Loss of WW domain-containing oxidoreductase (Wwox) expression has been observed in breast cancer (BC). However, its regulatory effects are largely unknown, especially in triple-negative breast cancer (TNBC). Herein, gene expression profiling revealed that JAK/STAT3 pathway was one of the most differentially modulated pathways in basal-like BC cells. The lower expression of Wwox was significantly correlated with high activation of STAT3 in basal-like cells and TNBC tissues. Overexpression of Wwox markedly inhibited proliferation and metastasis of BC cells by suppressing STAT3 activation, which is to interact with JAK2 to inhibit JAK2 and STAT3 phosphorylation. Furthermore, Wwox limited STAT3 binding to the interleukin-6 promoter, repressing expression of the IL-6 cytokine. Altogether, our data established that Wwox suppresses BC cell metastasis and proliferation by JAK2/STAT3 pathway. Targeting of Wwox with STAT3 could offer a promising therapeutic strategy for TNBC.

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globally, breast cancer (BC) is the most frequent malignancy and the leading cause of cancer-associated mortality in women. Based on the presence/absence of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor-2 (Her2), BC patients can be classified into the following categories: luminal A, luminal B, HER2 overexpression, and triple-negative subtypes. Triple-negative breast cancer (TNBC) tumors often are more aggressive, are less sensitive to typical endocrine therapies, have a poorer prognosis, and have a higher rate of distant recurrence compared to other subtypes. Less than 30% of patients with metastatic TNBC tumors survive more than 5 years after diagnosis. Patients with TNBC are difficult to treat due to the heterogeneity of the tumors and the lack of well-defined molecular targets. Defining the unique characteristics of an individual patient’s tumors is beneficial to the development of therapeutic schemes that will be most effective for individual patients.

The WW domain-containing oxidoreductase (Wwox) is a 46 kDa protein consisting of two N-terminal WW domains and a C-terminal short-chain dehydrogenase/reductase domain, and is encoded by a locus that spans FRA16D, one of the most active common fragile sites involved in cancer. The genomic location of the Wwox-encoding gene makes the locus susceptible to loss of heterozygosity and homozygous deletions, either of which results in reduced gene expression. Loss of Wwox expression has been observed in cancers of many organs, including breast, lung, esophageal, and gastric carcinomas. Loss of Wwox heterozygosity has been observed in 70% or more of the pre-invasive stages of BC samples. The level of Wwox expression was shown to correlate with ER and PR status, such that expression of Wwox is higher in ER- and PR-positive tumors than in tumors that are negative for these receptors. Conversely, knockdown of Wwox in MCF-10A significantly increased cell mobility compared with control cells.

Results

Wwox is negatively correlated with STAT3 activity in BC.

Firstly, we characterized the level of Wwox protein in BC cells, and Wwox protein was expressed at much lower levels in basal-like cells than in luminal cells (Fig. 1a). RNA sequencing (RNA-seq) experiments were performed to identify potential differences in gene expression levels between these two BC subtypes. A total of 7920 protein-encoding genes appeared to be deregulated in basal cells (Fig. 1b). Analysis using gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses revealed that the JAK/STAT3 pathway was one of the most differentially modulated canonical pathways in basal BC cells. We therefore examined the phosphorylation state of STAT3 in different BC cells. We observed that STAT3 was persistently phosphorylated in basal BC cells; the expression of Wwox was detected only at very low levels in these cells. In contrast, Wwox was highly expressed in luminal BC cells that exhibited very low levels of phosphorylated STAT3 (p-STAT3).

To determine whether increased expression of Wwox might inhibit STAT3 activation, we overexpressed Wwox into basal-like cells (SUM159, HBL100, and MDA-MB-231), and found that exogenous Wwox suppressed STAT3 activation (Fig. 1d-f). In contrast, STAT3 was activated when we knocked down Wwox in luminal cells (T47D, SKBR-3, MCF-7, and MCF-10A) (Supplementary Fig. 1c-f). Notably, IHC analyses revealed that Wwox expression was rarely detected in virtually all of the 90 TNBC samples (Fig. 1g, h); however, STAT3 was dramatically activated in these TNBC samples. We also examined the expression patterns of Wwox and p-STAT3 in 30 BC tissues and their paired adjacent normal tissues. IHC analyses showed that the low expression of Wwox correlated with increased STAT3 activation in BC tissues (Supplementary Fig. 1g, h). Taken together, these results suggested that the expression of the Wwox protein inversely correlates with the activation of STAT3, implying that Wwox may negatively regulate STAT3 activation.

Wwox inhibits tumor growth by suppressing STAT3 activity.

To characterize the effect of Wwox deficiency on the tumorigenicity of BC cells, we employed several cell culture models. The transwell migration assay revealed that cellular migration was attenuated upon overexpression of Wwox in SUM159, MDA-MB-231 (Fig. 2a, b), and HBL100 cells (Supplementary Fig. 2a, b). Conversely, knockdown of Wwox in MCF-10A significantly increased cell mobility compared with control cells (Supplementary Fig. 2c, d). In separate experiments, we used three-dimensional (3D) culture conditions; this cell culture technique recreates the architecture of epithelial tissue growth in vitro. In contrast to control cells, Wwox-silenced cells formed larger spheroid-like structures. Moreover, further inhibition of STAT3 activation in MCF-10A shWwox cells suppressed the growth of these spheroid-like structures (Supplementary Fig. 2e). These results suggested that Wwox can inhibit cell migration in vitro.

Next we investigated the effect of Wwox on BC proliferation and metastasis in vivo by utilizing orthotopic xenograft transplantation into the mouse mammary fat pad. We found that animals implanted with Wwox-overexpressing cells exhibited dramatically reduced tumor size, tumor growth rate, tumor weight, and number of liver metastases compared with control animals (Fig. 2f-h). As expected, the expression of STAT3-regulated genes was reduced in Wwox-overexpressed cells (Fig. 2i). In addition, the p-STAT3 levels were significantly decreased in Wwox-expressing MDA-MB-231 tumors (Fig. 2j). Thus, both in vitro and in vivo assays demonstrated that Wwox inhibits metastasis of BC cells.

To obtain direct evidence that Wwox inhibits STAT3 phosphorylation during tumor growth, we overexpressed either STAT3C, a constitutively active form of STAT3, or v-SRC, a protein widely shown to transform NIH3T3 cells by inducing constitutive STAT3 phosphorylation. Overexpression of Wwox dramatically decreased the level of p-STAT3 in the STAT3C- or v-SRC-transformed cells (Supplementary Fig. 3a). Migration and p-STAT3-dependent colony formation of the transformed cells was attenuated when Wwox was overexpressed (Supplementary Fig. 3b-e). In the xenograft transplantation tumor model, these cell lines yielded smaller tumors when Wwox was overexpressed compared with control cells. Both tumor growth rate and tumor weight were reduced in transformed cells transplanted with Wwox (Supplementary Fig. 3f-k).

STAT3 has been shown to be constitutively phosphorylated in B16 murine melanoma cells. Intriguingly, the level of p-STAT3 was downregulated when Wwox was expressed in B16 cells (Supplementary Fig. 4a). To examine the effect of Wwox on cell transformation, a soft agar colony formation assay was
performed. B16 cells with Wwox overexpression exhibited a decreased ability to form colonies (Supplementary Fig. 4b, c). We next investigated the tumorigenic capacity of Wwox-overexpressing B16 cells. Tumor size, tumor growth rate, and tumor weight were reduced in mice harboring B16 cells transfected with Wwox (Supplementary Fig. 4d–f). We also investigated the effect of Wwox on metastasis in pulmonary metastasis model. The results showed that Wwox impeded metastasis (Supplementary Fig. 4g–i). Taken together, our data suggested that Wwox negatively regulates migration in vitro and suppresses metastasis in vivo by targeting p-STAT3.

**Wwox interacts with STAT3.** Wwox has been defined as a partner of multiple transcription factors, including p73 and Jun. We therefore hypothesized that there might be an interaction between Wwox and STAT3, a possibility that we investigated via immunoprecipitation (IP) experiments (Fig. 3a). The interaction of endogenous Wwox and STAT3 was confirmed (Fig. 3b). Co-IP results additionally revealed that Wwox interacted with STAT5 and with STAT1 (Supplementary Fig. 5a, b). It appeared that the association between Wwox and STAT3 occurred via the WW1 domain of Wwox (Supplementary Fig. 5c, d) and the coiled-coil domain of STAT3 (Supplementary Fig. 5e, f).

We further investigated interactions between Wwox and different forms of STAT3. The results revealed that Wwox interacted with STAT3-Y705F, which is an inactive form of STAT3 that localizes primarily to the cytoplasm. In addition, Wwox retained affinity for STAT3C,
a constitutively activated and dimeric form of STAT3. A derivative of STAT3C that was further mutated at Y705 interacted more weakly with Wwox than did wild-type STAT3 (Supplementary Fig. 5g).

In the Wwox protein, tyrosine 33 (Y33), located in the first WW domain, was previously reported to abolish the Wwox interaction with p7322. We found that a Y33R mutant Wwox protein, in which Tyr33 is replaced with an Arg residue, was still able to interact with STAT3 (Supplementary Fig. 5h). IP experiments using deletion mutants of STAT3 showed that the coiled-coil domain of STAT3 is responsible for the interaction with Wwox (Supplementary Fig. 5i). Collectively, these results demonstrated that Wwox interacts with STAT3 via sequence-specific recognition.

Wwox impedes STAT3 phosphorylation. To address whether Wwox affects STAT3 transcriptional activity, we performed luciferase reporter assays using the STAT3-specific binding element APRE. IL-6-induced STAT3 transcriptional activity was inhibited by transient overexpression of Wwox, but was enhanced by knockdown of Wwox using short interfering RNA (siRNA; Fig. 3c, d, Supplementary Fig. 6a, b). Furthermore, the Wwox effect of IL-6 on APRE-driven transcriptional activity
was dose dependent (Supplementary Fig. 6c). Therefore, we concluded that Wwox inhibits the transcriptional activity of STAT3.

To clarify the molecular mechanism of Wwox inhibition of STAT3 activity, we performed an electrophoretic mobility shift assay (EMSA) to examine whether Wwox affected STAT3 DNA-binding ability. Under standard conditions, incubation of cells with IL-6 results in DNA binding by STAT3. However, exogenous expression of Wwox attenuated IL-6-induced DNA binding by STAT3 (Fig. 3e, Supplementary Fig. 6d). Conversely, depletion of Wwox cells resulted in enhanced STAT3 DNA-binding activity (Fig. 3e, Supplementary Fig. 6d). Thus, Wwox inhibits STAT3 transcriptional activity by blocking STAT3 DNA-binding activity.

We also observed that IL-6-induced accumulation of p-STAT3 was attenuated when Wwox was overexpressed in SUM159 cells (Fig. 3f). Conversely, IL-6-induced accumulation of p-STAT3 was potentiated in MCF-7 (Fig. 3g) and MCF-10A cells (Supplementary Fig. 6e) depleted for Wwox. Notably, Wwox protein levels were unchanged when STAT3 was depleted by siRNAs (Supplementary Fig. 6f). Neither the Wwox protein levels nor STAT3 activation were changed when ER was overexpressed (Supplementary Fig. 6g).

To distinguish whether Wwox inhibits the phosphorylation of STAT3 or accelerates STAT3 dephosphorylation, we employed cytokine stimulation assays. Western blot analysis showed that the maximum levels of p-STAT3 increased following incubation of Wwox knocked-down MCF-7 cells with IL-6 for increasing time intervals (Fig. 3h). In contrast, the maximum levels of IL-6-stimulated p-STAT3 were decreased in SUM159 (Fig. 3i) or in B16 (Supplementary Fig. 6h) cells with Wwox overexpression. We further tested the mechanism of Wwox effect on STAT3 activation using cytokine addition–withdrawal assays; p-STAT3 levels did not differ between cells that were depleted for Wwox or overexpressing Wwox in comparison to control cells (Supplementary Fig. 6i, j). Taken together, these data indicated that the effect of Wwox on the accumulation of p-STAT3 is mediated via STAT3 phosphorylation.

**Wwox inhibits JAK2 phosphorylation and JAK2 binding to STAT3.** The JAK2/STAT3 pathway has been widely studied in BC and other cancer types. Using co-IP, we confirmed that Wwox interacts with JAK2 (Fig. 4a, b). In addition, Wwox was also shown to interact with other members of the JAK kinase family (Supplementary Fig. 7a, b). Using a series of plasmids encoding truncated JAK2 and Wwox proteins, the association between JAK2 and Wwox occurs via the WW1 domain of Wwox and the TycKc domain of JAK2 (Fig. 4c, Supplementary Fig. 7c, d). We next tested whether Wwox overexpression induced
**Fig. 4** Wwox inhibits the interaction of JAK2 and STAT3. **a** Co-immunoprecipitation of Wwox and JAK2 shows Wwox-JAK2 interaction. 293T cells were transfected with plasmids expressing Myc-tagged full-length Wwox or HA-tagged full-length JAK2, as indicated. Whole-cell lysates were used in immunoprecipitation (IP) with anti-Myc antibody or anti-HA antibody. **b** Endogenous Wwox and JAK2 interact in vivo. Lysates of MCF-10A and T47D cells were subjected to IP with anti-JAK2 antibodies. **c** Wwox interacts with JAK2 mainly through the WW1 domain. IP and immunoblot (IB) of cell lysates from 293T cells expressing HA-tagged STAT3 and Myc-tagged truncated Wwox proteins (illustrated in Supplementary Fig. 5). Whole-cell lysates were immunoprecipitated with anti-HA and immunoblotted with anti-Myc antibody. **d** Wwox reduces p-STAT3 and p-JAK2 levels. MDA-MB-231 cells were transfected with Wwox plasmids and were treated with IL-6 for 30 min. The whole-cell lysates were immunoblotted with the indicated antibodies. **e** Interaction pattern of Wwox and JAK2 under IL-6 stimulation. **f** Wwox inhibits the interaction of JAK2 with STAT3. MCF-7 cells were transfected with Wwox siRNA, along with IL-6 stimulation; cell lysates were subjected to IP and IB analyses.

JAK2 activation. The results revealed that Wwox attenuated IL-6 induced JAK2 phosphorylation (Fig. 4d). Nonetheless, the interaction between JAK2 and Wwox was enhanced when cells were stimulated by exposure to IL-6 for different time intervals (Fig. 4e).

To further address whether Wwox suppresses the association of JAK2 with STAT3, we examined the interaction between JAK2 and STAT3 in cells performing knockdown of Wwox. The interaction between JAK2 and STAT3 was increased upon downregulation of Wwox (Fig. 4f). We also found that the interaction between JAK2 and STAT3 was attenuated upon overexpression of Wwox (Supplementary Fig. 7e). In this context, we identified several potential phosphorylation sites in the WW1 domain of Wwox and found that the interaction between JAK2 and Wwox was decreased when residue S14 or T49 of Wwox was mutated (Supplementary Fig. 7f). Notably, the association of JAK2 with STAT3 was restored when Wwox S14A or T49A was overexpressed (Supplementary Fig. 7g), in contrast to the effect seen upon overexpression of wild-type Wwox. Overexpression of S14- or T49-mutant Wwox proteins did not inhibit the IL-6-mediated induction of STAT3 transcriptional activity (Supplementary Fig. 7h). Together, these results indicated that Wwox decreases STAT3 activation by inhibiting JAK2 phosphorylation.

Wwox inhibits STAT3-mediated induction of IL-6. STAT3 functions as a transcription factor, and so we hypothesized that Wwox may have an effect on STAT3-mediated transcriptional activity function. We therefore investigated the potential role of Wwox in the secretion of the cytokines by basal BC cells. The cytokine profiles of conditioned medium were analyzed using Cytokine Antibody Array. The levels of secreted IL-6 exhibited the largest decrease in CM from SUM159 Wwox-overexpressing cells (Fig. 5a). We further confirmed that the levels of both IL-6 messenger RNA (mRNA) and secreted IL-6 protein were attenuated in the Wwox-transfected SUM159 cells (Fig. 5b, Supplementary Fig. 8a). The decreased levels of secreted IL-6 protein were confirmed (using enzyme-linked immunosorbent assay (ELISA)) in Wwox-transfected MDA-MB-231, HBL100, and BT-549 cells (Supplementary Fig. 8b-d); similarly, the levels of IL-6 mRNA were decreased in Wwox-transfected MDA-MB-231 and BT-549 cells (Supplementary Fig. 8e, f).

STAT3 activation is known to be required for IL-6 production, and the JAK2/STAT3, nuclear factor (NF)-κB, and p38 signaling pathways have been shown to promote IL-6 induction in human cells. To determine whether Wwox regulates IL-6 transcription, we assayed IL-6 transcription using a luciferase-encoding reporter under the control of the human IL-6 promoter. We observed that Wwox overexpression led to decreased IL-6 promoter-driven luciferase production (Fig. 5d). We speculated that STAT3 might mediate the induction of IL-6 expression via the partial IL-6 promoter on loss of Wwox in BC cells. Chromatin immunoprecipitation (ChIP) confirmed that STAT3 directly bound to the IL-6 promoter. Furthermore, forced expression of Wwox in
The level of Wwox has prognostic implications in BC. We examined Wwox expression levels in clinical specimens. In silico analyses showed that Wwox mRNA expression levels were much lower in basal BCs compared to those in normal tissues, and that Wwox mRNA levels in basal BCs were even significantly lower than those in luminal BCs. In addition, in silico analyses of three other independent datasets, obtained from Oncomine, revealed a very similar result in breast carcinoma patient samples and paired normal tissues. Furthermore, we employed a scoring approach based on the annotation of the IHC images. Specifically, IHC staining of 30 paired normal and BC tissues found that the protein levels of Wwox were lower in the BC specimens compared to normal tissues. IHC staining of 25 paired normal and TNBC tissues revealed that Wwox protein levels were dramatically lower in the TNBC specimens.

To investigate the correlation of Wwox expression with clinical features of BCs, we examined the protein expression levels of Wwox in 150 BC patient tissues by IHC. Kaplan–Meier analysis of specimens from human patients with BC revealed that patients harboring tumors with high Wwox protein levels (n = 83, median survival time = 116 months) had longer overall survival times than did patients harboring tumors with low Wwox protein levels (n = 67, median survival time = 82 months; p < 0.001) (Fig. 6d). As expected, all the TNBC cases are present Wwox low expression pattern in this cohort. We note that all the median survival time of TNBC patients is much shorter than other subtypes of BC patients (Fig. 6e). Moreover, the decreased level of Wwox protein was associated with larger tumor size and higher tumor grade level in patients (Supplementary Table 1). We further utilized the databases from the online Kaplan–Meier plotter website to perform in silico analysis of Wwox mRNA expression data. The frequency of relapse-free survival (RFS) was worse in patients with low Wwox expression levels (n = 1977) than in patients with high Wwox expression levels (n = 1974, respectively; p < 0.001) (Supplementary Fig. 8f). These clinical findings indicated that low Wwox expression level may be prognostic for the malignant progression of BC.

A proposed model for the Wwox regulatory role is summarized schematically in Fig. 6f. As shown in this model, luminal cells exhibit abundant Wwox expression. During IL-6 stimulation, Wwox is capable of competitive interaction with JAK2, leading to the inhibition JAK2 and STAT3 phosphorylation. In contrast, basal cells exhibit low levels of Wwox, permitting the activation of JAK2, the interaction of JAK2 with STAT3, and STAT3 phosphorylation. The p-STAT3 translocates into the nucleus, where p-STAT3 trans-activates IL-6 transcription; the resulting IL-6 secretion subsequently may promote further constitutive STAT3 activation.

**Discussion**

As reported here, we demonstrated that the level of Wwox protein negatively correlates with STAT3 activation, not only in BC cells, but also in clinical BC specimens. Additionally, we showed that overexpression of Wwox inhibits STAT3 activity in basal BC cells. Moreover, we demonstrated that Wwox inhibits tumor
growth and metastasis by basal-like BC cells. We further demonstrated that Wwox inhibits JAK2 phosphorylation and impedes the association of JAK2 with STAT3, thereby inhibiting STAT3 phosphorylation. In addition, Wwox suppresses IL-6 mRNA expression and IL-6 production by inhibiting the binding of STAT3 at the IL-6 promoter. Furthermore, we showed that patients harboring tumors with higher Wwox protein levels exhibited longer RFS and overall survival times. In summary, our studies suggested that Wwox is an inhibitor of the malignant progression of BC, and that this regulatory effect is mediated through the IL-6/JAK2/STAT3 axis.

Many studies have shown that Wwox is frequently deleted or altered in multiple malignant cancers. Decreased Wwox expression is frequently observed in TNBC and invasive BC subtypes that are associated with high local recurrence rates, lack of effective target therapies, distant metastases, and poor disease-free survival. Previous studies have shown that Wwox functions predominantly through its first WW domain, which physically interacts with PPxY-containing proteins, including AP-2γ and SMAD3, sequestering the PPxY-containing proteins in the cytoplasm to suppress their transcriptional functions. In the present study, we found that the low expression of Wwox is significantly correlated with highly activated STAT3 in basal cancer cells and in TNBC tissues. Decreased Wwox expression is associated with the triple-negative subtype and a poor disease-free survival rate for BC patients. Wwox interacts with STAT3 or JAK2 via Wwox first WW domain, and this interaction is independent of PPxY motifs. Phosphorylation of the Y33 residue of Wwox has been shown to enhance recognition of the PPxY motif and to promote the WW–PPxY interaction. However, in the present work, the interaction of Wwox with STAT3 was not affected when the Y33 residue of Wwox was mutated. Intriguingly, we identified some potential phosphorylation sites in the Wwox protein sequence and showed that mutation of these residues affects Wwox interaction with JAK2. These candidate sites included Wwox S14, a residue that has previously been shown to be important for protein–protein interactions. However, we did not identify a cognate kinase responsible for phosphorylation of the Wwox S14 residue. We did not find the evidence that ER can directly regulate Wwox–STAT3 signaling pathway. However, it was reported that S-glutathionylation of cysteine residues in ER protein influences the cellular consequences and cytoxic effects in dendritic cells. Further investigations will be needed to explore the role that ERa plays in the Wwox function and the role of Wwox phosphorylation in BC.

Persistent STAT3 activation has frequently been linked to more malignant cancer behaviors, including proliferation, invasion, and metastasis. Aberrant JAK2/STAT3 signaling has been detected in a variety of tumor types, indicating that STAT3 inhibitors might be widely effective as anticancer therapies. STAT3 activity is tightly regulated by phosphorylation activators and phosphorylation inhibitors. The protein inhibitors of activated STATs and the suppressors of cytokine signaling proteins (SOCSs) act as negative feedback regulators to prevent further JAK/STAT signal activation. Among these negative regulators, SOCS3 is widely recognized for its ability to attenuate IL-6-induced STAT3 activation. The accumulation of SOCS3 protein has been shown to enhance SOCS3 association with JAK2, which then promotes the ubiquitination and degradation of JAK2, resulting in a loss
of STAT3 phosphorylation and function, indicating that Wwox and SOCS3 negatively regulate STAT3 activity by distinct regulatory mechanisms. In the previous study, conditional deletion of Wwox in the mouse mammary gland does not result in tumorigenicity, although activation of STAT3 in the mouse mammary gland was observed. However, the precise mechanisms linking Wwox to tumorigenesis have not been explored. In the present study, we demonstrated that Wwox impedes the association of JAK2 with STAT3, thereby inhibiting STAT3 phosphorylation and p-STAT3-dependent cancer cell growth in vivo. Using NIH3T3 fibroblasts with STAT3 constitutive activation, we further proved that Wwox allows to suppress in vivo tumor growth depends on STAT3 activation. Our study provides new insights into how Wwox serves as an important negative regulator by suppressing STAT3 activation in the pathogenesis of BC.

Increased cytokine receptor signaling, such as that transduced by the IL-6 receptor, has been implicated as one of the reasons for prolonged STAT3 activation, given that such cytokines are continuously released in an autocrine or paracrine manner. IL-6 levels are dramatically increased in metastatic diseases, and elevated levels of serum IL-6 are associated with poor disease outcome and prognosis in BC patients. Deletion of IL-6 has been shown to yield reductions in tumor burden and metastasis in experimental models. We data suggested that persistently activated STAT3 contributes to IL-6 production in basal cells. However, when Wwox protein levels are elevated in luminal cells, STAT3 phosphorylation is decreased, suppressing IL-6 transcription. Other studies have reported that STAT3 can bind directly to the IL-6 promoter in human cells. However, we found that STAT3 binds to the IL-6 promoter at a site distinct from that identified in the previous studies. Given that the NF-κB and p38 signaling pathways are known to promote IL-6 induction, we cannot exclude the possibility that the Wwox induction of IL-6 expression in our system depends on one or both of these pathways. In addition, we observed Wwox-mediated induction of IL-8 and growth-regulated oncogene (GRO) in SUM159. Further investigations will be needed to address the potential effects of IL-8 and GRO on tumor growth.

In conclusion, our analyses have revealed that Wwox inhibits STAT3 phosphorylation, thereby serving as a potential suppressor in the progression and metastasis of BC. Notably, our data may provide a novel perspective on the role of Wwox in the progress of TNBC. These findings are expected to contribute to our understanding of the function of Wwox in tumor development, while also defining Wwox as a promising therapeutic target for the treatment of BC.

Methods
Plasmids and constructs. The Wwox and Wwox Y33R plasmids were kindly provided by Dr. Rami I. Aqeilan (The Hebrew University, Israel). The Flag-STAT3, Flag-STAT5, HA-STAT1, and STAT3 luciferase reporter plasmids were generously provided by Dr. Rami I. Aqeilan (The Hebrew University, Israel). The Flag-STAT3, Flag-STAT5, HA-STAT1, and STAT3 luciferase reporter plasmids were generously provided by Dr. Rami I. Aqeilan (The Hebrew University, Israel). The Flag-STAT3, Flag-STAT5, HA-STAT1, and STAT3 luciferase reporter plasmids were generously provided by Dr. Rami I. Aqeilan (The Hebrew University, Israel).

Luciferase assay. Luciferase assays were performed by co-transfection of the STAT3 luciferase reporter hSE/APRE and the expression plasmids or siRNAs indicated in the figure legends. In all experiments, pRL-TK vector (Promega, Madison, WI) was transfected as an internal control. Transfected cells were treated with 30% FBS without IL-6 for 6 h. The luciferase activities of the reporters were measured using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer’s protocol.

mRNA-seq and real-time quantitative PCR. For the mRNA-seq assay, samples were submitted to Shanghai Biotechnology Corporation for RNA-seq. Poly(A) RNA was purified from total RNA, then converted to double-stranded cDNA. For real-time quantitative PCR, cDNA samples were subjected to reverse transcription using the standard protocols. The sequencing reads were mapped to the human genome using tophat (version: 1.0.13). Avadis NGS (version: 1.3) was used to calculate reads per kilobase per million mapped reads (RPKM) values. Differentially expressed genes were called at fold-changes using RPKM. GO and KEGG analyses were performed using DAVID (Database for Annotation, Visualization and Integrated Discovery: http://david.abcc.ncifcrf.gov/). For real-time PCR, total RNA was isolated using Trizol (Invitrogen), then cDNA was generated by reverse transcription of

Antibodies and reagents. Antibodies and reagents were obtained as follows: anti-Wwox (sc-373846, WB, 1:1000; IHC, 1:100) antibody, STAT3 inhibitor Stattic, and all siRNAs from Santa Cruz Biotechnology; anti-STAT3 (9139S, western blot (WB), 1:1000; IP, 1:400), anti-p-STAT3 (9138S, WB, 1:1000; IHC, 1:200), anti-JAK2 (3230S, WB, 1:1000; IP, 1:200), anti-p-JAK2 (3771S, WB, 1:1000), anti-Myc (2272S, WB, 1:1000), and anti-HA (3724S, WB, 1:1000) primary antibodies and horseradish peroxidase (HRP)-conjugated secondary antibodies (7074S, 7076S, WB, 1:2000) from Cell Signaling Technology; anti-GAPDH (M2006M, WB, 1:5000) and anti-β-actin (M2011M, WB, 1:3000) antibodies from Abmart Company; anti-FLAG (SAB120678, WB, 1:5000) antibody from Sigma-Aldrich; human recombinant IL-6 from R&D Systems; and Matrigel from BD Pharimingen.

Cell culture transfection. Cells lines MCF-7, MDA-MB-231, MDA-MB-468, MDA-MB-435, MDA-MB-453, ZR-75, T47D, SKBR-3, BT-474, 547, HBL100, SUM159, B16, NIH3T3, and 293T were obtained from the American Type Culture Company; anti-FLAG (SAB1306078, WB, 1:5000) antibody from Sigma-Aldrich; anti-HE (3724S, WB, 1:1000) antibody from Cell Signaling Technology; Flag-STAT3, Flag-STAT5, HA-STAT1, and STAT3 luciferase reporter plasmids were generously provided by Dr. Rami I. Aqeilan (The Hebrew University, Israel). The Flag-STAT3, Flag-STAT5, HA-STAT1, and STAT3 luciferase reporter plasmids were generously provided by Dr. Rami I. Aqeilan (The Hebrew University, Israel). The Flag-STAT3, Flag-STAT5, HA-STAT1, and STAT3 luciferase reporter plasmids were generously provided by Dr. Rami I. Aqeilan (The Hebrew University, Israel). The Flag-STAT3, Flag-STAT5, HA-STAT1, and STAT3 luciferase reporter plasmids were generously provided by Dr. Rami I. Aqeilan (The Hebrew University, Israel).
aliquots of RNA using the Takara PrimeScript RT Reagent Kit (Takara) according to the manufacturer's instructions. The resulting cDNA was used for real-time PCR with SYBR Premix Ex Taq Kit (Takara) in a StepOne Real-Time PCR Detection System (Life Technologies). All expression data were normalized to β-actin-encoding transcript levels. Primers used for real-time PCR are shown in Supplementary Table 2.

**MTT proliferation assay.** Cells were seeded at 4000 cells per well in 96-well plates in triplicate, and incubated for indicated time points. Then, 20 µL MTT (Thiazolyl Blue Test Solution, 5 mg/ml, Sigma) was added to each well and plates were incubated for 4 h at 37 °C. The supernatant was aspirated and dimethyl sulfoxide (Sigma) was added 100 µl per well and the plates were incubated for 30 min at 37 °C. The absorbance was measured at 570 nm in a SpectraMax 190 microplate reader (Molecular Devices, USA).

**3D cell culture.** Aliquots of 4000 cells were seeded into each well of 8-well chamber slides coated with 70 µl per well of Matrigel; fresh medium containing Matrigel was added every 3 days. Phase images were captured on an IX51 Microscope (Olympus).

**Cell migration assay.** The cell migration assay was performed using modified Boyden chambers in 24-well dishes with Transwell filter inserts provided with 8 µm pore membranes (Corning Inc., Corning, NY, USA). Aliquots of 4 x 10^4 cells were seeded into each upper chamber of the insert in serum-free medium, and complete medium was added to the lower chamber. After 12 or 24 h, cells were fixed with 4% paraformaldehyde and stained using 0.1% crystal violet. Cells in the upper chamber were carefully removed, and the cells that migrated through the lower side of the filter were imaged (Olympus IX81) and quantified with ImageJ.

**Soft agar assay.** Aliquots of 3 x 10^3 NIH3T3 v-SRC, NIH3T3 STAT3C, or B16 cells were inoculated into 0.3% agar containing 1x DMEM and seeded in each well of a 6-well plate containing 0.6% agar in 1x DMEM. Cells were grown at 37 °C for 21 days and stained with crystal violet (0.5% w/v).

**Detection of tumor-derived cytokines using antibody arrays.** The Human Cytokine Antibody Arrays C5 kit (Raybiotech) was used according to the manufacturer's instructions. Briefly, each array was blocked and incubated overnight at 4 °C with 1 mL of undiluted condition medium (CM). Samples were aspirated; the array was washed five times, incubated with biotin-conjugated antibodies (1:250) for 2 h at room temperature, and then incubated with HRP-linked secondary antibody (1:1000) for 2 h at room temperature. The membranes were incubated with chemiluminescent substrate and imaged within 10–15 min, since chemiluminescent signals fade over time.

**Detection of human IL-6 concentration by ELISA.** Human IL-6 ELISA Kit (Raybiotech) was used according to the manufacturer's instructions. Briefly, an aliquot (100 µL) of standard or sample was added to each well, and plates were incubated for 2.5 h at room temperature. An aliquot of 100 µL of prepared biotin antibody was added to each well and plates were incubated for 1 h at room temperature. An aliquot of 100 µL of TMB One-Step Substrate Reagent was added to each well and plates were incubated for 45 min at room temperature. An aliquot of 100 µL of TB buffer was added to each well and plates were incubated for 30 min at room temperature. Finally, an aliquot of 50 µL of Stop Solution was added to each well and plates were read immediately by measuring absorbance at 450 nm.

**Electrophoretic mobility shift assay.** EMSA was performed using the LightShift Chemiluminescent EMSA Kit (Thermo) according to the manufacturer's protocols. The biotin-conjugated human high-affinity siss inducible element (hsIE) (5'-AGC TCCATTT CCCGTAATAC CCTAAAGG-3') was synthesized at Beyotime (Raybiotech) and annealed into a double-strand. Briefly, for the EMSA, nuclear extracts were incubated with biotin-labeled oligonucleotide probe in binding buffer (50% glycerol, 100 mM MgCl2, 1% NP-40, 0.1 µg/µl poly (dI-dC), 100 mM Tris, 500 mM KC1, 10 mM DTT) for 20 min at room temperature. The DNA–protein complexes were resolved by electrophoresis on 5% non-denaturing polyacrylamide gel in 0.5x TBE buffer at 100 V for 1.5 h, then transferred to a nylon membrane at 380 mA (~100 V) for 30 min. The membrane was crosslinked, blocked in blocking buffer for 15 min, and incubated with HRP-linked streptavidin (1:300) for 15 min with gentle shaking. The membranes were incubated with chemiluminescent substrate and exposed to X-ray film for 2–5 min.
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Author contributions
L.Z. and R.C. designed the research; R.C., L.S., Y.X., Y.W., C.D., X.W. and X.S. performed the research; Y.H. performed pathological diagnoses; W.L. and X.Z. shared the reagents; R.C. analyzed the data; and L.Z. and R.C. wrote the manuscript.

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