

14-3-3 ζ Cooperates with ErbB2 to Promote Ductal Carcinoma In Situ Progression to Invasive Breast Cancer by Inducing Epithelial-Mesenchymal Transition

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SUMMARY

ErbB2, a metastasis-promoting oncoprotein, is overexpressed in ~25% of invasive/metastatic breast cancers, but in 50%–60% of noninvasive ductal carcinomas in situ (DCIS). It has been puzzling how a subset of ErbB2-overexpressing DCIS develops into invasive breast cancer (IBC). We found that co-overexpression of 14-3-3 ζ in ErbB2-overexpressing DCIS conferred a higher risk of progression to IBC. ErbB2 and 14-3-3 ζ overexpression, respectively, increased cell migration and decreased cell adhesion, two prerequisites of tumor cell invasion. 14-3-3 ζ overexpression reduced cell adhesion by activating the TGF- β /Smads pathway that led to ZFH1B/SIP-1 upregulation, E-cadherin loss, and epithelial-mesenchymal transition. Importantly, patients whose breast tumors overexpressed both ErbB2 and 14-3-3 ζ had higher rates of metastatic recurrence and death than those whose tumors overexpressed only one.

INTRODUCTION

ErbB2 overexpression is found in approximately 25% of invasive breast cancers (IBC) and is strongly associated with poor patient survival (Slamon et al., 1989). Overexpression of ErbB2 has been demonstrated to promote breast cancer invasion and metastasis (Yu and Hung, 2000). However, ErbB2 is overexpressed in 50%–60% of ductal carcinomas in situ (DCIS) in general and 60%–70% of high-grade DCIS (Nofech-Mozes et al., 2005). DCIS, a precursor of IBC, consists of clonal proliferation of malignant cells within the lumen of mammary ducts, with no evidence of invasion through the basement membrane into the surrounding

stroma (Burstin et al., 2004). The apparent paradox that ErbB2, the well-known metastasis-promoting oncoprotein, is more frequently overexpressed in noninvasive DCIS than in IBC has been puzzling.

This stimulated debate about whether ErbB2 overexpression alone is sufficient to promote progression from noninvasive DCIS to IBC. The limited number of studies that have used patient follow-up data on invasive recurrence of primary DCIS have yielded ambiguous results. Some studies indicated that ErbB2-overexpressing DCIS had an increased risk of invasive recurrence (Provenzano et al., 2003), while others suggested the opposite (Perin et al., 1996; Ringberg et al., 2001). Interestingly,

SIGNIFICANCE

More than 90% of breast cancer-related deaths are caused by metastasis not primary tumor. For effective reduction of cancer mortality, it is extremely important to predict the risk of, and to intervene in, the critical transition from noninvasive ductal carcinomas in situ (DCIS) to life-threatening invasive breast cancer (IBC). Here, we discovered that 14-3-3 ζ overexpression is a “second hit” or “risk factor” facilitating a subset of ErbB2-overexpressing DCIS transition into IBC and identified molecular mechanisms/pathways through which ErbB2 and 14-3-3 ζ co-overexpression promotes invasion. This study identified biomarkers that allow selection of high-risk DCIS patients for more aggressive treatments at early stages of cancer development, while saving low-risk patients from ablative clinical procedures. Moreover, it provided promising targets for future intervention strategies to prevent DCIS progression to IBC.

Table 1. Expression of ErbB2 and 14-3-3 ζ in 25 DCIS Cases with up to 7 Years of Follow-Up Data and the Incidence of Metastatic Recurrence

	Number of Cases (Number of Metastatic Recurrence)		Total
	14-3-3 ζ (<3+)	14-3-3 ζ (3+)	
ErbB2 (<3+) 5 (0)		6 (0)	11(0)
ErbB2 (3+) 6 (0)		8 (4)	14 (4)
Total	11(0)	14 (4)	25 (4)

ErbB2 and 14-3-3 ζ expression levels were examined by IHC staining in 25 DCIS patients' samples. Fisher's exact test showed that ErbB2 and 14-3-3 ζ co-overexpression (3+/3+) in this cohort significantly ($p < 0.05$) correlated with distant site metastasis.

studies using 3D culture of mammary epithelial cells (MECs) showed that ErbB2 activation in preformed, growth-arrested mammary acini led to disruption of the well-organized acinar structure that shared several properties with DCIS in vivo, including uncontrolled cell proliferation, luminal filling, and no invasion (Muthuswamy et al., 2001). Moreover, transgenic mice expressing *neu* (rat homolog of human ErbB2) under its endogenous promoter developed DCIS-like mammary tumors after a long latency with rare metastasis (Andrechek et al., 2003).

These indicate that ErbB2 activation/overexpression may be involved in DCIS formation and that ErbB2 overexpression alone is not sufficient to drive invasion/metastasis. It was suggested that greater ErbB2 activity or additional genetic/epigenetic events ("second hits") are needed for MECs to gain invasive capability and for a subset of ErbB2-overexpressing DCIS to transition into IBC (Muthuswamy et al., 2001). However, it remained unclear as to what the second hits are.

The transition from a normal cell to a malignant cell is a multi-step process, and at least six hallmark alterations in cell physiology collectively drive the malignant progression (Hanahan and Weinberg, 2000). 14-3-3 is a family of evolutionally conserved proteins that can bind to many target proteins involved in each of these cancer hallmark alterations (Tzivion et al., 2006; Wilker and Yaffe, 2004). It is conceivable that deregulation of 14-3-3 may contribute to cancer development. Generally, 14-3-3 proteins are divided into two subgroups: 14-3-3 σ is a tumor suppressor, whereas the other 14-3-3 isoforms may have oncogenic functions. Increased 14-3-3 ζ expression was observed in several tumor types and in the early stages of breast diseases such as DCIS (Danes et al., 2008). This raised the interesting possibility that 14-3-3 ζ overexpression might contribute to DCIS progression to IBC.

The epithelial-mesenchymal transition (EMT) is a process during which epithelial cells convert to a mesenchymal cell phenotype after losing cell polarity, disassembling cell-cell adhesion machinery, and subsequently acquiring cell motility (Guarino, 2007). EMT promotes tumor invasion and metastasis by facilitating escape of tumor cells from the original rigid constraints of the surrounding tissue architecture (Guarino, 2007). The EMT-mediated increase in invasion/metastasis is largely contributed by loss of E-cadherin function because E-cadherin is essential for the maintenance of adherent junctions between neighboring cells and thus confers physical integrity on epithelial cells (Beavon, 2000; Guarino, 2007). E-cadherin loss has been shown to increase cell invasion in multiple in vitro models and

has been correlated with increased metastasis in several epithelial tumor types (Strathdee, 2002). Therefore, E-cadherin is considered a suppressor of tumor invasion.

Given that ErbB2 overexpression alone in DCIS is not sufficient for progression to IBC, we explored whether 14-3-3 ζ overexpression in DCIS may serve as a second hit that cooperates with ErbB2 to drive a subset of ErbB2-overexpressing DCIS progression into IBC.

RESULTS

ErbB2 and 14-3-3 ζ Co-overexpression in DCIS Is Associated with Increased Invasion Potential

To investigate whether 14-3-3 ζ overexpression cooperates with ErbB2 to drive a subset of ErbB2-overexpressing DCIS progression to IBC, we initially examined DCIS samples from 25 patients for whom up to 7 years of follow-up data was available. We analyzed the expression of ErbB2 and 14-3-3 ζ by immunohistochemistry (IHC) staining. Fourteen of the 25 cases (56%) showed a high level of ErbB2 expression (Table 1), which is consistent with previous reports of ErbB2 overexpression in 50%–60% of DCIS cases (Nofech-Mozes et al., 2005). Eight of the 25 cases (32%) exhibited high levels of both ErbB2 and 14-3-3 ζ (Table 1 and see Figure S1 available online). Strikingly, four of these eight patients had disease recurrence with distant site metastasis, whereas none of the 17 DCIS patients whose tumors did not overexpress both proteins developed distant metastasis (Table 1). Thus, ErbB2 and 14-3-3 ζ co-overexpression in this small cohort significantly ($p < 0.05$) correlated with distant site metastasis, suggesting that 14-3-3 ζ cooperates with ErbB2 to promote the progression from DCIS to IBC and metastasis.

MCF10A, a nontransformed human MEC line, is an excellent in vitro model in 3D culture for studying breast cancer progression as it forms well-organized acinar structures that mimic the normal mammary end bud in vivo (Debnath et al., 2003). Here, we used the MCF10A 3D culture model system to study whether and how 14-3-3 ζ cooperates with ErbB2 to gain invasiveness. We established multiple stable MCF10A sublines overexpressing ErbB2 (10A.ErbB2), HA-tagged 14-3-3 ζ (10A.14-3-3 ζ), or both ErbB2 and HA-tagged 14-3-3 ζ (10A.ErbB2. ζ), with 10A.Vec as the control (Figure 1A). We found that only the 10A.ErbB2. ζ cells formed soft agar colonies, whereas 10A.ErbB2, 10A.14-3-3 ζ , and 10A.Vec MECs did not (Figure 1B). The data indicated that ErbB2 or 14-3-3 ζ overexpression alone was not sufficient to induce a full transformation in MCF10A MECs, but ErbB2 and 14-3-3 ζ co-overexpression could cooperatively induce full transformation, an important step for cancer invasion/metastasis.

Strikingly, the four sublines showed distinct acinar structures when grown in 3D matrigel (Figures 1C and 1D and Figure S2). 10A.ErbB2 cells formed highly proliferative, but noninvasive, DCIS-like structures characterized by impaired proliferation suppression and luminal cell apoptosis resistance, similar to a previous report (Muthuswamy et al., 2001). 10A.14-3-3 ζ cells developed into abnormal acinar structures with no lumen formation, but no growth advantage, as we recently reported (Danes et al., 2008). 10A.ErbB2. ζ cells, however, demonstrated severe disruption of the acinar architecture, characterized by increased acinar size and no lumen formation (Figure 1C and 1D). The most distinct feature of the 10A.ErbB2. ζ acini was the gain of invasive

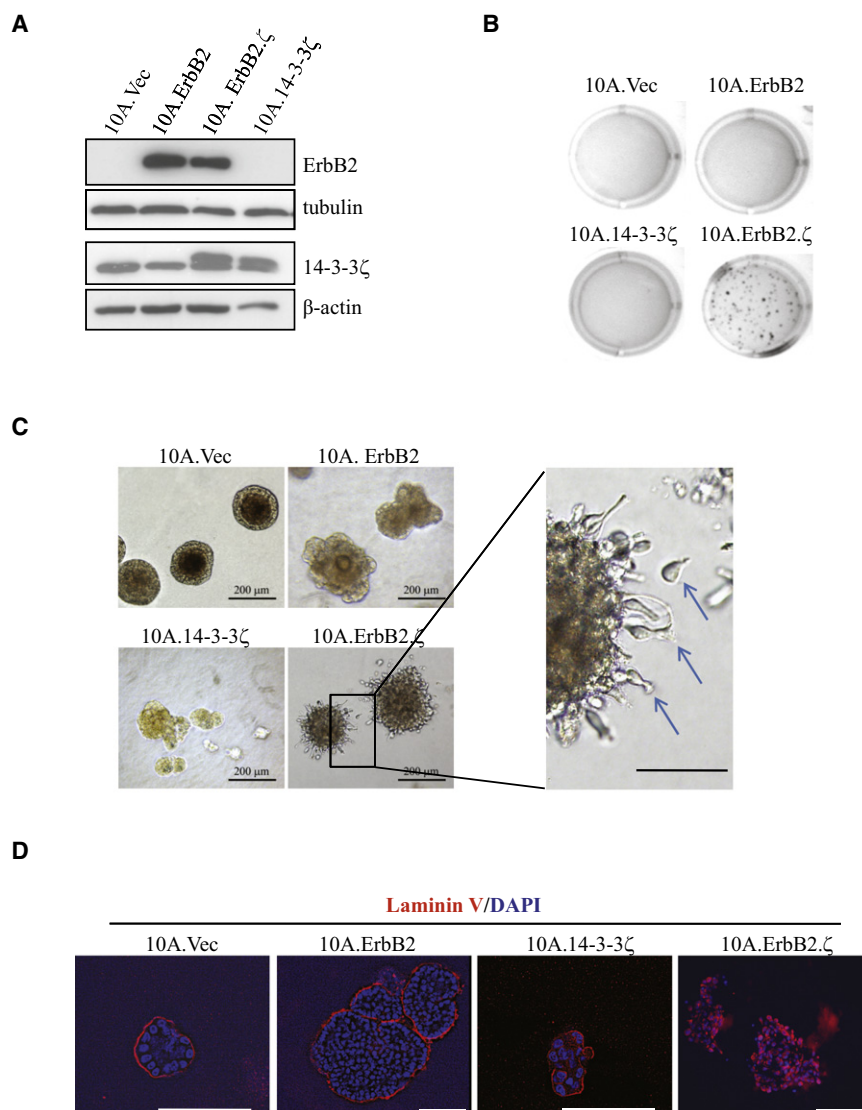


Figure 1. ErbB2 and 14-3-3 ζ Co-overexpression Is Associated with Increased Invasion Potential in an In Vitro DCIS Model

(A) Establishment of MCF10A stable cell lines overexpressing ErbB2 alone, 14-3-3 ζ alone, or both ErbB2 and 14-3-3 ζ . Multiple stable clones were established for each subline and most experiments were repeated with different clones to rule out clonal effects. Immunoblot analysis of one representative of each subline for the indicated proteins is shown.

(B) Co-overexpression of ErbB2 and 14-3-3 ζ led to anchorage-independent growth of MCF10A cells in soft agar assay.

(C) Distinct acinar structures of MCF10A sublines in 3D culture. The top panel shows phase-contrast images of acinar structures (19 days); the scale bar represents 200 μm. The inset shows an amplified view of individual 10A.ErbB2.ζ cell invading into surrounding matrigel; the scale bar represents 100 μm.

(D) Loss of basement membrane integrity in 10A.ErbB2.ζ acini. MCF10A sublines were cultured in 3D matrigel for 28 days and then stained for laminin V (red) and DAPI (blue). Representative images are shown. The scale bar represents 100 μm.

capacity, as many cells escaped from 10A.ErbB2.ζ acini and invaded the surrounding matrix (Figures 1C and 1D). An important feature of the noninvasive DCIS is the intact basement membrane that surrounds it, whereas invasive carcinomas are defined by loss of basement membrane integrity (Rizki and Bissell, 2004). Indeed, we observed that individual cells in 10A.ErbB2.ζ acini were patched by diffuse basement membrane protein laminin V, whereas laminin V formed a continuous basement membrane layer surrounding acini from 10A.ErbB2, 10A.14-3-3ζ, and 10A.Vec MECs (Figure 1D). Together, co-overexpression of ErbB2 and 14-3-3ζ in MCF10A MECs conferred invasiveness, whereas overexpression of ErbB2 or 14-3-3ζ alone did not.

Invasion Is the Collective Effect of ErbB2-Mediated Increase of Cell Migration and 14-3-3ζ-Mediated Decrease of Cell-Cell Adhesion via EMT

Tumor cell invasion is a multistep process, of which the key events include increased migration, increased protease secretion,

and altered adhesion to allow dissemination from primary tumor sites (Liotta and Stetler-Stevenson, 1991). We detected no significant difference in matrix metalloproteinase levels among the four MCF10A sublines (data not shown). However, migration and wound healing assays showed that both 10A.ErbB2 and 10A.ErbB2.ζ cells had increased cell motility, whereas 10A.14-3-3ζ cells had a low motility similar to that of 10A.Vec (Figures 2A and 2B). Thus, the increased cell motility was largely contributed by ErbB2 overexpression,

not by 14-3-3ζ overexpression. Multiple ErbB2 downstream signaling pathways can be involved in ErbB2-mediated cell motility, including PI3K, PAK1, Rac1, and Src activation (Feldner and Brandt, 2002). We found that Src phosphorylation is specifically increased in the two ErbB2-overexpressing MCF10A sublines compared to the two ErbB2 low-expressing MCF10A sublines (Figures S3A and S3B). Moreover, treatment with a Src kinase inhibitor (Saracatinib or AZD0530) significantly inhibited the motility of 10A.ErbB2 and 10A.ErbB2.ζ cells, whereas Rac1 and PI3K inhibitors had no significant effect (Figure S3C).

Reduced cell-cell adhesion is another prerequisite for individual cell invasion and EMT has been implicated in tumor invasion partly by reducing cell-cell adhesion (Guarino, 2007). In contrast to 10A.Vec and 10A.ErbB2 cells that had a cobblestone-like epithelial morphology in 2D culture, 10A.ErbB2.ζ and 10A.14-3-3ζ cells displayed a spindle-like shape and exhibited a scattered distribution, indicating loss of cell-cell contact and EMT (Figure 2C). The morphological changes during EMT are

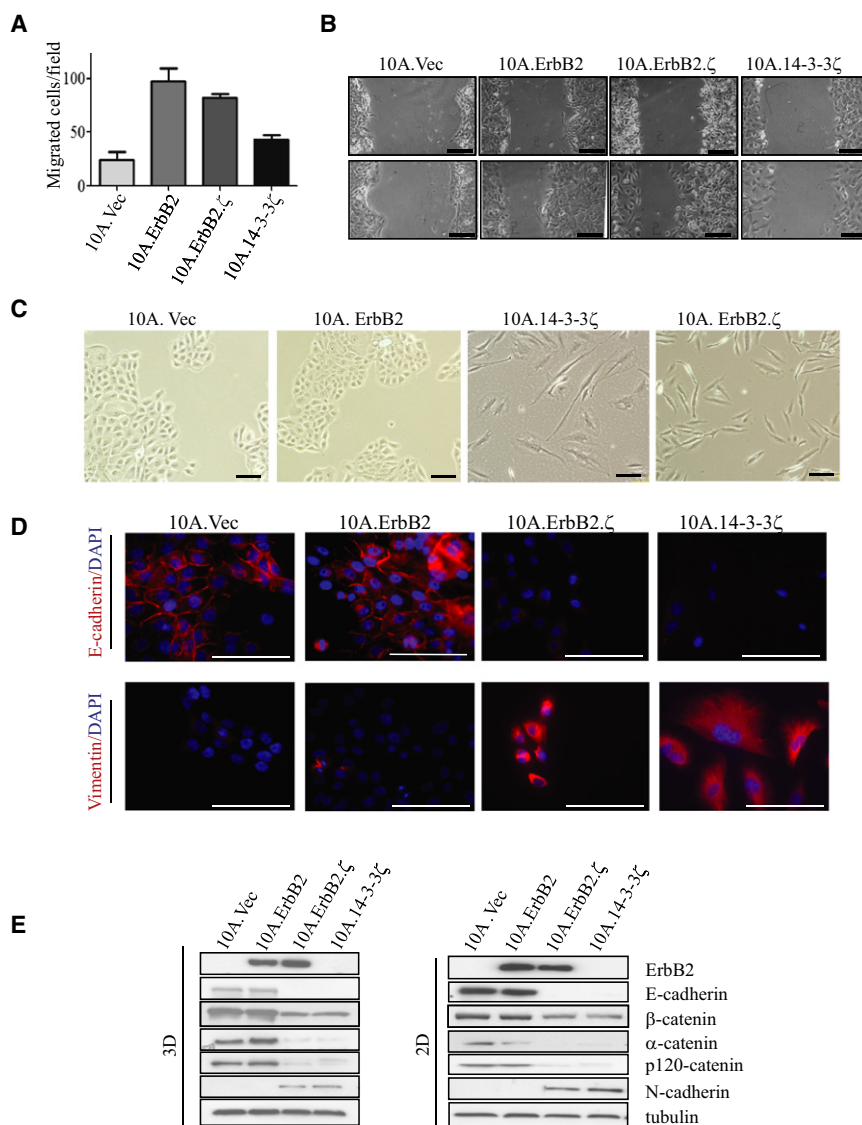


Figure 2. ErbB2 Overexpression Led to Increased Cell Migration and 14-3-3 ζ Overexpression Led to Decreased Cell-Cell Adhesion as a Result of EMT

(A) Transwell migration assay of the indicated MCF10A sublines. Cells that migrated to the bottom of the chamber were counted in five fields under 20 \times magnification. Experiments were done three times with triplicates and error bar represents SEM.

(B) Wound healing assay of the indicated MCF10A sublines. Wound closures were photographed at 0 and 6 hr after wounding. The scale bar represents 200 μ m.

(C) The four MCF10A sublines exhibited different morphologies in 2D culture. The scale bar represents 100 μ m.

(D) IFS of EMT markers in MCF10A sublines. E-cadherin (top, red), vimentin (bottom, red), and DAPI (blue) are shown. The scale bar represents 100 μ m.

(E) Immunoblot analysis of the indicated EMT markers in MCF10A sublines. Expression of epithelial cell markers (E-cadherin, β -catenin, α -catenin, and p120 catenin) and mesenchymal cell marker (N-cadherin) were examined by immunoblot analysis in both 2D and 3D culture cell lysates.

E-Cadherin Loss, a Key Event of EMT, Is Mediated by ZFH1B in 10A.ErbB2.ζ Cells

We next investigated how 14-3-3 ζ overexpression led to E-cadherin loss, a key event of EMT resulting in decreased cell-cell adhesion. RT-PCR analysis showed that E-cadherin mRNA level was dramatically lower in 10A.ErbB2.ζ and 10A.14-3-3 ζ cells than in 10A.Vec and 10A.ErbB2 cells (Figure 3A). E-cadherin mRNA loss could result from hypermethylation of its promoter (Strathdee, 2002), but we detected no significant

driven by a number of molecular and cellular alterations, including loss or decrease of epithelial cell markers (e.g., E-cadherin, β -catenin, α -catenin, and p120-catenin) and de novo expression of mesenchymal markers (e.g., N-cadherin, vimentin, and fibronectin). Certainly, we found that 10A.Vec and 10A.ErbB2 cells expressed high levels of E-cadherin, β -catenin, α -catenin, and p120-catenin, but minimal levels of N-cadherin and vimentin. 10A.ErbB2.ζ and 10A.14-3-3 ζ cells, however, showed E-cadherin loss, dramatically reduced β -catenin, α -catenin, and p120-catenin, and de novo expression of N-cadherin and vimentin (Figures 2D and 2E). Similarly, 14-3-3 ζ overexpression in HMEC-hTERT cells, immortalized by the telomerase reverse transcriptase catalytic subunit, also led to EMT (Figure S4). Thus, 14-3-3 ζ overexpression contributed to the loss of cell-cell adhesion and the EMT phenotype. Together, a collective effect of ErbB2-mediated increase of cell migration and 14-3-3 ζ -mediated decrease of cell-cell adhesion conferred 10A.ErbB2.ζ acini invasiveness.

differences in E-cadherin promoter methylation status among the four MCF10A sublines (data not shown). Another major mechanism of E-cadherin mRNA loss is direct transcriptional repression by repressors, including snail, slug, twist, E12, E47, ZFH1B (also named SIP1), and deltaEF1 (Peinado et al., 2004). These transcriptional repressors have been found to induce EMT in vitro and their overexpression in a variety of human tumors is associated with increased tumor invasion/metastasis and poor prognosis. We examined the expression levels of snail, slug, twist, E12, E47, and deltaEF1 and found they were not significantly different among the four MCF10A sublines (Figure 3B). Interestingly, expression of ZFH1B was dramatically higher in 10A.ErbB2.ζ and 10A.14-3-3 ζ cells than in 10A.Vec and 10A.ErbB2 cells at both mRNA and protein levels (Figure 3C).

ZFH1B is a two-handed zinc-finger protein that binds to the E boxes in the E-cadherin proximal promoter to repress E-cadherin transcription (Comijn et al., 2001). To examine whether the

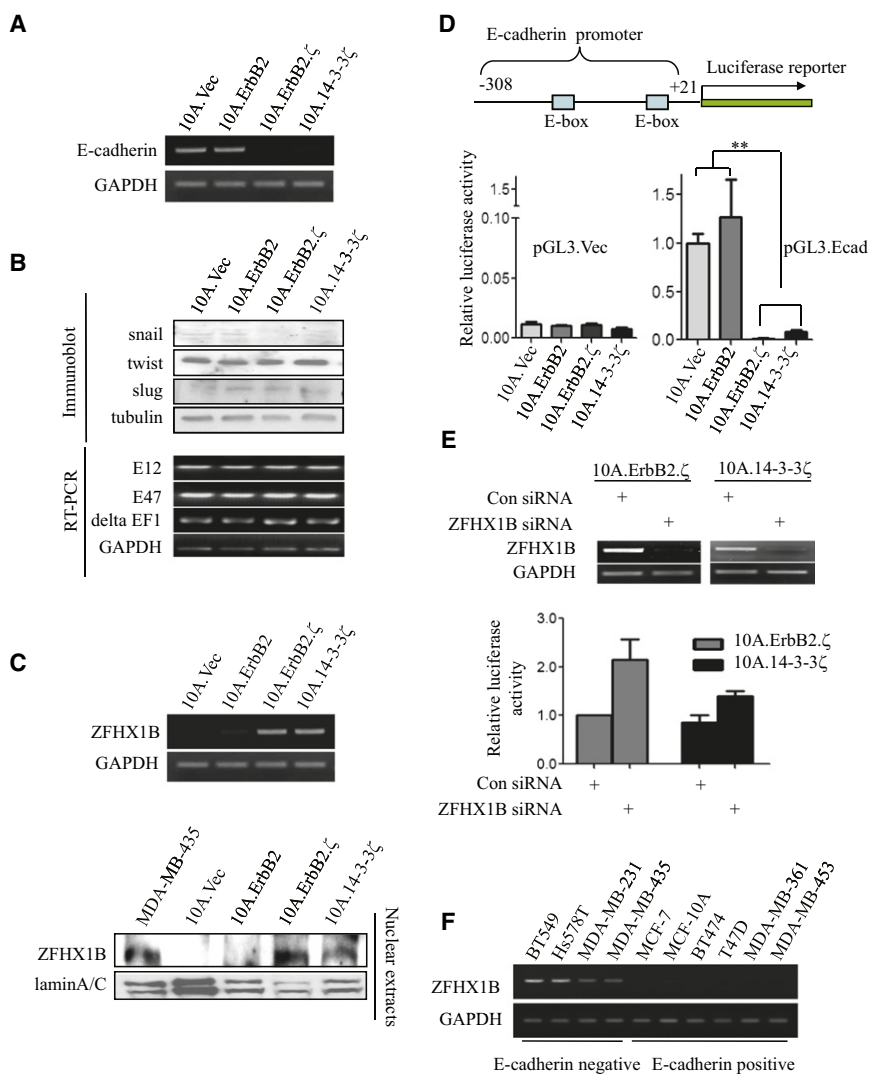


Figure 3. EMT Phenotype in 10A.ErbB2.ζ and 10A.14-3-3ζ Cells Was Mediated by ZFH1B Upregulation

(A) E-cadherin mRNA was dramatically reduced in 10A.ErbB2.ζ and 10A.14-3-3ζ cells.

(B) Expression of EMT regulators in the four MCF10A sublines. The top panel shows immunoblot analysis of EMT regulators, including snail, twist, and slug. The bottom panel shows RT-PCR analysis of EMT regulators, including E12, E47, and delta EF1.

(C) RT-PCR and immunoblot analysis of ZFH1B levels in the four MCF10A sublines. MDA-MB-435 cells served as the positive control for ZFH1B protein expression.

(D) 14-3-3ζ overexpression led to transcriptional repression of E-cadherin promoter activity. The top panel shows a schematic representation of the luciferase reporter driven by E-cadherin proximal promoter containing two ZFH1B binding E-box motif: pGL3.Ecad. The bottom panel shows relative luciferase activity of pGL3.Ecad in the four MCF10A sublines. $p < 0.05$. Error bars indicate SEM.

(E) Downregulation of ZFH1B in 14-3-3ζ-overexpressing MCF10A sublines by siRNA partially relieved suppression of E-cadherin promoter-driven luciferase activity. As shown in the top panel, control siRNA and ZFH1B siRNA were transfected into 10A.ErbB2.ζ cells and 10A.14-3-3ζ cells. After 48 hr, downregulation of ZFH1B was examined by RT-PCR with GAPDH as internal control. As shown in the bottom panel, cells were pretransfected with ZFH1B siRNA or control siRNA. After 48 hr, pGL3.Ecad was cotransfected with pRL.TK plasmid as transfection efficiency control. Relative luciferase activity was determined 36 hr later. Error bars indicate SEM.

(F) A reverse correlation between ZFH1B and E-cadherin expression in breast cancer cell lines. RNA was extracted from four E-cadherin-negative breast cancer cell lines (BT549, Hs578T, MDA-MB-231, and MDA-MB-435), five E-cadherin-positive breast cancer cell lines (MCF-7, BT474, T47D, MDA-MB-361, and MDA-MB-453), and a non-transformed breast epithelial cell line (MCF10A), and then reverse transcribed into cDNA; this was followed by PCR with ZFH1B-specific primers. GAPDH served as loading control.

E-cadherin loss in 10A.ErbB2.ζ and 10A.14-3-3ζ cells was due to transcriptional repression by the upregulated ZFH1B, we cloned a fragment of E-cadherin promoter (containing two consensus ZFH1B binding motifs: CANNTG) upstream of a luciferase reporter plasmid (pGL3.Ecad) and compared its activity among the MCF10A sublines (Figure 3D). Indeed, pGL3.Ecad luciferase activities were significantly repressed ($p < 0.05$) in 10A.ErbB2.ζ and 10A.14-3-3ζ cells versus 10A.Vec and 10A.ErbB2 cells (Figure 3D). Moreover, the repression of E-cadherin promoter-driven luciferase activity was partially relieved in 10A.ErbB2.ζ and 10A.14-3-3ζ cells when ZFH1B expression was inhibited by small interfering RNA (siRNA; Figure 3E). Therefore, ZFH1B upregulation contributed to the transcriptional repression of E-cadherin in 10A.ErbB2.ζ and 10A.14-3-3ζ cells. In addition, examination of ZFH1B expres-

sion in six E-cadherin-positive and four E-cadherin-negative breast cancer cell lines showed a general correlation between ZFH1B expression and E-cadherin loss (Figure 3F).

ZFH1B Is Upregulated by 14-3-3ζ through Upregulation of TGF-β Receptor I and Activation of TGF-β/Smads Pathway

Next, we investigated the mechanism of ZFH1B upregulation in 10A.ErbB2.ζ and 10A.14-3-3ζ cells. Because TGF-β/Smads pathway activation was shown to induce EMT and was also known to be involved in ZFH1B upregulation (Zavadil and Bottinger, 2005), we examined whether ZFH1B upregulation by 14-3-3ζ might be due to increased TGF-β/Smads signaling. Expression of the TβRI protein, but not RNA, was dramatically increased in 10A.ErbB2.ζ and 10A.14-3-3ζ cells, whereas TβRII

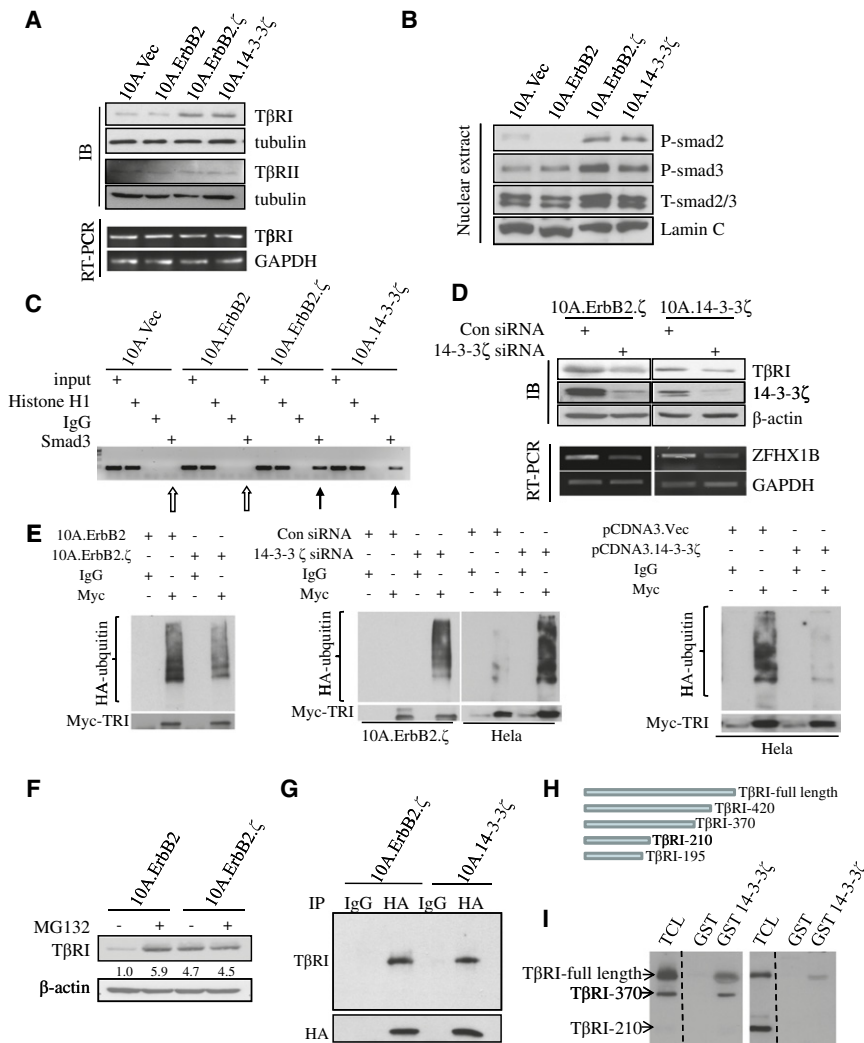


Figure 4. 14-3-3 ζ Overexpression Led to TGF- β /Smads Pathway Activation in MCF10A Cells via Increasing T β RI Expression

(A) Immunoblot and RT-PCR analysis of T β RI and T β RII expression in MCF10A sublines.

(B) Immunoblot analysis of nuclear p-smads (p-smad2/p-smad3) and T-smad2/3, with lamin C as nuclear fraction loading control.

(C) ChIP assay with the indicated antibodies showed smad3 binding with ZFH1B promoter in 10A.ErbB2. ζ and 10A.14-3-3 ζ cells (arrows), not in 10A.Vec and 10A.ErbB2 cells (empty arrows).

(D) Knockdown of 14-3-3 ζ by siRNA reduced T β RI and ZFH1B expression in 10A.ErbB2. ζ and 10A.14-3-3 ζ cells. T β RI level and ZFH1B level were analyzed by immunoblot and RT-PCR, respectively, 48 hr after siRNA transfection.

(E) 14-3-3 ζ inhibited T β RI ubiquitination. 10A.ErbB2. ζ and 10A.ErbB2 cells were cotransfected with vectors expressing myc-T β RI and HA-ubiquitin (left), 10A.ErbB2. ζ and HeLa cells were cotransfected with myc-T β RI, HA-ubiquitin, and control/14-3-3 ζ siRNA (middle), and HeLa cells were cotransfected with myc-T β RI, HA-ubiquitin, and pcDNA3.Vec/pcDNA3.14-3-3 ζ (right). After 48 hr, cell lysates were collected and subjected to immunoprecipitation and immunoblot with myc and HA antibodies.

(F) 10A.ErbB2 and 10A.ErbB2. ζ cells were treated with DMSO or 20 μ g/ml MG132 for 6 hr; T β RI levels were analyzed by immunoblot.

(G) 14-3-3 ζ associated with T β RI. 10A.ErbB2. ζ and 10A.14-3-3 ζ cell lysates were immunoprecipitated by anti-HA antibody, followed by immunoblot analysis of T β RI.

(H) Schematic representation of different T β RI mutants.

(I) 14-3-3 ζ binds to T β RI at its kinase domain between amino acids 210 and 370. 10A.ErbB2. ζ cells were infected with lentivirus expressing different T β RI mutants as in (H). Then the cell lysates were subjected to pull-down assay with GST or GST-14-3-3 ζ , followed by immunoblot with T β RI antibody.

protein levels were similar among the four MCF10A sublines (Figure 4A). Consistently, we also observed increased T β RI level in 14-3-3 ζ -overexpressing HMEC-hTERT-HA-14-3-3 ζ cells accompanied by upregulation of ZFH1B (Figure S4C). The increased T β RI protein levels led to increased TGF- β /Smads activation, as indicated by the increased nuclear phospho-smad2/sm3 and total smad2/sm3 levels in 10A.ErbB2. ζ and 10A.14-3-3 ζ cells (Figure 4B). Moreover, chromatin immunoprecipitation (ChIP) assay detected binding of nuclear smad3 to the ZFH1B promoter in 10A.ErbB2. ζ and 10A.14-3-3 ζ cells, but not in 10A.Vec or 10A.ErbB2 cells (Figure 4C). These data indicate that 14-3-3 ζ -mediated TGF- β /Smads activation contributed to ZFH1B transcriptional upregulation. Indeed, blocking 14-3-3 ζ by siRNA reduced T β RI protein expression, which also led to reduced ZFH1B expression (Figure 4D and Figure S5A).

T β RI protein level is mainly regulated by its internalization, followed either by trafficking back to the cell membrane after

engulfed in early endosome or by ubiquitination-mediated degradation when engulfed in lipid raft-caveolae-1 vesicles (Di Guglielmo et al., 2003; Lin et al., 2004). To investigate the mechanisms of 14-3-3 ζ -mediated T β RI protein upregulation, we first investigated whether it is contributed by reduced T β RI ubiquitination. Indeed, ubiquitination of Myc-tagged T β RI in 10A.ErbB2. ζ cells was reduced compared to 10A.ErbB2 cells when HA-tagged ubiquitin was coexpressed (Figure 4E, left). 14-3-3 ζ knockdown by siRNA in 10A.ErbB2. ζ cells and in HeLa cells (endogenous 14-3-3 ζ) led to a consistent increase in T β RI ubiquitination, whereas T β RI ubiquitination was inhibited when 14-3-3 ζ was overexpressed (Figure 4E, middle and right). Furthermore, treatment with MG132, a proteasome inhibitor, led to greater accumulation of T β RI in 10A.ErbB2 cells than in 10A.ErbB2. ζ cells (Figure 4F), indicating a more rapid T β RI ubiquitination and proteasome-mediated degradation in 14-3-3 ζ low-expressing 10A.ErbB2 cells. Next, we examined whether 14-3-3 ζ inhibited T β RI ubiquitination and degradation by binding to T β RI. Indeed,

14-3-3 ζ and T β RI coexisted in the same complex (Figure 4G) and the binding region is between amino acid 210 and 370 in the kinase domain of T β RI (Figures 4H and 4I). Immunofluorescence staining (IFS) also detected diffuse staining of both 14-3-3 ζ and T β RI proteins both in the cytosol and on the cell membrane (Figure S5B). The data are consistent with previous reports that T β RI is constantly recycled between membrane and cellular vesicles, resulting in \sim 20% localization to the cell membrane and \sim 80% remaining in the cytosol (Di Guglielmo et al., 2003). Most importantly, the binding of 14-3-3 ζ protects T β RI from degradation because the T β RI-210 that cannot bind to 14-3-3 ζ has a much shorter half-life compared to the T β RI-370 that binds to 14-3-3 ζ (Figure S5C). Furthermore, when 14-3-3 ζ expression is knocked down by siRNA, the half-life of T β RI-370 is dramatically reduced, while the half-life of T β RI-210 is not affected (Figure S5D and data not shown). These results indicated that overexpressed 14-3-3 ζ in 10A.ErbB2. ζ and 10A.14-3-3 ζ cells bound to T β RI and inhibited the proteasome-mediated T β RI degradation, leading to increased T β RI protein level and TGF- β /Smads pathway activation.

To further confirm the role of TGF- β /Smads pathway activation in the induction of the EMT phenotypes, we treated the 10A.Vec and 10A.ErbB2 cells with TGF- β 1 to activate the TGF- β /Smads pathway. The treatment induced smad3 phosphorylation, ZFH1B upregulation, and morphological features of EMT, with corresponding downregulation of E-cadherin and upregulation of vimentin and fibronectin (Figure S6). Therefore, activation of the TGF- β /Smads pathway was sufficient to induce EMT in MCF10A cells.

Inhibition of TGF- β Receptor Activation Partially Reversed EMT and Inhibited the Invasiveness of 10A.ErbB2. ζ Acini

To determine whether activation of the TGF- β /Smads pathway is required for the EMT and invasive phenotype of the 10A.ErbB2. ζ cells, we inhibited TGF- β /Smads pathway activation by treating 10A.ErbB2. ζ cells with a TGF- β receptor I/II kinase inhibitor, LY2109761 (Melisi et al., 2008). LY2109761 treatment reduced smad2/3 phosphorylation and total smad3, but had no significant effect on the phosphorylation of Akt (p-Akt) or p42-MAPK (p-P42) (Figure 5A). Interestingly, LY2109761-treated 10A.ErbB2. ζ cells adhered to neighboring cells to form cell islands, indicating improved cell-cell adhesion (Figure 5B, left). More importantly, the invasive phenotype of 10A.ErbB2. ζ acini in 3D matrigel culture was dramatically inhibited by LY2109761 treatment compared to control treatment (Figure 5B, middle). In contrast, LY2109761 treatment had no significant impact on acini development and maintenance in the other MCF10A sublines (Figure S7). Consistent with the partial reversal of EMT morphology of the cells in 2D culture and reduced invasiveness in 3D culture, there was increased epithelial protein expression, such as E-cadherin and α -catenin, after LY2109761 treatment. E-cadherin was specifically located in the membrane regions forming cell-cell contacts, a prerequisite for adherent junction formation (Figure 5B, right). Prolonged treatment also led to decreased mesenchymal protein expression (Figure 5C). Collectively, these data indicate that 14-3-3 ζ -mediated TGF- β /Smads pathway activation plays a critical role in the EMT phenotype and gain of invasiveness in 10A.ErbB2. ζ cells.

Reintroduction of E-Cadherin in 10A.ErbB2. ζ Cells Inhibits Invasion

Inhibition of TGF- β /Smads pathway by LY2109761 partially recovered E-cadherin expression that inhibited the invasion of 10A.ErbB2. ζ acini, indicating that E-cadherin loss was a key event in the gain of invasiveness during EMT. To further determine the critical role of E-cadherin loss in invasion, we restored E-cadherin expression in the 10A.ErbB2. ζ cells (named 10A.ErbB2. ζ .Ecad) to levels similar to those in the 10A.Vec cells (Figure 5D). The restored E-cadherin expression led to the recovery of other epithelial proteins, such as α -catenin, β -catenin, and p120-catenin, and reduced mesenchymal proteins, such as N-cadherin and vimentin (Figure 5E). Moreover, the cells with recovered E-cadherin expression showed a dramatic increase in cell adhesion (Figures S8A and S8B). Importantly, 10A.ErbB2. ζ .Ecad cells formed acinar structures with fewer individual cells invading into surrounding matrigel, in contrast to the highly invasive acinar structures of 10A.ErbB2. ζ .Vec cells (Figure 5F and Figure S8C). Thus, re-expression of E-cadherin in 10A.ErbB2. ζ cells efficiently increased cell-cell adhesion and inhibited, at least partially, the invasive phenotype in 3D culture. Therefore, E-cadherin loss played a critical role in inducing invasiveness of 10A.ErbB2. ζ cells.

14-3-3 ζ Overexpression Is Associated with High Levels of T β RI Expression in Both Human DCIS and IBC

We have identified T β RI upregulation as a major mechanism of 14-3-3 ζ overexpression-induced invasiveness in MCF10A.ErbB2. ζ cells. To evaluate the biological relevance of these findings, we investigated whether there is a correlation between T β RI and 14-3-3 ζ expression in patients' samples. Because we did not have enough of the DCIS samples shown in Table 1 remaining for these staining, we stained 138 DCIS samples from patients with recently diagnosed disease and 100 IBC with clinical follow-up. We found that 14-3-3 ζ overexpression significantly ($p < 0.05$) correlated with increased T β RI levels in both populations (Figure 6A). Moreover, IHC staining for 14-3-3 ζ , T β RI, ErbB2, E-cadherin, vimentin, and N-cadherin on the DCIS samples showed that co-overexpression of 14-3-3 ζ and T β RI significantly ($p < 0.05$) correlated with (at least two) EMT marker alterations (reduced expression of epithelial marker E-cadherin and expression of mesenchymal markers vimentin and/or N-cadherin; Table S1 and Figure S9). Importantly, high 14-3-3 ζ and T β RI expression levels plus two EMT marker alterations in DCIS were significantly ($p < 0.05$) associated with high-grade DCIS phenotype, which correlates with a higher risk of invasive recurrence (Table S2). Representative images of multiple markers' expressions in a pure low-grade DCIS and in a DCIS sample with microinvasion are shown in Figure 6B. Together, 14-3-3 ζ overexpression in DCIS lesions correlated with T β RI upregulation and induced EMT that could contribute to a higher risk of invasive recurrence.

Co-overexpression of ErbB2 and 14-3-3 ζ Is Associated with Higher Metastatic Potential in Mice and Increased Metastatic Disease Recurrence and Death in Breast Cancer Patients

The above findings demonstrated that co-overexpression of ErbB2 and 14-3-3 ζ increased the invasiveness of MECs in 3D

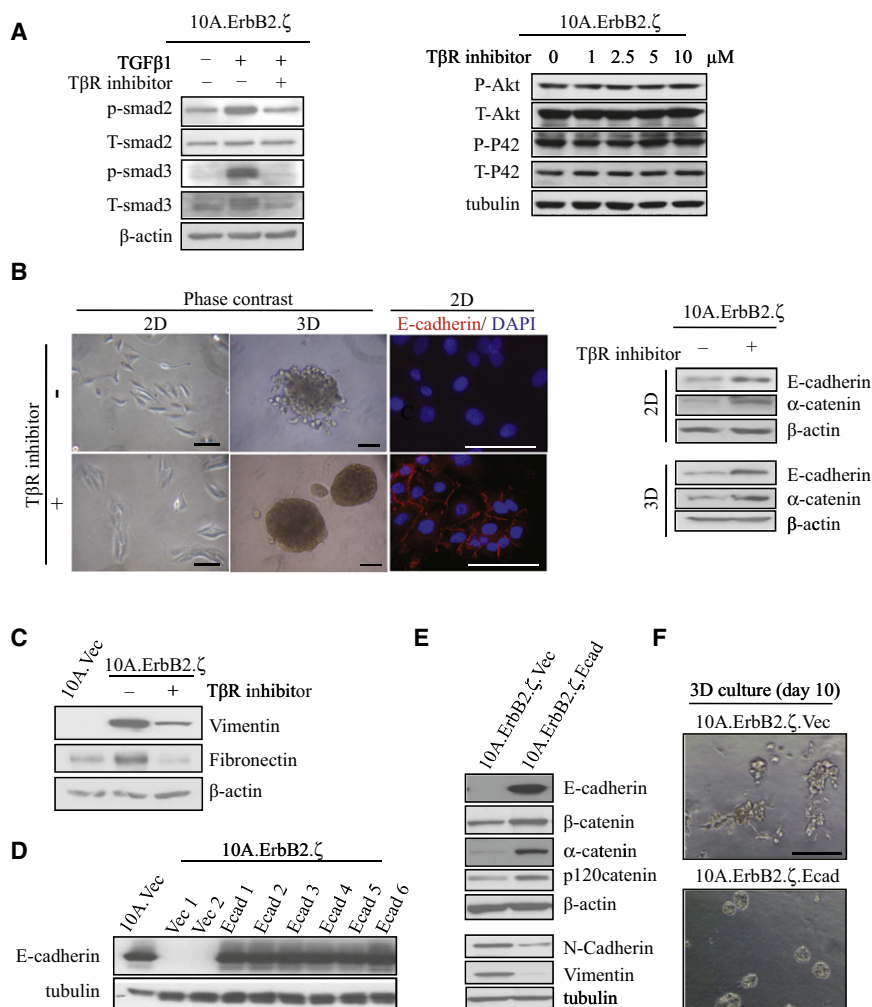


Figure 5. Inhibition of TGF- β /Smads Pathway Partially Reversed EMT of 14-3-3 ζ -Overexpressing MCF10A Cells and Inhibited Invasion of 10A.ErbB2.ζ Cells

(A) LY2109761 efficiently inhibited smad2/3 phosphorylation in 10A.ErbB2.ζ cells. Cells were treated with TGF- β 1 (5 ng/ml) or TGF- β 1 (5 ng/ml) plus TβR inhibitor LY2109761 (10 μM) for 60 min and then analyzed by immunoblotting with indicated antibodies (left). Cells were treated with increasing dose of LY2109761 and then analyzed by immunoblotting for p-Akt, T-Akt, p-P42, T-P42, and β-actin (right).

(B) Treatment of 10A.ErbB2.ζ cells with TGF- β receptor inhibitor partially reversed the EMT morphology in 2D culture and inhibited the invasiveness of acini in 3D culture. As shown in the left panel, cells were treated with LY2109761 for 48 hr, and images were photographed under a phase-contrast microscope. As shown in the middle panel, 10A.ErbB2.ζ cells were cultured on 3D matrigel for 13 days to form invasive acinar structures, and then treated with DMSO or LY2109761 for 6 days. As shown in the right panel, 10A.ErbB2.ζ cells were treated with DMSO or LY2109761 and subjected to IFS of E-cadherin. The scale bar represents 100 μm. Cells lysates were also collected after treatment and subjected to immunoblot with E-cadherin, α-catenin, and β-actin antibodies.

(C) Prolonged LY2109761 treatment (15 days) reduced mesenchymal protein expression (fibronectin and vimentin).

(D) E-cadherin expression was stably reintroduced into 10A.ErbB2.ζ cells.

(E) E-cadherin restoration led to increased epithelial protein expression (α-catenin, β-catenin, and p120-catenin) and decreased mesenchymal protein expression (N-cadherin and vimentin).

(F) E-cadherin restoration inhibited 10A.ErbB2.ζ cell invasion. Phase-contrast microscopy of acinar structures of 10A.ErbB2.ζ.Vec and 10A.ErbB2.ζ.Ecad cells in 3D culture. The scale bar represents 200 μm.

culture. To determine whether co-overexpression of ErbB2 and 14-3-3 ζ may increase invasion/metastasis in vivo, we stably overexpressed 14-3-3 ζ in TM15 cells, a mouse mammary tumor cell line from a MMTV-Cre/flox-neoNeuNT mouse that expresses the transforming *neu* under an endogenous promoter. We established the TM15.14-3-3 ζ cell line with TM15.Vec as our controls. The two sublines were injected into mammary fat pads of nude mice to establish xenografts and mice were monitored for metastatic lesions. Mice injected with the TM15.14-3-3 ζ cells definitely had more lung metastasis than mice with TM15.Vec cells (Figure 6C). To further investigate the impact of co-overexpression of ErbB2 and 14-3-3 ζ on breast cancer progression, especially metastatic disease recurrence and death of breast cancer patients, we performed IHC analysis to examine ErbB2 and 14-3-3 ζ expression in 107 cases of IBC in consecutive slides. Remarkably, 23 of the 107 patients had breast tumors co-overexpressing both ErbB2 and 14-3-3 ζ , and these patients had significantly shorter overall survival ($p < 0.05$) and disease-free survival ($p < 0.05$) than patients whose tumors overexpressed either one or neither (Figure 6D).

In addition, in this patient cohort, multivariate analysis demonstrated that co-overexpression of ErbB2 and 14-3-3 ζ in breast tumors can predict poor prognosis (Table S3). Because a majority of these patients died of recurrent metastatic disease, these data indicated that breast cancers overexpressing both ErbB2 and 14-3-3 ζ are more aggressive and have greater metastatic potential.

DISCUSSION

14-3-3 ζ Is a Biomarker for Patients with ErbB2-Overexpressing DCIS Who Have a Higher Risk of Progression to IBC

Both clinical and experimental data support that ErbB2 overexpression plays a critical role in DCIS but is not sufficient to drive progression of the noninvasive DCIS to IBC. It has been puzzling as to what other alterations may cooperate with ErbB2 to allow a subgroup of ErbB2-overexpressing DCIS to progress to life-threatening invasive/metastatic breast tumors. Here, we identified 14-3-3 ζ as a molecule that, when

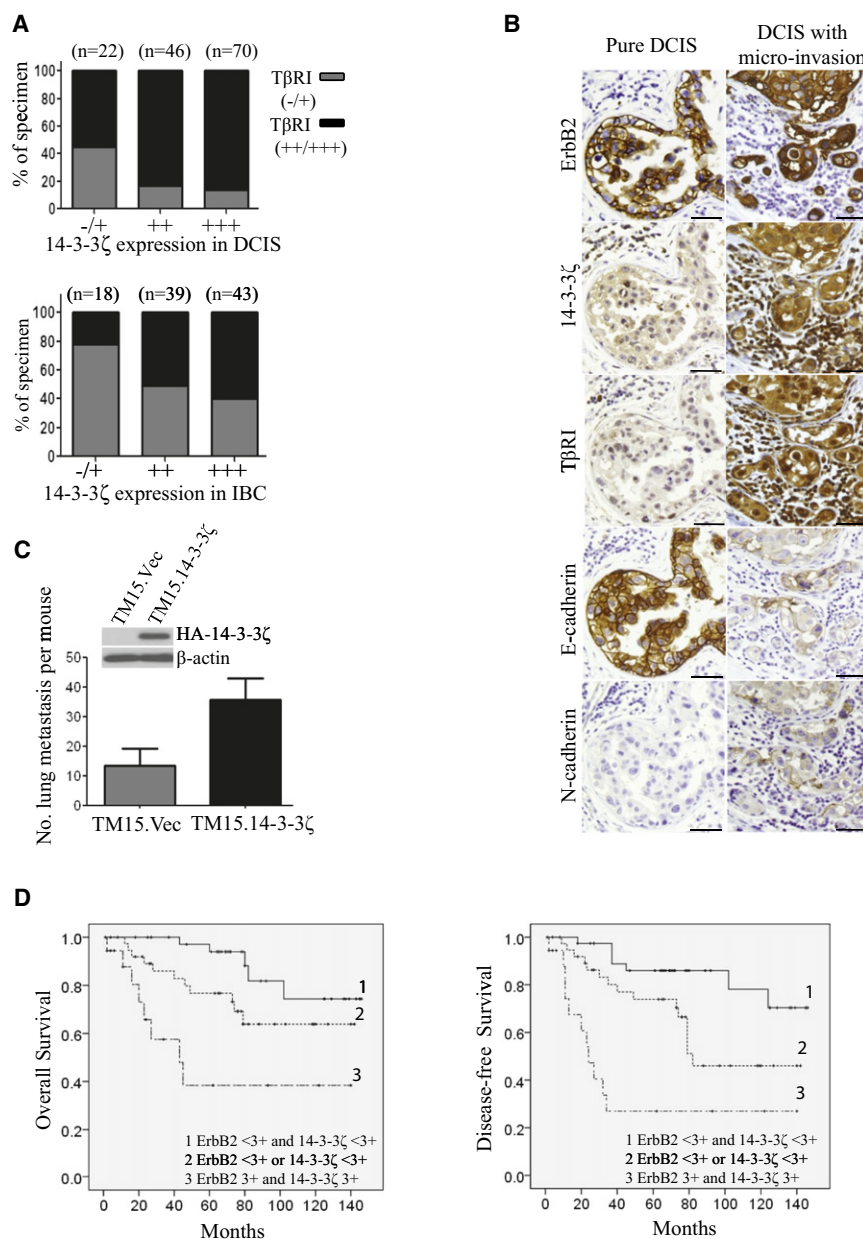


Figure 6. Co-overexpression of ErbB2 and 14-3-3 ζ Promotes Invasion and Metastasis

(A) 140 DCIS (top) and 107 IBC (bottom) samples were subjected to IHC staining for T β RI and 14-3-3 ζ , of which 138 DCIS and 100 IBC cases were satisfactory for analysis. Chi-square test indicated a correlation between T β RI and 14-3-3 ζ expression in both cohorts ($p < 0.05$).

(B) Representative IHC staining for ErbB2, 14-3-3 ζ , T β RI, E-cadherin, and N-cadherin in a DCIS case with microinvasion and a low-grade DCIS. The scale bar represents 50 μ m.

(C) 14-3-3 ζ overexpression in TM15 cells increased lung metastasis. TM15 cells were stably transfected with HA-14-3-3 ζ or empty vector. TM15.Vec and TM15.14-3-3 ζ cells were injected into mammary fat pad of nude mice (four mice in each group). When the primary tumors reached the size of 150 mm³, mice were sacrificed and their lungs underwent histological analysis to measure metastasis ($p < 0.05$). Error bar indicates SEM. Experiment was repeated twice and similar results were observed.

(D) Co-overexpression of ErbB2 and 14-3-3 ζ in IBC correlated with higher rates of death and disease recurrence. Tumor samples from 107 breast cancer patients were IHC stained for ErbB2 and 14-3-3 ζ . Kaplan-Meier plots of overall survival (left) and disease-free survival (right) according to ErbB2 and 14-3-3 ζ expression are shown. $p < 0.05$ (log-rank test).

co-overexpressed with ErbB2, increases the potential of DCIS to progress to IBC.

Individual tumor cell invasion is a highly complicated process that requires malignant cells to obtain at least both the “capability” (migration) and the “freedom” (dissemination) to escape from the constraint of tissue structure. We found that ErbB2 overexpression alone promoted cell migration via Src activation, but not invasion, whereas 14-3-3 ζ overexpression alone had no effect on cell motility but was sufficient to reduce cell-cell adhesion via inducing EMT. Therefore, the increased invasive potential in cells overexpressing both the ErbB2 and the 14-3-3 ζ proteins is the collective effect of ErbB2-mediated increase in migration plus 14-3-3 ζ -mediated decrease in cell-cell adhesion (Figure 7A). This finding is likely to have broader implications. Other genetic or epigenetic alterations that facilitate the

higher risk of progressing to IBC and therefore influence the therapeutic decision.

14-3-3 ζ Contributes to the Increased Invasive Ability of ErbB2-Overexpressing MECs by Inducing EMT

Accumulating evidence supports the role of EMT in promoting tumor invasion (Guarino, 2007). Pathological examination shows that malignant cells have often detached from the tumor mass at the periphery or at the invading front of the tumor. Moreover, EMT has recently been associated with “cancer stem cell” traits, suggesting a role for EMT in the initiation of recurrent tumors from disseminating cancer cells (Mani et al., 2008). However, the involvement of EMT in invasion and metastasis under a clinical setting remains controversial because of the transient and elusive nature of EMT in vivo. In this study, we detected

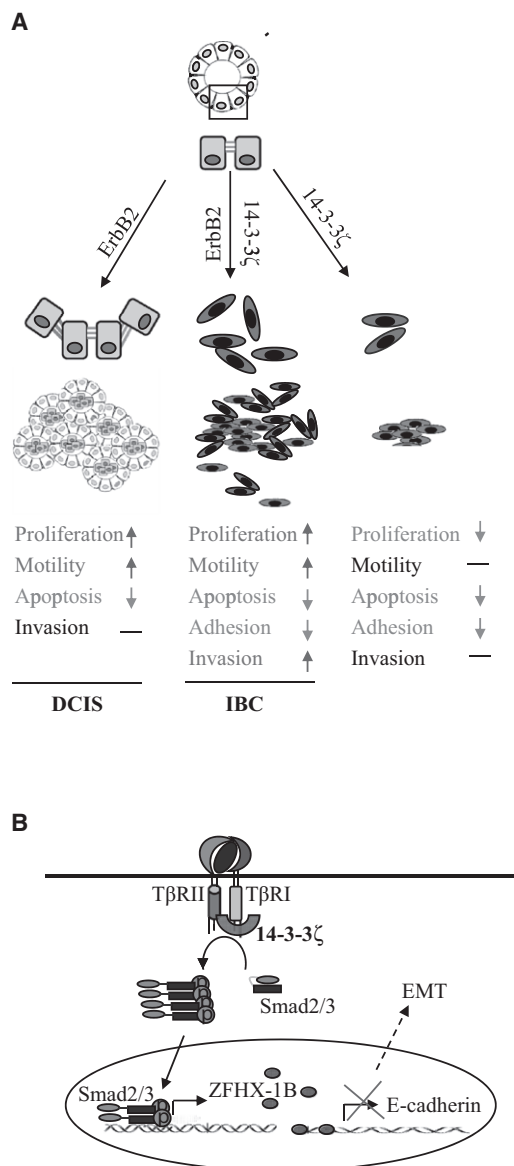


Figure 7. Models Represent How Co-overexpression of ErbB2 and 14-3-3 ζ Promotes Invasion

(A) A model of how co-overexpression of ErbB2 and 14-3-3 ζ promotes invasion. ErbB2 overexpression increased cell proliferation and motility; 14-3-3 ζ overexpression decreased cell-cell adhesion via induction of EMT. Collectively, co-overexpression of ErbB2 and 14-3-3 ζ promoted cell invasion. Co-overexpression of ErbB2 and 14-3-3 ζ may promote progression from DCIS to IBC via this mode of cooperation. ↑, increase; ↓, decrease; —, no significant effect.

(B) A model of 14-3-3 ζ -mediated E-cadherin repression. 14-3-3 ζ interacts with and stabilizes T β RI, leading to smad2/3 phosphorylation and translocation to the nucleus, where smads bind to ZFH1B promoter to increase its transcription. ZFH1B then represses E-cadherin transcription by binding to its promoter.

deregulation of EMT markers more frequently in DCIS overexpressing 14-3-3 ζ and T β RI, which significantly associated with higher grade DCIS that had a greater risk of developing invasive recurrence. These findings strongly support the involvement of EMT in DCIS progression toward invasive/metastatic disease.

Clearly, further studies in larger cohorts are needed and may guide the design of strategies for intervention in the progression from noninvasive DCIS to life-threatening IBC.

EMT-mediated invasion has been largely attributed to the loss of E-cadherin, a tumor invasion suppressor (Beavon, 2000). Indeed, restoration of E-cadherin expression increased cell-cell adhesion and reduced invasion in 3D culture of the invasive 10A.ErbB2. ζ cells (Figure 5). A key mechanism of E-cadherin loss downstream of 14-3-3 ζ overexpression is ZFH1B upregulation (Figure 3). ZFH1B, like other E-cadherin transcriptional repressors, has been implicated in regulation of EMT during embryogenesis (Van de Putte et al., 2003), and elevated level of ZFH1B mRNA has been reported to associate with metastasis of ovarian (Eloul et al., 2005), gastric (Rosivatz et al., 2002), and pancreatic tumors (Imamichi et al., 2007). Our findings that ZFH1B suppressed E-cadherin in 10A.ErbB2. ζ and 10A.14-3-3 ζ cells and that high level of ZFH1B expression correlated with E-cadherin loss in multiple breast cancer cell lines indicate a role for ZFH1B in breast cancer cell invasion.

14-3-3 ζ Overexpression Promotes TGF- β /Smads Pathway Activation

14-3-3 ζ upregulated ZFH1B by binding to T β RI and inhibiting the ubiquitin-proteasome pathway-mediated T β RI degradation, resulting in increased T β RI level, which subsequently led to TGF- β /Smads pathway activation and ZFH1B upregulation (Figure 7B). Interestingly, overexpression of 14-3-3 ζ in 293T cells has no discernable effect on ubiquitination of receptor interacting protein (data not shown), which indicates that the effect of 14-3-3 ζ on T β RI ubiquitination is selective rather than an overall deregulation of the ubiquitination machinery. Furthermore, 14-3-3 protein binding can both positively and negatively regulate the stability of distinct target proteins. For example, 14-3-3 ζ has been previously found to promote MDMX's ubiquitination and degradation (LeBron et al., 2006). One possible explanation for the different effects of 14-3-3 binding is that the binding on different target proteins may either expose or mask an additional signaling motif that is essential for triggering the degradation process. Further investigation is needed to elucidate the detailed mechanism.

There are seven 14-3-3 isoforms and 14-3-3 ζ can form heterodimers with other 14-3-3 isoforms. Therefore, it is possible that overexpression of other isoforms may have an effect on T β RI ubiquitination. Consistently, *Schistosoma mansoni* 14-3-3 ϵ was found to interact with SmRK1, a divergent type I TGF- β receptor, and positively regulated its signaling (McGonigle et al., 2001). In contrast, despite the highly conserved sequence and tertiary structure of 14-3-3 proteins, they appear to have distinct binding specificity and affinity to various target proteins. For example, 14-3-3 σ has a unique tumor suppressor function partially by directly binding and stabilizing p53 in response to DNA damage, whereas none of other 14-3-3 isoforms share this mode of regulation (Yang et al., 2003). Therefore, further systematic studies are clearly needed to investigate the effect of other 14-3-3 isoforms on the TGF- β /Smads pathway.

The TGF- β /Smads pathway can both positively and negatively regulate tumor development (Bachman and Park, 2005). TGF- β /Smads pathway is a tumor suppressor prior to and during early tumor progression, mainly through inhibiting proliferation

(Bachman and Park, 2005). Consistently, 10A.14-3-3 ζ cells with increased T β RI expression proliferated at a slower rate than 10A.Vec cells (data not shown) and formed smaller acini than 10A.Vec cells. The inhibition of proliferation may result from upregulation of cell cycle inhibitors downstream of TGF- β /Smads activation in the nontransformed MCF10A cells. In contrast, the overexpressed ErbB2 in 10A.ErbB2. ζ cells can activate various downstream signals to counter the growth-inhibitory effect of TGF- β /Smads activated by 14-3-3 ζ . However, during the later stages of tumor progression, the TGF- β /Smads pathway can function as a tumor invasion promoter via induction of EMT (Bachman and Park, 2005). Intriguingly, 14-3-3 ζ overexpression alone in MCF10A cells led to TGF- β /Smads pathway activation and EMT (as in 10A.ErbB2. ζ cells), although without increased invasion. These data indicate that 14-3-3 ζ -mediated EMT is necessary but not sufficient to promote cell invasion because of its lack of intrinsic migration ability, whereas migration is promoted by ErbB2 overexpression in 10A.ErbB2. ζ cells that become invasive. Our findings are consistent with a previous report that ErbB2 activation can cooperate with TGF- β treatment to promote invasion (Seton-Rogers et al., 2004). Conversely, bitransgenic mice that expressed *MMTV-neu* and a soluble antagonist of TGF- β had a significant reduction of metastasis (Yang et al., 2002). Our findings on the synergistic effect of ErbB2 overexpression and 14-3-3 ζ -mediated activation of TGF- β /Smads pathway shed light on molecular mechanisms of gain of invasiveness during ErbB2-overexpressing DCIS progression, which is contributed by ErbB2-induced motility and proliferation plus 14-3-3 ζ -mediated loss of cell-cell adhesion via inducing EMT. Recently, the TGF- β /Smads pathway was implicated to play a critical role in the “communication” of MECs with their “natural invasion suppressors,” myoepithelial cells (Hu et al., 2008). The impact of ErbB2 and 14-3-3 ζ co-overexpression on myoepithelial cells will be investigated in future studies.

Our findings that ErbB2 and 14-3-3 ζ co-overexpression in DCIS predicts a higher risk of progression to IBC also provide molecular targets for designing combination therapies to intervene in DCIS progression. Targeting 14-3-3 ζ may be challenging at the current stage because 14-3-3 ζ regulates many important proteins that are essential for homeostasis. Identification of the TGF- β /Smads pathway as a downstream event of 14-3-3 ζ overexpression in promoting invasion represents an opportunity for therapeutic intervention. Currently, the TGF- β /Smads pathway is under intensive investigation as a therapeutic target (Dumont and Arteaga, 2003; Yingling et al., 2004). Given the dichotomous role of the TGF- β /Smads pathway in tumor development, it is critical to dissect the TGF- β /Smads downstream signals and their crosstalk with other signaling networks, such as ErbB2 signaling, in order to specifically activate its tumor-suppressing role or specifically inhibit its tumor-promoting role. Our findings suggest the potential therapeutic benefit of inhibiting the TGF- β /Smads pathway in the context of ErbB2 and 14-3-3 ζ co-overexpressing breast cancers.

EXPERIMENTAL PROCEDURES

Tissue Specimens

Twenty-five DCIS and 107 IBC specimens were obtained from the Cancer Hospital, FuDan University (Shanghai, China). An additional 140 DCIS were

collected at The University of Texas M.D. Anderson Cancer Center (MDACC; Houston, TX). Patient samples were collected and processed in compliance with protocols approved by the MDACC Institutional Review Board and by the Cancer Hospital/Cancer Institute, FuDan University Institutional Review Board. Detailed clinic-pathological information of these patient cohorts are provided in Tables S4–S6.

Cells, Constructs, Antibodies, and Reagents

The MCF10A cell line was a kind gift from Dr. Robert Pauley (Karmanos Cancer Institute, Detroit, MI) and was cultured in 3D culture as previously described (Debnath et al., 2003). The HMEC-hTERT cell line was kindly provided by Dr. Victoria Seewaldt (Duke University, Durham, NC). ErbB2, HA-14-3-3 ζ , and E-cadherin genes were cloned into pLPCX, pLNCX2, and pLHCX vectors (Clontech), respectively. Retroviral infection was done as previously described (Danes et al., 2008). Stable clones were selected with 400 μ g/ml neomycin, 800 ng/ml puromycin, and 100 μ g/ml hygromycin, respectively. Multiple stable clones were used to rule out potential clonal effects. HA-14-3-3 ζ was also cloned into pRV3 (with GFP in the backbone) and pLOVE lentiviral vectors. Lentivirus production and infection, and reagents and antibodies used, are described in the Supplemental Experimental Procedures.

Soft Agar Colony Formation Assay, Cytoplasm and Nuclear Protein Fractionation, Immunoblotting, IHC, IFS, Immunoprecipitation, RNA Extraction, RT-PCR, and Quantitative RT-PCR, Luciferase Reporter Assay, Migration Assay, Wound Healing Assay, Cell Adhesion Assay, and Cell Aggregation Assay

The detailed procedures are described in the Supplemental Experimental Procedures. IFS analysis of monolayer cell cultures was done as previously described (Debnath et al., 2003). For IFS of 3D cultures, acini were embedded in sucrose and frozen in Tissue-Tek OCT (Sakura Finetek), and 5 μ m frozen sections were cut and subjected to analysis (Weaver et al., 1997).

siRNA Transfection and ChIP

ON-TARGET plus SMART Pool siRNA for 14-3-3 ζ , ON-TARGET plus control siRNA and ZFH1B siRNA were purchased from Dharmacon. Transfection was done as previously described (Danes et al., 2008). ChIP assay was performed with ChIP-IT kit from Active Motif according to the manufacturer's instructions. The DNA pulled down by antibodies was amplified with ZFH1B promoter-specific primers.

Cell Adhesion Assay and Cell Aggregation Assay

For the cell adhesion assay, 96-well plate was coated with fibronectin (10 ng/ml) before use. Ten thousand cells were resuspended as single-cell suspension in 200 μ l DMEM/F12 media containing 0.5% BSA and added to the coated plate for incubation at 37°C for 1 hr. Nonadherent cells were washed away with DMEM/F12 media containing 0.1% BSA. Adherent cells were detected by MTS assay according to the manufacturer's instruction. For aggregation assay, a six-well plate was coated with poly 2-hydroxyethyl methacrylate (polyHEMA) before use. Fifty thousand cells were resuspended to single cell suspension in 500 μ l DMEM/F12 containing 0.5% BSA, added to the polyHEMA-coated plates, and incubated at 37°C on a rotating platform (150 rpm) for 30 min. Cells were then fixed with methanol and photographed under a phase-contrast microscope.

Statistical Analysis

Statistical tests used to analyze data included Fisher's exact test, log-rank test, Chi-square test, and Student's t test. Multivariate statistical analysis was performed using a Cox regression model. Statistical analysis was performed using SPSS for Windows (16.0; SPSS, Inc.) and GraphPad Prism (Prism 5.0; GraphPad Software Inc.) packages. A p value < 0.05 was considered significant.

SUPPLEMENTAL DATA

Supplemental Data include ten figures, six tables, and Supplemental Experimental Procedures and can be found with this article online at [http://www.cell.com/cancer-cell/supplemental/S1535-6108\(09\)00256-6](http://www.cell.com/cancer-cell/supplemental/S1535-6108(09)00256-6).

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