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14-3-3 ζ Turns TGF- β 's Function from Tumor Suppressor to Metastasis Promoter in Breast Cancer by Contextual **Changes of Smad Partners from p53 to Gli2**

Graphical Abstract



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In Brief

Xu et al. provide molecular insight into how 14-3-3ζ coordinates inhibition of TGF-β tumor suppressor function in mammary epithelial cells and promotion of TGF-β-induced bone metastasis in breast cancer.

Highlights

- 14-3-3 ζ switches TGF- β 's function by providing contextual partners for Smads
- 14-3-3ζ inhibits YAP1-induced 14-3-3σ to disrupt p53/Smads complex
- 14-3-3ζ stabilizes Gli2/Smads complex to activate PTHrP and induce bone metastasis
- 14-3-3ζ is associated with TGF-β's functional switch during breast cancer development

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14-3-3 ζ Turns TGF- β 's Function from Tumor Suppressor to Metastasis Promoter in Breast Cancer by Contextual Changes of Smad Partners from p53 to Gli2

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SUMMARY

Transforming growth factor β (TGF- β) functions as a tumor suppressor in premalignant cells but as a metastasis promoter in cancer cells. The dichotomous functions of TGF- β are proposed to be dictated by different partners of its downstream effector Smads. However, the mechanism for the contextual changes of Smad partners remained undefined. Here, we demonstrate that 14-3-3 ζ destabilizes p53, a Smad partner in premalignant mammary epithelial cells, by downregulating 14-3-3 σ , thus turning off TGF- β 's tumor suppression function. Conversely, 14-3-3 ζ stabilizes Gli2 in breast cancer cells, and Gli2 partners with Smads to activate PTHrP and promote TGF- β -induced bone metastasis. The 14-3-3 ζ -driven contextual changes of Smad partners from p53 to Gli2 may serve as biomarkers and therapeutic targets of TGF- β -mediated cancer progression.

INTRODUCTION

TGF- β manifests multifunctional and sometimes dichotomous roles in numerous biological processes, such as embryonic stem cell self-renewal and differentiation, homeostasis of differentiated mammalian cells, and different stages of cancer progression. In premalignant cells, TGF- β is primarily a tumor suppressor that inhibits cell proliferation or induces apoptosis (Massagué, 2008). In the later stages of cancer progression, however, TGF- β functions as a metastasis promoter by inducing epithelial-mesenchymal transition (EMT), leading to increased invasion of cancer cells, and also by inducing genes that facilitate metastatic colonization of secondary organ sites (Massagué, 2008). Although the opposing functions of TGF- β in early- versus late-stage cancer have been known for decades, how and when TGF- β switches its functional roles are long-standing questions with no clear answer. TGF- β binds to its receptor, TGF β R1/2, on the cell membrane and induces a signaling cascade by phosphorylating Smad2/3. Phosphorylated Smad2/3 forms a complex with Smad4 and translocates from the cytoplasm to the nucleus to activate the transcription of downstream targets. An emerging notion is that the unique cellular context

Significance

TGF- β 's critical role in cancer has motivated numerous efforts toward developing TGF- β -targeting anti-cancer therapeutics, some of which have been clinically tested. However, TGF- β 's dichotomous roles during cancer development have been major obstacles for effective TGF- β -targeting therapies because systemic inhibition of TGF- β signaling also blocks TGF- β 's cytostatic function in normal tissues. This study identified 14-3-3 ζ as a molecular switch turning TGF- β from tumor suppressor to metastasis promoter by altering Smad partners from p53 in premalignant cells to Gli2 in cancer cells. Thus, 14-3-3 ζ and downstream Smad partners could serve as (i) biomarkers for patient selection and timing of anti-TGF- β therapy administration and (ii) therapeutic targets to selectively block TGF- β signaling in cancer without impeding TGF- β 's tumor suppressor function in normal tissues.



(e.g., Smad binding partners and their modifiers) dictates the complicated and even converse biological responses to TGF- β (Massagué, 2012). However, which specific Smad partners determine TGF- β 's tumor suppressor versus metastasis promoter functions is unclear. More importantly, what factor(s) triggers the change of Smad partners in early- versus late-stage cancer is also unclear.

The critical role of TGF- β in cancer, especially in the process of metastasis, has spurred the development of antagonists that target TGF- β signaling as cancer therapeutics (Akhurst and Hata, 2012). Disappointingly, many of the current TGF- β -targeting drugs showed limited clinical efficacy. Considering the opposing functions of TGF-β in cancer development (Massagué, 2008, 2012), it is not surprising that general inhibition of the TGF-^β pathway may have deleterious consequences (Bierie and Moses, 2009). Inhibiting TGF- β may accelerate the progression of preneoplastic lesions in which TGF-ß still acts as a tumor suppressor. For example, conditional knockout of Tgfbr2, which encodes the type II TGF- β receptor (TGF β R2), in the mammary gland of mice expressing the polyoma viral middle T antigen (PyVmT) at puberty before mammary tumors are established resulted in shortened tumor latency and increased pulmonary metastases (Forrester et al., 2005). In contrast, a short induction of TGF- β expression after mammary tumors were established in PyVmT mice accelerated metastatic progression (Muraoka-Cook et al., 2004). Thus, it is essential to identify the disease stage at which patients will benefit from TGF- $\!\beta$ antagonists and to develop biomarkers to guide the selection of patients for TGF-\beta-targeting therapies and the evaluation of therapeutic efficacy.

RESULTS

14-3-3ζ Inhibits TGF-β Cytostatic Program by Downregulation of 14-3-3σ, p53, and p21

To understand how TGF- β functions are governed during different disease stages, we first used TGF- β -responsive cell models representing distinct breast disease stages. Since 14-3-3 ζ enhances TGF- β signaling and induces early transformation and EMT of human mammary epithelial cells (HMECs) (Lu et al., 2009), we used nontransformed MCF10A HMECs stably transfected with a control vector (10A.Vec) or 14-3-3 ζ cDNA (10A. ζ). In 3D culture, the 10A.Vec cells produced normal acini,

while the 10A.² cells generated atypical ductal hyperplasia (ADH)-like acini. Initially, we performed reverse phase protein array (RPPA) proteomic profiling of 10A.Vec and 10A.C cells cultured with fresh media containing TGF-B or vehicle for 2 hr (Figure 1A and Figure S1A available online). 14-3-3^c inhibited TGF-β-induced expression of p21, a key effector of TGF-β's cytostatic program, but not other known effectors (e.g., p27/ Kip1) (Massagué, 2008). Compared to 10A.Vec cells, both the basal and TGF-β-induced p21 protein and mRNA expression were inhibited in 10A. ζ cells (Figures 1A–1C). Consequently, TGF- β inhibited the proliferation and immediate entry into the S phase of 10A.Vec cells, but not 10A. ζ cells (Figures 1D and S1B-S1D). Similar results were found in MCF12A HMECs (Figures S1E and S1F), indicating that 14-3-3ζ blocks the proliferation-suppression function of TGF- β in HMECs. To test whether p21 is the key executor of TGF- β 's cytostatic tumor suppression function in these HMECs, we knocked down p21 in both MCF10A and MCF12A cells (Figures 1E and S1G). Indeed, p21 knockdown abrogated TGF-β's proliferation suppression function in both cell lines (Figures 1F, S1H, and S1I), highlighting that p21 is the key executor required for TGF-B's cytostatic tumor suppression function and 14-3-3ζ-mediated p21 downregulation can block TGF- β 's cytostatic program in HMECs.

TGF-β-induced p21 expression in HMECs requires specific cellular context-dependent Smad partners, e.g., p53 (Cordenonsi et al., 2003) or FOXO3a (Muñoz-Espín et al., 2013). We found that p53 complexed with Smad2 in TGF-β-treated 10A.Vec and 12A.Vec cells, while FOXO3a did not bind to Smads (Figures 1G and S1J–S1L). Interestingly, 14-3-3ζ downregulated p53 and diminished p53's interaction with Smad2 in both 10A.ζ and 12A.ζ cells (Figures 1G and S1J). Furthermore, silencing p53 in both MCF10A and MCF12A cells reduced TGF-β-induced p21 expression (Figures S1M and S1N), indicating that p53 is the contextual determinant of Smads for TGF-β-induced p21 expression in these HMECs and that 14-3-3ζ inhibits TGF-β-induced p21 expression by reducing p53 level.

Since p53 downregulation reduces TGF- β -induced p21 expression and cytostatic function, and p53 regulation occurs at multiple levels (Golubovskaya and Cance, 2013), we dissected the mechanisms of p53 downregulation by 14-3-3 ζ . We compared gene expression profiles of 3D-cultured 10A.Parental (10A.P), 10A.Vec, and 10A. ζ cells by cDNA microarray. We discovered that 14-3-3 σ (SFN), a tumor suppressor that

Figure 1. 14-3-3 ζ Inhibits TGF- β Cytostatic Program by Downregulation of 14-3-3 σ , p53, and p21

(A) Reverse phase protein array (RPPA) analysis of 10A.Vec and 10A. ζ cells treated with vehicle (–) or 5 ng/ml TGF- β (+) for 2 hr.

Error bars represent SD, *p < 0.05, **p < 0.01, ***p < 0.001. See also Figure S1.

⁽B and C) Immunoblotting (IB) (B) and quantitative reverse transcriptase PCR (qRT-PCR) (C) analysis of p21 expression in 10A.Vec and 10A. ζ cells treated with vehicle (–) or 5 ng/ml TGF- β (+) for 2 hr.

⁽D) Cell growth inhibition assay analysis of 10A.Vec or 10A. ζ cells treated with 5 ng/ml TGF- β .

⁽E) IB of p21 in p21-knockdown MCF10A cells (10A.shp21-233 and 10A.shp21-535) and control cells transfected with control shRNA (10A.shCtrl).

⁽F) Cell growth inhibition assay analysis of 10A.shCtrl or 10A.shp21 cells treated with 5 ng/ml TGF-β.

⁽G) Immunoprecipitation (IP) of Smad2 and IB analysis of p53, p-Smad2 in indicated cells treated with vehicle (–) or 10 ng/ml TGF-β (+) for 2 hr.

⁽H) Gene expression profiling of 10A.P, 10A.Vec, and 10A.ζ cells by cDNA microarray. Heatmap depicts 14-3-3ζ-induced top gene alterations. *SFN. (I and J) gRT-PCR (I) and IB (J) analysis of 14-3-3σ expression in 10A.P, 10A.Vec and 10A.ζ cells.

⁽K) IB analysis of 14-3-3σ and p53 expression in 14-3-3σ-knockdown cells compared to 10A.P and 10A.shCtrl cells.

⁽L) Cell growth inhibition assay analysis of 10A.shσ (-128 and -130) cells compared to 10A.shCtrl cells treated with 5 ng/ml TGF-β.

⁽M) IB of indicated proteins of TGF-β cytostatic program in 10A.shCtrl and 10A.shσ-128 cells treated with vehicle (–) or 5 ng/ml TGF-β (+) for 2 hr.

⁽N) IB analysis of protein expressions of TGF- β 's cytostatic program in indicated cells.

⁽O) BrdU incorporation assay analysis of TGF- β -treated 10A.Vec, 10A. ζ , and 10A. ζ . σ cells.



Figure 2. 14-3-3ζ Represses 14-3-3σ Transcription by Cytosolic Sequestration of YAP1

(A) Luciferase activity assay of 14-3-3 σ promoter (-1221)-driven luciferase (pGL3-14-3-3 σ) expression in indicated cells 48 hr posttransfection. The pRL-TK vector was used as an internal control.

(B) Schematic representation of sequential deletions of the 14-3-3 σ promoter cloned in the upstream of the luciferase reporter (top) and mutations (M1 to M5) of putative transcription factor binding sites in the -922 to -741 region of the 14-3-3 σ promoter (bottom).

(C) Relative luciferase activity driven by sequential deletions of 14-3-3 or promoter (-1221, -922, and -741) in 10A.Vec and 10A. cells.

(D) Relative luciferase activity driven by binding site mutations of $14-3-3\sigma$ promoter (-922) in 10A.Vec cells.

(E) ChIP assay of YAP1 binding to 14-3-3 or promoter in 10A.Vec and 10A. cells with indicated antibodies.

stabilizes p53 protein through cytoplasmic retention of the E3 ligase for p53-MDM2 (Lee and Lozano, 2006), was downregulated by 14-3-3 ζ (Figure 1H). 14-3-3 σ downregulation by 14-3-3ζ was confirmed at both mRNA and protein levels in both10A.ζ and 12A.ζ cells (Figures 1I, 1J, S1O, and S1P). Additionally, overexpression of 14-3-3 ζ reduced 14-3-3 σ level, whereas knockdown of 14-3-3 ζ increased 14-3-3 σ level in multiple human breast cancer cell lines (Figure S1Q), suggesting that 14-3-3 ζ -mediated regulation of 14-3-3 σ is common in breast cancer. Furthermore, silencing of 14-3-3 reduced p53 level and blocked TGF-β-induced p21 expression and proliferation suppression in both MCF10A and MCF12A cells (Figures 1K-1M and S1R–S1U). Conversely, reintroducing 14-3-3 σ rescued p53 expression and restored TGF-β-induced p21 expression and cytostatsis in 10A.² cells (Figures 1N and 1O). This indicated that 14-3-3^c and 14-3-3^o play opposing roles in regulating TGFβ-induced p21 expression and proliferation suppression and 14-3-3 ζ downregulated p53 primarily by repressing 14-3-3 σ . Altogether, 14-3-3 ζ -mediated 14-3-3 σ downregulation led to loss of p53, the specific Smad partner for TGF-B-induced p21 expression in HMEC, and, consequently, switched off TGF-β's cytostatic tumor suppression function in premalignant cells.

14-3-3 ζ Represses 14-3-3 σ Transcription by Cytosolic Sequestration of YAP1

To investigate how 14-3-3 ζ downregulates 14-3-3 σ expression, we first compared 14-3-3 σ mRNA stability and its promoter methylation between 10A. ζ and 10A.Vec cells and found no significant difference (Figures S2A and S2B). Further, DNA methylation inhibitor (5-Aza-2'-deoxycytidine) treatment did not restore 14-3-3 σ expression in 10A. ζ cells (Figure S2C). This suggested that 14-3-3 ζ did not affect 14-3-3 σ mRNA stability or its epigenetic regulation but instead inhibited its transcription. Indeed, when we transfected a luciferase reporter driven by the 14-3-3 σ promoter into 10A.parental (10A.P), 10A.Vec, and 10A. ζ cells, we found that 14-3-3 σ promoter activity was repressed in 10A. ζ cells compared to 10A.P and 10A.Vec cells (Figure 2A).

To uncover the specific DNA element in the 14-3-3 σ promoter responsible for 14-3-3 ζ -mediated trans-repression, we generated a series of 14-3-3 σ promoter 5'-deletion driving luciferase-reporter constructs (Figure 2B). We found that a 181 bp (-922 bp to -741 bp) promoter region was mostly responsible for 14-3-3 σ transcription, which was repressed by 14-3-3 ζ (Figure 2C). TESS (http://www.cbil.upenn.edu/tess) analysis revealed several putative transcription factor binding sites within this 181 bp region (Figure S2D). Mutation analyses (M1–M5) revealed that the M2 site, a predicted binding site for the YAP1 transcriptional coactivator, was primarily responsible for 14-3-3 σ transactivation (Figures 2B, 2D, and S2E). Indeed, YAP1 bound efficiently to the 14-3-3 σ promoter in 10A.Vec cells,

whereas the binding was significantly inhibited by 14-3-3 ζ overexpression (Figure 2E). This inhibition was due to 14-3-3 ζ binding to and sequestering phospho-YAP1 outside of the nucleus (Figures 2F–2I and S2F and S2G), while 14-3-3 ζ had no significant effect on YAP1 protein level (Figure S2H). Moreover, 14-3-3 σ protein and mRNA levels were reduced in YAP1-knockdown cells (Figures 2J and 2K), indicating that YAP1 is a critical transactivator of 14-3-3 σ in HMECs, while 14-3-3 ζ binds to phospho-YAP1, sequesters it in the cytoplasm, and prevents it from transactivating 14-3-3 σ .

14-3-3 ζ -Linked Gene Signature Is Associated with TGF- β -Regulated Genes in Bone Metastasis

The above findings indicate that 14-3-3^{\(\zeta\)} inhibits the TGFβ-induced cytostatic program in premalignant HMECs by cytoplasmic retention of YAP1, thereby repressing 14-3-3 o that leads to downregulation of p53, a Smad partner for p21 transcription. To explore whether 14-3-3ζ may also switch on TGF-β's metastasis promoter function in cancer cells, we generated derivatives of MDA-MB-231, a highly metastatic human breast cancer cell line that expresses a high level of 14-3-3^{\(\zeta\)}, with either 14-3-3^{\(\zeta\)} shRNA (231.shζ) or control shRNA (231.shCtrl) (Figure 3A). First, cDNA microarray analysis of 231.shCtrl cells treated with vehicle or TGF- β revealed a TGF- β -induced 84-gene signature (Figure S3A). Analyses of these TGF-β-induced genes in the data set available in the Gene Expression Omnibus (GEO) database under accession number GSE14020, generated from metastatic tumors of human breast cancers to the major metastatic organ sites (Zhang et al., 2009), revealed that many of these genes were expressed at a higher level in bone metastases than in metastases to other organs (Figures S3B and S3C). Second, comparing 231.sh ζ and 231.shCtrl without TGF- β treatment revealed an 85-gene 14-3-3ζ signature, which was also highly expressed in breast cancer bone metastases compared to metastases to other organs (Figures 3B and 3C). Consistently, a 14-3-3ζ-induced gene signature generated from cDNA microarrays of 10A. compared to 10A. Vec cells was also predominantly expressed in bone metastases (Figures 1H and 3D). To see whether patients with 14-3-3 ζ^{high} breast cancers are prone to development of bone metastasis, univariate analysis was performed on a cohort of 253 breast cancer patients with clinically annotated data for development of bone metastasis in 5 years (EMC-286 data set) (Bos et al., 2009). It revealed that patients with 14-3-3 ζ^{high} breast tumors had a significantly (p = 0.034) reduced bone metastasis-free survival (Figure 3E). Additionally, among patients who developed bone metastasis, patients with 14-3-3 ζ^{high} breast tumors have a significantly earlier bone metastasis onset than those with 14-3-3 ζ low-expressing tumors (p < 0.05; Figure S3D). Together, these data indicated that elevated 14-3-3^{\zet} is associated with breast cancer bone metastases.

(J and K) IB (J) and qRT-PCR (K) analysis of 14-3-3 σ expression in YAP1-knockdown cells compared to 10A.shCtrl or 12A.shCtrl cells. Error bars represent SD, *p < 0.05, **p < 0.01, ***p < 0.001. See also Figure S2.

⁽F) IP of HA-14-3-3 ζ and IB analysis of indicated proteins in 10A. ζ cells.

⁽G) IP of HA-14-3-3ζ and IB analysis of indicated proteins in 12A.Vec and 12A.ζ cells. IP Ab, antibodies used for IP.

⁽H) Immunofluorescence staining analysis of 14-3-3ζ and YAP1 in 10A.Vec and 10A.ζ cells. Arrows indicate colocalization of 14-3-3ζ and YAP1 in the cytosol of 10A.ζ cells. Scale bars represent 10 μm.

⁽I) Cell fractionation of 10A.Vec and 12A.Vec or 10A.ζ and 12A.ζ cells and IB of indicated proteins. Tubulin serves as cytoplasmic marker; Lamin B serves as nuclear marker.



Figure 3. 14-3-3ζ-Linked Gene Signature Is Associated with TGF-β-Regulated Genes in Bone Metastasis
(A) IB of indicated proteins in 231.shCtrl and 231.shζ cells treated with vehicle or TGF-β (5 ng/ml, 2 hr).
(B) Expression heatmap of 14-3-3ζ 85-gene signature in 231.shCtrl cells compared to 231.shζ cells.
(C) Significance score distribution of 14-3-3ζ signature from MDA-MB-231 cells in breast cancer metastases of indicated organs.

Finally, the 231.sh ζ cells with 14-3-3 ζ knockdown manifested a partially impaired TGF- β -induced gene signature (Figure 3F), and many of these impaired genes were highly expressed in bone metastasis (Figure 3G). This suggested that 14-3-3 ζ may switch on TGF- β 's bone metastasis promoter function by regulating TGF- β 's transcriptomic profile in breast cancer.

14-3-3 ζ Promotes TGF- β -Induced Breast Cancer Bone Metastasis by Activating PTHrP

After we found that 14-3-3ζ-linked genes were associated with TGF-β's bone metastasis promoter function in breast cancer, we next set to determine whether 14-3-3 ζ plays an essential role in TGF- β -mediated bone metastasis. Using 231-1566 cells, a MDA-MB-231 bone metastasis derivative that expresses GFP and luciferase (Khotskaya et al., 2014), we introduced either 14-3-3ζ shRNA (1566.shζ) or control shRNA (1566.shCtrl) (Figure S4A, left). We then compared their bone metastasis potential by injection into the left ventricle of nude mice. Mice injected with 1566.sh^{\z} had significantly longer bone metastasis-free survival compared to mice in the 1566.shCtrl group, even though 1566.shCtrl and 1566.shζ cells showed no discernable difference in cell proliferation in vitro (Figures 4A, 4B, and S4B). Bioluminescence imaging (BLI) revealed that 100% of mice injected with 1566.shCtrl cells developed bone metastases by 35 days, when only 25% of mice injected with 1566.sht cells had bone lesions (Figure 4A), and these bone lesions had an incomplete $14-3-3\zeta$ knockdown (Figure 4B). Similar trends were observed in another derivative (BoM-1833) from MDA-MB-231-induced bone metastasis (Kang et al., 2003; Zhang et al., 2009) (Figures S4A-S4D).

Breast cancer cell growth in the bone is a critical step for bone metastasis formation. To further investigate whether 14-3-32 promotes breast cancer cell growth in the bone, we injected GFP- and luciferase-labeled 231.shCtrl (14-3-3 c high) and 231.sh(14-3-3(low) cells intratibially into nude mice to compare their bone colonization ability. Mice injected with 231.shζ cells showed significantly prolonged bone tumor-free survival and delayed outgrowth of bone tumors compared to the 231.shCtrl group (Figures 4C and 4D). The difference in bone tumor burden between the 231.shCtrl and 231.sh groups was also clearly demonstrated by BLI, X-ray analysis, and hematoxylin and eosin (H&E) and 14-3-3^{\zeta} immunohistochemical (IHC) staining (Figure 4D). Additionally, we examined whether 14-3-3ζ plays an essential role in TGF-\beta-mediated bone metastasis outgrowth in a syngeneic mouse model using the highly metastatic 4T1 mouse mammary tumor cell line that expresses a high level of 14-3-3ζ. We generated 4T1 derivatives transfected with either 14-3-32 shRNA (4T1.shζ) or control shRNA (4T1.shCtrl) (Figure S4A, right) and injected 4T1.shCtrl and 4T1.sh cells expressing GFP and luciferase intratibially into BALB/c mice. Mice injected with 4T1.sh ζ cells had significantly reduced bone tumor burdens with smaller osteolytic lesions compared to mice injected with the 4T1.shCtrl cells as quantified by the BLI signal, X-ray analysis, H&E and 14-3-3 ζ IHC staining, although 4T1.shCtrl and 4T1.sh ζ cells showed no apparent difference in cell proliferation in vitro (Figures S4E–S4G). Overall, these data demonstrated that elevated 14-3-3 ζ expression in human breast cancer and mammary tumor cells with increased TGF- β activity promotes their outgrowth in the bone and increases bone metastasis.

TGF-ß promotes breast cancer bone metastasis via a "vicious cycle": briefly, osteoclast-induced osteolysis releases TGF- β in the bone microenvironment, stimulating metastatic cancer cell proliferation and secretion of parathyroid hormone-related protein (PTHrP), which stimulates osteoblasts to release RANKL, which in turn promotes osteoclast maturation and function, ultimately releasing more TGF-B (Mundy, 2002; Padua and Massagué, 2009). To investigate whether and how 14-3-3 c promotes bone metastasis via TGF-ß's "vicious cycle," we cocultured 231.shCtrl and 231.sh² cells with preosteoclasts (RAW264.7) and osteoblasts (MC3T3) under vehicle or TGF-B treatment (triple coculture). We examined osteoclast maturation and breast cancer cell proliferation since both osteolysis by mature osteoclasts and breast cancer cell growth in the bone are critical events for bone metastasis formation. TGF- β treatment of triple co-cultured 231.shCtrl cells stimulated RAW264.7 preosteoclast differentiation and maturation, indicated by tartrate-resistant acid phosphatase-positive (TRAP⁺) staining, a marker of mature osteoclasts; this was inhibited by 14-3-3ζ knockdown (Figures 4E and 4F). Meanwhile, proliferation of 231.shCtrl, but not 231.sh ζ , was increased by TGF- β treatment in triple coculture (Figures 4G and S4H). Notably, osteoclast maturation and 231.shCtrl and 231.sh cell proliferation were similar in vehicletreated cocultures and in TGF-β-treated single cultures (Figures 4E-4G, S4H, and S4I), indicating that 14-3-3ζ does not itself affect osteoclast maturation or cancer cell proliferation, but rather modulates TGF-B's effects on the bone microenvironment. Collectively, 14-3-3ζ in breast cancer cells promotes TGF-β-induced breast cancer cell proliferation and osteoclast maturation, thereby enhancing TGF-β-induced osteolysis, breast cancer outgrowth, and bone metastasis.

Several of TGF- β 's downstream targets, including PTHrP, IL-11, and Jagged-1, can promote breast cancer metastasis to, and survival in, the bone microenvironment (Kang et al., 2003; Sethi et al., 2011; Waning and Guise, 2014). To identify which TGF- β downstream target(s) mediates 14-3-3 ζ -driven bone metastasis promotion in vivo, we measured PTHrP, IL-11, and Jagged-1 protein levels in the bone lesions. We found that only the expression of PTHrP, and not IL-11 or Jagged-1, was reduced in 1566.sh ζ and 231.sh ζ bone metastases compared

⁽D) Significance score distribution of 14-3-3 ζ -induced gene signature from MCF10A cells in metastases of breast cancer at different organs. Red dashed line indicates the significance level of p = 0.0001.

⁽E) Kaplan-Meier curves for bone metastasis-free survival according to 14-3-3^c expression level in a subset of 253 breast tumors in the EMC-286 cohort. p value was calculated based on log rank test.

⁽F) Gene expression heatmap of 34 TGF- β -induced genes inhibited by 14-3-3 ζ knockdown.

⁽G) Significance score distribution of TGF-β-induced bone metastasis genes (10 of the genes identified in Figure 3F) inhibited by 14-3-3ζ knockdown in metastases of indicated organs (accession number GSE14020).

Box plots show the distribution of scores. The thick line in the middle of the box shows median value. The top and bottom of the box represent upper (third) and lower (first) quartile values, respectively, and the top and bottom of the line show maximum and minimum values, respectively. See also Figure S3.



Figure 4. 14-3-3ζ Promotes TGF-β-Induced Breast Cancer Bone Metastasis by Activating PTHrP

(A) Kaplan-Meier survival analysis of mice injected intracardially with 1 \times 10⁵ 1566.shCtrl or 1566.sh ζ cells.

(B) Representative BLI, X-ray, and H&E images of bone metastatic lesions of mice in (A) at the indicated time. 14-3-3ζ expression level was shown by IHC staining. Arrows indicate osteolytic bone lesions. Abbreviations: T, tumor; B, bone tissue; BLI, bioluminescence imaging. Scale bars represent 50 μm. (C) Kaplan-Meier survival analysis of mice injected intratibially with 231.shCtrl or 231.shζ cells.

(D) Representative BLI, X-ray, and H&E images of bone lesions (day 49) of mice in (C). 14-3-3² expression level was shown by IHC staining. Arrows indicate osteolytic bone lesions. Abbreviations: T, tumor; B, bone tissue. Scale bars represent 50 µm.

to 1566.shCtrl and 231.shCtrl metastases, respectively (Figures 4H, 4I, S4J, and S4K). Similarly, PTHrP expression was also low in 4T1.sh ζ bone lesions compared to 4T1.shCtrl bone metastases (Figures S4L and S4M). Furthermore, TGF- β -induced PTHrP mRNA expression was inhibited by 14-3-3 ζ knockdown in 1566.sh ζ , 231.sh ζ , and 4T1.sh ζ cells (Figures 4J, S4N, and S4O), indicating that 14-3-3 ζ contributes to TGF- β -induced PTHrP mRNA expression in breast cancer cells.

14-3-3ζ Enhances TGF-β-Induced PTHrP mRNA Expression by Stabilizing Gli2

PTHrP is a key mediator of TGF-_β's bone metastasis program, and TGF-^β has been reported to induce PTHrP mRNA expression primarily through canonical Smad signaling (Kakonen et al., 2002). Smads are known to transcriptionally upregulate Gli2, which in turn transactivates PTHrP mRNA expression (Dennler et al., 2007). This TGF-β/Smad/Gli2 signaling axis was shown to be important for PTHrP mRNA expression and bone metastasis (Javelaud et al., 2011). To investigate how 14-3-3 contributes to TGF- β -induced PTHrP mRNA expression, we first examined whether 14-3-3ζ affects Smad2/3 or Gli2 expression and Smad2/3 phosphorylation. Compared to 1566.shCtrl, 231.shCtrl, or 4T1.shCtrl cells, silencing of 14-3-3ζ significantly decreased Gli2 protein level in vivo and in vitro without altering Smad2/3 expression or phosphorylation in 1566.sht, 231.sht, and 4T1.shζ cells (Figures 5A-5C and S5A-S5E). Furthermore, overexpression of 14-3-3 ζ in 10A. ζ , 12A. ζ , and MCF7. ζ cells increased Gli2 protein levels to turn on TGF-B's bone metastasis program (upregulation of PTHrP) while concomitantly turning off TGF- β 's cytostatic program (downregulation of 14-3-3 σ , p53, and p21), compared to 10A.Vec, 12A.Vec, and MCF7.Vec cells (Figures S5F and S5G). Unexpectedly, Gli2 mRNA levels were similar between control and 14-3-3 knockdown cells with or without TGF-β treatment (Figures S5H–S5J), indicating that the TGF-β-induced increase of Gli2 protein in 14-3-3ζ overexpression cells was independent of the previously reported TGF-B/ Smad-mediated Gli2 transcription (Dennler et al., 2007). At the protein level, 14-3-3^{\zeta} knockdown did not affect Gli2 translation, but reduced protein stability by increasing proteasome-mediated degradation (Figures 5D and S5K-S5M). Compared to 231.shCtrl or 4T1.shCtrl cells, Gli2 ubiquitination was increased in 231.sh ζ or 4T1.sh ζ cells with 14-3-3 ζ knockdown under TGF- β treatment (Figures 5E and S5N-S5Q). To further dissect the mechanism of 14-3-3ζ-mediated inhibition of Gli2 ubiquitination, we compared the binding of Gli2 to its E3 ligase, β-TrCP, in TGFβ-treated 231.shCtrl and 231.shζ cells (Bhatia et al., 2006). In 231.shCtrl cells, both endogenous Gli2 and ectopically expressed Myc-tagged Gli2 bound robustly to 14-3-3 c but scarcely to β-TrCP; conversely, Gli2 binding to β-TrCP was evident in 231.shζ cells, wherein Gli2 could not bind to 14-3-3ζ (Figures 5F and S5R). Additionally, GST-tagged 14-3-3 ζ proteins efficiently competed with β -TrCP for binding to Gli2 (Figure 5G), indicating that 14-3-3 ζ blocked Gli2 binding to β -TrCP, thereby increasing Gli2 stability. Moreover, the MG132 treatment that stabilized Gli2 in 231.sh ζ also rescued TGF- β -induced PTHrP mRNA expression (Figure 5H), indicating that Gli2 stabilization by 14-3-3 ζ enhances TGF- β -induced PTHrP mRNA expression.

These unexpected findings of 14-3-3 ζ -induced Gli2 protein stabilization in response to TGF- β differ from previously reported TGF- β /Smad-induced Gli2 transactivation (Dennler et al., 2007). To gain insight on how TGF- β -activated Smads cooperate with 14-3-3 ζ -stabilized Gli2 to transactivate PTHrP in MDA-MB-231 cells, we tested whether Gli2 functioned as a Smad binding partner in the same transcriptional complex. Obviously, TGF- β treatment induced Gli2 binding to Smads in 231.shCtrl, but not in 231.sh ζ cells (Figures 5I, S5S, and S5T), indicating that Gli2, stabilized by 14-3-3 ζ , is a decisive contextual determinant of Smads for transactivation of PTHrP expression in breast cancer cells.

14-3-3 ζ Overexpression Is Associated with the Loss of TGF- β Tumor Suppressor Function and Gain of Its Metastasis Promoter Functions in Patients

Our result so far indicate that 14-3-3ζ blocks TGF-β's tumor suppressor function by reducing 14-3-3 σ and p21 but switches on TGF-B's metastasis-promoting function by stabilizing Gli2 in breast cancer. To determine the clinical relevance and validity of our findings, we examined the expression of key components of the TGF- β signaling program (Smads, YAP1, 14-3-3 σ , p21, and Gli2) and their relationship with 14-3-3 in patient-derived tissue samples. We performed IHC analysis on different stages (40 cases each) of breast disease and cancer, including atypical ductal hyperplasia (ADH), ductal carcinoma in situ (DCIS), and invasive ductal carcinoma (IDC), with normal breast tissue as controls. TGF- β downstream targets p-Smad2 and p-Smad3 were positive in normal tissues and ADH, a very early stage of breast disease, and significantly increased at the later stages of DCIS and IDC; at the same time, total Smad2/3 levels were positive but constant (Figures S6A-S6D). Together, these findings indicated that TGF- β signaling was functional and further activated during breast cancer initiation and progression. Notably, increased p-Smad2 and p-Smad3 levels significantly correlated with 14-3-3^c overexpression, which started at ADH and increased at DCIS and IDC (Figures 6A, S6E, and S6F), indicating that 14-3-3 ζ enhanced TGF- β /Smad signaling as we previously reported (Lu et al., 2009).

We also found that YAP1 expression and nuclear localization were significantly decreased relative to normal breast tissues starting from ADH stage (Figures 6A, S6G, and S6H), which is consistent with previous reports (Tufail et al., 2012; Yuan et al., 2008). Moreover, 14-3-3 σ and p21 expression decreased

(J) qRT-PCR analysis of PTHrP mRNA in 231.shCtrl or 231.shζ cells.

⁽E and F) Representative staining images (E) and quantification (F, three repeats) of TRAP⁺ mature osteoclasts culture with MC3T3 osteoblasts and 231 breast cancer cells treated with vehicle or TGF-β. The arrows indicate TRAP⁺ mature osteoclasts. Scale bars represent 200 µm.

⁽G) Quantification of indicated GFP-labeled cancer cells grown under triple coculture. Cells were trypsinized from each triple coculture group and recultured in 10 cm plates for counting. TGF-β treatment (5 ng/ml, 6 days).

⁽H and I) Representative IHC images (H) and quantification (I) of PTHrP protein expression in mouse bone lesions. Note that four mice in the 1566.sh² group and five mice in the 231.sh² group had no bone lesions and were excluded from PTHrP protein analysis. Scale bars represent 50 µm.

Error bars represent SD, *p < 0.05, **p < 0.01, ***p < 0.001. See also Figure S4.



Figure 5. 14-3-3ζ Enhances TGF-β-Induced PTHrP mRNA Expression by Stabilization of Gli2

(A and B) Representative IHC images (A) and quantification (B) of Gli2 expression in the indicated mouse bone lesions. Scale bar represents 50 µm.

(C) IB analysis of Gli2 expression in 231.shCtrl and 231.sh ζ (-4 and -5) cells treated by vehicle or TGF- β (5 ng/ml).

(D) IB analysis of Gli2 protein in 231.shCtrl and 231.sh ζ cells treated with TGF- β (5 ng/ml) and MG132 (10 μ M) for indicated times.

(E) IP of Myc-Gli2 and IB analysis of Gli2 ubiquitination in indicated cells treated with TGF-β (5 ng/ml) and MG132 (10 μM).

(F) IP of Myc-Gli2 and IB analysis of indicated proteins in cells treated with TGF- β (5 ng/ml) and MG132 (10 μ M).

(G) IP of Myc-Gli2 and IB analysis of indicated proteins. 231.sh ζ cells were transfected with Myc-Gli2 and treated with TGF- β (5 ng/ml) and MG132 (10 μ M). Cell lysates were incubated with or without GST (1 μ M) or GST-14-3-3 ζ (200 nM or 1 μ M) overnight.

(H) qRT-PCR analysis of PTHrP mRNA level in indicated cells with indicated treatment.

(I) IP of Smad2 and IB analysis of indicated proteins in indicated cells.

IP Ab, antibodies used for IP. Error bars represent SD, *p < 0.05, **p < 0.01, ***p < 0.001. See also Figure S5.

significantly starting at ADH stage and nuclear YAP1, 14-3-3 σ , and p21 levels inversely correlated with 14-3-3 ζ overexpression during breast cancer progression (Figures 6A–6C and 6E). In contrast, Gli2 was significantly increased starting at the ADH stage and correlated with 14-3-3 ζ overexpression at IDC stage (Figures 6A, 6D, and 6E).

DISCUSSION

TGF-β's complicated biological responses have been proposed to be governed by the different cellular contextual determinants of Smads (Massagué, 2012), including a wide-ranging complement of DNA-binding transcription factors, involving p53 and

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members of the bHLH, Forkhead box (e.g., Foxo3a), and zinc finger protein families (e.g., Gli2) in different cells and various biological processes (Cordenonsi et al., 2003; Feng and Derynck, 2005). However, the specific Smad partners determining TGFβ's tumor suppressor versus metastasis promoter functions were elusive, and the factors triggering the contextual changes in Smad determinants are also not clear. Here, we unveiled that (1) p53 is the critical Smad partner for TGF-β-induced p21 expression and tumor suppression in premalignant HMECs, whereas Gli2 is the decisive Smad partner for TGF-β-induced PTHrP expression and metastasis promotion in breast cancer cells, and (2) 14-3-3 ζ switches TGF- β 's function by triggering the contextual change of Smad partners from p53 in premalignant HMECs to Gli2 in breast cancer. Mechanistically, 14-3-32 overexpression destabilizes p53 by cytoplasmic sequestration of YAP1, leading to reduced transcription of 14-3-3 σ , a tumor suppressor that stabilizes p53 protein, while 14-3-3 stabilizes Gli2 by blocking Gli2's binding with its E3 ligase β-TrCP (Figure 7). Therefore, our study provided answers to the long-standing questions of how and when TGF-ß switches its functional roles from a tumor suppressor to a metastasis promoter. Moreover, TGF-^β has multifunctional roles in numerous biological processes. Our study may facilitate more discoveries on cellular events that switch the contextual determinants of Smads, which will enable comprehensive understanding of TGF-B's multifunctional and even opposing roles not only in cancer development but also in TGF-β's various general biological and pathological responses.

Previously, it was reported that 4 hr of TGF- β treatment induced Gli2 mRNA transcription in MDA-MB-231 cells, which subsequently transactivated PTHrP (Dennler et al., 2007). Here, we found that TGF- β induced an immediate (in < 2 hr) increase of Gli2 protein via 14-3-3ζ-mediated Gli2 stabilization, indicating that TGF-β induces Gli2 expression at multiple levels. In addition, we found that TGF- β -activated Smads can directly bind to Gli2 protein to upregulate PTHrP transcription. Thus, TGF-B-activated Smads can induce PTHrP expression both indirectly via transactivating Gli2, which in turn induces PTHrP transcription, and directly by binding to Gli2 to turn on PTHrP transcription, which is empowered by 14-3-3ζ. Interestingly, 14-3-3ζ was reported to bind to and stabilize β -catenin in the cytoplasm in response to Wnt signaling (Tian et al., 2004), suggesting that 14-3-3ζ may regulate multiple other signaling pathways by a similar mode of binding to and stabilizing downstream key regulators of these pathways.

The 14-3-3 proteins are a family of evolutionarily conserved and ubiquitously expressed proteins in eukaryotes with seven mammalian isoforms: β , γ , ε , η , σ , τ , and ζ . These 29-31 kDa acidic proteins form heterodimers or homodimers to bind to specific motifs on target proteins in a phosphorylation-dependent manner, subsequently altering their subcellular localization, stability, enzymatic activity, etc. As a result, 14-3-3 proteins are involved in many important cellular processes (Reinhardt and Yaffe, 2013). Here, we found that 14-3-3 σ overexpression led to transcriptional repression of 14-3-3 σ . Although in the same family, 14-3-3 ζ and 14-3-3 σ have opposite roles in several biological functions, including PI3K-Akt signaling, p53 stability, cell polarity, and invasion (Danes et al., 2008; Ling et al., 2010; Lu et al., 2009; Neal et al., 2012). Among the 14-3-3 family members, 14-3-3 σ is known as a tumor suppressor gene, which is lost in multiple types of cancer (Akahira et al., 2004). The unique biology of 14-3-3 σ may be explained by its different structure from the rest of 14-3-3 family members (Reinhardt and Yaffe, 2013; Wilker et al., 2005). An imbalance between 14-3-3 σ and other 14-3-3 family members may result in cancer or other disorders. However, the mechanism underlying the imbalance between 14-3-3 σ and other 14-3-3 σ and other

14-3-3 σ and 14-3-3 ζ have opposing functions on p53. 14-3-3 σ can interact with and stabilize p53 by blocking Mdm2-mediated p53 ubiquitination and nuclear export (Yang et al., 2003, 2007). We found that 14-3-3 ζ can destabilize p53 protein partially through PI3K-Akt activation of Mdm2 (Danes et al., 2008) and by downregualtion of 14-3-3 σ . As restoration of 14-3-3 σ fully recovered p53 expression in 10A. ζ cells, 14-3-3 σ is likely a major regulator of p53 stability, while 14-3-3 ζ destabilizes p53 primarily through downregulating 14-3-3 σ .

The Hippo-YAP pathway plays very important roles in controlling organ size and tumorigenesis, and activation of the Hippo pathway leads to phosphorylation and inhibition of transcriptional coactivators, including YAP (Harvey et al., 2013). There have been controversial reports on the role of YAP1 in cancer development (Bertini et al., 2009; Luk and Guan, 2014; Zhao et al., 2011). Initially, the YAP1 gene was found to be amplified in human hepatocellular carcinomas and induce transformation (Zender et al., 2006). Recently, however, YAP1 was defined as an apoptosis-inducing tumor suppressor that is silenced in multiple types of cancer (Bai et al., 2013; Barry et al., 2013; Cottini et al., 2014; Lapi et al., 2008; Yu et al., 2013). Moreover, mammary epithelia-specific hyperactivation of YAP1 in transgenic mice did not increase mammary cell proliferation or induce tumors (Chen et al., 2014), indicating that YAP1 does not have an oncogenic function in mammary glands. Here, we found that nuclear YAP1 transactivates the 14-3-3 σ tumor suppressor gene in HMECs. Thus, 14-3-3 σ is a target gene of YAP1 and mediates YAP1 tumor suppressor function in HMECs. As a transcriptional coactivator, YAP1 has oncogenic functions when it binds to TEA domain family transcription factors (Harvey et al., 2013), while it functions as a tumor suppressor when bound to p73 or p53BP2 (Basu et al., 2003; Cottini et al., 2014; Fausti et al., 2013). In future studies, it will be interesting to identify the partner transcription factors of YAP1 for 14-3-3 o transactivation and to test the functional role of cytosolic YAP1 retained by 14-3-3ζ in HMECs.

YAP1 nuclear localization was inhibited by 14-3-3 ζ both in tissue samples and in cell models. Previously, it was found that YAP1 expression is downregulated in breast cancers compared to normal breast tissues (Tufail et al., 2012; Yuan et al., 2008). Here, we also found that YAP1 protein levels in breast tissues were decreased as cancer progressed and 14-3-3 ζ expression increased. However, YAP1 protein levels in 10A. ζ and 12A. ζ cells were similar to that in their control cells. This suggested that other 14-3-3 ζ -independent mechanisms may regulate YAP1 protein stability (Lapi et al., 2008) or YAP1 protein translation



Е

		ADH				DCIS			IDC			
		14-3-3ζ expression				14-3-3ζ expression			14-3-3ζ expression			
Markers		Low(%)	High(%) p	Value Pe	arson's R	Low(%)	High(%) p	Value Pearson's F	R Low(%)	High(%) p	Value Pea	rson's R
Nuclear YAP1	Low High	17(34.7%) 32(65.3%)	16(80%) 4(20%)	p=0.001	R=-0.41	21(47.7%) 23(52.3%)	26(89.7%) 3(10.3%)	p<0.001 R=-0.43	16(41%) 23(59%)) 38(84.4%)) 7(15.6%)	p<0.001	R=-0.45
14-3-3σ	Low High	9(18.8%) 39(81.2%)	13(65%) 7(35%)	p<0.001	R=-0.45	5(11.4%) 39(88.6%)	16(51.6%) 15(48.4%)	p<0.001 R=-0.44	5(12.5%) 35(87.5%)) 30(68.2%)) 14(31.8%)	p<0.001	R=-0.56
p21	Low High	15(32.6%) 31(67.4%)	18(94.7%) 1(5.3%)	p<0.001	R=-0.57	12(28.6%) 30(71.4%)	30(93.8%) 2(6.2%)	p<0.001 R=-0.65	8(21.1%) 30(78.9%)) 36(94.7%)) 2(5.3%)	p<0.001	R=-0.75
Gli2	Low High								23(62.2%) 14(37.8%)) 15(38.5%)) 24(61.5%)	p=0.039	R=0.24



Figure 7. Diagram of 14-3-3ζ-Driven Contextual Changes of Smad Partners from p53 in Premalignant Cells to Gli2 in Cancer Cells

In premalignant human MECs, YAP1 is a critical transcription coactivator for 14-3-3 σ , and 14-3-3 σ stabilizes p53, a Smad partner for transactivation of p21, which is the key executor of TGF-\beta's cytostatic and tumor suppression program (left). In breast cancer cells, Gli2 is a Smad partner for transactivation of PTHrP, which is a key facilitator of TGF-B's bone osteolysis and bone metastasis program (right). 14-3-3ζ overexpression in premalignant cells turns off TGF-B's cvtostatic tumor suppressor function by binding to YAP1 and sequestering YAP1 in the cytosol, thereby blocking YAP1-induced transactivation of 14-3-3 or: conversely, 14-3-3ζ overexpression in breast cancer cells turns on TGF- β 's metastasis promoter function by blocking $\beta\text{-TrCP}$ binding to, and ubiquitination of, Gli2, thus stabilizing Gli2. Together, 14-3-3^{\zet} serves as a molecular switch turning TGF- β from a tumor suppressor to a metastasis promoter by altering cellular contextual determinants of Smads. Solid lines represent findings described in this study, and dashed lines represent previously described links.

Current therapies targeting the TGF- β pathway are mainly focused on targeting TGF- β ligands and/or receptors, e.g., TGF- β -neutralizing antibodies, TGF- β receptor kinase inhibitor, and TGF- β ligand

(Yu et al., 2013) in human breast cancer in vivo that were not recapitulated by 14-3-3 ζ overexpression cell models in vitro.

The crucial role of TGF- β in cancer and other diseases has motivated numerous efforts on developing therapeutics targeting the TGF- β pathway, some of which have been tested in the clinic (Akhurst and Hata, 2012). In phase I and II trials for treating cancers that generally overexpress TGF- β , some patients with latestage cancers received a marginal benefit from TGF- β inhibitors (Bogdahn et al., 2011). A major obstacle for effective therapies targeting the TGF- β pathway has been the complex nature and opposing roles of TGF-B in different stages of cancer development. Thus, understanding the molecular mechanism by which TGF- β switches its role could guide more effective TGF- β -targeting strategies. In this regard, our findings (Figure 7) suggest that the TGF- β functional switch by 14-3-3 ζ and the contextual changes of Smad partners (p53 and Gli2) could be utilized as biomarkers to aid in selecting appropriate cancer patients who will benefit from TGF- β antagonists and determining optimal treatment timing when TGF- β -targeted therapy inhibits cancer.

trap (Akhurst and Hata, 2012). These therapies downmodulate excessive levels of TGF- $\!\beta$ ligands or block the entire TGF- $\!\beta$ signaling cascade. However, a systemic application of anti-TGFβ therapies may bring significant side effects, such as widespread inflammation (Shull et al., 1992), autoimmunity, or cardiovascular defects (Larsson et al., 2001), because TGF-B plays important roles in various physiological functions. Thus, more specific drugs that selectively target the downstream signaling in cancer cells without compromising other systemic homeostatic functions of TGF-B would be superior. Our findings in this study suggest targeting TGF-β's functional switch or the downstream cancer-specific Smad partners (e.g., Gli2) may be better alternatives to targeting TGF-β ligand and/or receptor for cancer treatment. This will inspire the development of drugs to selectively target the TGF- β signaling in cancer and metastasis without impeding TGF-ß physiological functions in normal tissues. Taken together, our findings may steer biomarker-guided selection of patients who will most likely respond to second generation selective therapies to effectively target TGF- β signaling in the era of personalized medicine.

(B–D) Percentage of 14-3-3 σ (B), p21 (C), and Gli2 (D) IHC score distribution in the indicated breast tissues. Unpaired t test with Welch's correction was used to compare IHC score in ADH, DCIS, and IDC to normal tissue. *p < 0.05, **p < 0.01, ***p < 0.001, by two-tailed t test.

Figure 6. High 14-3-3ζ Is Associated with TGF-β's Functional Switch during Breast Cancer Development

⁽A) Representative IHC staining of 14-3-3ζ, YAP1, 14-3-3σ, p21, and Gli2 in normal breast tissue, ADH, DCIS, and IDC. Scale bars represent 50 µm.

⁽E) The association between 14-3-3ζ expression and the expression level of nuclear YAP1, 14-3-3σ, p21, and Gli2 in ADH, DCIS, and IDC compared to normal tissue. Number of cases and percent of positive staining in the corresponding group (%) are shown in the table. p values were determined by chi-square analysis. See also Figure S6.

EXPERIMENTAL PROCEDURES

Antibodies and reagents, immunoblotting, cell proliferation assay and BrdU incorporation assay, immunoprecipitation assay, immunofluorescence staining, cytoplasm and nuclear protein fractionation, RPPA, cell cycle analysis, mRNA stability assay, bisulfite genomic sequencing, 5-Aza-2'-deoxycytidine treatment, luciferase reporter assay, chromatin immunoprecipitation (ChIP) assay, generation of knockdown and overexpression cells, cDNA micro-array analysis, significance score distribution analysis, expression heatmap, clinical samples and bioinformatics analysis, RNA extraction, RT-PCR, triple coculture assay, ubiquitination assay, immunohistochemical staining, and statistics are described in detail in the Supplemental Experimental Procedures.

Cell Line and Cell Culture

MCF10A and MDA-MB-231 cell lines and their modified variants were obtained, generated, and cultured as previously described (Khotskaya et al., 2014; Li et al., 2009; Lu et al., 2009). The 231-1566 cell line is clone 1566 of the MDA-231-LUC-Met cell line, which was obtained from Dr. Mien-Chie Hung (Khotskaya et al., 2014). The BoM-1833 cell line was obtained from Dr. Xiang H.-F. Zhang (Zhang et al., 2009). The MCF12A cell line was obtained from American Type Culture Collection (ATCC) and cultured in MCF10A medium. The 4T1 cell line was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4.5 g/l glucose, L-glutamine, sodium pyruvate, and 10% FBS. The murine osteoblast cell line MC3T3 was obtained from Dr. Sue-Hwa Lin (MD Anderson Cancer Center) and maintained in a MEM medium with 10% FBS. The murine preosteoclast cell line RAW 264.7 from ATCC was maintained in DMEM with 10% FBS for regular culture and supplemented with 30 ng/ml RANKL for osteoclastogenesis assay. For in vitro assays on TGF- β functions, cells were all treated with fresh medium containing TGF- β (+) or vehicle (-) for 2 or 4 hr after removal of the original culture medium.

Bone Metastasis Assay, Bone Tumor Grafts, and Bioluminescence Analysis

For bone metastasis studies, two different routes of injection were used. For the intracardiac injection model, $1 \times 10^5 231-1566$ or 5×10^4 BoM-1833 cells were injected into the left ventricle of anesthetized female athymic Ncr-nu/nu mice. For the intratibial injection model, 2×10^5 MDA-MB-231 cells were injected into the tibia of anesthetized female athymic Ncr-nu/nu mice, or 1×10^3 4T1 sublines were injected into the tibia of anesthetized female athymic Ncr-nu/nu mice, or 1×10^3 4T1 sublines were injected into the tibia of anesthetized female BALB/c mice. Development of bone metastases was monitored by bioluminescence imaging (BLI). Anesthetized mice were intraperitoneally injected with 75 mg/kg D-luciferin. Bioluminescence images were acquired with a Xenogen IVIS 200 imaging system. Analysis was performed with live imaging software by measuring photon flux in the hindlimbs of mice. Data were normalized to the signal on day 4. Bone metastasis-free survival curves represent the time point at which each mouse developed bone metastasis by threshold BLI signals in the hind limbs. All mice used were of the same age and similar body weight.

X-Ray Analysis and Quantification

Osteolysis was assessed by X-ray radiography. Anesthetized mice were exposed to X-ray at 40 mm for 1 min and were directly imaged by Carestream Molecular Imaging software using the Kodak In-Vivo Multispectral Imaging System. Osteolytic lesions were identified on radiographs as demarcated radiolucent lesions in the bone and quantified using ImageJ software (NIH).

Hematoxylin and Eosin and Immunohistochemical Staining

Hindlimb bones were excised from mice at the end point of each experiment, fixed in 10% neutral-buffered formalin, decalcified in 10% EDTA for 2 weeks, and embedded in paraffin for H&E and immunohistochemical staining. Histo-morphometric analysis was performed on H&E-stained bone metastasis samples using the Zeiss Axiovert 200 microscope and AxioVision software version 4.6.3 SP1. Immunohistochemical analysis was performed with heat-induced antigen retrieval. Primary antibodies used are indicated in the table of the anti-body list. Biotinylated secondary antibody was used with Vectastain ABC Kit (Vector Laboratories) and DAB detection kit (Zymed) to reveal the positively stained cells with nuclei counterstained with hematoxylin.

Tissue Specimens

Tissue specimens representing 160 cases were collected at The University of Texas MD Anderson Cancer Center (UTMDACC) and Southern Medical University (Guangzhou, China), including normal breast tissue, ADH, DCIS, and IDC (40 cases each). Patient samples were collected and processed in compliance with protocols (LAB10-0995) approved by the UTMDACC Institutional Review Board and by the Southern Medical University Institutional Review Board. We used archived samples. At time of tissue collection, informed consent was obtained from all patients.

Animal Experiment

All procedures involving mice and experimental protocols (ACUF ID #07-04-03437, and ACUF ID #02-12-02331) were approved by the Institutional Animal Care and Use Committee (IACUC) of University of Texas MD Anderson Cancer Center.

ACCESSION NUMBERS

The raw and normalized microarray data have been deposited in the GEO database under accession numbers GSE52032 and GSE52066.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at http://dx.doi.org/ 10.1016/j.ccell.2014.11.025.

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