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ORIGINAL ARTICLE

A potential dichotomous role of ATF3, an adaptive-response gene, in cancer development

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Activating transcription factor 3 (ATF3) is a member of the ATF/cyclic AMP response element-binding family of transcription factors. We present evidence that ATF3 has a dichotomous role in cancer development. By both gain- and loss-of-function approaches, we found that ATF3 enhances apoptosis in the untransformed MCF10A mammary epithelial cells, but protects the aggressive MCF10CA1a cells and enhances its cell motility. Array analyses indicated that ATF3 upregulates the expression of several genes in the tumor necrosis factor pathway in the MCF10A cells but upregulates the expression of several genes implicated in tumor metastasis, including TWIST1, fibronectin (FN)-1, plasminogen activator inhibitor-1, urokinase-type plasminogen activator, caveolin-1 and Slug, in the MCF10CA1a cells. We present evidence that ATF3 binds to the endogenous promoters and regulates the transcription of the TWIST1, FN-1, Snail and Slug genes. Furthermore, conditioned medium experiments indicated that ATF3 has a paracrine/autocrine effect, consistent with its upregulation of genes encoding secreted factors. Finally, ATF3 gene copy number is >2 in ~80% of the breast tumors examined ($N = 48$) and its protein level is elevated in ~50% of the tumors. These results provided a correlative argument that it is advantageous for the malignant cancer cells to express ATF3, consistent with its oncogenic roles suggested by the MCF10CA1a cell data.

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Introduction

During cancer progression, the cells encounter many stress signals; all along, the cells have built-in mechanisms to restrain or eliminate themselves (a review, Hanahan and Weinberg, 2000). However, the successful cancer cells manage to foil the hardwired stress response to eliminate themselves. Emerging evidence indicates that some of the genes that under normal conditions would function to eliminate the cancer cells—the tumor suppressors—appear to be co-opted to become oncogenes. One well-known example of this dichotomy is the transforming growth factor (TGF)-β gene, which induces apoptosis or cell cycle arrest in normal or less transformed cells, but facilitates metastasis in advanced tumors (Massague, 2000; Derynck et al., 2001; Roberts and Wakefield, 2003). This 'Jekyll-and-Hyde' conversion is an intriguing but largely unresolved issue in cancer biology. In this study, we present evidence suggesting that activating transcription factor 3 (ATF3) is a new dichotomous molecule in cancer development.

ATF3 is a member of the ATF/cyclic AMP response element-binding family of transcription factors, which share the basic region/leucine zipper DNA-binding motif and bind to the CRE/ATF consensus sequence TGACGTCA. Overwhelming evidence indicates that the ATF3 mRNA level is not detectable in most cells, but greatly increased by a variety of stress signals, including anoxia (Ameri et al., 2007), hypoxia, DNA damage and carcinogens (Hai et al., 1999; Hai and Hartman, 2001). However, recent literature indicates that some of the signals that can induce ATF3 gene expression do not fit the conventional definition of stress signals (Hai, 2006). Thus, the characterization of ATF3 as a stress-inducible gene is overly simplistic. We suggested characterizing ATF3 as an adaptive-response gene that participates in cellular processes to adapt to extra- and/or intracellular changes (Hai, 2006; Lu et al., 2006a). Three clues suggested that ATF3 may have a dichotomous role in cancer development. First, ATF3 has been demonstrated to play a role in apoptosis and proliferation, two cellular processes critical for cancer progression. However, the literature shows conflicting results: ATF3 can either promote or suppress these processes (Hai, 2006).

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Second, ATF3 has been demonstrated to be either a tumor suppressor or an oncogene in xenograft injection models using various cell lines (Ishiguro and Nagawa, 2000; Bottone et al., 2005; Bandyopadhyay et al., 2006; Lu et al., 2006b). Third, Massagué and colleagues demonstrated that TGF-β induces the expression of ATF3; its gene product in turn interacts with Smad3, resulting in a functional repressor complex on the Id-1 promoter (Kang et al., 2003). Since TGF-β is well known for its dichotomy, this in combination with the conflicting data on ATF3 in the literature promoted us to hypothesize that ATF3 has a dichotomous role in cancer progression. One drawback of the literature on ATF3 is that it was derived from vastly different cell lines and models, making it difficult to deduce the reasons for the apparent contradictions. Thus, we used a breast cancer cell system developed by Miller and colleagues (Santner et al., 2001) to test the dichotomy hypothesis. These cells have the same genetic backgrounds except the genetic and/or epigenetic alterations that allow them to have varying degrees of malignancy. In this study, we describe our findings supporting a dichotomous role of ATF3.

Results

We used two human breast cancer cell lines: MCF10A and MCF10CA1a. MCF10A is a spontaneously immortalized mammary epithelial line and exhibits many characteristics of normal breast epithelium (Soule et al., 1990; Debnath et al., 2003). MCF10CA1a is a high-grade malignant cell line derived from MCF10A after transformation and repeated selections via xenograft injections and passages in culture (Santner et al., 2001). We will refer to MCF10A as M-I cells and MCF10CA1a as M-IV cells, following the previous nomenclature (Tang et al., 2003).

ATF3 has opposite effects on the untransformed M-I cells and the malignant M-IV cells
We generated stable derivatives from M-I and M-IV cells to express ATF3 or a control vector, and will refer to them as M-I/Vec, M-I/ATF3, M-IV/Vec and M-IV/ATF3 in the rest of the report. The protein level of the exogenous ATF3 in these cells was comparable to that of the endogenous ATF3 induced by TGF-β as shown by immunoblot (Figure 1a). Immunofluorescence indicated that ATF3 was expressed in the majority of the cells (Supplementary Figure S1a). To reduce the possibility of changes in cells due to prolonged passages, we used cells within 10 passages after establishment, and all results presented in this report were confirmed from at least three independent transductions. As shown in Figure 1b, serum withdrawal reduced the numbers of vector control cells. Significantly, ectopic expression of ATF3 in M-I cells further reduced the cell numbers upon serum withdrawal, indicating that ATF3 is deleterious. In contrast, ectopic expression of ATF3 in M-IV cells protected the cells from serum withdrawal-induced reduction in cell numbers (Figure 1b). Thus, ATF3 has opposite effects on the untransformed M-I cells and the malignant M-IV cells.

ATF3 is pro-apoptotic in untransformed breast epithelial cells
The deleterious effect of ATF3 on the M-I cell number (under serum withdrawal, Figure 1b) could be due to increased apoptosis, increased cell cycle arrest or both. Fluorescence-activated cell sorting (FACS) and immunoblot analyses showed that serum withdrawal-induced apoptosis in the vector control cells as evidenced by the cell population with $<2N$ DNA content (Figure 1c) and the activation of caspases 3 and 8 (Figure 1d). Significantly, ATF3 enhanced the apoptotic response in M-I cells (Figures 1c and d). Analysis of the cell morphology showed rounding up of the M-I/ATF3 cells at 6 hours after serum withdrawal (Supplementary Figure S1b). We also examined the cells for DNA replication by bromodeoxyuridine (BrdU) incorporation upon serum withdrawal. Under this condition, the cells did not show much labeling by BrdU, consistent with the FACS profile. No difference was observed between vector control and ATF3-expressing cells (data not shown). Thus, the primary reason for the lower cell number of the M-I/ATF3 cells than the M-I/Vec cells upon serum withdrawal (Figure 1b) was enhanced apoptosis.

Since serum withdrawal also induced the expression of the endogenous ATF3 gene (Figure 1d), we asked whether ATF3 is necessary for serum withdrawal to induce apoptosis in the control cells. As shown in Figure 1e, ATF3 knockdown reduced caspase activation. Control small interfering RNA (siRNA) did not affect ATF3 expression and did not affect serum withdrawal-induced caspase activation. Analysis using pathway-specific array (apoptosis microarray) indicated that several genes in the tumor necrosis factor (TNF) pathway were upregulated in the M-I/ATF3 cells under normal growth conditions: TNF-SF7, TNF-SF10 and TNFR-SF12A. SYBR green-based reverse transcription coupled with quantitative PCR (RT–qPCR) confirmed their differential expression (Figure 3a). We also examined the genes encoding their cognate ligands and receptors by RT-qPCR and found no upregulation by ATF3 (data not shown). This result provides a partial explanation for the lack of obvious apoptosis in the unstressed M-I/ATF3 cells.

ATF3 protects aggressive breast cancer cells from stress-induced cell cycle arrest and promotes their cell motility and invasiveness in vitro
To address whether ATF3 protected the aggressive breast cancer cells by inhibiting apoptosis, cell cycle arrest or both, we analysed the cells by FACS analysis and caspase activation as above. As shown in Figure 1f, serum withdrawal increased G_1 cell population in the M-IV/Vec cells but did not increase the cell population with $<2N$ DNA content. Thus, the aggressive M-IV cells responded to serum withdrawal differently than the untransformed M-I cells: instead of an apoptotic response, the cells exhibited G_1 arrest (albeit incomplete). Importantly,
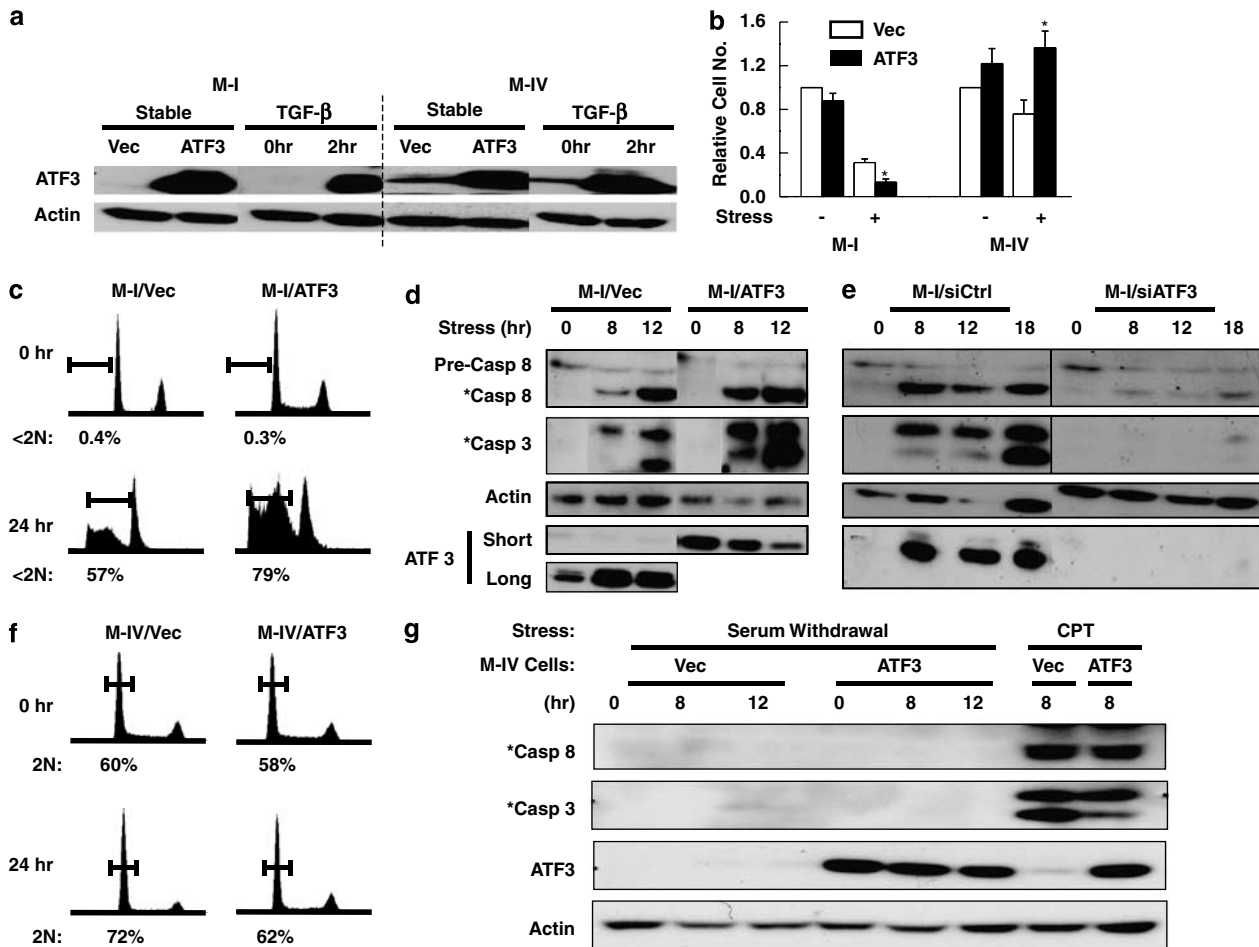
Figure 1 Activating transcription factor 3 (ATF3) has opposite effects on the untransformed M-I and the malignant M-IV cells. (**a**) The indicated stable cells were analysed by immunoblot. The level of ATF3 induced by transforming growth factor (TGF)-β (2.5 ng ml^{-1}) in the parental cells were analysed in parallel. (**b**) The indicated stable cells were seeded into normal growth media (−stress) or serum-free media (+ stress), grown for 72 h and analysed for cell viability by crystal violet assay. The values from vector control cells in normal growth media were defined as 1. Mean ± s.d. from three experiments (*$P < 0.05$, versus M-I/Vec). (**c**) M-I/Vec and M-I/ATF3 cells cultured in serum-free media for the indicated times were analysed by FACS. Representative data from three independent experiments are shown, with the percentage of cells with <2N DNA indicated. (**d**) M-I/Vec and M-I/ATF3 cells were cultured in serum-free media (Stress) and analysed by immunoblot. Two exposures for ATF3 are shown (*Casp, activated caspase). (**e**) M-I cells were transfected with control (siCtrl) or ATF3 (siATF3) siRNAs for 72 h and then cultured under serum-free media (Stress), followed by immunoblot. (**f**) M-IV/Vec and M-IV/ATF3 cells were cultured and analysed by FACS as in (**c**), with the percentage of G$_1$ cells indicated. (**g**) M-IV/Vec and M-IV/ATF3 cells were cultured in serum-free media and analysed by immunoblot. Camptothecan (CPT) at 30 μM was used to induce apoptosis as a control.

ATF3 protected the cells from this response (Figure 1f). We also assayed apoptosis by caspases 3 and 8 activation. As shown in Figure 1g, serum withdrawal did not induce their activation, consistent with the FACS analysis. This lack of apoptotic response in M-IV cells was not due to the defects in their apoptotic machinery, since camptothecan, a DNA-damaging agent, induced caspase activation (Figure 1g). Thus, ATF3 protected the M-IV cells from serum withdrawal primarily by inhibiting cell cycle arrest.

We next examined the ability of ATF3 to affect cell motility using Boyden chamber and found that M-IV/ATF3 cells had higher motility than the control cells either in the absence or presence of TGF-β (Figure 2a). A representative picture of the motility assay is shown in Supplementary Figure S2a. ATF3 also increased the cell invasion when matrigel-coated membrane was used (Figure 2a). Since the endogenous level of ATF3 in the M-IV cells was detectable (Figure 1a), we asked whether ATF3 plays a necessary role in its basal motility. Significantly, siRNA knockdown of ATF3 reduced the cell motility of M-IV cells (Figure 2b), indicating that ATF3 is necessary to enhance M-IV cell migration. Taken together, both the gain- and loss-of-function approaches indicated that ATF3 promotes cell migration in the M-IV aggressive breast cancer cells. For a comparison, we examined whether ATF3 affects the cell motility of the untransformed M-I. As shown in Supplementary Figure S2b, ectopic expression of ATF3 also increased its cell motility. Since the basal expression level of ATF3 in M-I cells was usually undetectable, we did not carry out the knockdown experiments. We note

that ATF3 was demonstrated to increase cell motility in a melanoma line and a colon cancer line (Ishiguro et al., 1996, 2000). Thus, the ability of ATF3 to affect cell motility is not limited to breast epithelial cells.
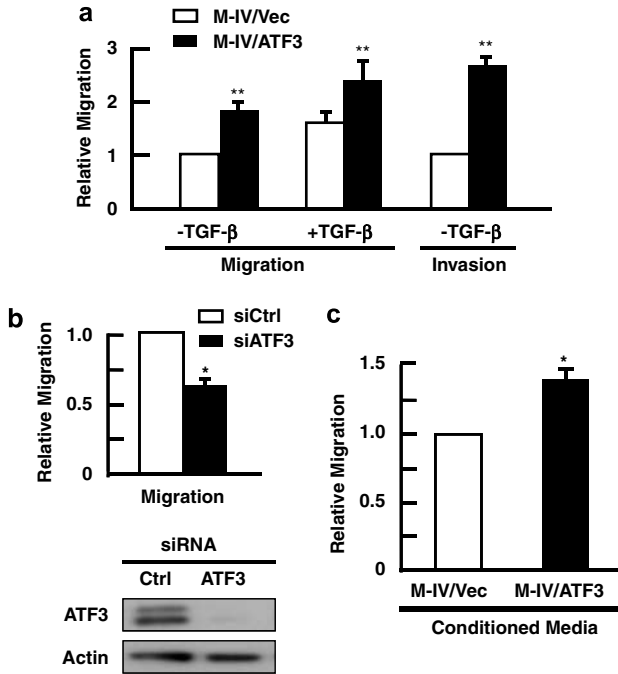

Figure 2 Activating transcription factor 3 (ATF3) enhances cell mobility and invasiveness in malignant breast carcinoma cells. (**a**) M-IV/Vec and M-IV/ATF3 cells were analysed by Boyden chamber assay for migration or invasion in the absence or presence of transforming growth factor (TGF)-β (2.5 ng ml^{-1}). A_{595} reading from M-IV/Vec cells in the absence of TGF-β was defined as 1. (**b**) M-IV cells were transfected with control (siCtrl) or ATF3 (siATF3) small interfering RNA (siRNA) for 72 h, and assayed for cell migration (upper) or protein (bottom). (**c**) M-III cells were assayed for migration in the presence of conditioned media (CM) from M-IV/Vec or M-IV/ATF3 cells. A_{595} reading from the cells incubated with the M-IV/vector CM was defined as 1. Mean ± s.d. from three independent experiments (*$P < 0.05$, **$P < 0.01$, versus M-IV/Vec).

ATF3 modulates the expression of genes known to regulate cell motility in aggressive breast cancer cells

We carried out array analysis using the Metastasis Microarray, which contains 113 genes known to modulate cell–cell or cell–extracellular matrix (ECM) interactions—key processes affected in metastasis. Several genes were modulated by ATF3 in repeated array analysis: fibronectin-1 (FN-1), secretory leukocyte peptidase inhibitor (SLPI), collagen IVa2 (COL4A2) and caveolin-1 (CAV1). RT-qPCR confirmed their differential expression (Figure 3b). In addition, we examined a few candidate genes known to modulate cell motility but are not included in the array, and found that urokinase-type plasminogen activator (uPA, also called PLAU), plasminogen activator inhibitor-1 (PAI-1), TWIST1 and Slug are upregulated in the M-IV/ATF3 cells (Figure 3b). ATF3 expression also upregulated the Snail steady-state mRNAs. However, the fold change was small (1.6-fold) and not statistically significant. Either the number of experimental repeats ($N = 3$) is not high enough or ATF3 by itself is not sufficient to upregulate Snail expression. Many of the ATF3 target genes identified here encode molecules involved in epithelial-to-mesenchymal transition, a process implicated in cancer cell motility and/or metastasis (Lee et al., 2006; Thiery and Sleeman, 2006). Thus, these target genes provide a mechanistic explanation for the ability of ATF3 to increase cell motility. Intriguingly, several of these genes—FN-1, SLPI, uPA, PAI-1 and COL4A2—encode secreted factors and are thought to play a role in remodeling the ECM. We thus carried out a conditioned medium (CM) experiment using the low-grade carcinoma MCF10CA1h (M-III) cells. Figure 2c shows that CM from M-IV/ATF3 cells promoted the M-III cells to migrate, indicating that ATF3 expression in the cancer epithelial cells can promote cell motility via a paracrine (or autocrine) effect.

Differential regulation of gene expression by ATF3 in M-I versus M-IV cells

To determine whether ATF3 differentially regulates gene expression in M-I versus M-IV cells, we carried out qRT-PCR analyses of both M-I and M-IV cell for all the genes
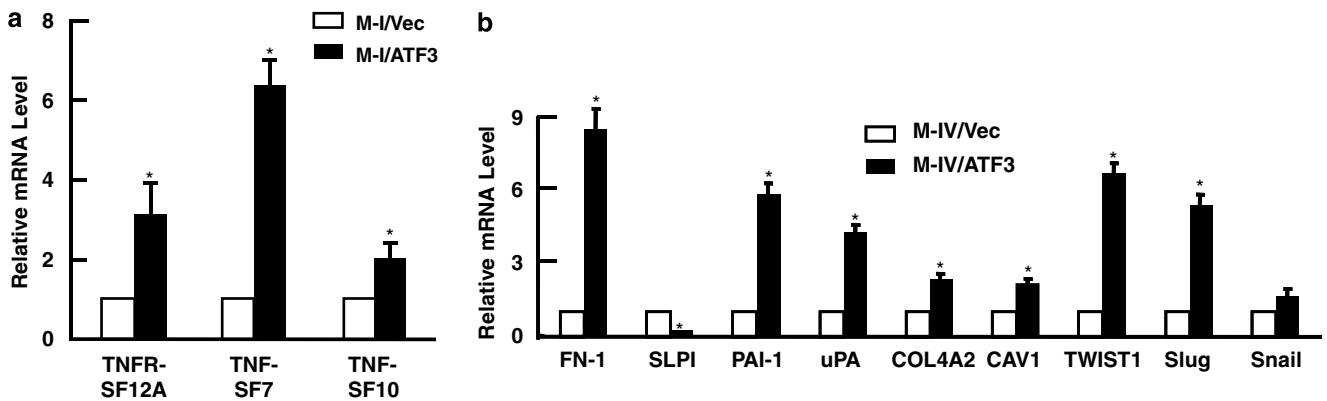

Figure 3 Ectopic expression of activating transcription factor 3 (ATF3) modulates the expression of various genes. (**a**) M-I/Vec and M-I/ATF3 cells were analysed by RT-qPCR for the indicated mRNAs. The levels in M-I/Vec cells were defined as 1. Mean ± s.d. from three independent experiments (*$P < 0.01$, versus M-I/Vec). (**b**) Same as (**b**) except the indicated M-IV cells were analysed (*$P < 0.05$, versus M-IV/Vec).

identified above. Table 1 summarizes the results and shows that these genes can be classified into three groups. (1) Group 1 are genes that are regulated similarly in M-I and M-IV cells: SLPI, PAI-1, uPA, TWIST1 and COL4A2. (2) Group 2 are genes downregulated in M-I cells but upregulated in M-IV cells: FN-1, Slug and CAV1. (3) Group 3 are genes upregulated in M-I cells but not changed in M-IV cells: Snail, TNFR-SF12A, TNF-SF7 and TNF-SF10. The potential significance of these findings is discussed (below).

Direct target genes of ATF3
Although the above results show an altered expression of several genes in the ATF3-expressing cells, they do not indicate whether these are direct target genes of ATF3. To address this issue, we first analysed their promoter sequences using the MotifScanner. As shown in Supplementary Figure S3, all of them contain consensus CRE/ATF or CRE/ATF-like sequences. However, for some promoters the sites are more than 2 kilobases (kb) upstream from the transcriptional start (+1) site. Although this *per se* does not rule out the possibility that they are direct target genes of ATF3, we focused our efforts on FN-1, TWIST1 and slug, which have the potential ATF3-binding sites within 200 base pair (bp) from the +1 site. We also analysed Snail, which has a potential binding site at around -1.7 kb. As shown by chromatin immunoprecipitation (ChIP) assay, ATF3 bound to these promoters in both M-I and M-IV cells (Figure 4a). The binding to the FN-1 promoter was obvious even in the M-IV/Vec cells, which do not have exogenous ATF3. Presumably, this was due to the binding by the endogenous ATF3, which was detectable by immunoblot (Figure 1a).

Table 1 Classification of potential ATF3 target genes based on their differential expression in M-I versus M-IV cells

Group	Gene name	M-I (fold)	M-IV (fold)
1	SLPI	0.03	0.2
	PAI-1	9.5	5.7
	uPA	3.2	4.2
	TWIST1	9.6	6.6
	COL4A2	2.6	2.3
2	FN-1	0.4	8.4
	Slug	0.1	5.3
	CAV1	0.6	2.1
3	Snail*	7.1	No changes
	TNFR-SF12	3.0[a]	No changes
	TNF-SF7	6.3[a]	No changes
	TNF-SF10	2.1[a]	No changes

Abbreviations: CAV1, caveolin-1; COL4A2, collagen IVA2; FN-1, fibronectin-1; PAI-1, plasminogen activator inhibitor-1; SLPI, secretory leukocyte peptidase inhibitor; TNF, tumor necrosis factor; uPA, urokinase-type plasminogen activator. The numbers indicate the fold change: relative mRNA levels in ATF3-expressing cells to that in the corresponding vector control cells ($P<0.05$ for all numbers, except those indicated by [a] $P<0.01$). Group 1: genes similarly regulated by ATF3 in M-I and M-IV cells; group 2: genes downregulated by ATF3 in M-I cells but upregulated in M-IV cells; group 3: genes upregulated by ATF3 in M-I cells but not regulated in M-IV cells. *The fold change for Snail in M-IV cells was 1.6, but not statistically significant.

Since ATF3 is a transcription factor, its binding to these promoters suggests, but does not prove, that ATF3 regulates their transcriptional activity. To address the issue of transcription, we examined RNA polymerase II (Pol II) occupancy on these genes by ChIP using two primer sets (Figure 4b). (1) Primer set 1 targets to a region downstream (around 2–5 kb) from the +1 site. Pol II occupancy in these regions would indicate active transcription of the corresponding genes. (2) Primer set 2 targets to the proximal promoter region around the +1 site. ChIP signals from this primer set indicate the loading of Pol II on the promoters. We quantified ChIP signal by qPCR and compared ATF3-expressing cells to vector control cells by defining the signals from the control cells as 1. As shown by primer set 1, ATF3 upregulated TWIST-1 and Snail gene transcription but downregulated FN-1 and Slug transcription in M-I cells (Figure 4c). This pattern paralleled the effects of ATF3 on their steady-state mRNA levels (Table 1, qRT-PCR data). Although the fold change was not exactly the same, the trend was the same. For M-IV cells, ATF3 upregulated FN-1, TWIST-1 and Slug gene transcription, but did not significantly affect snail gene (Figure 4c). Again, this pattern paralleled the modulation of their steady-state mRNA levels by ATF3 (Table 1). In contrast to primer set 1, primer set 2 showed similar Pol II loading on these promoters in ATF3-expressing and control cells (Figure 4d), a pattern different from the steady-state mRNA data (Table 1). This result was observed for both M-I and M-IV cells, and indicated that Pol II loading on the promoters does not necessarily correlate with transcription. This lack of correlation between Pol II loading and gene transcription has been reported previously (Komarnitsky *et al.*, 2000; Cheng and Sharp, 2003); in fact, the accumulation of paused Pol II in the proximal promoter was suggested to be a common feature of mammalian transcriptional regulation (Cheng and Sharp, 2003). As a control for Pol II ChIP, we examined Pol II binding to the α-actin and β-actin promoters. The ChIP signals were found on the β-actin but not α-actin promoter (data not shown), consistent with the fact that α-actin, a muscle-specific gene, is not expressed in the epithelial cells. Taken together, our results indicate that FN-1, TWIST-1 and Slug are direct target genes of ATF3: ATF3 binds to their promoter and modulates their transcription in both M-I and M-IV cells. The situation for Snail is more complex. ATF3 binds to its promoter and regulates its transcription in M-I cells. However, ectopic expression of ATF3 does not modulate Snail expression in M-IV cells despite the evidence of ATF3 binding to its promoter, suggesting that some other factors are required to regulate the expression of Snail in M-IV cells. We have not examined the rest of the potential ATF3 target promoters identified above (such as PAI-1 and uPA) for their transcriptional activity or binding by ATF3; thus it is not clear whether they are direct or indirect targets.

ATF3 gene is amplified and upregulated in human breast tumors
The above results from the cell-based system strongly suggest that ATF3 plays an oncogenic role in malignant breast carcinoma cells. To examine the potential relevance
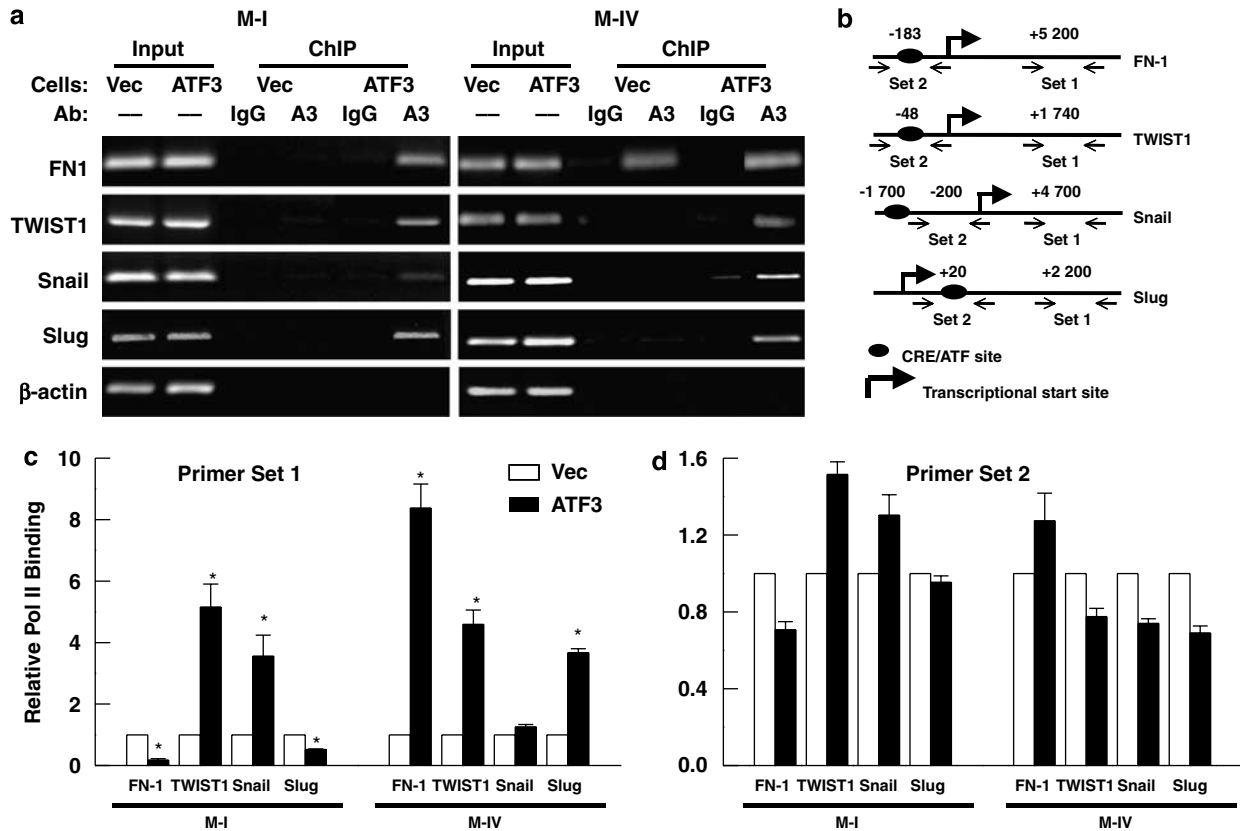
Figure 4 Fibronectin (FN)-1, TWIST1, Snail and Slug are direct target genes of activating transcription factor 3 (ATF3). (**a**) The indicated cells were analysed by chromatin immunoprecipitation (ChIP) for ATF3 binding to the indicated promoters using the ATF3 (A3) or control (immunoglobulin G, IgG) antibodies, with β-actin promoter as a negative control. Input: 5%. A representative of three experiments is shown. (**b**) Schematic of the indicated genes: the transcriptional start (+1), potential cyclic AMP response element (CRE)/ATF sites and the primer target sites are indicated. (**c**) The indicated cells were analysed by ChIP coupled with qPCR for Pol II occupancy using primer set 1. Signals in the vector cells (open bars) were defined as 1 to obtain the relative Pol II occupancy in the corresponding ATF3-expressing cells (close bars). Mean ± s.d. from three experiments (*$P<0.05$, versus open bars). (**d**) Same as (**c**), except primer set 2 was used.

of ATF3 to human breast cancer, we examined the expression of ATF3 by immunoblot in human breast tumors. Among the 48 samples examined, 23 (48%) expressed ATF3 at a level higher than their matching normal controls (Figure 5a). One caveat of comparing tumors to normal breast tissues is that adipocytes are the predominant cells in the normal breast tissues but epithelial cells are predominant in the tumors. Thus, it is possible that the elevated levels of ATF3 in the tumors are due to the differences in cell type rather than the differences in transformation status. This possibility was ruled out by the finding that the primary human mammary epithelial cells (HMECs) had no detectable ATF3 (Figure 5b). The HMECs we used were at early passages (<3) and proliferated well *in vitro*. Thus, the expression of ATF3 in the tumors reflects some features of the breast carcinoma, not simply their proliferation. In addition, the ATF3 protein levels increased upon the treatment of HMECs by TNF-α or TGF-β (Figure 5c), indicating that the programs to induce ATF3 expression are functional in the HMECs.

Intriguingly, ATF3 gene is localized on human chromosome at 1q32.3, within the 1q amplicon. 1q

was identified as the second most frequently amplified region of solid tumors, based on meta-analysis of 100 papers describing comparative genome hybridization findings in >2000 solid tumors of 27 cancer types (Rooney *et al.*, 1999). In breast tumor, 1q is the most frequently amplified region (in 53% tumors). We thus examined the copy number of ATF3 genes by qPCR in the tumors. As controls, we used M-I cells, which have two copies of 1q, and M-IV cells, which have three copies of 1q (Santner *et al.*, 2001). qPCR was repeated at least twice and a representative result is shown (Figure 6). Among the 48 samples, 38 (~80%) had ATF3 gene amplification (>2 copy). Since the tumor samples contained both the stroma and cancer epithelia, we isolated the cancer epithelia by laser capture microdissection (LCM) and analysed the ATF3 gene copy. As shown in Figure 6a inset, all four samples examined by this method showed ATF3 gene amplification. Although our LCM analysis was not comprehensive, our results—in the context of well-documented DNA amplification in cancer cells—support the notion that the ATF3 gene is amplified in the breast cancer epithelia.
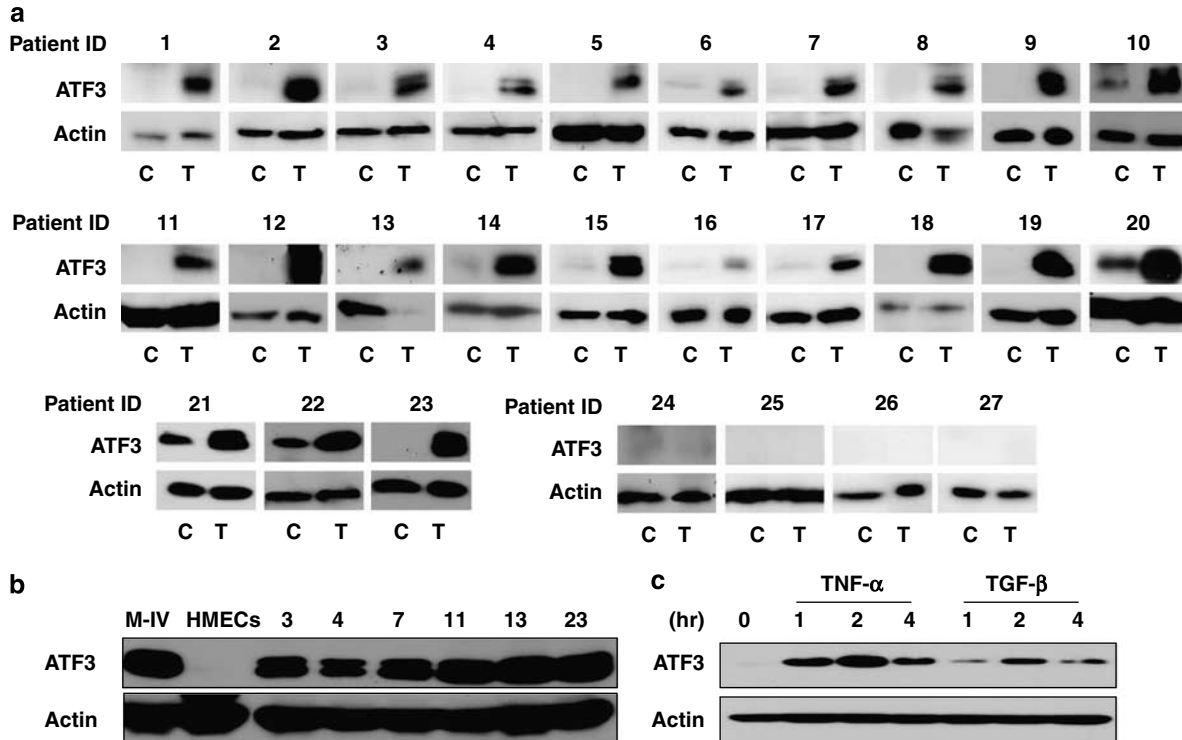
Figure 5 Activating transcription factor 3 (ATF3) expression is upregulated in human breast tumors. (**a**) Human breast tumors (T) and the matching adjacent breast controls (C) were analysed by immunoblot. Samples 1–23: tumors with elevated ATF3 protein levels compared to their corresponding controls (group A); samples 24–27: examples of the 25 tumors without elevated ATF3 expression (group B). (**b**) Proliferating primary human mammary epithelial cells (HMECs) were analysed by immunoblot, with M-IV cells and a few breast tumors for comparison. (**c**) HMECs were treated with transforming growth factor (TGF)-β (2.5 ng ml^{-1}) or tumor necrosis factor (TNF)-α (60 ng ml^{-1}) for the indicated times and analysed by immunoblot.

Since ATF3 is pro-apoptotic in the M-I-untransformed breast epithelium (above), it is possible that ATF3 may be mutated in the breast carcinoma. To address this issue, we sequenced the open reading frame of all 23 tumors that had elevated expression of ATF3. No mutation was found.

Discussion

In addition to the M-I and M-IV cells, we analysed the ability of ATF3 to affect the cell viability of two cell lines with intermediate tumorigenic features: M-II (MCF10AT1k) and M-III (MCF10CA1h). The ATF3-expressing M-II and M-III cells showed a similar trend as the M-IV counterpart: higher cell number than their corresponding vector control cells under serum withdrawal (Supplementary Figure S4). Although ATF3 has been demonstrated to have opposite functions in the literature (Introduction), our result is the first to demonstrate this dichotomy using cell lines with similar genetic backgrounds, and suggests that the degree of malignancy of the cells affects ATF3 function. However, since this phenomenon could also be due to some unidentified variables, it is important to examine its reproducibility using other pairs of cancer cells with varying degrees of malignancy.

As shown in Table 1, ATF3 differentially regulates several genes in the M-I and M-IV cells—the group 1 and group 2 genes. The significance of this finding is twofold. First, this differential gene regulation provides at least partial mechanistic explanations for the dichotomous role of ATF3. Second, since the M-IV cells are derived from the M-I cells, they share mostly the same genetic backgrounds, providing a tractable system to study the cellular contexts that allow ATF3 to differentially regulate gene expression. One possibility for the differential regulation is that the concentrations or properties of the ATF3-interacting partners are different in these cells. Another possibility is that ATF3 recruits different cofactors to its target promoters, such as FN-1 and Slug, in M-I versus M-IV cells, resulting in their differential regulation.

Among the 23 tumors with elevated ATF3 levels (referred to as group A in Figure 6), 21 (>90%) had ⩾3 copies of the gene. Among the 25 tumors without elevated ATF3 expression (group B), 17 of them (68%) had ⩾3 copies of the gene. Although the copy number of ⩾3 was higher than the normal diploid, the distribution in group B was toward the low copy numbers (no. 3–5)—in contrast to the tumors in group A, which had distribution toward the higher copy numbers (Figure 6b). These results suggest that gene amplification is potentially a contributing factor for the elevated ATF3 protein levels in group A. However, since
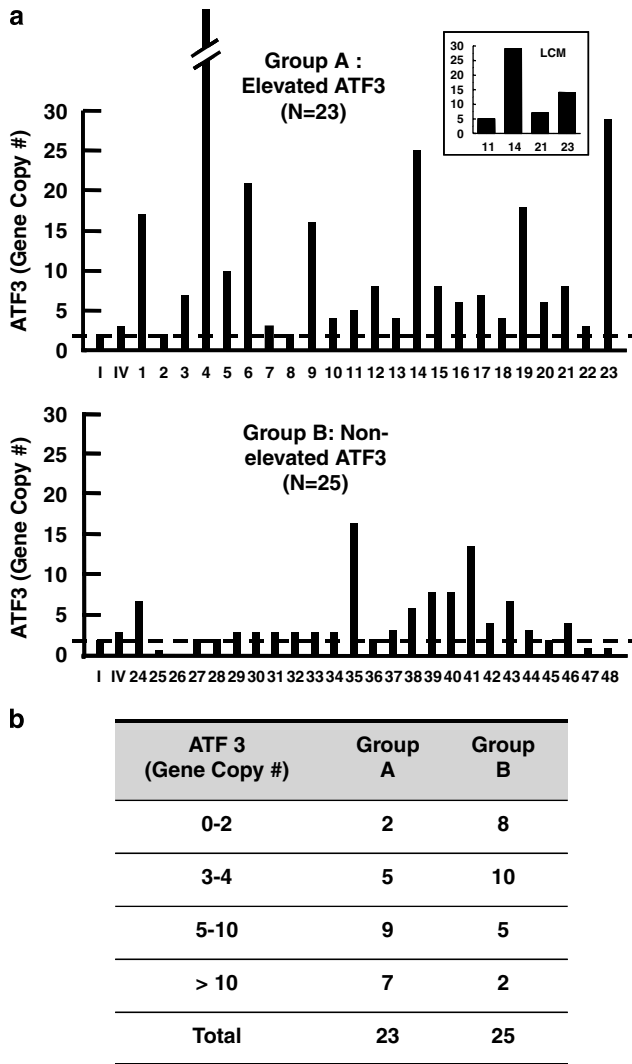
ATF 3 (Gene Copy #)	Group A	Group B
0-2	2	8
3-4	5	10
5-10	9	5
> 10	7	2
Total	23	25

Figure 6 Activating transcription factor 3 (ATF3) gene is amplified in human breast tumors. (**a**) Genomic DNAs from human breast tumors were analysed by qPCR to determine the ATF3 gene copy number. The signals from M-I cells (I) are defined as 2 (dashed lines) and that from M-IV cells (IV) as 3. Inset shows the ATF3 gene copy number of the cancer epithelia from the indicated tumors after isolation by LCM. (**b**) A summary of the ATF3 gene copy numbers in group A and B tumors.

some tumors in group B, which had no increase in ATF3 protein level, showed increased gene copy number (>2), gene amplification is not the only controlling factor. Clearly, the levels of protein can be regulated by various mechanisms, and the mechanisms for the elevated ATF3 expression remain to be determined.

Amplification of oncogenes occurs frequently in the late-stage solid tumors (some reviews, Albertson, 2003, 2006). Although ATF3 gene is localized in the 1q amplicon, due to the crude resolution of the methods to identify amplicons it was not clear whether the ATF3 gene is indeed amplified. Our report is the first to demonstrate the amplification of ATF3 gene in breast tumors. We note that the ERBB2 (HER2/NEU) gene, a well-known oncogene for breast cancer, is amplified in

25–30% of breast tumors (Pauletti et al., 1996; Pollack et al., 1999), a number lower than that for ATF3 (~ 80%). Although our data from the human tumors are descriptive in nature, they—in combination with the results from the cultured cells described in this report—strongly suggest that ATF3 is an oncogene, rather than merely a bystander gene in the amplicon. (1) ATF3 is functionally important: it promotes cell motility and protects the malignant breast cancer cells from stress-induced cell cycle arrest. (2) The ATF3 target genes (direct or indirect) in the malignant breast cancer cells identified here have all been implicated to play a role in cell–cell and/or cell–ECM interactions. Recently, ATF3 was shown to be downregulated by Drg-1, a tumor metastasis suppressor gene, in prostate cancer cells (Bandyopadhyay et al., 2006), again suggesting an oncogenic nature of ATF3 in cancer cells.

We note that some of the group B breast tumors (those without elevated ATF3 expression) had ATF3 gene loss rather than gain: no. 26 (zero copy), 25, 47 and 48 (one copy). The actin gene control was normal in these tumors. Although we have not ruled out the possibility that the ATF3 gene was rearranged so that the primers we used did not hybridize, this result is consistent with a dichotomous role of ATF3 in cancer cells. It is conceivable that some breast tumors were derived from cells that had deleted ATF3 (a tumor suppressor), whereas others from cells that had 'evolved' to co-opt ATF3 (to become an oncogene). This speculation fits with the observations that cancer cells are heterogenous in their genetic and epigenetic alterations (some reviews; Loeb, 2001; Fidler, 2003; Albertson, 2006). Although frequently amplified, 1q was documented to be lost in some tumors (Rooney et al., 1999), such as renal carcinoma, rhabdomyosarcoma and gastrointestinal tumors. In all these tumors, both 1q gain and 1q loss have been found, again supporting the notion that different paths can be taken by the cancer cells to succeed.

One potential clinical relevance of our findings is that ATF3 can be induced by compounds with antitumorigenic activities, including adriamycin (Yu et al., 1996; Mallory et al., 2005), nonsteroidal antiinflammatory drugs (Bottone et al., 2003), the phosphatidylinositol 3-kinase inhibitor LY294002 (Yamaguchi et al., 2006), progestin (Syed et al., 2005) and dietary polyphenols such as curcumin from the tumeric plant (Yan et al., 2005) and catecins in green tea (Baek et al., 2004). Some of these compounds have been used in clinical settings. The oncogenic activity of ATF3 in advanced cancer cells indicates that these antitumorigenic compounds may have undesired effects. Elucidating the molecular context that determines whether ATF3 is a tumor suppressor or an oncogene will likely to provide insights for rational designs of anticancer treatments in the future.

Materials and methods

Cell lines, primary HMECs and human specimens
MCF10A (M-I), MCF10AT1k (M-II), MCF10CA1 h (M-III) and MCF10CA1a (M-IV) cells were purchased from the

Barbara Ann Karmanos Cancer Institute (Detroit, MI, USA) and cultured according to the instructions from the suppliers. Primary HMECs were from Dr Martha Stampfer (Lawrence Berkeley National Laboratory, CA, USA). Human samples were obtained from the Ohio State University (OSU) Comprehensive Cancer Center Tissue Procurement shared resource with the approval from the Institutional Review Board.

Retroviruses and siRNAs
Retroviruses were generated in pan-tropic packaging cell line GP2-293 (Clontech, CA, USA). Viral transduction was followed by antibiotic selection to generate stable cells. The ATF3 siRNAs and the control siRNA (Dharmacon, CA, USA) were transfected according to the instructions from the manufacturers.

Cell viability assay, flow cytometry, immunoblot and immunofluorescence
Cell viability was measured by crystal violet assay as previously described (Lu *et al.*, 2006b). Flow cytometry was carried out after fixing and staining the cells with propidium iodide. Immunoblot and immunofluorescence were carried out using anti-ATF3 (Santa Cruz, CA, USA), anti-actin (Sigma, MO, USA), antiactivated caspase 3 and anti-caspase 8 (Cell Signaling, MA, USA) antibodies.

RNA isolation, RT-qPCR, SuperArray analysis and ChIP assay
See Supplementary Materials and methods.

Conditioned medium, Boyden chamber migration and invasion assay
Cells (2.5×10^5) were seeded on 8-µm cell culture insert without coating (to assay migration) or coated with matrigel (to assay invasion) (BD Biosciences, NJ, USA). TGF-β1 ($2.5\,\mathrm{ng\,ml^{-1}}$), vehicle control or CM was included in the lower chamber. To make the CM, 5×10^6 cells were cultured in 6 ml of media for 48 h and the media centrifuged to remove the debris. At 24 h after incubation in the Boyden chamber, cells on the upper side of the insert were removed by cotton swab and cells on the under side were stained with crystal violet and the A_{595} measured.

Gene copy number, LCM and mutation analysis
Genomic DNA isolated from the frozen tumors by the DNeasy Tissue Kit (Qiagen) was analysed by qPCR to determine the gene copy number. LCM was carried out by the OSU core facility. For sequencing, exons were amplified by PCR and the products sequenced at both strands. The primers for gene amplification and sequencing are listed in the Supplementary Table S1.

Statistics
All quantitative data are expressed as mean ± s.d. Comparisons were made by the Student's *t*-test and $P<0.05$ is considered significant.

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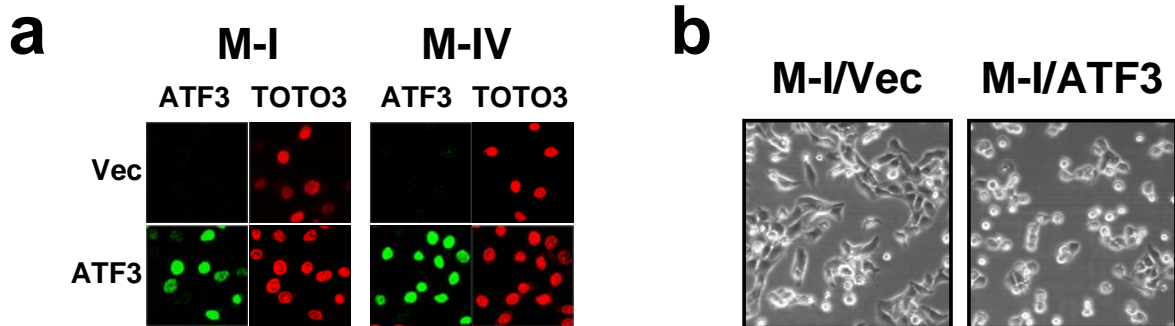Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc).

Figure S1. Characterization of stable M-I and M-II cells expressing ATF3 or the control vector. **(a)** M-I/ATF3, M-I/Vec, M-IV/ATF3 and M-IV/Vec cells were analyzed by immunofluorescence using the ATF3 antibodies (green). TOTO-3 (red) was used to indicate the nuclei. **(b)** M-I/Vec and M-I/ATF3 cells were seeded in serum-free media and photographed by phase contrast microscopy at 6 hr after seeding.
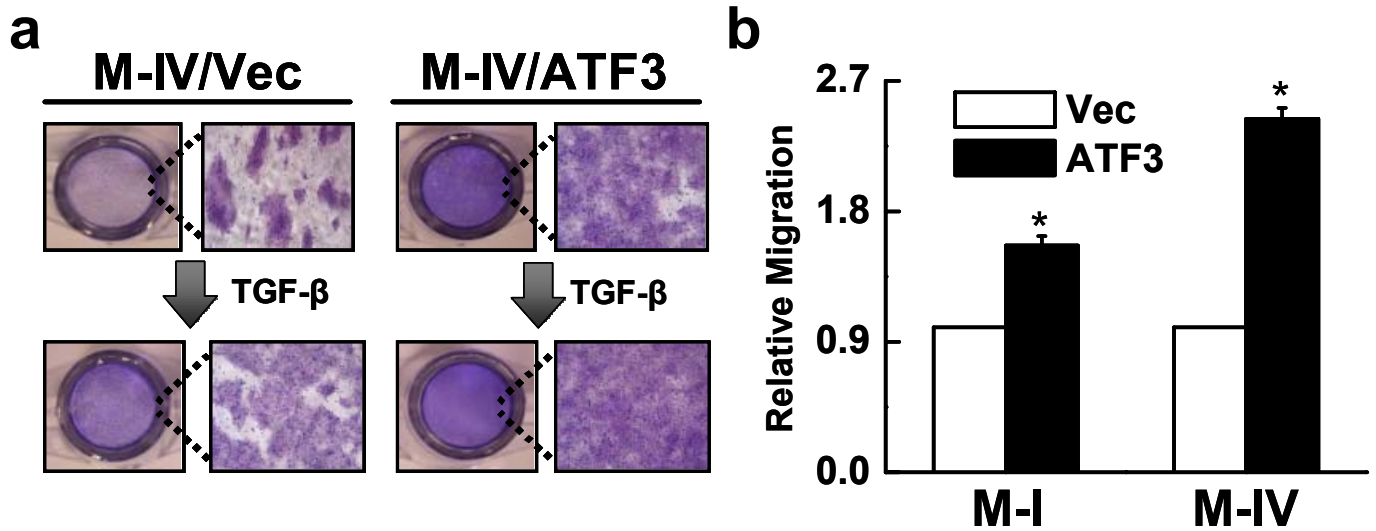
Figure S2. The effects of ATF3 expression on cell migration. **(a)** M-IV/Vec and M-IV/ATF3 cells were analyzed by Boyden chamber assay and the migrated cells were photographed under microscope after staining with crystal violet. **(b)** M-I/Vec, M-I/ATF3, M-IV/Vec and M-IV/ATF3 cells were analyzed by Boyden chamber assay for migration. A_{595} readings from the corresponding vector control cells were arbitrarily defined as 1. Shown are the mean ± S.D. from three independent experiments. * $p < 0.01$, versus Vec cells.

	CRE/ATF Site		
Gene	**Position**	**Sequence**	
FN-1	-183	TGACGTCA	
TWIST1	-48	TCACGTCA	
Snail	-1 700	CAATGTCA	
Slug	+20	TGACTTCA	
SLPI	-1 975	TGACCTCA	
	-2 930	TGACCTCA	
	-3 087	TGACCTCA	
PAI-1	-5 512	TGAGGTCA	
	-3 253	TGGCGTCA	
uPA	-4 708	TGACCTCA	
	-4 399	TGACGACA	
	-3 697	TGAGGTCA	
	-2 017	TGAAGTCA	
COL4A2	-2 111	TGAGGTCA	
CAV1	-903	TGACTGCA	
	-1 958	TGAGGCCA	
Consensus		**TGACGTCA**	

● CRE/ATF site
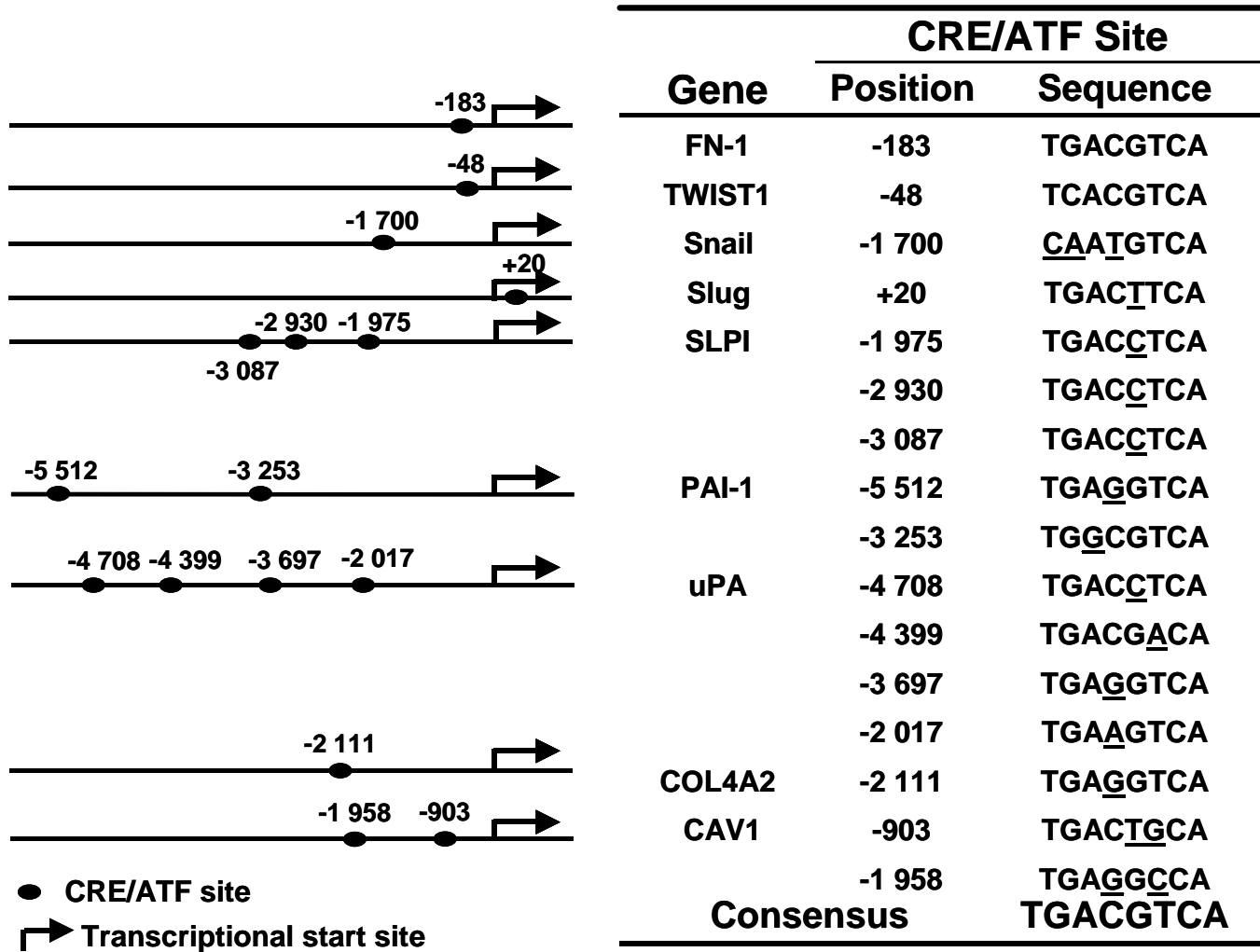
➤ Transcriptional start site

Figure S3. Potential ATF3 target promoters. Schematic representations of the promoters are shown on the left, with the CRE/ATF sites indicated. The corresponding CRE/ATF sequences are indicated on the right, with the deviations from the consensus sequence underlined.
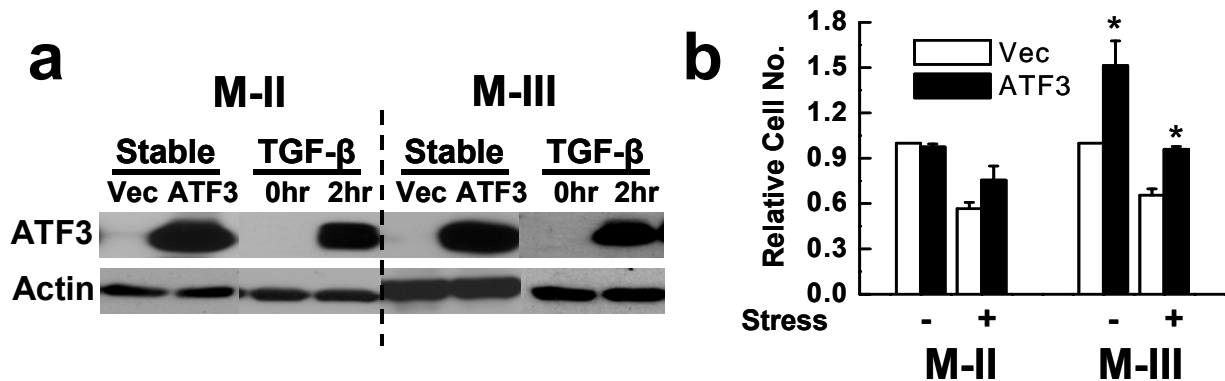
Figure S4. The effects of ATF3 expression on M-II and M-III cells. **(a)** Stable cells derived from M-II and M-III cells to express ATF3 or the control vector (Vec) were analyzed by immunoblot using the indicated antibodies. The endogenous ATF3 expression levels induced by TGF-β (2.5 ng/ml) in the parental M-II and M-III cells were analyzed in parallel. **(b)** ATF3-expressing and the corresponding control cells were seeded into normal growth (- stress) or serum-free media (+ stress) media and grown for 72 hr. Viable cells were analyzed by crystal violet assay. The values from vector control cells in normal growth media were arbitrarily defined as 1. Shown are the mean ± S.D. from three independent experiments (* p < 0.05, versus Vec).

Table S1. A list of the primers used in this study for the indicated assays.

Assays	Gene	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
RT-qPCR	ATF3	CTGCAGAAAGAGTCGGAG	TGAGCCCGGACAATACAC
	β-actin	AAGATCAAGATCATTGCTCCTC	GGACTCATCGTACTCCTG
	TNFR-SF12A	GTTCACCACCCCCATAGA	AGGCAGAGACTGGCTCTA
	TNF-SF7	TGCTTTGGTCCCATTGGT	GTGATTCAGCTGCAGCTC
	TNF-SF10	CCAGAGGAAGAAGCAACAC	GGAATGAATGCCCACTCC
	FN-1	GCAACTCTGTCAACGAAG	ACTTCCAAAGCCTAAGCA
	SLPI	CTGGAAAGTCCTTCAAAGCTG	CATTTGATGCCACAAGTGTC
	PAI-1	CATCATCAATGACTGGGTG	AGTAGAGGGCATTCACCA
	uPA	TACGGCTCTGAAGTCACC	CCAGTCAAAGTCATGCGG
	COL4A2	GAGGACTTGGTTTCTACGG	ATCTGGGTGGAAGGTGAC
	CAV1	GCAACATCTACAAGCCCA	CTTCAAAGTCAATCTTGACCAC
	TWIST1	GAGACCTAGATGTCATTGTTTCC	GCCCTGTTTCTTTGAATTTGGA
	Slug	ACACATACAGTGATTATTTCCC	ATGAGGAGTATCCGGAAAGAG
	Snail	ACTCTAATCCAGAGTTTACCTTCC	AGGACAGAGTCCCAGATGAG
ChIP for ATF3 binding	FN-1	TCCCTTTCCTCCCAGCCGCTTCC	AGCCGACCGCGCGCCGATTG
	TWIST1	GAGGGGGACTGGAAAGCGGAAA	TGGGCGAGAGCTGCAGACTTG
	Slug	AGGAGCTGAAATCTGAACCTCT	GTTGAAATGCTTCTTGACCAGG
	Snail	AAGGTCTGGGATGTTGGAGG	GTCCTCTCCTCAGCCAACTC
ChIP for RNA Pol II occupancy	FN-1 (Set 1)	CTGAGAAGTGTTTTGATCATGC	TTCTAGAAGTGCAAGTGATGC
	FN-1 (Set 2)	TCCCTTTCCTCCCAGCCGCTTCC	AGCCGACCGCGCGCCGATTG
	TWIST1 (Set 1)	GGCACCATCCTCACACCTCT	CGACCTCTTGAGAATGCATGC
	TWIST1 (Set 2)	GAGGGGGACTGGAAAGCGGAAA	TGGGCGAGAGCTGCAGACTTG
	Slug (Set 1)	TTCATGTATGATTGGCAGCAGTA	GCAAATGCTCTGTTGCAGTGA
	Slug (Set 2)	AGGAGCTGAAATCTGAACCTCT	GTTGAAATGCTTCTTGACCAGG
	Snail (Set 1)	CTTTGGACCCTGGCTGTGTGT	CTTCTTGACATCTGAGTGGGTC
	Snail (Set 2)	CGTCAGAAGCGCTCAGACCA	ACTCCTCCGAGGCGGGGTT
	β-actin	CGGCCAACGCCAAAACTCTC	AACTTTCGGAACGGCGCACG
	α-actin	GCAAACCCGCTAGGGAGACA	TGGGTGTTGGGCACTAGAGC
Gene copy # analysis	ATF3	ATGCCCCAGAGCCCCTGAAA	TACCTGTGATAGAACCCACC
	Actin	AAGATCAAGATCATTGCTCCTC	GGACTCATCGTACTCCTG
Genomic DNA sequencing	ATF3 Exon B	ATGCCCCAGAGCCCCTGAAA	TACCTGTGATAGAACCCACC
	ATF3 Exons C to E	GGTAGGTGGCATTTCTTACTGC	TCTCCAATGGCTTCAGGGTT

Supplemental Materials and Methods

RNA isolation, RT-qPCR and SuperArray analysis

Total RNAs were isolated using Trizol (Invitrogen, CA, USA) and analyzed by qRT-PCR using

the QuantiTect SYBR Green PCR Kit (Qiagen, CA, USA) in triplicate with ß-actin as an internal

control. In general, the threshold cycle numbers for actin in different cells are very close, and the

relative mRNA level for the gene of interest is calculated as $2^{[Ct(actin)-Ct(gene)]}$, where Ct is

threshold cycle number. The relative mRNA level of a given gene in the vector control cells is

then arbitrarily defined as 1. PCR primer sequences are listed in the Supplemental Table S1.

For pathway-specific array analysis, total RNAs were isolated using RNeasy kit (Qiagen) and

analyzed using the Apoptosis (OHS-012) or Metastasis (OHS-028) Microarray (SuperArray,

MD, USA).

Chromatin immunoprecipitation (ChIP) assay

ChIP assay was carried out as described previously (Lu et al., 2006). Anti-ATF3, anti-Pol II

antibodies and control IgG (Santa Cruz) were used for the assay. The anti-ATF3 antibody (Santa

Cruz), in general, does not give rise to non-specific bands in immunoblot against mammalian

whole cell extracts; in a limited survey of bZip proteins, it does not react with ATF1, 2, 4,

CREB, gadd153/Chop10, or Jun (unpublished results). PCR primer sequences are listed in the

Supplemental Table S1.