Interaction between STAT3 and GLI1/tGLI1 oncogenic transcription factors promotes the aggressiveness of triple-negative and HER2-enriched breast cancers

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Abstract

STAT3, GLI1, and truncated GLI1 (tGLI1) are oncogenic transcription factors playing important roles in breast cancer. tGLI1 is a gain-of-function GLI1 isoform. Whether STAT3 physically and/or functionally interacts with GLI1/tGLI1 has not been explored. To address this knowledge gap, we analyzed 47 node-positive breast cancer specimens using immunohistochemical staining and found that p-STAT3 (Y705), GLI1, and tGLI1 are co-overexpressed in the majority of triple-negative (64%) and HER2-enriched (68%) breast carcinomas, and in lymph node metastases (65%). Using Gene Set Enrichment Analysis, we analyzed 710 breast tumors and found that STAT3- and GLI1/tGLI1-activation signatures are co-enriched in triple-negative and HER2-enriched, but not in luminal subtypes of breast cancers. Patients with high levels of STAT3 and GLI1/tGLI1 co-activation in their breast tumors had worse metastasis-free survival compared to those with low levels. Since these proteins co-overexpress in breast tumors, we examined whether they form complexes and observed that STAT3 interacted with both GLI1 and tGLI1. We further found that the STAT3-GLI1 and STAT3-tGLI1 complexes bind to both consensus GLI1- and STAT3-binding sites using chromatin immunoprecipitation (ChIP) assay, and that the co-overexpression markedly activated a promoter controlled by GLI1-binding sites. To identify genes that can be directly co-activated by STAT3 and GLI1/tGLI1, we analyzed three ChIP-Seq datasets.
and identified 36 potential target genes. Following validations using RT-PCR and survival analysis, we identified three genes as novel transcriptional targets of STAT3 and GLI1/tGLI1, R-Ras2, Cep70, and UPF3A. Finally, we observed that co-overexpression of STAT3 with GLI1/tGLI1 promoted the ability of breast cancer cells to form mammospheres and that STAT3 only cooperates with GLI1 in immortalized mammary epithelial cells. In summary, our study identified novel physical and functional cooperation between two families of oncogenic transcription factors, and the interaction contributes to aggressiveness of breast cancer cells and poor prognosis of triple-negative and HER2-enriched breast cancers.

**Keywords**
STAT3; GLI1; tGLI1; breast cancer; HER2

**INTRODUCTION**

Breast cancer is classified into five major molecular subtypes, luminal A (ER+PR+), luminal B (ER+PR+ HER2+), HER2-enriched, normal breast-like, and triple-negative breast cancer. Triple-negative breast cancer is negative for ERα, PR, and HER2 with frequent BRCA1 mutations. 15–20% of all breast cancer belongs to the triple-negative histological subtype, affecting more young women and African American women compared to other breast cancer subtypes. Triple-negative breast cancer remains a heterogeneous disease that encompasses two major intrinsic subtypes, basal-like and claudin-low. Basal-like triple-negative breast cancer is highly proliferative and positive for CK4/7 and EGFR displaying the epithelial phenotype. Claudin-low triple-negative breast cancer is biologically closely related to mammary stem cells with low expression of genes involved in tight cell junctions (claudins and E-cadherin) and the mesenchymal phenotype. Women with triple-negative breast cancer have more aggressive diseases and unfavorable prognosis. Five-year survival rate is lower for triple-negative breast cancer than for other subtypes. Survival following metastatic relapse is shorter in triple-negative breast cancer compared to other breast cancer subtypes. Women with triple-negative breast cancer have high rates of recurrence and metastasis. 20–30% of all breast cancer overexpress HER2, including the HER2-enriched and luminal B subtypes. HER2 over-expression in breast tumors is associated with poor patient survival and high risks of relapse and metastasis. Most of the metastatic breast tumors that initially respond to trastuzumab-based combination treatments begin to progress within one year. Trastuzumab used as a single agent has been generally ineffective while trastuzumab resistance after initial responses is common.

Janus-activated kinase 2 (JAK2) is a non-receptor tyrosine kinase that is amplified and hyperactive in triple-negative and HER2-positive breast cancers. JAK2 serves as a signaling hub that connects oncogenic receptor tyrosine kinases and interleukin receptors to signal transducer and activator of transcription 3 (STAT3) oncogenic transcription factor. JAK2 phosphorylates STAT3 at Y705 to activate its nuclear import and transcriptional activity. Activated STAT3 binds to its target gene promoters and upregulates expression of genes involved in G1 cell cycle progression, proliferation, oncogenesis, anti-apoptosis, angiogenesis, and metastasis. We have reported STAT3’s ability to
upregulate expression of TWIST, iNOS, Cox-2, and STAT1. Dysregulated JAK2-STAT3 pathway is associated with poor clinical outcomes and is a breast cancer therapeutic target. Several JAK2 inhibitors have been developed. Ruxolitinib is the only FDA approved inhibitor for JAK2 (wild-type and V617F mutant) and is approved for psoriasis and myelofibrosis.

The smoothened-glioma oncogene homolog 1 (SMO-GLI1) pathway is a therapeutic target in triple-negative breast cancer, and possibly HER2-positive breast cancer. SMO is an oncogenic 7-transmembrane receptor that activates the GLI1 oncogenic transcription factors. The SMO-GLI1 pathway is important for differentiation and normal development, and is implicated in tumorigenesis, vascular development, and stem cell self-renewal. SMO is activated (de-repressed) following binding of sonic hedgehog, SHH, to its receptor patched, PTCH1, a SMO repressor. Three GLI isoforms have been identified with GLI1 and GLI2 as the activators and GLI3 as the repressor of the SHH-SMO pathway. GLI1 is frequently activated in breast cancer and associated with poor survival. Our laboratory discovered truncated GLI1 (tGLI1) as an alternatively spliced variant of GLI1 that contains a small in-frame deletion of 41 codons. tGLI1 retains all of the known functional domains of GLI1, undergoes nuclear import, responds to SHH and activates GLI1-targeted genes, but gains the propensity to activate genes that are not regulated by GLI1 leading to increased migration, invasion and angiogenesis. SMO has emerged as an important therapeutic target for several cancer types including breast cancer. Following a literature search, we did not find published reports that investigated breast cancer for co-overexpression of activated STAT3 with GLI1 and tGLI1. However, a recent study showed that p-STAT3 (Y705) co-expressed with GLI1 in non-small cell lung cancer. Hence, this study explored whether the JAK2/STAT3 and GLI1/tGLI1 pathways are concurrently activated and found them to be highly co-activated in the triple-negative and HER2-enriched subtypes of breast cancer. We further observed that patients with breast tumors expressing activated STAT3/GLI1/tGLI1 had poor survival. Our biochemical analysis indicated that STAT3 physically interacts with GLI1 and tGLI1, and that the interaction leads to activation of STAT3- and GLI1/tGLI1-targeted gene promoters, and promotes mammosphere-forming ability of breast cancer cells. Together, these novel observations point to a physical and functional cooperation between two oncogenic pathways that plays an important role in breast cancer aggressiveness and patient prognosis.

**RESULTS**

P-STAT3 (Y705), GLI1, and tGLI1 are co-overexpressed in the majority of triple-negative and HER2-enriched breast cancer specimens, and in lymph node metastases

Whether the STAT3 and GLI1/tGLI1 pathways are co-overexpressed in breast cancer has not been investigated. Here, we analyzed a tissue microarray (BR10010; USBiomax) consisting of 47 primary node-positive breast carcinomas with subtype information for p-STAT3 (Y705), GLI1, and tGLI1 by immunohistochemical staining, IHC. Since commercial GLI1 antibodies detect both GLI1 and tGLI1, we developed and validated GLI1- and tGLI1-specific antibodies and used them in multiple published studies. As summarized in...
Fig. 1A, we observed that the three proteins are co-overexpressed in 64% and 68% of triple-negative and HER2-enriched breast carcinomas, respectively, to a higher extent than luminal tumors (41%). Fig. 1B shows representative images. To determine whether there three proteins are present in metastases, we analyzed 60 lymph node metastases that unfortunately do not have subtype information (USBiomax). As shown in Fig. 1C, the three proteins are highly co-overexpressed in lymph node metastases (65%). These observations together indicate that p-STAT3 (Y705), GLI1 and tGLI1 are significantly co-expressed in triple-negative and HER2-enriched breast cancer specimens and also in lymph node metastases of breast cancer.

Co-activation of the STAT3 and GLI1/tGLI1 pathways is enriched in triple-negative and HER2-enriched breast cancers

Following observing co-expression of p-STAT3 with GLI1 and tGLI1, we asked whether their downstream target genes are accordingly regulated. For this, we retrieved a STAT3- and a GLI1-activation signature from two published studies. Of note, tGLI1 retains its activity to activate GLI1 target genes. Using these signatures, we then used the Gene Set Enrichment Analysis (GSEA) to analyze 710 breast tumors whose expression profiles were retrieved from the Gene Expression Omnibus/GEO database (GSE12276/2034/2603/5327/14020). We observed that the two pathways, individually and jointly, are more enriched in triple-negative (Fig. 2A) and HER2-enriched (Fig. 2B) breast cancers, compared to luminal A and B subtypes. We further generated heat maps via clustering analysis and the results showed that STAT3- and GLI1/tGLI1-activated genes are expressed at higher levels in the triple-negative and HER2-enriched breast cancers compared to the luminal tumors (Fig. 2C). Furthermore, we observed that triple-negative and HER2-enriched breast cancers have significantly higher levels of STAT3 activation and GLI1/tGLI1 activation than the ER+ luminal tumors (Fig. 2D). These results indicate that the STAT3 and GLI1/tGLI1 pathways are concurrently activated in the majority of triple-negative and HER2-enriched breast cancers to higher degrees than the luminal subtypes.

Co-activation of the STAT3 and GLI1/tGLI1 pathways is associated with shortened metastasis-free survival of triple-negative and HER2-enriched breast cancers

Whether co-activation of the two pathways is a prognostic indicator has not been reported. Here, we used the Kaplan-Meier analysis and the two activation signatures to analyze 672 patients whose expression profiles and survival data were obtained from the GEO database (GSE12276/2034/2603/5327/14020). The cohort was divided into four groups according to the extent of activation for both pathways. As shown in Fig. 3A, patients with co-activation of both pathways in their triple-negative tumors had the shortest time to develop metastases (1.26 yrs). As further shown in Fig. 3B, we found HER2-enriched tumors with co-activation of both pathways to associate with the shortest time to develop metastases (1.29 yrs). For the luminal subtypes of breast cancer, the co-activation is also associated with worst outcome; however, the median time to develop metastases (2.77 yrs) was longer than the triple-negative and HER2-enriched subtypes. To assess the importance of STAT3 and GLI1/tGLI1 pathways in metastasis-free survival, we carried out multivariate analysis using the same patient cohort used in Fig. 3A. Our results showed that within patients with the triple-negative (Fig. 3C) and HER2-enriched (Fig. 3D) tumors, those with activated STAT3 or
GLI1/tGLI1 had an increased likelihood of metastasis as indicated by an increased hazard ratio in both univariate and multivariate models. These data indicate that STAT3 signaling and GLI1/tGLI1 signaling are associated with metastasis-free survival independent of each other.

**Breast cancer cell lines and xenografts co-overexpress p-STAT3, GLI1, and tGLI1**

We analyzed a panel of breast cancer cell lines, and cell line-derived xenografts for levels of p-STAT3, GLI1, and tGLI1 using IHC. As shown in Fig. 4A, we have implanted MDA-MB-231 triple-negative cells into the mammary fat pad (mfp; top panel) of female nude mice, and MDA-MB-231-BrM cells (bottom panel), the brain-metastatic subline of MDA-MB-231 from Dr. Joan Massague, into the mouse brain, and then analyzed the tumors for expression of three proteins. The results showed that both xenografts co-overexpressed all three proteins. Images shown represent the results of 4 tumors. We further show in Fig. 4B that the three proteins are readily detected in breast cancer cell lines.

**STAT3 forms complexes with GLI1 and tGLI1, leading to gene expression activation**

Next, we asked whether STAT3 physically interacted with GLI1 and tGLI1, which has never been reported. Of note, GLI1 family of transcription factors function as monomers and they do not dimerize with each other. We have shown that tGLI1 can bind to the consensus GLI1-binding site. To address this question, we conducted immunoprecipitation-western blotting (IP-WB) and found that STAT3 interacted with both GLI1 and tGLI1 in MDA-MB-468 triple-negative cells (Fig. 5A). To determine whether phosphorylation of Y705 or S727 is required for the interaction of STAT3 with GLI1/tGLI1, we transfected stable HEK293-GLI1 and HEK293-tGLI1 cells with STAT3CA, STAT3-DN (Y705F), and STAT3(S727A), and conducted IP-WB. As shown in Fig. 5B, we observed no differences in the ability of these STAT3 mutants to interact with GLI1 or tGLI1. Next, we determined whether STAT3, GLI1, and tGLI1 bind to the GLI1/tGLI1-binding sites in the PTCH1 and GLI1 gene promoters and/or the STAT3-binding sites in the pGAS-Luc plasmid using chromatin immunoprecipitation (ChIP) assay. As shown in Fig. 5C, we found that STAT3 binds to the PTCH1 and GLI1 promoters, and the STAT3-binding sites in the pGAS-Luc plasmid (as expected). We also observed that both GLI1 and tGLI1 bind to the STAT3-binding sites in the pGAS-Luc plasmid, and the GLI1/tGLI1-binding sites in the PTCH1 and GLI1 gene promoters (as expected). Consistent with these data, we found co-overexpression of GLI1/tGLI1 with STAT3CA (constitutively active STAT3 with A661S that automatically dimerizes) markedly enhanced the activity of a promoter controlled by 8 copies of GLI1-binding sites in MDA-MB-468 cells (Fig. 5D). STAT3CA alone did not significantly enhance the promoter activity. We further found that the STAT3-binding sites-containing reporter was activated by SHH and EGF (as expected) and importantly, was further activated by the co-stimulations with EGF and SHH (Fig. 5E). We next examined where the STAT3 and GLI1/tGLI1 complexes were localized within the cells using immunofluorescence (IF) staining/confocal microscopy, and found that they were colocalized mainly in the nucleus with diffuse staining in the cytoplasm (Fig. 5F). These results indicate that STAT3 interacts with GLI1 and tGLI1, leading to gene expression activation.
Identification of genes co-activated by STAT3 and GLI1/tGLI1

The strategy we took to identify genes co-activated by STAT3 and GLI1/tGLI1 is outlined in Fig. 6A. To identify putative genes with the propensity to be recognized by both STAT3 and GLI1/tGLI1, we acquired the publicly available ChIP-seq data from Sequence Read Archive of NCBI (https://www.ncbi.nlm.nih.gov/sra), including a GLI1 ChIP-seq dataset and two STAT3 ChIP-seq datasets\(^{17,64}\). Using Fastqc software and alignment of the raw fastq files to mm9 genome by bowtie2\(^{35}\) followed by peak calling enabled by MACS2 software\(^{66}\), we identified 34 genes, including PTCH1 and GLI1, with reasonable distance between boundaries of the STAT3 and GLI1 peaks and the binding sites are located within 1,500 bp upstream of the transcription start sites or introns (Supplemental Table I). To further narrow down to genes that may play an important role in breast cancer, we performed Kaplan-Meier analysis and found 18 out of the 34 genes to correlate with shortened metastasis-free survival. We further conducted qRT-PCR of the 18 genes and found three genes (R-Ras2, Cep70, and UPF3A) to be significantly upregulated by co-overexpression of STAT3-GLI1 and/or STAT3-tGLI1 (Fig. 6B). Furthermore, we examined the impact of STAT3 and GLI1/tGLI1 downregulation on expression of target genes using shSTAT3 and shGLI1/tGLI1, and carried out qPCR. We observed that downregulation of both STAT3 and GLI1/tGLI1 significantly reduced expression of Cep70 and UPF3A (Supplemental Fig 1). All three genes contain regions that are bound to STAT3 and GLI1 as indicated by the ChIP-Seq data (Fig. 6C). High expression of R-Ras2, Cep70, or UPF3A genes is associated with worse metastasis-free survival (Fig. 6D). Gene expression profiles and survival data of 710 tumors were retrieved from the GEO database (GSE12276/2034/2603/5327/14020). Since we observed that R-Ras2 gene promoter was significantly activated by the co-overexpression of STAT3 with GLI1, and STAT3 with tGLI1, we examined whether the STAT3-GLI1 and STAT3-tGLI1 complexes bind to the R-Ras2 gene promoter using sequential ChIP assay with two antibodies sequentially, as we previously conducted\(^{25}\). ChIP and sequential ChIP data indicated that the STAT3-GLI1 and STAT3-tGLI1 complexes bind to the R-Ras2 gene promoter but not to the Slug gene promoter that does not have binding sites for STAT3 or GLI1/tGLI1 (Fig. 6E). In summary, we identified three genes that can be directly co-activated by STAT3-GLI1 and STAT3-tGLI1 cooperation.

STAT3 synergizes with GLI1/tGLI1 to promote mammosphere-forming ability of breast cancer cells and immortalized mammary epithelial cells

STAT3 and GLI1 transcription factors are known to promote cancer stem cell renewal\(^{2,9,45}\); however, whether they cooperate to promote cancer stemness has not been reported. To determine whether co-overexpression of STAT3 and GLI1/tGLI1 promote aggressive phenotypes of breast cancer cells, we transfected four cell lines with STAT3CA with and without GLI1/tGLI1 and then determined their ability to form mammospheres. Our results show that the co-overexpression significantly enhanced mammosphere-forming ability of two triple-negative breast cancer cell lines, MDA-MB-468 (Fig. 7A) and BT20 (Fig. 7B). Interestingly, the co-overexpression of STAT3CA with tGLI1 significantly promotes the ability of immortalized human mammary epithelial MCF-10A cells (Fig. 7C) and HMLE cells (Fig. 7D) to form mammospheres. These observations indicate that STAT3 synergizes...
with GLI1/tGLI1 to promote mammosphere-forming ability of breast cancer cells and immortalized mammary epithelial cells.

**DISCUSSION**

We report in this study, for the first time, that STAT3 undergoes protein-protein interactions with GLI1 and tGLI1, and that the interactions contribute to gene activation and stem-like phenotype of breast cancer. Our study also provides the first evidence that activated STAT3 is co-overexpressed with GLI1 and tGLI1 in the majority of triple-negative and HER2-enriched subtypes of breast cancer that are known to be more metastatic and associated with worse survival, compared to luminal subtypes of breast cancer. Our study further showed that STAT3- and GLI1/tGLI1-activation signatures are co-enriched in triple-negative and HER2-enriched, but not in luminal subtypes, and that patients with high levels of STAT3 and GLI1/tGLI1 co-activation in their breast tumors had worse metastasis-free survival compared to those with low levels.

We identified and validated three genes that can be directly activated by STAT3-GLI1 and/or STAT3-tGLI1 cooperation, namely, R-Ras2, Cep70, and UPF3A. R-Ras2 (also known as TC21) is a transforming GTPase that shares downstream effectors with Ras subfamily proteins. Constitutively activated mutants of the Ras-related protein R-Ras2 has been shown to promote tumorigenic transformation of NIH3T3 cells. R-Ras2 has a role in both breast tumorigenesis and the late metastatic steps of cancer cells in the lung parenchyma. R-Ras2 drives tumorigenesis in a phosphatidylinostiol-3 kinase-dependent and signalling autonomous manner. Interestingly, overexpression of R-Ras2 is commonly found in breast cancer and has been shown to potentially contribute to the development of human breast cancers. These findings suggest that R-Ras2 may be an important downstream effector of the STAT3-GLI1/tGLI1 pathway.

We identified Cep70 as a novel target for STAT3 and GLI1. Cep70 protein interacts with gamma-tubulin to localize at the centrosome playing a critical role in mitotic spindle assembly. Cep70 also regulates microtubule stability by interacting with HDAC6. Interestingly, Cep70 has been shown to contribute to angiogenesis through modulating microtubule rearrangement and stimulating cell polarization and migration, and STAT3/GLI1 can also promote angiogenesis and migration. Cep70 has been reported to enhance breast cancer lung metastasis. These findings suggest that STAT3 and GLI1/tGLI1 may promote breast cancer progression through activating expression of Cep70.

Our results indicate that UPF3A expression can be enhanced by co-expression of STAT3 and tGLI1. UPF3 (up-frameshift) is a mostly nuclear protein that shuttles between the nucleus and cytoplasm and interacts specifically with spliced mRNAs. UPF3 is a key factor in nonsense-mediated messenger RNA decay (NMD), a critical process of selective degradation of mRNAs that contain premature stop codons. UPF3 interacts with Y14, a component of post-splicing mRNA-protein complexes, as part of the complex that assembles near exon-exon junctions, allows it to serve as a link between splicing and NMD in the cytoplasm. UPF3A acts primarily as a potent NMD inhibitor that stabilizes hundreds of transcripts. Mice conditionally lacking UPF3A have been reported to exhibit “hyper”
NMD and display defects in embryogenesis and gametogenesis. In light of these observations, STAT3 and tGLI1 may cooperate to regulate NMD through UPF3A.

The novel physical and functional interactions of STAT3 and GLI1/tGLI1 we uncovered have important translational implications because both pathways can be targeted through inhibiting their upstream regulators. Our results support the combination of STAT3- and GLI1-targeted therapies for triple-negative and HER2-enriched breast cancers. One of STAT3’s upstream activating kinases is Janus-activated kinase 2 (JAK2), a non-receptor tyrosine kinase that is amplified and hyperactive in triple-negative and HER2-positive breast cancers. Several JAK2 inhibitors have been developed. Ruxolitinib is the only FDA approved inhibitor for JAK2 (wild-type and V617F mutant) and is approved for psoriasis and myelofibrosis. For triple-negative inflammatory breast cancer, a phase I/II trial with ruxolitinib in combination with paclitaxel is being conducted (NCT02041429). Ruxolitinib with capecitabine is evaluated in a phase II trial for advanced or metastatic HER2-negative breast cancer (NCT02120417). A phase II trial with ruxolitinib as single agent did not produce sufficient patient response (NCT01562873). For metastatic HER2-positive breast cancer, ruxolitinib in combination with Herceptin is in a phase I/II clinical trial (NCT02066532). Another JAK2 inhibitor pacritinib has equipotent activity against JAK2 (wild-type and mutant) and FLT3, and is being tested clinically for leukemias, and lung and colon cancers. There is currently no clinical trial with pacritinib for breast cancer.

SMO has emerged as an important therapeutic target for several cancer types including breast cancer. Two orally active SMO inhibitors, vismodegib and sonidegib, received FDA approval for advanced basal cell carcinoma. For triple-negative advanced breast cancer, vismodegib is being evaluated with chemotherapeutic agents, paclitaxel, epirubicin and cyclophosphamide, in a phase II trial (NCT02694224) while sonidegib is being tested in a phase I trial (NCT02027376). Additional SMO inhibitors are presently investigated in the clinic for other cancer types, including glasdegib, taladegib, and saridegib.

In summary, this study reports for the first time the physical and functional interactions of two families of oncogenic transcription factors in two highly aggressive subtypes of breast cancer, namely, triple-negative and HER2-enriched breast cancers. Our findings shed new light into the malignant biology of these aggressive and hard-to-treat breast cancers. Our findings also provide novel rationale to simultaneously inhibit the JAK2-STAT3 and the SMO-GLI1/tGLI1 pathways to achieve optimal clinical efficacy for triple-negative and Herceptin-refractory HER2-enriched breast cancers.

**MATERIALS AND METHODS**

**Cell lines, tumor tissues, and plasmids**

All cell lines were purchased from ATCC (Manassas, VA) except the brain-metastatic MDA-MB-231-BrM cells and HMLE immortalized human mammary epithelial cells. All cell lines were cultured, according to the instructions provided by the ATCC. The breast cancer tissue microarray (BR10010) was obtained from US Biomax (Rockville, MD). GLI1 and tGLI1 plasmids were generated in our laboratory. STAT3CA, STAT-DN and STAT3-S727A plasmids were generous gifts from Dr. Keping Xie at the University of Texas-MD.
Anderson Cancer Center. The GLI1-binding sites-driven luciferase construct, 8x3′ Gli-BS Luc, was generously provided by Dr. Hiroshi Sasaki at Osaka University, Japan.

**Immunohistochemistry (IHC), immunoblotting, and immunoprecipitation (IP)**

IHC and immunoblotting were performed as we previously described. Antibodies for IHC included p-STAT3 (Y705) antibody (Cell Signaling; #9145; 1:50), and rabbit polyclonal GLI1 and tGLI1 antibodies that we developed and validated. Antibodies for immunoblotting included p-STAT3 (Y705) antibody (Cell Signaling, 1:1,000), STAT3 rabbit polyclonal antibody (Santa Cruz; C20; 1:1,000), α-tubulin and β-actin mouse monoclonal antibodies (Sigma), and rabbit polyclonal GLI1/tGLI1 antibody (Cell Signaling, 1:1,000). For IP, cells were transfected with flag-tagged GLI1, tGLI1 or STAT3 mutants, stimulated with 100 ng/ml EGF and 100 ng/ml SHH for 2 hrs, washed, fractionated, and precleared with protein G-agarose (Sigma; A2220). Precleared lysates were incubated with anti-flag M2 affinity gel or mouse IgG at 4°C overnight with gentle agitation. Pellets were collected, washed and subjected to SDS-PAGE and WB analysis. STAT3 IP was performed using antibodies from Santa Cruz (C-20). GLI1 and tGLI1 immunoprecipitation was performed using a GLI1 antibody (Santa Cruz, H-300) that recognizes the COOH-terminal end and, therefore, pulls down GLI1 and tGLI1.

**Immunofluorescence (IF) staining/confocal microscopy**

Isogenic MDA-MB-231 cells stably overexpressing GLI1 or tGLI1 with an RFP were immunostained with STAT3 antibody (Cell Signaling; #12640; 1:1000), Alexa Fluor 488 conjugated secondary (Cell Signaling; #4412; 1:200), stained with DRAQ5 (ThermoFisher; 62251; 5 μM), and immediately subjected to confocal microscopy.

**Gene Set Enrichment Analysis (GSEA), Heat Map Generation and statistical analyses**

GSEA was performed by generating the Gene MatriX file (.gmx) by using published signatures for Angiogmatrix. The Gene Cluster Text file (.gct) was generated from the GEO database (GSE12276/2034/2603/5327/14020). The Categorical Class file (.cls) was generated based on the STAT3 activation and GLI1/tGLI1 activation scores. The number of permutations for GSEA was set to 1,000. For generation of heat maps, patients were divided into high or low STAT3 and GLI1 scores, and the genes included in the map were genes within the published signatures for the indicated breast cancer subtypes. Heat maps were generated using Morpheus software developed by the Broad Institute. Student-test and univariate/multivariate analysis were done using Excel.

**Chromatin Immunoprecipitation (ChIP) Assay**

This was performed using a ChIP Assay Kit (Upstate Millipore), as we described previously. A GLI1 antibody (Santa Cruz, H-300) that recognizes the COOH-terminal region present in both GLI1 and tGLI1 proteins was used for immunoprecipitation of cells expressing TGLI1, GLI1, or vector. STAT3 antibody used for ChIP was purchased from Santa Cruz (C-20). The primers used for PCR following ChIP are located in Supplemental Table II.
Quantitative RT-PCR

Total RNA was isolated using Promega SV Total RNA Isolation kit and subjected to quantitative PCR as we previously described. Primers for PCR are described in Supplemental Table III.

Promoter Reporter Assay

All transfections were performed with cells in exponential growth using XtremeGene HP (Roche). GLI1-binding sites-driven luciferase construct, 8x3′Gli-BS and a STAT3-binding site containing luciferase reporter plasmid (Strategene) were used. An additional renilla luciferase expression vector, pRl-CMV was used to control for transfection efficiency. 48 hr after transfection, cells were lysed and luciferase activity was measured using the Firefly and Renilla luciferase activity kit (Biotium), as we previously described. Relative promoter activity was computed by normalizing the Firefly luciferase activity against that of the Renilla luciferase.

Identification of genes co-activated by STAT3 and GLI1/tGLI1 via mining ChIP-seq datasets

We acquired the publicly available ChIP-seq data from Sequence Read Archive of NCBI (https://www.ncbi.nlm.nih.gov/sra). The GLI1 ChIP-seq data were derived from the project of PRJNA179206 with GEO accession of GSE42132. It included 7 runs (SRR611923 - SRR611929). The STAT3 ChIP-seq data were downloaded from two projects: PRJNA129289 with a single run of SRR271642, and PRJNA142287 with a single run of SRR091648. The raw fastq files were derived from SRA, by the fastq-dump tool with the run ids. We checked the data quality of the fastq files by using Fastqc software (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and identified all of the fastq files passed the quality control. Then we aligned the raw fastq files to mm9 genome by bowtie2. The peak calling was enabled by MACS2 software using default parameters. We have compared the peaks of GLI1 called MACS2 with the high-confidence GLI1 peaks in the supplementary table of Peterson et al. They are highly consistent. We combined the peaks of STAT3 from the two studies as aforementioned. The co-binding analysis was implemented by a python script that processed the bed files from the peak calling of ChIP-seq data of GLI1 and STAT3. The co-binding region was defined by the distance between boundaries of the two peaks. We used the gene annotation from mm9 reference to check if the co-binding regions are located to specific regions of a gene. We utilized IGV software to check the regions of interest manually, such as, those for CEP70, Rras2, and UPF3a.

Mammosphere Assay

Cells were seeded (500–1000 cells/well) in 24-well ultra-low attachment plates (Corning) with DMEM/F12 (Gibco) containing 2% B27 (Gibco), 20 ng/mL EGF (Sigma), 4 ug/mL insulin (Sigma), and 100 ng/mL SHH (Sigma). Mammospheres were cultured for 7–14 days and supplemented with fresh mammosphere medium (100–200 uL) every 3–4 days. The number of spheres per well were counted under 5x objective.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.
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ABBREVIATIONS

- CEP70: centrosome protein 70
- EGF: epidermal growth factor
- EGFR: epidermal growth receptor
- ER: estrogen receptor
- HER2: human epidermal growth factor-2
- GEO: Gene Expression Omnibus
- GSEA: Gene Set Enrichment Analysis
- GLI1: glioma-associated oncogene homolog-1
- JAK2: Janus-activated kinase 2
- NMD: nonsense-mediated messenger RNA decay
- PTCH1: patched 1
- PR: progesterone receptor
- SHH: sonic hedgehog
- SMO: smoothened
- STAT3: signal transducer and activator-3
- STAT3CA: constitutively active STAT3
- tGLI1: truncated glioma-associated oncogene homolog-1
- UPF3A: up-frameshift 3A

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Figure 1. Significant co-expression of p-STAT3 (Y705), GLI1 and tGLI1 in triple-negative and HER2-enriched node-positive breast carcinomas and in lymph node metastases

A) A TMA of 47 node-positive breast carcinomas tumors with subtype information was analyzed for expression levels of p-STAT3 (Y705), GLI1, and tGLI1 using IHC. >20% nuclear staining denotes positivity. *,** denote p<0.05.

B) Representative images.

C) Sixty lymph node metastases without subtype information were analyzed via IHC for p-STAT3 (Y705), GLI1, and tGLI1. All slides were scored by a pathologist.
Figure 2. Co-activation of the STAT3 and GLI1/tGLI1 pathways is enriched in triple-negative and HER2-enriched breast cancers

GSEA was used to determine if activated STAT3 (A) and GLI1 (B) signatures were enriched in 710 breast tumors. Expression profiles of 710 tumors were retrieved from the GEO database (GSE12276/2034/2603/5327/14020). NES, normalized enrichment score. C) Heat maps were generated via GraphPad Prism. D) Triple-negative and HER2-enriched breast cancers have significantly higher levels of STAT3 activation and GLI1/tGLI1 activation than the ER+ liminal tumors.
Figure 3. Co-activation of the STAT3 and GLI1/tGLI1 is associated with shortened metastasis-free survival of triple-negative and HER2-enriched breast cancers

Using the Kaplan-Meier analysis, log-rank analyses, univariate and multivariate analyses, and the STAT3 and GLI1/tGLI1 activation signatures, we analyzed 672 patients whose expression profiles and survival data were obtained from the GEO database (GSE1276/2034/2603/5327/14020). The cohort was divided into four groups according to the extent of activation for both pathways. Median survival years are indicated. A) Triple-negative breast cancer cohort. B) HER2-enriched breast cancer cohort. C) Univariate and multivariate analyses of triple-negative breast cancer cohort. D) Univariate and multivariate analyses of Her2-enriched breast cancer cohort.
Figure 4. Breast cancer cell lines and xenografts co-overexpress p-STAT3 (Y705), GLI1, and tGLI1

A) Breast cancer cell lines were analyzed by western blots for levels of p-STAT3, GLI1, and tGLI1. B) Cell line-derived xenografts were analyzed for p-STAT3 (Y705), GLI1, and tGLI1 via IHC. MDA-MB-231 triple-negative cells into the mammary fat pad (mfp; top panel) of female nude mice, and the brain-metastatic MDA-MB-231-BrM cells (bottom panel) into the mouse brain.
Figure 5. STAT3 physically interacts and functionally cooperates with GLI1 and tGLI1
A) STAT3 interacts with both GLI1 and tGLI1 as shown by IP-WB using MDA-MB-468 cells transfected with flag-tagged GLI1 or tGLI1 plasmids. A flag antibody (Ab) was used in IP. IgG was used as negative IP controls. B) STAT3 mutants (Constitutively Active (CA), S727A, and Y705F) similarly interacted with both GLI1 and tGLI1 as shown by IP-WB using isogenic HEK293-GLI1 or -tGLI1 cells transfected with flag-tagged STAT3 mutant plasmids. C) ChIP assay and sequential ChIP assay were used to examine the binding of STAT3, GLI1, and/or Gli1 and their complexes to bind to the GLI1/tGLI1-binding sites in the PTCH1 and GLI1 gene promoters and/or the STAT3-binding sites (in the pGAS-Luc plasmid). D) Co-overexpression of STAT3CA with GLI1 or tGLI1 enhanced the activity of a promoter controlled by GLI1-binding sites in MDA-468 cells. 8x3′Gli-BS Luc was used with a control Renilla luciferase reporter. E) The STAT3-binding sites-containing reporter (pGAS-Luc) was activated by SHH, EGF, or in combination. Transfected cells were starved for 16 hrs and then treated with SHH (100 ng/ml) and/or EGF (100 ng/ml) for 4 hrs followed by determination of luciferase activity. F) Representative IF staining images depicting nuclear colocalization of ectopic STAT3 (GFP) and GLI1 or tGLI1 (RFP) in isogenic MDA-MB-231 cells. All experiments were done three times to derive means and standard deviations. Student t-test was used to compute p values (*, p<0.05).
Figure 6. Identification of genes co-activated by STAT3 and GLI1/GLI1

A) Strategy to identify genes co-activated by STAT3 and GLI1/GLI1. B) Three of the 18 potential gene targets were significantly upregulated by co-overexpression of STAT3-GLI1 and/or STAT3-tGLI1. qRT-PCR was used. tGLI1, TG. C) R-Ras2, Cep70, and UPF3A gene promoter and enhancer regions that are bound to STAT3-GLI1 and/or STAT3-tGLI1 complexes. GLI1/tGLI1-bound regions are marked by green peaks (top panels). STAT3-bound regions are marked by blue peaks (bottom panels). D) High expression of R-Ras2, Cep70, or UPF3A genes is associated with worse metastasis-free survival. Gene expression profiles a survival data of 710 tumors were retrieved from the GEO database (GSE12276/2034/2603/5327/14020). E) STAT3-GLI1 and STAT3-tGLI1 complexes bind to the R-Ras2 gene promoter but not to the promoter of the internal reference control (Slug gene promoter) as shown by sequential ChIP assay with two antibodies sequentially.
Figure 7. STAT3 synergizes with GLI1/tGLI1 to promote mammosphere-forming ability of breast cancer cells and immortalized mammary epithelial cells
We transected three cell lines with STAT3CA with and without GLI1/tGLI1 and then determine their ability to form mammospheres. A) Triple-negative MDA-MB-468 cells. B) Triple-negative BT20 cells. C) Immortalized human mammary epithelial MCF-10A cells. D) Immortalized human mammary epithelial HMLE cells. All experiments were done three times to derive means and standard deviations. Student t-test was used to compute p values (*, p<0.05).