Abstract

Triple-negative breast cancer (TNBC) is an aggressive clinical subtype accounting for up to 20% of all breast cancers, but its malignant determinants remain largely undefined. Here, we show that in TNBC the overexpression of Fra-1, a component of the transcription factor AP-1, offers prognostic potential. Fra-1 depletion or its heterodimeric partner c-Jun inhibits the proliferative and invasive phenotypes of TNBC cells in vitro. Similarly, RNAi-mediated attenuation of Fra-1 or c-Jun reduced cellular invasion in vivo in a zebrafish tumor xenograft model. Exploring the AP-1 cistrome and the AP-1–regulated transcriptome, we obtained insights into the transcriptional regulatory networks of AP-1 in TNBC cells. Among the direct targets identified for Fra-1/c-Jun involved in proliferation, adhesion, and cell–cell contact, we found that AP-1 repressed the expression of E-cadherin by transcriptional upregulation of ZEB2 to stimulate cell invasion. Overall, this work illuminates the pathways through which TNBC cells acquire invasive and proliferative properties.

Introduction

Breast cancer is a biologically and clinically heterogeneous disease. Studies of breast cancers using gene expression profiling have identified several major molecular subtypes, including luminal A, luminal B, HER2 (also known as ERBB2)-overexpressing, normal breast tissue-like, and basal-like (1). In contrast, triple-negative breast cancers (TNBC) are defined merely by a lack of expression of estrogen receptor (ER) and progesterone receptor (PR) as well as the absence of HER2 overexpression (2, 3). There is substantial overlap between TNBC and the basal-like subtype of breast cancer, approximately 80% of TNBCs are basal-like, and therefore the two terms describe a broadly similar group of cancers (3). TNBCs account for some of the most aggressive types of breast cancers, marked by high rates of relapse, visceral metastases, and poor prognosis (2). Clinical advances have led to significant progress in the development of targeted therapies for patients with ER-positive or HER2-positive diseases, for example, endocrine therapies or HER2-directed agents such as trastuzumab (4). However, TNBC lacks a targeted therapy, has a poor prognosis, and represents a major unmet medical need.

The AP-1 transcription factor is a key component of many signal transduction pathways. The AP-1 family of transcription factors consists of dimeric complexes of either homodimers of Jun family members (c-Jun, JunB, and JunD) or heterodimers of Jun and Fos family members (c-Fos, FosB, Fra-1, and Fra-2). AP-1 has been shown to regulate the expression of AP-1 target genes by binding to consensus DNA-regulatory elements, known as 12-O-tetradecanoylphorbol-13-acetate (TPA) response elements (TRE; ref. 5). Previous studies showed that Fos/Jun heterodimers are more stable and efficient in driving transcriptional activation than Jun/Jun homodimers, with c-Jun being the most potent transcriptional activator in its group (5). Accumulating evidence has implicated AP-1 in the regulation of a variety of cellular processes, including proliferation, differentiation, growth, apoptosis, cell migration, and transformation (6). For example, AP-1 has been shown to promote proliferation of ER-positive breast cancer cells (7). In the ER-positive breast cancer cell line MCF-7, upregulated AP-1 activity has been associated with tamoxifen resistance and increased invasiveness (8). However, despite increasing knowledge about the physiologic functions of AP-1, our understanding of how AP-1 governs transcriptional regulatory networks in
breast cancer, and how AP-1 regulates the growth of breast cancer, including TNBC, remains largely unknown. Assaying expression of AP-1 family members in a limited number of breast cancer samples, we previously observed that Fra-1 expression was higher in TNBCs compared with ER-positive breast cancers (9). In the present study, expression pattern of AP-1 members across distinct molecular subtypes, luminal A, luminal B, HER2-enriched, basal-like, and normal-like, reveals the overexpression of Fra-1 in basal-like subtype and its prognostic value. Furthermore, we use an integrative analysis combining the genomic landscape of the AP-1 components, Fra-1 and c-Jun, with loss-of-function transcriptome analysis to comprehensively decipher the role of AP-1 in TNBC. In addition, we examine the upstream signaling pathway that regulates AP-1 expression and transcriptional activity and identify potential mediators of the proliferative and invasive phenotypes associated with AP-1 signaling.

Materials and Methods

Cell lines

T47D, MCF-7, MDA-MB-175, ZR-751, SK-BR-3, HCC1569, MDA-MB-453, HCC202, HCC1954, MDA-MB-231, BT549, Hs578T, Sum159, and MDA-MB-157 cells were obtained from the American Type Culture Collection. Whole-cell lysates from MDA-MB-361 and HCC70 were purchased from Biomiga.

Analysis of breast tumor microarray data

Four publicly available breast tumor microarray datasets were analyzed. The TCGA RNA-seq data were downloaded from the UCSC Cancer Genomics Browser (10, 11), where gene-level transcript levels are estimated by the RNA-Seq by Expectation Maximization (RSEM) algorithm. The K-M dataset was downloaded from the Kaplan–Meier plotter website (12). Molecular subtypes for this data were determined with the R package genefit (http://www.bioconductor.org/packages/release/bioc/html/genefit.html), using its robust subtype clustering model for PAM50 subtypes. Data from Wang and colleagues (13) were downloaded from NCBI’s Gene Expression Omnibus (GEO), accession GSE2034. PAM50 subtypes determined as described above. Data from Yau and colleagues (14) were downloaded from the UCSC Cancer Genomics Browser. Kaplan–Meier plots were generated and log-rank tests were performed in R using the package survival.

Chromatin immunoprecipitation and chromatin immunoprecipitation sequencing

Chromatin immunoprecipitation (ChIP) was performed as previously described (15). The antibodies used in ChIP experiments are anti-Fra-1 (R-20): sc-605 and anti-c-Jun (H-79): sc-1694 from Santa Cruz Biotechnology. ChIP DNA was prepared using the INTERFERin siRNA transfection reagent (Polyplus) according to the manufacturer’s instructions. Total RNA was isolated 72 hours posttransfection. Total RNA from three biologic replicates was hybridized to Affymetrix Human Gene 1.1 ST array. The data were analyzed as previously described (17). Analysis of enrichment of Gene Ontology biologic processes was carried out with the Pathway Studio software suite (Ariadne Genomics). The microarray raw data are deposited into GEO (accession number GSE46440).

Gene expression microarray analysis

BT549 cells were cultured to 50% confluency and transfected with Fra-1 ON-TARGET plus SMARTpool (Thermo Scientific; L-004341-00-0010), c-Jun ON-TARGET plus SMARTpool (Thermo Scientific; L-003268-00-0010), or nontargeting pool (Thermo Scientific; D-001810-10-20) using the INTERFERin siRNA transfection reagent (Polyplus) according to the manufacturer’s instructions. Total RNA was isolated 72 hours posttransfection. Total RNA from three biologic replicates was hybridized to Affymetrix Human Gene 1.1 ST array. The data were analyzed as previously described (17). Analysis of enrichment of Gene Ontology biologic processes was carried out with the Pathway Studio software suite (Ariadne Genomics). The microarray raw data are deposited into GEO (accession number GSE46440).

Western blotting

Western blot analyses were performed as previously described (15). Antibodies used were anti-Fra-1 (R-20): sc-605, anti-c-Jun (H-79): sc-1694 from Santa Cruz Biotechnology, anti-E-cadherin (610182) from BD Biosciences, anti-FLAG (M2) from Sigma, anti-Akt (#9272), anti-phospho-Akt (Ser473; #9271), anti-ERK, and anti-phospho-ERK were from Cell Signaling Technology.

qPCR

qPCR was performed as previously described (15).

Cell proliferation assay

Cell proliferation was measured using a WST-1 kit (Roche Applied Science).

Invasion assay

Cells were seeded in the upper chamber of 24-well BD BioCoat growth factor–reduced Matrigel Invasion Chambers (8.0 μm pore; Becton Dickinson) with 0% FBS media. Media containing 10% FBS were added to the lower chamber. After 24 hours, cells in the upper chamber were removed by scraping. Cells that migrated to the lower chamber were stained and counted under a microscope. ZEB2 siRNA (sc-38641) and CDH1 siRNA (sc-270050) were purchased from Santa Cruz Biotechnology.

Zebrafish metastatic model

Tumor invasion and metastasis experiments in zebrafish were performed according to our previously published methods (18, 19). Briefly, BT549 cells were labeled with 1,1′-dioctadecyl-3,3′,3′,3′-tetramethylindocarbocyanine perchlorate (DiI; Fluka), followed by transfection with siRNAs on the next day. After 24 hours, cells were harvested for implantation into zebrafish embryos 2 days after fertilization. Injected embryos were kept at 28°C and were examined for tumor invasion using a fluorescent microscope (Nikon; Eclipse C1).
Reporter gene assays
BT549, MDA-MB-231, and Hs578T cells were transfected with the 2xTRE luciferase reporter plasmid or the PGL3-promoter vector using Lipofectamine 2000 (Invitrogen; Life Technologies). Luciferase activity was measured using the Dual-Luciferase Reporter Assay (Promega). To control for transfection efficiency, luciferase activity was normalized to Renilla luciferase activity.

Results
Fra-1 is overexpressed in basal-like breast cancers and associated with poor prognosis
Gene expression profiling has become an important tool for accurate tumor classification, prognosis, and as a guide to therapeutic intervention. We analyzed publicly available gene expression profiling datasets from breast tumor samples (11–14) to gain an overall view of the gene expression patterns of AP-1 family members, across the molecular subtypes luminal A, luminal B, HER2-enriched, basal-like, and normal-like, as defined by the 50-gene signature (PAM50)-based subtype classification (1, 20). Among all AP-1 family members, we observed that Fra-1 (FOSL1) is overexpressed in the basal-like subtype (Fig. 1A). Notably, within the ER-negative subgroup that contains both basal-like and HER2-enriched tumors, Fra-1 is not highly expressed in the HER2-enriched subtype (Fig. 1A). Moreover, survival analysis of the datasets for which information about distant metastasis–free survival (DMFS) was available revealed that Fra-1 was associated with poor clinical outcome (Fig. 1B; ref. 12, 14).

In a panel of 16 breast cancer cell lines, including five ER-positive, five HER2-positive, and six basal-like TNBC cell lines, Fra-1 protein was highly expressed in all TNBC cell lines (Fig. 1C), which are models of basal-like breast cancer (21). As Fra-1 requires heterodimerization with Jun family members for efficient transactivation, and c-Jun is the most potent transcriptional activator in its group; we also examined the expression of c-Jun in these cell lines. We observed high levels of c-Jun protein expression in all TNBC cell lines (Fig. 1C). Moreover, the mRNA expression of both Fra-1 and c-Jun was higher in TNBC cell lines in comparison with ER-positive and HER2-positive cell lines (Fig. 1C). These data provide a basis for continued studies of the molecular mechanisms of AP-1 signaling in TNBC cells.

Fra-1 and c-Jun cistromes in TNBC cells
We chose to pursue the cistrome and transcriptome analyses in the BT549 cell line because it belongs to the basal-like subtype based on global gene expression analysis (22).
combined with the ability to achieve high transfection efficiency of siRNA in this cell line allowing significant inhibition of Fra-1 or c-Jun expression.

To begin to dissect AP-1 signaling networks in TNBC, we determined genome-wide Fra-1 and c-Jun binding regions in the BT549 cells by ChIP-Seq. We identified 11,670 Fra-1 binding regions and 14,201 c-Jun binding regions, with 84% of the Fra-1 cistrome overlapping with the c-Jun cistrome (Fig. 2A), consistent with the notion that Fra-1 binds to DNA as an obligate heterodimer with Jun proteins. In contrast, the c-Jun cistrome included a large fraction (31%) of unique binding regions (Fig. 2A), in agreement with the ability of c-Jun homodimers to bind to DNA. Examples of shared and unique binding regions for Fra-1 and c-Jun are shown in Fig. 2B. The Fra-1 and c-Jun cistromes could consistently be verified by independent ChIP-qPCR experiments (Supplementary Fig. S1). Sequence conservation analysis of Fra-1 and c-Jun binding regions across different species showed that sequences near the center of the binding regions tend to be more evolutionarily conserved than flanking sequences (Fig. 2C), suggesting a conserved regulatory role.

We observed that Fra-1 and c-Jun binding regions are distributed throughout the genome with a preponderance of binding regions occurring within intergenic and intronic regions with only 10% of Fra-1 binding sites and 17% of c-Jun binding sites being detected in the proximal 5′ region of the nearest gene (Fig. 2D). To further characterize binding site locations relative to a gene transcription start site (TSS),

![Figure 2. Fra-1 and c-Jun cistromes in TNBC cells. A, Venn diagram showing the overlap between Fra-1 and c-Jun cistromes. B, representative examples of ChIP-Seq results showing binding regions shared by Fra-1 and c-Jun in the CSRNP1 gene, a Fra-1 unique binding region upstream of UBE2D3 and a c-Jun unique region upstream of DUSP16. C, averaged conservation profiles using phastCons scores for Fra-1 and c-Jun binding sites, compared with random regions. D, genomic positions of Fra-1 and c-Jun binding sites relative to the nearest gene. E, kernel density plot illustrating enrichment of Fra-1 and c-Jun peaks close to the TSS of their most proximal gene. F, top-enriched DNA motifs, prospective families of DNA binding transcription factors, and percentage of peaks containing the motif identified by de novo motif analysis of Fra-1 and c-Jun binding sites.](image-url)
the distribution of binding sites relative to the most proximal TSS was determined in a window of 25 kb upstream and downstream of TSS. As shown in Fig. 2E, this distribution reveals that the binding of Fra-1 and c-Jun is highly enriched in close proximity to the TSS. 

De novo DNA motif analysis revealed several highly enriched motifs. As expected, consensus AP-1 motifs were identified in 58% of Fra-1 binding regions and in 47% of c-Jun binding regions (Fig. 2F), in line with direct DNA binding of AP-1 members to these regions. These results also indicate that AP-1 family members interact indirectly with DNA sequences of some target sites through mechanisms such as tethering.

**Fra-1- and c-Jun–regulated transcriptomes in TNBC Cells**

To gain further insight into AP-1–regulated transcriptional networks in TNBC cells, we determined global gene expression profiles for TNBC cells upon Fra-1 or c-Jun knockdown. Protein and mRNA levels of Fra-1 and c-Jun were efficiently depleted by Fra-1 siRNA and c-Jun siRNA, respectively (Supplementary Fig. S2A and S2B). We identified differential expression of 419 and 690 genes upon Fra-1 and c-Jun knockdown (Fig. 3A), respectively. Of these, 180 genes were induced and 42 genes were repressed by both Fra-1 and c-Jun knockdown (Fig. 3A), indicating that they are regulated by Fra-1/c-Jun heterodimers. Assigning these genes to functional categories, we found that genes associated with cytokine-mediated signaling, cell adhesion, immune response, cell junction assembly and inflammatory response, among others, were highly enriched (Fig. 3B). This is consistent with the established role of AP-1 in regulating immunity and inflammation (23). We used qPCR to confirm the expression of 10 Fra-1/c-Jun common target genes identified in the gene expression profiling analysis (Supplementary Fig. S2C).

Combining data from the cistrome and transcriptome analysis, 56% (233 of 419) of Fra-1–regulated genes were identified as including Fra-1 binding regions and 60% (411 of 690) of c-Jun–regulated genes were identified as including c-Jun binding regions within 50 kb upstream or downstream of their TSSs, suggesting that they represent direct AP-1 target genes. Among these, 101 genes were induced and 24 genes were repressed by both Fra-1 and c-Jun knockdown, suggesting that they represent direct target genes of Fra-1/c-Jun heterodimers (Fig. 3C).

Combining data from the cistrome and transcriptome analysis also demonstrates that c-Jun and Fra-1 binding regions were enriched within 50 kb of the TSS of c-Jun- and Fra-1–regulated genes (Fig. 3D). Furthermore, this analysis showed that c-Jun binding sites were highly enriched near the c-Jun–upregulated genes as compared with its repressed genes (Fig. 3D). Interestingly, it has also been shown that the binding of nuclear receptors such as AR, ESR2, HNF4A, NR3C1, PGR, and VDR were enriched in regions associated with upregulated genes compared with repressed genes (24).

**Fra-1 and c-Jun common target genes regulating cell proliferation**

Because of the importance of AP-1 in regulating tumor cell proliferation (25), we assessed the function of Fra-1 and c-Jun in TNBC cell proliferation. Both Fra-1 and c-Jun knockdown markedly inhibited the proliferation of BT549 breast cancer cells (Fig. 3E), implying that AP-1 increases cell proliferation of TNBC cells. Inhibition of proliferation by Fra-1 and c-Jun siRNA was confirmed using an independent siRNA pool (Supplementary Fig. S3A), suggesting that this effect is specific to Fra-1 and c-Jun silencing. Targeted knockdown of Fra-1 in an additional TNBC cell line, Hs578T, also led to a significant inhibition of cell proliferation compared with control siRNA, whereas no such effect was observed in MCF-7 breast cancer cell line with low endogenous Fra-1 expression (Supplementary Fig. S3B). Furthermore, overexpression of Fra-1 in BT549 cells depleted of Fra-1 by siRNA largely reversed the effect of Fra-1 depletion on cell proliferation (Fig. 3F).

We next identified Fra-1- and c-Jun–regulated genes with known regulatory functions in cell proliferation. Using this approach, 20 genes that repress cell proliferation were identified to be upregulated upon Fra-1 and c-Jun knockdown, whereas seven genes that promote cell proliferation were identified to be downregulated upon Fra-1 and c-Jun knockdown (Fig. 3G). Putative Fra-1 and c-Jun direct target genes were identified from the Fra-1 and c-Jun cistromes. This analysis identified 16 potential AP-1 direct transcriptional target genes encoding regulators of cell proliferation, including ABTSBP, BIRC3, CLCA2, DCL1, IL22RA2, ITGB5, KCNIP1, KRT18, NFR3, PAD12, TIMP3, MEF2C, MTF1, NFATC2, NPYT, and NTRK2 (Fig. 3G). Moreover, survival analysis of the K-M dataset (12) revealed that this list of 16 genes has prognostic significance in ER-negative patients (Supplementary Fig. S4). Among these, CLCA2 (chloride channel accessory 2) was identified as the most upregulated transcript upon Fra-1 and c-Jun knockdown. Interestingly, reduced expression of CLCA2 was frequently observed in breast cancer (26). In addition, CLCA2 was shown to play a crucial role in inhibition of cancer cell migration and invasion (27, 28). We confirmed by qPCR that CLCA2 expression was substantially elevated after Fra-1 or c-Jun depletion (Fig. 3H). As shown in Fig. 3I, Fra-1 and c-Jun binding regions were observed within intron 3 of the CLCA2 gene. We further validated, by ChIP–qPCR, the binding of Fra-1 and c-Jun to this region in BT549 cells (Fig. 3H). To determine the influence of CLCA2 overexpression on cell proliferation, we generated BT549 cells stably expressing CLCA2. Consistent with previous findings (27), CLCA2-overexpressing cells exhibited reduced cell proliferation, as compared with mock-transfected cells (Fig. 3J and Supplementary Fig. S5A). Collectively, these findings reveal molecular mechanisms by which AP-1 induces cell proliferation.

**AP-1 promotes TNBC cell invasion through transcriptional upregulation of the E-cadherin (CDH1) repressor ZEB2**

Following our observation of a significant enrichment of genes involved in cell adhesion among AP-1 target genes (Fig. 3B), we examined whether AP-1 may regulate the invasive capacity of TNBC cells. To address this, we performed Transwell cell invasion assays for BT549 cells and observed that knockdown of Fra-1 or c-Jun expression significantly reduced
Figure 3. Fra-1– and c-Jun–regulated transcriptomes and target genes related to cell proliferation in TNBC cells. A, overlap of differentially expressed genes upon Fra-1 or c-Jun knockdown, separated by up- and downregulation. B, overrepresented gene ontology biologic processes for common genes (overlaps from A), C, overlap of genes with Fra-1 or c-Jun binding regions (within 50 kb upstream or downstream of their TSS) also identified as differentially expressed genes by microarray analysis. D, bar plot showing fractions of differentially expressed genes with binding sites within 50 kb of their TSS. (Continued on the following page.)
the invasion ability (Fig. 4A). This is consistent with AP-1 promoting cell invasion in TNBC cells.

Screening of the genes involved in the ‘cell adhesion’ gene ontology category revealed significant upregulation of E-cadherin (CDH1) upon Fra-1 and c-Jun knockdown (Fig. 4B). E-cadherin is widely acknowledged for its role in cell-cell adhesion (29). We validated that knockdown of Fra-1 and c-Jun substantially elevated the mRNA level of E-cadherin using qPCR (Fig. 4B). Western blot analysis showed that knockdown of Fra-1 and c-Jun significantly increased E-cadherin protein levels (Fig. 4C). Conversely, treatment of cells with TPA, which is a potent activator of AP-1, decreased E-cadherin levels (Fig. 4C). Down-regulation of E-cadherin by Fra-1 and c-Jun was further validated in another TNBC cell line Hs578T (Supplementary Fig. S5B and S5C).

Notably, the Fra-1 and c-Jun cistromes did not reveal any AP-1 binding regions within or proximal to the E-cadherin gene locus. The expression of E-cadherin is regulated by several known transcriptional repressors, including SNAIL, SLUG, ZEB1, ZEB2, and TWIST (30). Interestingly, a Fra-1 and c-Jun binding region was observed in one intron of the ZEB2 gene (Fig. 4D). A highly conserved sequence with high homology to the AP-1 consensus motif was identified in this binding region (Fig. 4D). This region seems to be an active enhancer, marked by enrichment of H3K4me1.
H3K4me4, and H3K27Ac (Fig. 4D). An independent ChIP–qPCR assay confirmed that Fra-1 and c-Jun were directly bound to this genomic region (Fig. 4E). Furthermore, Fig. 4E shows that ZEB2 expression was downregulated in Fra-1- or c-Jun–depleted cells, with c-Jun depletion showing a predominant effect. Analysis of ZEB2 expression in the TNBC cell line HS578T confirmed downregulation of ZEB2 mRNA following knockdown of Fra-1 or c-Jun (Supplementary Fig. S5D). These data suggest that AP-1 directly activates ZEB2 transcription. Moreover, we noted a statistically significant negative association between c-Jun and E-cadherin expression levels in a published study of breast cancer samples from 286 individuals (ref. 13; Fig. 4F), further supporting the in vivo relevance of the ability of AP-1 to promote tumor invasion in human breast tumors.

**ZEB2 mediates downregulation of E-cadherin and promotes cell invasion**

To further establish a role of ZEB2 in cell invasion in TNBC cells, we knocked down the expression of ZEB2 in BT549 cells (Fig. 5A). Knockdown of ZEB2 expression greatly inhibited the invasiveness of BT549 cells (Fig. 5B). In line with the suppression of cell invasion, E-cadherin protein levels were increased upon ZEB2 depletion (Fig. 5C). To establish the relationship between ZEB2 expression and an invasive breast cancer phenotype, we examined ZEB2 expression in nine human breast cancer cell lines, including three TNBC cell lines and six non-TNBC breast cancer cell lines. High levels of ZEB2 mRNA were detected only in cell lines representing the invasive TNBC subtype (Fig. 5D). These data link ZEB2, a direct target gene of AP-1, to downregulation of E-cadherin and enhanced cell invasion in TNBC cells (Fig. 7D).

To determine the role of E-cadherin in the regulation of cell invasiveness downstream of Fra-1, we examined whether simultaneous E-cadherin and Fra-1 knockdown would render cells insensitive to Fra-1 knockdown. The reduced invasiveness of BT549 cells upon Fra-1 knockdown was reversed by knockdown of E-cadherin (Fig. 5E and Supplementary Fig. S5E), demonstrating the importance of the Fra-1–E-cadherin axis.

**Knockdown of AP-1 inhibits invasion of TNBC cells in vivo**

To study the role of Fra-1 and c-Jun in regulating TNBC cell invasion and metastasis in vivo, we used a zebrafish tumor model (18, 19). Tumor-implanted fish embryos were scored for the dissemination of tumor cells at day 4 after injection. Implantation of control-siRNA–treated BT549 tumor cells led to broad dissemination of tumor cells in different regions of the fish body, whereas BT549 cells silenced for Fra-1 or c-Jun mostly remained in the perivitelline space (Fig. 6A and B). Thus, Fra-1 or c-Jun knockdown inhibits the invasiveness of TNBC cells in vivo.

**The PI3K/Akt and MAPK/ERK pathways positively regulate Fra-1 and c-Jun expression and are associated with increased AP-1 transcriptional activity in TNBC cells**

Our results demonstrated high Fra-1 and c-Jun levels in TNBC cell lines (Fig. 1C). PI3K/Akt signaling has been shown to be enhanced in TNBC compared with other breast tumor subtypes (31). Furthermore, activation of the ERK signaling pathway induces expression of AP-1 components (32, 33). To determine whether these two signaling pathways mediated upregulation of AP-1 members in TNBC cells,
we used two pharmacologic inhibitors, LY294002 and PD98059, to specifically block the PI3K/Akt and MAPK/ERK pathways, respectively. A decrease in phosphorylated Akt upon LY294002 treatment led to a significant downregulation of Fra-1 and c-Jun proteins at 6 and 24 hours (Fig. 7A and Supplementary Fig. S6A). These results were further validated in two other TNBC cell lines, Hs578T and MDA-MB-231 (Supplementary Fig. S6B). Similarly, PD98059 treatment led to a significant downregulation of Fra-1 and c-Jun proteins at 2, 6, and 24 hours (Fig. 7B and Supplementary Fig. S6C). PD98059 inhibition of ERK activity was confirmed by assessing the level of ERK1/2 phosphorylation (Fig. 7B). These data suggest that both PI3K/Akt and MAPK/ERK signaling can increase the expression of Fra-1 and c-Jun in TNBC cells.

In addition, we assessed whether these two pathways are involved in the enhancement of AP-1 transactivation in TNBC cells. In accordance with the reduced AP-1 protein levels, the AP-1 transcriptional activity was attenuated by LY294002 and PD98059 treatment (Fig. 7C). Thus, activation of the PI3K/Akt and MAPK/ERK pathways is associated with increased AP-1 transactivation in TNBC cells.
Discussion

Understanding the molecular basis of TNBC is of pivotal importance in light of the current lack of therapeutic options and the poor clinical outcome for this class of breast cancers. We report that TNBC cell lines and primary tumors express high levels of Fra-1, and that Fra-1 expression is associated with poor prognosis. On the basis of these observations, we pursue a comprehensive characterization of AP-1 signaling in TNBC and suggest how this contributes to the proliferative and invasive phenotypes. Our results suggest both PI3K/Akt and MAPK/ERK signaling as important regulators of AP-1 transcriptional activity in TNBC cells and reveal novel mechanisms underlying AP-1-mediated regulation of cell proliferation and invasion in TNBC cells (Fig. 7D).

We demonstrate overexpression of Fra-1 in basal-like breast cancer (Fig. 1A) and basal-like breast cancer cell lines (Fig. 1C), one of the most aggressive types of breast cancer. It is notable that Fra-1 was expressed at a relative high level in luminal MDA-MB-175 cells (Fig. 1C). However, MDA-MB-175 cells have been demonstrated to have much higher pAkt and pERK1/2 expression than other luminal cell lines such as MCF-7 and T47D (34), and Fra-1 has been shown to be activated by PI3K/Akt or MAPK/ERK signaling (Fig. 7A and B; ref. 33). Our analysis also reveals that Fra-1 levels correlate with poor outcome in breast tumors (Fig. 1B). Consistent with our findings, one recent study showed that the Fra-1-dependent transcriptome has prognostic potential in human breast cancer (35). It has long been suspected that Fra-1 plays an important role in tumor metastasis. Consistent with this hypothesis, we further show that Fra-1 and c-Jun promote cell invasion both in vitro and in vivo (Fig. 4A and Fig. 6). In line with this, Fra-1 has been shown to be involved in the migratory or invasive capabilities of various cancer cell lines (36, 37). Furthermore, in rodent model systems, Fra-1 was identified as a key regulator of metastasis (35). On the basis of these data, intervention of Fra-1–mediated signaling may be explored for therapeutic options in basal-like breast cancer.

Our results indicate that high PI3K/Akt or MAPK/ERK activity increases AP-1 signaling and ultimately cell invasion for TNBC cells. In TNBCs, these two pathways are known to be
activated and are associated with a poor prognosis (38, 39). A recent study suggested that a combination regimen with MEK/ERK and PI3K inhibitors might effectively be used to treat basal-like breast cancers (40). Our data further suggest that such an approach in combination with intervention of AP-1 signaling could be beneficial in TNBCs.

In recent years, cistrome analyses have shown that AP-1 cooperates with nuclear receptors such as ER (41, 42), liver X receptor (43), and glucocorticoid receptor (44) in orchestrating gene expression programs. However, the cistromes of AP-1 members have not been described for human breast cancer cells. Using ChIP-Seq, 11,670 Fra-1 binding regions and 14,201 c-Jun binding regions were identified in BT549 TNBC cells, the majority of which (about 75%) were distal to the promoter in intergenic and intronic regions, consistent with the pattern for nuclear receptor binding to DNA (42, 45). The majority of de novo Fra-1 or c-Jun binding sites are, therefore, either not functional or affect transcription through long-range promoter–enhancer interactions. Indeed, long-range chromatin interactions have been proposed as a model for regulation of gene expression by distal ER binding sites (46). It is of interest to note that there are many more Fra-1 and c-Jun binding sites in the genome than differentially expressed genes, as has been observed for several nuclear receptors (45, 47, 48). This suggests that multiple sites are involved in the regulation of a single gene, and/or that binding alone does not necessarily drive gene expression. In addition, as our analyses were conducted at a single time point, they will provide only a snapshot of the transcriptional consequences of Fra-1 or c-Jun knockdown. Dynamic changes in gene expression, along with potential differences in the kinetics of individual siRNA, may explain some of the discordance between the number of Fra-1- or c-Jun-regulated genes and common genes regulated by both Fra-1 and c-Jun.

So far, the understanding of molecular events underlying the aggressiveness of TNBC is limited, but the TNBC subtype is frequently associated with apparent epithelial–mesenchymal transition (EMT). One of the hallmarks of EMT is the downregulation of the cell–cell junction protein E-cadherin (49). The gene encoding E-cadherin is considered as an invasion-suppressor gene and downregulation of E-cadherin expression correlates with malignancy in human cancers. ZEB2 represses E-cadherin transcription through its direct binding to conserved E2 boxes present in the E-cadherin promoter (50). In this study, we have shown that AP-1 directly activates ZEB2 expression through binding to a regulatory region of the ZEB2 gene. This in turn leads to downregulation of E-cadherin. Moreover, analysis of clinical breast tumor data reveals a significant negative correlation between the expression level of c-Jun and E-cadherin. These findings highlight a crucial AP-1–mediated signaling cascade promoting invasion in TNBC.

In summary, we provide the first genome-wide map of AP-1 binding in TNBC cells and demonstrate overexpression of Fra-1 in basal-like breast tumors. Our results reveal novel mechanisms underlying AP-1–mediated regulation of cell proliferation and invasion in TNBC cells (Fig. 7D). Knockdown of Fra-1 reduces cellular invasion both in vitro and in vivo, suggesting that Fra-1 may be useful as a therapeutic target for patients with invasive breast tumors. One of the future challenges will be to identify specific Fra-1 inhibitors and demonstrate their efficacy in inhibiting cell proliferation and invasion of TNBC cell lines and relevant animal models.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Zhao et al.


Genome-wide Profiling of AP-1–Regulated Transcription Provides Insights into the Invasiveness of Triple-Negative Breast Cancer

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