**SUPPLEMENTARY INFORMATION.**

**Activating transcription factor 4 modulates TGFβ-induced aggressiveness in triple negative breast cancer via SMAD2/3/4 and mTORC2 signaling**

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**SUPPLEMENTARY MATERIALS AND METHODS**

**Reagents**

Inhibitors LY2157299 (TGFβR1 Kinase inhibitor), U0126 (MEK1/2 inhibitor), SB203580 (P38-MAPK inhibitor), BKM120 (PI3K inhibitor, p110α/β/δ/γ catalytic isoforms), LY294002 (PI3K inhibitor, p110α/β/δ catalytic isoforms), GSK690693 (pan-AKT inhibitor), Rapamycin (mTOR inhibitor), GSK2334470 (PDK1 inhibitor), BI-D1870 (pan-RSK inhibitor), GSK650394 (SGK1/2 inhibitor), CHIR-99021 (GSK3α/β inhibitor), ISRIB trans-isomer (ATF4, and integrated stress response, or ISR, inhibitor) were purchased from MedChem Express and (5Z)-7-Oxozeaenol from (TAK1 inhibitor) Sigma. Human recombinant Transforming Growth Factor-β1 (TGFβ1) was obtained from Peprotech. Trilencer-27 Human siRNAs for *ATF4* and scrambled control (SCR) were purchased from Origene. siRNAs for *SMAD2/3*, *SMAD4*, *PERK*, *PKR*, *GCN2*, *HRI*, eIF2α, *RPTOR*, *RICTOR* and *TAK1* were purchased from Santa Cruz Biotechnology. *RAS*-siRNA was from Sigma. For *in vivo* studies, *ATF4*-siRNA#2 and SCR control (sequences from Origene) were synthesized by Sigma. DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine) was purchased from Avanti Polar Lipids (Alabaster). Docetaxel was obtained from the Houston Methodist Hospital pharmacy. Thapsigargin was purchased from Santa Cruz Biotechnology.

**Data mining of Chromatin ImmunoPrecipitation (ChIP-Seq)-Sequencing**

Data mining of published ChIP-Seq datasets (1) was carried out. ChIP-Seq bigwig files of genome-wide binding profiles of SMAD2/3 in BT549 upon TGFβ1 stimulation were downloaded from gene expression omnibus (accession number GSE83788) and analyzed using Integrative genomics viewer version 2.3.

**ChIP-qPCR**

ChIP was performed in triplicates as described previously (2) with the following modifications: 2x106 BT549 cells were used for each IP reaction using anti-SMAD2/3 (R&D Systems, AF3797). Immunoprecipitated DNA was purified by phenol-chloroform extraction and analyzed by qRT-PCR using locus-specific primers and GoTaq qPCR Master Mix (Promega, A6002).

Primers used were: *SERPINE1*\_Fw: GCAGGACATCCGGGAGAGA and *SERPINE1*\_Rv: CCAATAGCCTTGGCCTGAGA, *MMP2*\_Fw: TCCCAGGCCTGCCCATGTCA and *MMP2*\_Rv: GGAGCTGGTGGGTGGAAAGCC, *ATF4*\_Fw: CGTCCCCATAGAGACGAAGTC and *ATF4*\_Rv: GGCGGGCAAAGTAGAAATG, *LAMB3*\_Fw: TTGCCCTGCACTACAACACA and *LAMB3*\_Rv: GTAACACACCAGGCCCACTT, *HPRT1*\_Fw: TGTTTGGGCTATTTACTAGTTG and *HPRT1*\_Rv: ATAAAATGACTTAAGCCCAGAG.

**Mammosphere-forming efficiency (MSFE)**

MSFE was determined as previously described to minimize clumping (3,4). Briefly, cells transfected with *ATF4*-siRNA and treated with TGFβ1 for 24 h in a 6-well plate, were harvested and cultured in ultra-low attachment plates at 500cells/cm2 in MammoCult medium and 0.5% methylcellulose (MethoCult) (STEMCELL Technologies). Mammospheres were counted after 72 h using GelCount colony counter (Oxford Optronix).

**Quantitative RT-PCR**

Total RNA was extracted using the EZNA MicroElute Total RNA kit (Omega Bio-tek) and reverse transcription was performed with the qScript cDNA Synthesis Kit (Quantabio), according to the manufacturer’s instructions. Quantitative RT-PCR was performed using iTaq Universal SYBR Green Supermix (Bio-Rad) on a QuantStudio 6 Flex Real-Time PCR System (Thermo Fisher Scientific).

Primers used were: *ATF4*\_Fw: CCTTCACCTTCTTACAACCT and *ATF4*\_Rv: GTAGTCTGGCTTCCTATCTC, *NANOG*\_Fw: GTCAAGAAACAGAAGACCAG and *NANOG*\_Rv: GCCACCTCTTAGATTTCATTC, *SOX2*\_Fw: ATAATAACAATCATCGGCGG and *SOX2*\_Rv: AAAAAGAGAGAGGCAAACTG, *OCT4*\_Fw: GATCACCCTGGGATATACAC and *OCT4*\_Rv: GCTTTGCATATCTCCTGAAG, *NOTCH1*\_Fw: AAGATATGCAGAACAACAGG and *NOTHC1*\_Rv: TCCATATGATCCGTGATGTC, *CXCL10*\_Fw: AAAGCAGTTAGCAAGGAAAG and *CXCL10*\_Rv: TCATTGGTCACTTTTAGTG, *GADPH*\_Fw: ATCACCATCTTCCAGGAGC and *GADPH* \_Rv: CATGGTTCACACCCATGAC.

**Western blotting**

Western blotting was performed as described previously (3). Protein bands were detected with the ImageQuantLAS4000 digital imager. Densitometric analysis of protein bands was done with the ImageJ software.

Primary antibodies to ATF4 (CREB-2, C-20 and B-3), SMAD4 (B-8), TWIST1 (Twist2C1a), BCL2 (C-2), MCL1 (22), CD44 (HCAM, DF1485), SOX2 (E-4), HRI (D12), RAPTOR (10E10), RICTOR (H-11), TAK1 (H-5) and RHOA (26C4) were from Santa Cruz. β-Actin, phospho-SMAD2 (Ser465/467)/3(Ser423/425) (D27F4), SMAD2/3, N-Cadherin (D4R1H), ZEB1 (D80D3), SNAIL (C15D3), SLUG (C19G7), NANOG (D73G4), Cleaved NOTCH1 (Val1744) (D3B8), OCT4, PERK (C33E10), PKR, GCN2, eIF2α, phospho-AKT (Ser473), phospho-P70S6K (Thr389), phospho-NDRG1 (Thr346), and RAS were from Cell Signaling and RAC1 (102) from BD Biosciences. Secondary anti-rabbit and anti-mouse antibodies were from Cell Signaling.

**SBE-reporter assay**

SBE-HEK293 cells were transfected with *ATF4*-siRNA and treated with TGFβ1 for 24 h. SBE activity was analyzed with the ONE-Step Luciferase Assay System (BPS Bioscience), according to the manufacturer’s instructions.

**Cell proliferation assay**

Cells were transfected and treated with TGFβ1 for 24 h (MDA-MB-231 for 72 h) in 96-well plates. Cell proliferation was determined by the WST-1 assay as described by adding premixed WST-1 reagent (Roche). After incubation at 37°C for 3 hours, absorbance was read at 450nm (reference wavelength 690nm).

**Migration and invasion assays**

Migration rate was analyzed by the wound healing assay in cells transfected with *ATF4*-siRNA for 48h and treatment TGFβ1 with for 24h in growth medium. A wound was done in the cell monolayer with a 100µl pipette tip and images were taken at 0, 14 or 24h. Invasion was assayed with the Cultrex BME Cell Invasion Assay kit (Trevigen) according to the manufacturer’s instructions. Transfected cells were treated with TGFβ1 for 24 h (MDA-MB-231 cells for 72 h) and seeded onto a 1X-BME-coated transwell chamber (500,000 cells/mL) in 50 µL of medium without FBS and supplemented with TGFβ1 (10 ng/mL). In the bottom well, growth medium with 10% FBS was added. Medium without FBS was used as negative control. After 16-h incubation, the number of invading cells was quantified using Calcein AM at 485 nm excitation and 520 nm emission wavelength, by comparison with a standard curve.

**Inhibitors treatment**

Inhibitors LY2157299 (5µM), U0126 (10µM), SB203580 (10µM), BKM120 (5µM), LY294002 (10µM), GSK690693 (10µM), Rapamycin (10µM), GSK2334470 (10µM), BI-D1870 (10µM), GSK650394 (10µM), CHIR-99021 (10µM), ISRIB (5µM) and (5Z)-7-Oxozeaenol (1µM), were added 1 h before the treatment with TGFβ1 for 24 h and 72 h. Cells in growth medium were treated with thapsigargin at 24, 48 and 72h at a concentration of 100nM (BT549) and 50nM (SUM159PT and MDA-MB-231).

**Animal experiments**

***RNA-Seq and data analysis***

The RNA sequencing was performed by NeoGenomics Laboratories (Houston, TX). Total RNA extracted from each PDX tissue underwent sample quantity and quality assessment using the NanoDrop ND-1000 Spectrophotometer and Agilent 2100 BioAnalyzer. Library preparation was performed using the Illumina TruSeq Stranded mRNA Library prep Kit. The generated indexed libraries were quantified using the Invitrogen Quant-iT dsDNA Assay Kit for concentration and Agilent 2100 BioAnalyzer for library size and quality. The libraries were pooled equally and sequenced on a HiSeq instrument with 1% PhiX spike-in control at 2x101 cycles. ~2x20 million reads with read length of 100bp was generated for each sample.

Raw RNA-Seq data for PDXs was first processed to remove mouse reads. The tool BBSplit was used with mouse genome FASTA sequence as reference to remove all mice reads. Parsed RNAseq data was then aligned to Human reference genome (Version hg19 from UCSC) using STAR (version 2.4.2). Aligned reads were then quantified against the reference annotation (hg19 from UCSC) to obtain FPKM (Fragments/Kilobase/Million) using CuffLinks (v 2.2.1). The RNA-seq data has been deposited and the accession number is GSE113362.

***Validation of ATF4 as a target in the metastatic PDX model of TNBC***

The PDX model BCM-3887 metastasizes to lungs (5), and we used it to develop a highly metastatic model (3887-LM) by serial transplantation of a metastatic nodule from lungs. A fragment of BCM-3887 tumor tissue was transplanted into the mammary fat pad of the NSG mice. When tumors grew to 200-250 mm3, they were removed and mice were maintained alive until the appearance of morbidity signs or body weight loss. After euthanasia, lungs were removed and all metastatic nodules were resected, placed into RPMI supplemented with 10% FBS, and serially transplanted into the mammary fat pad of new 4-5-week-old NSG mice. This process was repeated until the second generation, designated as 3887-LM, and this model was used for further studies. Briefly, a tissue fragment was transplanted in the mammary fat pad of the NSG mice (*n*=10). When tumors reached 150-200 mm3 in size, we removed them and mice were allowed 1 week of recovery from surgery. Afterward, mice were randomized into two groups (*n*=5/group): 1) SCR and 2) siRNA#2. Each siRNA was administered intraperitoneally twice weekly for 6 weeks at a dose of 5 µg/mouse. Mice were euthanized upon signs of metastatic morbidity or weight loss. Lungs and livers were macroscopically assessed for metastases and preserved in formalin for further immunohistochemical (IHC) analyses.

***Liposomal nanoparticle preparation***

siRNA#2 and SCR for *in vivo* delivery were incorporated into DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine) liposomes as previously described (6). siRNAs were mixed with DOPC in the presence of excess tertiary butanol (1:10, w/w), and Tween 20 was subsequently added. The mixture was frozen in an acetone/dry ice bath and lyophilized. Before *in vivo* administration, the lyophilized preparation was hydrated with phosphate-buffered saline (PBS) at 25 μg/mL concentration.

***Flow cytometry***

Aldehyde dehydrogenase 1 (ALDH1) activity changes were analyzed with the Aldefluor (ALDF) assay (STEMCELL Technologies) and a LSR Fortessa (BD Biosciences) flow cytometer, using the single cells isolated from PDX tumors as previously described (7).

**Immunohistochemistry**

Evaluation of lung and liver metastases was performed by Ki67 (Dako, M7240) staining (1:100 dilution). PDX tissue was stained for ATF4 (Abcam, ab28830) at 1:50 dilution (8).

**Supplementary statistical analysis**

***Meta-analysis study***

The correlation of high gene expression with RFS and OS in breast cancer patients (ER+/–, PR+/–, HER2+/–, and the intrinsic subtypes basal, luminal A and B, and HER2) was analyzed by Kaplan-Meier plotter, and the hazard ratio with 95% confidence intervals and the log-rank *P* value were calculated. Patients were separated by computing all percentiles of the expression between the lower and upper quartiles, and the best performing threshold was used as a cutoff in the Cox regression analysis (9). ATF4 expression (probe 200779\_at) on OS (All\_BC): expression range 2601 – 13519 (expression ≥ 7321 correlated with poorer OS). ATF4 expression on RFS: 1) All\_BC: expression range 1033 – 25273 (expression ≥ 7025 correlated with poorer RFS), 2) ER+: expression range 1403 – 17883 (expression ≥ 6733 correlated with poorer RFS), 3) ER–: expression range 1592 – 25273 (expression ≥ 8282 correlated with poorer RFS), 3) TNBC: expression range 2601 – 13519 (expression ≥ 8346 correlated with poorer RFS).

***Gene signature***

Effects of the mechanism-based eight-gene signature on RFS of patients with breast cancer were determined by Kaplan-Meier plotter using the multigene classifier option and computing mean gene expressions. Median survival of the high gene-expression cohort was normalized to the low gene-expression cohort as the percentage, and the survival fold change was calculated as follows: fold change (%) = survival of “high-expression” cohort – 100 (baseline). Multiple testing correction was performed using a step-up method (9). The performance of the eight-gene signature and multiple-gene analysis was evaluated by the leave-one-out cross-validation (LOOCV) method (10). Correlation analyses were performed using Person’s correlation.

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**SUPPLEMENTARY FIGURE LEGENDS**

**Supplementary Figure 1. Patient survival with high *ATF4* expression in ER+ patients. ROC curve. Differential ATF4 expression induced by TGFβ1 and ERS. SBE motifs in ATF4 promoter and ChiP-Seq analysis. A)** Kaplan-Meier showing that high *ATF4* expression correlates with a poorer relapse-free survival (RFS) in estrogen receptor positive (ER+, *n*=2061, *P*=0.0011) breast cancer patients. Follow-up threshold was set at 10 years. **B)** ROC curve analysis to determine the best performing threshold of ATF4 score. Score ≥1 was the cutoff selected (1-Specificity: 0.517. Sensitivity: 0.833. **C)** Differential ATF4 protein expression induced by TGFβ1 (10ng/ml) and thapsigargin (TG) at 24, 48 and 72h in BT549 (100nM), SUM159PT and MDA-MB-231 (50nM) cells. **D)** Human *ATF4* promoter sequence (from -1050 to +1000 bp) showing SBE motifs (CAGAC, CAGA, GTCT, GGCGC, GGCCG). **E)** ChIP-Seq analysis of SMAD2/3 binding to the *ATF4* promoter. Positive and negative control regions of SMAD2/3 binding were *SERPINE1* and *MMP2,* *HBB* and *HPRT1* promoters, respectively. The TGFβ1 responsive genes *ID1, JUN* and *CDKN1A* were analyzed as additional positive controls.

**Supplementary Figure 2. Target efficiency of *ATF4*-siRNA, effects of *ATF4* knockdown on tumor cell migration, and ATF4 expression in mammospheres. A)** Efficiency of two siRNA sequences on *ATF4* mRNA levels in SUM159PT and BT549 cells for 48 h, treated or not with TGFβ1 for 24 h. **B)** Representative images of wound healing assay after *ATF4* depletion in SUM159PT, BT549 and MDA-MB-231 cells. **C)** ATF4 protein levels in mammospheres (MS) compared with attached cells (Att.) at 24, 48, 72 and 96 h in SUM159PT and MDA-MB-231 cells. \*\* *P*<0.01, \*\* *P*<0.001

**Supplementary Figure 3. Screening of non-canonical molecular pathways and OncoPrint analysis of the eight-gene signature. A)** ATF4 expression in the initial screening with the pharmacological inhibitors U0126 (MEK1/2), BKM120 (PI3K), SB203580 (P38-MAPK), (5Z)-7-Oxozeaenol (TAK1) in SUM159PT and BT549 cells co-treated with TGFβ1 for 24 h. **B)** ATF4 levels with the inhibitors BKM120, GSK2334470 (PDK1), GSK690693 (pan-AKT), rapamycin (mTOR), CHIR-99021 (GSK3α/β), BI-D1870 (pan-RSK), GSK650394 (SGK1/2), ISRIB (ATF4, integrated stress response) in SUM159PT, BT549 and MDA-MB-231 cells co-treated with TGFβ1 for 24 and 72 h. **C)** Decreased ATF4 levels in SUM159PT, BT549 and MDA-MB-231 treated with LY294002 (PI3K inhibitor) with TGFβ1 for 24 and 72 h. **D)** Western blot showing ATF4 expression in SUM159PT and BT549 cells after *RAS* knockdown for 48 h with/without TGFβ1 for 72 h. **E)** Protein levels of RHOA and RAC1 in SUM159PT, BT549 and MDA-MB-231 after *ATF4* knockdown with/without TGFβ1 at different time points. **F)** Percentage of alterations (amplification, deep deletion, mRNA up- and down-regulation) in *ATF4*, *TGFBR1*, *SMAD4*, *PIK3CA*, *RPTOR*, *EIF4EBP1*, *RICTOR* and *NDRG1* genes in a cohort of 2509 breast cancer patients by OncoPrint.