDH. qPCR primer sequences were obtained from the Massachusetts General Hospital/Harvard Medical School PrimerBank (<http://pga.mgh.harvard.edu/primerbank/>).

Chromatin Immunoprecipitation (ChIP)

ChIP was performed using ChIP-IT Express Chromatin Immunoprecipitation kit (Active Motif). Antibodies used were TEAD4 (N-G2, Santa Cruz Biotechnology) and TAZ (70148S, Cell Signaling Technologies). The following qPCR primer sequence was used to amplify the region of the TEAD4 peak in the β 2-chimaerin promoter identified in ENCODE: Forward primer 5'-GCTTACAGCTGGCTTCACTT-3' Reverse Primer 5'-GGCCGAGAGAGAGAGAGTTT-3'.

Immunofluorescence Confocal Microscopy

TAZ localization was performed by fixing cells with paraformaldehyde (4%) and permeabilizing them with Triton X-100 (0.1%) as described (23, 32). Cells were blocked with 1% BSA and horse serum (2.5%) and incubated with TAZ antibody (1:100 dilution; sc-48805, Santa Cruz Biotechnology) overnight at 4°C. Subsequently, cells were washed with 1X PBS and incubated with fluorochrome-conjugated secondary antibodies. Images were captured at 20X magnification using a confocal microscope (Zeiss).

ENCODE Data Analysis

Encode TEAD4 binding signals were downloaded from www.encodeproject.org in bigwig format. The coverages of duplicate samples were pooled and then plotted along the promoter region of the $\beta 2$ -chimaerin (CHN2) gene.

cBioPortal Analysis

cBioPortal (www.cbioportal.org) was utilized to compare the mRNA expression (RNA Seq V2 RSEM) of TAZ, VEGFA, NRP2 and $\beta 2$ -chimaerin using the TCGA invasive breast carcinoma provisional dataset (68, 69). In addition, the mRNA expression (RNA Seq V2 RSEM) of TAZ and $\beta 2$ -chimaerin was compared in the TCGA glioblastoma provisional dataset and the TCGA colorectal adenocarcinoma Nature 2012 dataset. The cancer cell line encyclopedia (Novartis/Broad, Nature 2012) was used to compare the expression of TAZ and $\beta 2$ -chimaerin across various cell lines using the mRNA expression z-scores microarray. To determine whether the expression of two genes are inversely correlated, we performed the mutual exclusivity analysis with a z-score threshold of ± 1.5 as expressed, and calculated the log odds ratio between the two genes and *p* value using Fisher exact *t*-test. In addition, we stratified breast cancer patients in the TCGA Cell 2015 database based on their ER status, and compared $\beta 2$ -Chimaerin expression in the ER⁺ and ER⁻ subgroups using Welch *t*-test.

Microarray Data Analysis

The microarray dataset from GEO (GSE48204) (72) was download using the Bioconductor package *GEOquery* (version 2.41.0). Moderated *t*-test was used to identify differentially expressed genes between TGF- β induced EMT cells and NMuMG cells treated with vehicle. Genes with an adjusted *p*-value of ≤ 0.05 using B-H method were considered significant (84).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1: VEGF-NRP2 Signaling Contributes to TAZ Activation.

Expression of NRP2 (A) and VEGF (B) was diminished in MES cells and the impact on TAZ abundance was quantified by immunoblotting. Representative blots are shown. Densitometric values were processed using ImageJ and are provided as bar graphs (mean $\pm$ SEM) of $n = 3$ biological replicates. C.) TAZ localization (cytoplasm, nucleus/cytoplasm and nucleus) in control and VEGF-depleted MES cells was determined by immunofluorescence confocal microscopy. Data are mean of $n = 3$ biological replicates. D.) mRNA expression of the indicated TAZ target genes was quantified by qPCR in NRP2-depleted MES cells. Data

are mean $\pm$ SEM of $n = 3$ biological replicates. *E.*) MES cells were treated with the indicated concentrations of a function-blocking NRP2 antibody for 6 hours and the impact on TAZ abundance was quantified by immunoblotting. Representative blots are shown. Densitometric values are provided as bar graphs (mean $\pm$ SEM) of $n = 3$ biological replicates of MES cells treated with 3 $\mu\text{g/mL}$ of the NRP2 function-blocking antibody. *F.*) NRP2 expression was diminished in MDA-MB-231 cells and the impact on TAZ abundance was quantified by immunoblotting. Representative blots are shown. Densitometric values are provided as bar graphs (mean $\pm$ SEM) of $n = 3$ biological replicates. *G.*) mRNA expression of the indicated TAZ target genes was quantified by qPCR in NRP2-depleted MDA-MB-231 cells. Data are mean $\pm$ SEM of $n = 3$ biological replicates. *H.*) NRP2-depleted MDA-MB-231 cells were transfected with an 8XGTIIIC-luciferase reporter construct and assayed for TEAD transcriptional activity. Data are mean $\pm$ SEM of $n = 3$ biological replicates. * $p < 0.05$ by two-tailed t test.

Figure 2: VEGF-NRP2 Signaling Activates Rac1.

A.) Expression of VEGF was diminished in MES cells and the impact on Rac1 activity was assessed using a GST fusion protein containing the Rac/cdc42 binding domain of PAK (PBD). Representative blots are shown. Densitometric values are provided as bar graphs (mean ± SEM) of n = 3 biological replicates. B.) MES cells were treated with 3 µg/mL of a function blocking NRP2 antibody for 6 hours and assayed for Rac1 activity. Representative blots are shown. Densitometric values are provided as bar graphs (mean ± SEM) of n = 3 biological replicates. C.) NRP2 expression was diminished in MDA-MB-231 cells and the impact on Rac1 activity was assessed. Representative blots are shown. Densitometric values

are provided as bar graphs (mean $\pm$ SEM) of $n = 3$ biological replicates. *D.*) MDA-MB-231 cells were serum-starved for 24 hours, treated with 50 ng/mL of VEGF for 30 minutes and assayed for Rac1 activity. Representative blots are shown. Densitometric values are provided as bar graphs (mean $\pm$ SEM) of $n = 3$ biological replicates. *E.*) MES cells were treated with 2 μ M of FAK14 for the indicated time points and assayed for Rac1 activity. Representative blots are shown. Densitometric values are provided as bar graphs (mean $\pm$ SEM) of $n = 3$ biological replicates of MES cells treated with FAK14 for 6 hours. *F.*) NRP2 expression was diminished in MDA-MB-231 cells that were then transfected with constitutively active V12 Rac1-GST. GST expression was quantified by immunoblotting. mRNA expression of the indicated TAZ target genes was quantified by qPCR. Data are mean $\pm$ SEM of $n = 3$ biological replicates. *G.*) MES cells were treated with 50 μ M of the Rac inhibitors EHT1864 or NSC23766 and assayed for self-renewal by serial passage mammosphere formation (P1: passage 1; P2: passage 2). Data are mean $\pm$ SEM of $n = 3$ biological replicates. * $p < 0.05$ by two-tailed t test.

Figure 3: Rac1 Facilitates VEGF-NRP2 Activation of TAZ through Inhibition of LATS.

A.) MES cells were treated with the indicated concentrations of the Rac inhibitor EHT1864 for 2 hours and the impact on pSer⁸⁹ TAZ and TAZ abundance was quantified by immunoblotting. Representative blots are shown. Densitometric values are provided as bar graphs (mean ± SEM) of n = 3 biological replicates. **B.)** MES cells were treated with the Rac inhibitor EHT1864 (100 μM), lysed at the indicated time points and the impact on pThr¹⁰⁷⁹ LATS1, LATS1 and TAZ abundance was quantified by immunoblotting. Data are representative of n = 2 biological replicates. **C.)** MES cells were transfected with dominant

negative N17 Rac1-HA and the impact on TAZ abundance was quantified by immunoblotting. Data are representative of $n = 2$ biological replicates. *D.*) MES cells were treated with the indicated concentrations of a function-blocking NRP2 antibody for 6 hours and the impact on pSer⁸⁹ TAZ and TAZ abundance was quantified by immunoblotting. Representative blots are shown. Densitometric values are provided as bar graphs (mean $\pm$ SEM) of $n = 3$ biological replicates of MES cells treated with 3 $\mu\text{g/mL}$ of the NRP2 function-blocking antibody. *E.*) NRP2 expression was diminished in MDA-MB-231 cells and the impact on pSer⁸⁹ TAZ and TAZ abundance was quantified by immunoblotting. Representative blots are shown. Densitometric values are provided as bar graphs (mean $\pm$ SEM) of $n = 3$ biological replicates. *F.*) NRP2 expression was diminished in MDA-MB-231 cells and the impact on pThr¹⁰⁷⁹ LATS1 and LATS1 was quantified by immunoblotting. Data are representative of $n = 2$ biological replicates. *G.*) NRP2-depleted MDA-MB-231 cells were transfected with empty vector, wild-type TAZ or S89A TAZ and assayed for self-renewal by serial passage mammosphere formation. Data are mean $\pm$ SEM of $n = 3$ biological replicates. *H.*) VEGF-depleted MES cells were transfected with empty vector, wild-type TAZ or S89A TAZ and assayed for self-renewal by serial passage mammosphere formation. Error bars indicate standard deviation from 3 technical replicates. Data are representative of $n = 2$ biological replicates. * $p < 0.05$ by two-tailed t test.

Figure 4: VEGF-NRP2 Signaling Represses the Rac GAP β2-chimaerin.

A.) Abundance of β2-chimaerin was quantified by immunoblotting in EPTH and MES cells. Representative blots are shown. Densitometric values are provided as bar graphs (mean ± SEM) of n = 3 biological replicates. **B.)** Expression of NRP2 was diminished in MES cells and β2-chimaerin mRNA expression was quantified by qPCR. Data are mean ± SEM of n = 3 biological replicates. **C.)** β2-chimaerin abundance was quantified by immunoblotting in NRP2-depleted MES cells. Representative blots are shown. Densitometric values are provided as bar graphs (mean ± SEM) of n = 3 biological replicates. **D.)** MES cells were

treated with the indicated concentrations of a function blocking NRP2 antibody for 6 hours and abundance of β 2-chimaerin was quantified by immunoblotting. Representative blots are shown. Densitometric values are provided as bar graphs (mean $\pm$ SEM) of $n = 3$ biological replicates of MES cells treated with 3 μ g/mL of the NRP2 function-blocking antibody. * $p < 0.05$ by two-tailed t test.

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Figure 5: TAZ Activates Rac1 by Repressing $\beta 2$ -chimaerin.

A.) TAZ expression was diminished in MES cells and the impact on $\beta 2$ -chimaerin mRNA expression was quantified by qPCR. Data are mean $\pm$ SEM of $n = 3$ biological replicates. (B and C) $\beta 2$ -chimaerin abundance (B) and Rac1 activity (C) were assessed in TAZ knockdown MES cells. Representative blots are shown. Densitometric values are provided as bar graphs (mean $\pm$ SEM) of $n = 3$ biological replicates. D.) TAZ expression was diminished in MDA-MB-435 cells and the impact on $\beta 2$ -chimaerin mRNA expression was quantified by qPCR. Data are mean $\pm$ SEM of $n = 3$ biological replicates. E.) TAZ expression was diminished in

MDA-MB-231 cells and the impact on $\beta 2$ -chimaerin mRNA expression was quantified by qPCR. Data are mean $\pm$ SEM of n = 3 biological replicates. *F.*) MDA-MB-231 cells were transfected with TAZ and the impact on $\beta 2$ -chimaerin mRNA expression was quantified by qPCR. Data are mean $\pm$ SEM of n = 3 biological replicates. *G.*) MES cells were treated with the indicated concentrations of the Rac inhibitor EHT1864 for 2 hours and $\beta 2$ -chimaerin mRNA expression was quantified by qPCR. Data are mean $\pm$ SEM of n = 3 biological replicates. * $p < 0.05$ by two-tailed *t* test.

Figure 6: TEAD Mediates Repression of β2-chimaerin by TAZ.

A.) Using ENCODE, TEAD4 binding signals were analyzed from ChIP-seq datasets from h1-hESCs (human embryonic stem cells), HCT116 (colon cancer), Ishikawa (endometrial adenocarcinoma) and SK-N-SH (neuroblastoma) cells in the promoter region of the β2-chimaerin gene. B.) Binding of TEAD4 and TAZ on the β2-chimaerin promoter was analyzed using ChIP in MES cells. Error bars indicate standard deviation from 3 technical replicates. Data are representative of n = 2 biological replicates. C.) TEAD 1/3/4 expression was diminished in MES cells and the impact on β2-chimaerin mRNA expression was

quantified by qPCR. Data are mean $\pm$ SEM of n = 3 biological replicates. (*D* and *E*) $\beta 2$ -chimaerin abundance (*D*) and Rac1 activity (*E*) were assessed in TEAD 1/3/4 knockdown MES cells. Representative blots are shown. Densitometric values are provided as bar graphs (mean $\pm$ SEM) of n = 3 biological replicates. *F*) $\beta 2$ -chimaerin mRNA expression was quantified by qPCR in MDA-MB-231 cells expressing either a control vector or dominant-negative TEAD4. Data are mean $\pm$ SEM of n = 3 biological replicates. * $p < 0.05$ by two-tailed *t* test.

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Figure 7: β2-chimaerin Repression Contributes to Enhanced TAZ activity.
A.) MDA-MB-231 cells were transfected with Myc-tagged β2-chimaerin and Rac1 activity and TAZ abundance were assessed. Representative blots are shown. Densitometric values are provided as bar graphs (mean ± SEM) of n = 3 biological replicates. B.) mRNA expression of the indicated TAZ target genes was quantified by qPCR in MDA-MB-231 cells transfected with Myc-tagged β2-chimaerin. Data are mean ± SEM of n = 3 biological replicates. C.) Expression of β2-chimaerin was diminished in EPTB cells and the impact on Rac1 activity and TAZ abundance were assessed. Representative blots are shown.

Densitometric values are provided as bar graphs (mean $\pm$ SEM) of $n = 3$ biological replicates. *D.*) mRNA expression of the indicated TAZ target genes was quantified by qPCR in $\beta 2$ -Chimaerin-depleted EPTH cells. Data are mean $\pm$ SEM of $n = 3$ biological replicates. *E.*) $\beta 2$ -chimaerin-depleted EPTH cells were assayed for self-renewal by serial passage mammosphere formation (P1: passage 1; P2: passage 2). Data are mean $\pm$ SEM of $n = 3$ biological replicates. *F.*) MDA-MB-231 cells were transfected with empty vector, wild-type TAZ or S89A TAZ with and without Myc-tagged $\beta 2$ -chimaerin and assayed for self-renewal by serial passage mammosphere formation. Error bars indicate standard deviation from 3 technical replicates. Data are representative of $n = 2$ biological replicates. *G.*) cBioPortal for cancer genomics was used to compare TAZ expression with $\beta 2$ -chimaerin, NRP2 and VEGFA expression in the invasive breast carcinoma dataset from the Cancer Genome Atlas (TCGA). The expression of TAZ vs. $\beta 2$ -chimaerin was also compared in the TCGA glioblastoma and colorectal adenocarcinoma databases as well as the cancer cell line encyclopedia (967 cell lines). Log odds ratios and p values were calculated using fisher exact t test with the mutual exclusivity tool on cBioPortal. *H.*) cBioPortal for cancer genomics was used to analyze the expression of $\beta 2$ -chimaerin in ER⁺ vs. ER⁻ breast cancer patients from the TCGA invasive breast carcinoma database. * $p < 0.05$ by two-tailed t test; *** $p < 0.0001$ by Welch t test.

Figure 8: Model depicting the major findings of this study.

VEGF-NRP2 signaling promotes FAK-mediated Rac1 activation, which inhibits LATS. Consequently, TAZ is located in the nucleus where it associates with TEAD and represses the Rac GAP β2-chimaerin to maintain elevated Rac1 activity in a positive feedback loop.

Table 1:
mRNA screen of Rac GEFs and GAPs in EPTH and MES cells.

The expression of the indicated Rac GEFs and GAPs was compared in EPTH and MES cells using qPCR. Table 1 shows fold change in mRNA expression upon normalization with EPTH cells, which was set as 1. NE indicates not expressed. Numbers in parentheses indicate standard deviation.

	EPTH	MES
GEFs		
PRex1	NE	NE
PRex2	NE	NE
Tiam1	1 (0.13)	0.52 (0.04)
Tiam2	NE	NE
SOS1	1 (0.01)	0.62 (0.04)
ARHGEF7	1 (0.08)	1.1 (0.01)
Dock1	1 (0.08)	0.56 (0.13)
VAV1	NE	NE
VAV2	1 (0.07)	0.63 (0.02)
VAV3	1 (0.1)	0.69 (0.17)
Trio	1 (0.05)	0.76 (0.02)
GAPs		
β2-chimaerin	1 (0.08)	0.16 (0.07)
ARHGAP10	1 (0.05)	0.7 (0.05)