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Original Article

14-3-3ζ loss impedes oncogene-induced mammary tumorigenesis and metastasis by attenuating oncogenic signaling

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Abstract: The 14-3-3ζ protein belongs to the 14-3-3 family of regulatory eukaryotic proteins that modulate signaling by binding to wide variety of signaling molecules. 14-3-3ζ expression is amplified in over 40% breast cancer patients and is associated with a poor prognosis. Various *in vitro* and xenograft models have suggested that attenuating 14-3-3ζ expression may provide therapeutic benefits but there has been no study looking at tumor onset and metastasis in breast cancer mouse models with a targeted deletion of 14-3-3ζ. We generated a 14-3-3ζ knockout mouse model to characterize the role of 14-3-3ζ in breast cancer progression. Crossing 14-3-3ζ-/- mice with MMTV-PyMT and MMTV-Neu transgenic mice revealed that loss of 14-3-3ζ prolonged tumor latency and reduced lung metastasis as compared to MMTV-PyMT and MMTV-Neu mice. Mechanistically, loss of 14-3-3ζ suppressed tumor proliferation and angiogenesis and promoted apoptosis by suppressing the Akt and Erk pathway and upregulated the expression of the tumor suppressor p53. Our results provide evidence showing that attenuating 14-3-3ζ expression/activity in mammary tumors can provide a therapeutic benefit.

Keywords: 14-3-3ζ, mammary tumors, metastasis, oncogenic signaling

Introduction

The 14-3-3 family of proteins is ubiquitously expressed in eukaryotes and includes seven isoforms in mammals (β, τ, ε, ζ, η, γ and σ). They play an important role in regulating diverse biological processes by binding to target proteins with specific phospho-serine/threonine containing motifs and thereby regulating the conformation, enzymatic activity, protein stability and subcellular localization of the target proteins [1]. 14-3-3 proteins are known to interact with multiple proteins involved in diverse biological processes such as the regulation of cell cycle, DNA damage repair, cell proliferation, cell polarity, programmed cell death and cell metabolism [2-6]. Despite the high homology in the protein sequence as well as protein structure [7], distinct 14-3-3 isoforms exhibit unique protein binding specificities [8]. Knockout mouse models of different 14-3-3 family members have revealed distinctive biological functions for the different isoforms [1, 9, 10].

We previously demonstrated that 14-3-3ζ is overexpressed in greater than 40% of breast cancer patients and is a strong indicator of poor prognosis [11]. Enhanced expression of 14-3-3ζ in breast cancer contributes to tumor initiation and progression by downregulating p53 [12], activating the phosphoinositide 3-kinase (PI3K) pathway [13], co-operates with ErbB2 in promoting epithelial to mesenchymal transition (EMT) by activation of the TGFβ/SMAD pathway [14], and promotes the metastasis promoting activity of TGF-β [15]. Despite the importance of 14-3-3ζ in human malignancies, the studies on 14-3-3ζ functions have been primarily performed using cell line models or have been limited to correlative studies in human tissues.

Systematic in-depth analysis of 14-3-3ζ function *in vivo* will significantly enhance our knowledge regarding the functional mechanisms of 14-3-3ζ in various diseases and provide new opportunities to develop more effective therapeutic approaches.

We generated 14-3-3ζ hypomorpohic mice using a gene trap approach in the FVB background. A detailed description and characterization of the mice will be reported elsewhere (JY, SJ, DY: unpublished). The mice were then crossed with transgenic mouse strains of mouse mammary tumor virus long terminal repeat (MMTV-LTR) driven Polyoma middle T antigen (MMTV-PyMT) or activated Neu oncogene (MMTV-Neu). The loss of 14-3-3ζ prolonged tumor latencies and attenuated lung metastases compared with their wild-type counterparts. The mammary tumors formed from the 14-3-3ζ knockout mice exhibited reduced cell proliferation, increased apoptosis and decreased angiogenesis. Reverse Phase Protein Array (RPPA) analysis showed that loss of 14-3-3ζ lead to attenuated activation of the PI-3K (phosphoinositol-3-kinase)/Akt and ERK (Extracellular regulated kinase) kinase pathways. The critical role of 14-3-3ζ in tumor progression indicates that 14-3-3ζ may serve as a potential anti-cancer therapeutic target.

Materials and methods

Maintenance and generation of mice

14-3-3ζ-/- mice were generated using a gene trap approach on the 129Ola/B6 hybrid background. The heterozygous mice were then backcrossed into a FVB/NJ congenic background as determined by genome scan using a panel of simple sequence length polymorphism (SSLP) (microsatellite) markers and maintained thereafter. The 14-3-3ζ mice on the FVB/NJ background were crossed with the MMTV-PyMT and MMTV-Neu mice and maintained thereafter. The MMTV-PyMT mice and the FVB/NJ mice were obtained from the Jackson Laboratory (Bar Harbor, ME). MMTV-Neu transgenic mice have been previously described [16]. All animal work was performed under an IACUC-approved protocol. University of Texas MD Anderson Cancer Center is an AAALAC accredited institution.

Mammary gland whole mount staining

The number 4 mammary glands on the left side of the mice were spread onto microscopic slides, fixed in Carnoy's fixative overnight, hydrated, and stained with carmine alum stain (Sigma, St. Louis, Mo) overnight; they were then dehydrated through sequential ethanol, treated with xylene to remove fat, and mounted with Secure Mount (Fisher Scientific, Pittsburgh, PA) and cover slips. Mammary gland tissue samples were collected during all phases of the estrous cycle. The samples were imaged using a Zeiss Discovery V20 dissection microscope and Axiom imaging software supplied the microscope.

Tissue collection and histological analysis

Mammary tumor formation was monitored by palpation twice a week. Upon formation of a palpable tumor, the mice were observed for 3-4 weeks for tumor progression. When the tumor diameter reached 15 mm, the mammary tumors and lungs were harvested. Tissues were fixed in 10% Neutral buffered formalin for 12-18 h. The samples were stored in 70% ethanol and then embedded in paraffin. Paraffin sections (5 μm) were stained with hematoxylin and eosin. Mammary tumor histology and lung metastasis were independently evaluated by two pathologists (Y.X., W.H. and Q.Z.). Immunohistochemistry (IHC) was performed as previously described [14]. Antibodies used were Ki67 (DAKO, Carpentaria, CA M7249), TUNEL (Roche, Indianapolis, IN), CD34 (eBioscience, San Diego, CA 14-0341), 14-3-3ζ (C-16, Santa Cruz, Santa Cruz, CA sc-1019). For IHC analysis and quantification, 10 fields were randomly chosen at 200× magnification. The total number of cells and positive cells were counted, and the average percentage of positive cells was determined.

Immunoblotting

Tissues and mammary tumors were collected from the mice. Protein extracts were prepared by homogenizing samples in tissue lysis PBSTDS buffer (10 mmol/L sodium phosphate (pH 7.3), 154 mmol/L NaCl, 5% sodium deoxycholate, 1% SDS) using a tissue grinder, followed by centrifugation to remove particulate matter and lipids. Immunoblotting was performed as previously described (Lu et al., 2009). Antibodies used were anti-HA high affinity (clone 3F10, Roche 11867423001), 14-3-3ζ (C-16, Santa Cruz sc-1019), Neu (C-18, Santa Cruz sc-284), Erk (Cell Signaling 4695), phosphor-Erk (T202/Y204, Cell Signaling 4370),

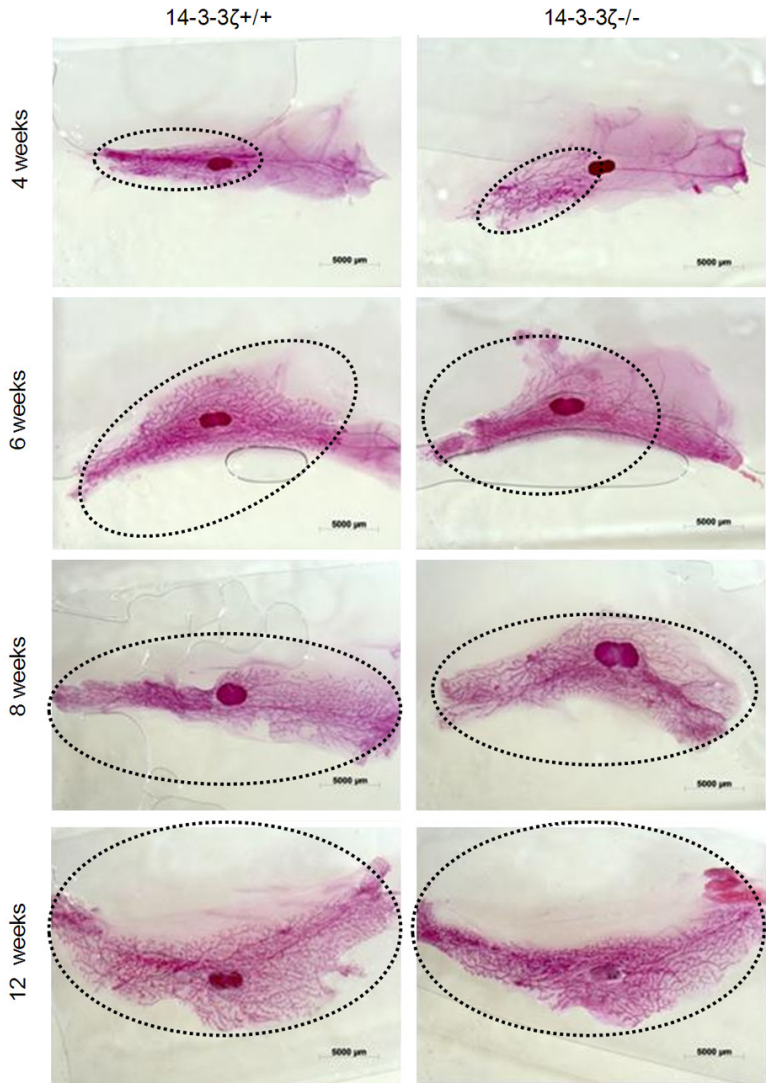

Figure 1. Loss of 14-3-3ζ attenuates early mammary gland development. Whole-mount staining of mammary glands dissected at 4, 6, 8, and 12 weeks from 14-3-3ζ wild-type (+/+, left panel) and homozygous mutant (-/-, right panel) mice.

Mek1 (Cell Signaling 9126), phosphor-Mek1 (S221, Cell Signaling 2338), Akt (Cell Signaling 9272), phosphor-Akt (S473, Cell Signaling 3787), p53 (DO-1, Santa Cruz sc-126), Bax (B-9, Santa Cruz sc-7480), VEGF (A-20, Santa Cruz sc-152), PyMT(Novus Biologicals NB100-2749), β-actin (Sigma A5441), and tubulin (Sigma T5168). The antibodies were mainly used at a 1:1000 dilution.

Assessment of proliferation, apoptosis, and angiogenesis

To assess the proliferation rate or apoptosis, 1,000 tumor cells were counted to determine the percentage of Ki67 or TUNEL positive-staining cells by investigators blinded to the identity of the mice. Angiogenesis was evaluated by CD-34 IHC staining and by counting blood vessels in three areas of the section at 200× magnification. The blood vessel index was expressed as the mean number of vessels in the three areas.

Reverse-phase protein array (RPPA)

RPPA of MMTV-PyMT 14-3-3ζ+/+ and MMTV-PyMT 14-3-3ζ-/- mammary tumors was performed in the MDACC Functional Proteomics core facility as previously described [17].

Statistical analyses

Analysis of mammary tumor latency was performed using the Kaplan-Meier method while the differences in survival were determined using log-rank test. Other statistical differences were assessed with Student's t-test or one-way ANOVA. Values of $P < 0.05$ were considered statistically significant. *P < 0.05; **P < 0.01; ***P < 0.001.

Results

Loss of 14-3-3ζ delayes mammary gland development and inhibits mammary tumorigenesis

14-3-3ζ overexpression is known to play a pro-oncogenic role in breast cancer [11, 12, 18] but its role in mammary gland development is unknown. Mammary gland development was examined by dissecting mammary glands from the wild-type and 14-3-3ζ-/- mice in the FVB/NJ genetic backgrounds at 4, 6, 8, and 12 weeks of age. Whole-mount staining of the mammary glands indicated that the ductal outgrowth in the 14-3-3ζ-/- mice was delayed at 4 and 6 weeks of age but by 8 and 12 weeks was simi-

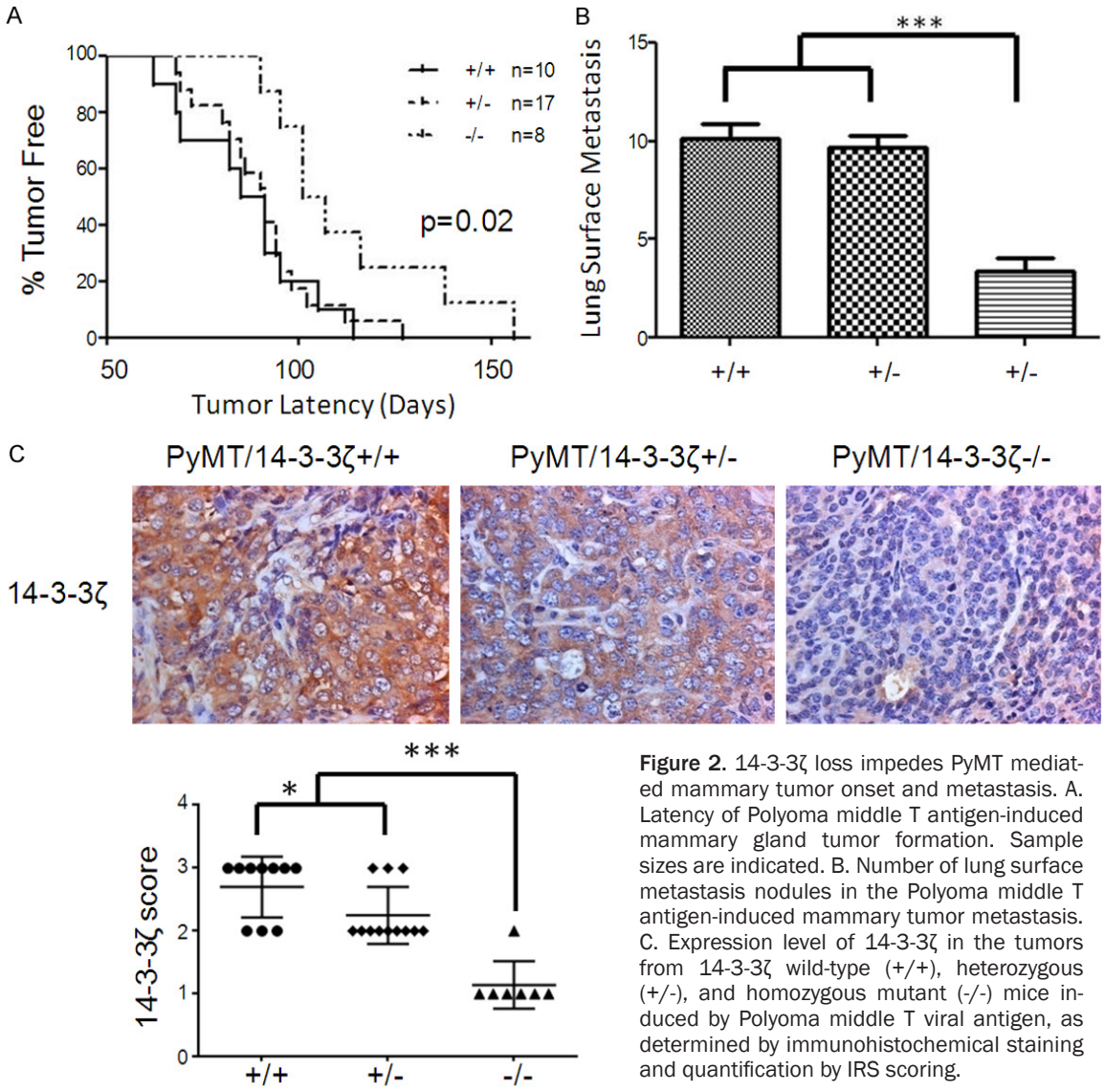

Figure 2. 14-3-3ζ loss impedes PyMT mediated mammary tumor onset and metastasis. A. Latency of Polyoma middle T antigen-induced mammary gland tumor formation. Sample sizes are indicated. B. Number of lung surface metastasis nodules in the Polyoma middle T antigen-induced mammary tumor metastasis. C. Expression level of 14-3-3ζ in the tumors from 14-3-3ζ wild-type (+/+), heterozygous (+/-), and homozygous mutant (-/-) mice induced by Polyoma middle T viral antigen, as determined by immunohistochemical staining and quantification by IRS scoring.

lar to that of wild-type mice (**Figure 1**). Thus 14-3-3ζ plays an important role in early mammary gland development.

To determine the impact of 14-3-3ζ-loss on mammary tumorigenesis, 14-3-3ζ-/- mice on a FVB/NJ background were crossed with the MMTV-PyMT mice, a well-characterized mammary tumor mouse model that develops mammary tumors with the mean tumor latency ranging from ~37 to 80 days [19]. The PyMT/14-3-3ζ-/- mice had significantly (p=0.02) longer mammary tumor latency as compared to PyMT/14-3-3ζ+/+ and PyMT/14-3-3ζ+/- mice (**Figure 2A**). When tumors reached 15 mm in diameter, the mice were sacrificed and examined for metastasis. The PyMT/14-3-3ζ-/- mice had a significantly lower lung metastases as compared to PyMT/14-3-3ζ+/+ and PyMT/14-3-3ζ+/- mice ($p < 0.0001$ and $p < 0.0001$ respectively) (**Figure 2B**). Reduced mammary tumorigenesis and metastasis in the PyMT/14-3-3ζ-/- mice correlated with a significantly reduced 14-3-3ζ expression in the mammary tumors compared with that in the PyMT/14-3-3ζ+/+ and PyMT/14-3-3ζ+/- mice (**Figure 2C**). Thus 14-3-3ζ has an essential role in PyMT-induced mammary tumorigenesis and metastasis.

We also crossed the 14-3-3ζ-/- mouse on a FVB/NJ background with the MMTV-driven Neu-NDL2-5 (MMTV-Neu) transgenic mouse strain expressing a mammary gland-restricted

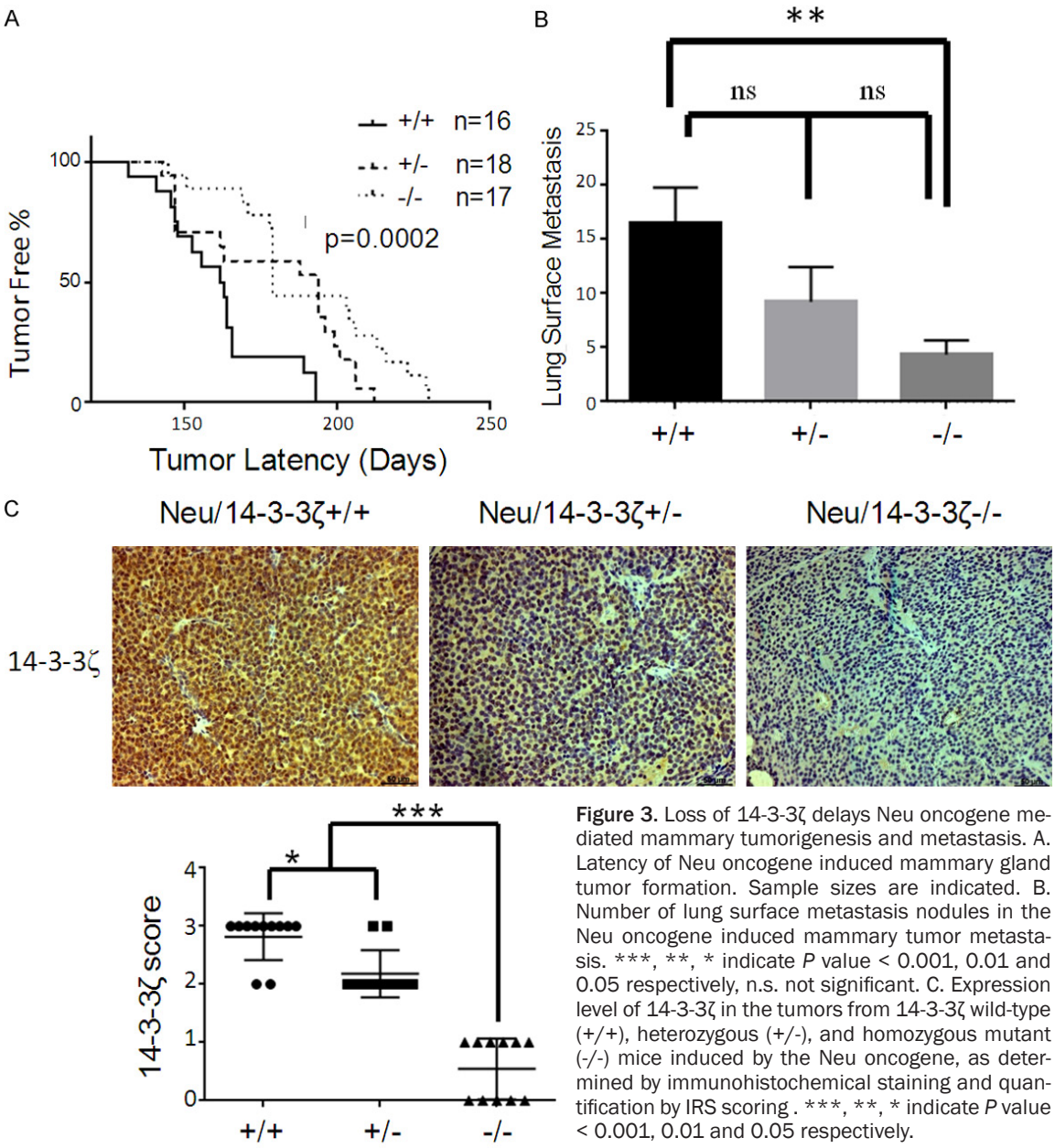

Figure 3. Loss of 14-3-3ζ delays Neu oncogene mediated mammary tumorigenesis and metastasis. A. Latency of Neu oncogene induced mammary gland tumor formation. Sample sizes are indicated. B. Number of lung surface metastasis nodules in the Neu oncogene induced mammary tumor metastasis. ***, **, * indicate *P* value < 0.001, 0.01 and 0.05 respectively, n.s. not significant. C. Expression level of 14-3-3ζ in the tumors from 14-3-3ζ wild-type (+/+), heterozygous (+/-), and homozygous mutant (-/-) mice induced by the Neu oncogene, as determined by immunohistochemical staining and quantification by IRS scoring . ***, **, * indicate *P* value < 0.001, 0.01 and 0.05 respectively.

constitutively active Neu oncogene; mice with this transgene develop spontaneous mammary tumors around 6 months of age [20]. The MMTV-Neu/14-3-3ζ-/- mice had a significantly (P=0.0002) prolonged tumor latency as compared to the MMTV-Neu/14-3-3ζ+/+ mice (**Figure 3A**). Similarly, the Neu/14-3-3ζ-/- mice exhibited significantly (p=0.0026) decreased lung metastases as compared to Neu/14-3-3ζ+/+ mice (**Figure 3B**), suggesting that 14-3-3ζ plays a critical role in Neu induced lung metastasis. Reduced mammary tumorigenesis and metastasis in the Neu/14-3-3ζ-/- mice correlated with a significantly reduced tumor 14-3-3ζ expression as compared with that in the Neu/14-3-3ζ+/+ and Neu/14-3-3ζ+/- mice (**Figure 3C**). Thus 14-3-3ζ facilitates oncogene induced mammary tumorigenesis and metastasis in both the well-characterized mammary tumor mouse models.

Loss of 14-3-3ζ affects multiple aspects of tumor biology via different signaling pathways

To determine the mechanism of 14-3-3ζ loss mediated anti-oncogenic properties, tumor cell

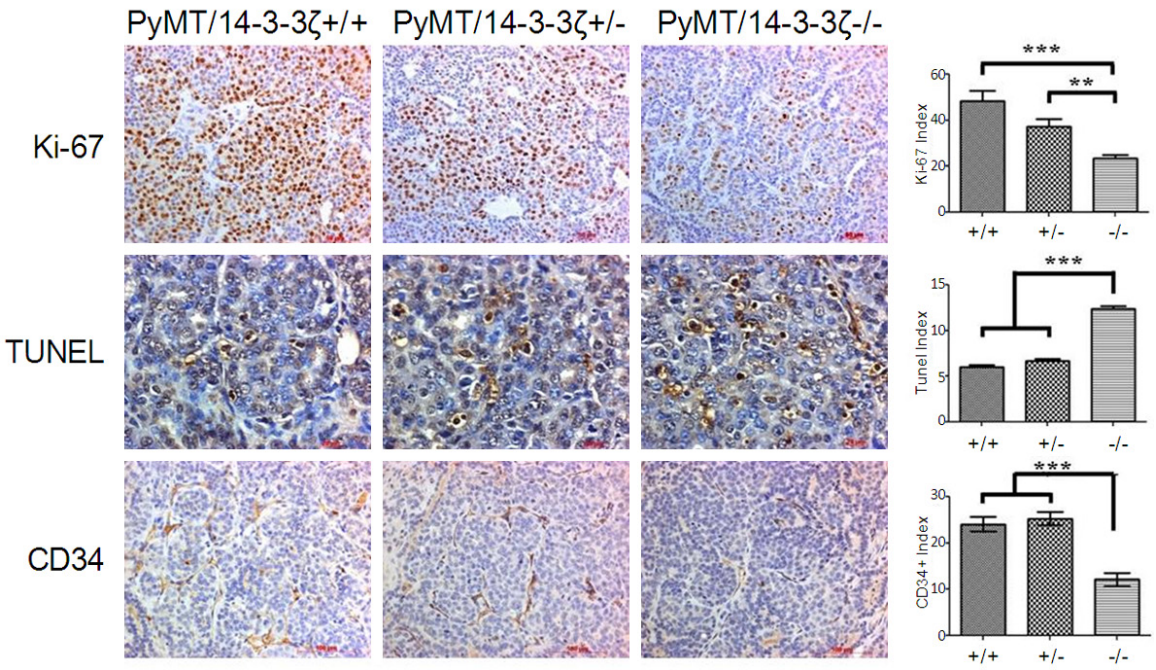

Figure 4. Loss of 14-3-3ζ attenuates proliferation, angiogenesis and enhances apoptosis in PyMT induced mammary tumors. Left: Representative IHC staining of Ki67, TUNEL and CD34 on PyMT-induced mammary tumors of different 14-3-3ζ genotypes as indicated. Right: The quantification of the IHC stainings. ***, **, * indicate *P* value < 0.001, 0.01 and 0.05 respectively. Length of the scale bar (represents 50 μM) is indicated in each panel.

proliferation, apoptosis, and angiogenesis in mammary tumors was assessed by IHC staining for Ki-67, TUNEL, and CD34, respectively. The mammary tumors from the PyMT/14-3-3ζ-/- mice had significantly reduced proliferation (decreased Ki-67 staining) and increased apoptosis (increased TUNEL positive signals) compared to their wild-type and heterozygous counterparts (**Figure 4**). PyMT/14-3-3ζ-/- tumors exhibited reduced angiogenesis (decreased CD34 staining) as compared to the wild-type and the heterozygous counterparts (**Figure 4**). Similarly, the MMTV-Neu/14-3-3ζ-/- tumors exhibited decreased proliferation (**Figure 5**, top) and increased apoptosis (**Figure 5**, middle) as compared to the MMTV-Neu/14-3-3ζ+/+ tumors. Tumors from both MMTV-Neu/14-3-3ζ-/- and MMTV-Neu/14-3-3ζ+/- mice showed significant reductions of angiogenesis compared to the wild-type counterparts (**Figures 4** and **5**, bottom). These results indicated that loss of 14-3-3ζ inhibits mammary tumorigenesis and metastasis by altering multiple mechanisms critical for tumor progression and metastasis.

To investigate the molecular mechanisms underlying these biological changes in mammary tumors of the 14-3-3ζ-/- mice, we performed reverse-phase protein array (RPPA) analyses on MMTV-PyMT driven. Multiple signaling pathways were found to be altered in the mammary tumors of 14-3-3ζ-/- mice compared with their wild-type counterparts (**Figure 6A**). Both PyMT and Neu oncogenes are known to effectively activate downstream Ras/Raf/Erk and PI3K/Akt signaling pathways [21-23]. Consistently, RPPA data revealed that phospho-Raf-S388, phospho-Mek1-S217, phospho-Akt-T308, and phospho-Akt-S473 are dramatically reduced in 14-3-3ζ-/- mammary tumors compared to their wild-type counterparts, indicating Ras/Raf/Erk and PI3K/Akt signaling pathway activities were significantly inhibited by the loss of 14-3-3ζ (**Figure 6A**). Immunoblotting further validated these findings in mammary tumors from both PyMT/14-3-3ζ-/- and Neu/14-3-3ζ-/- mouse models (**Figure 6B**, **6C**). Additionally, RPPA and western blot analyses showed increased p53 levels in the 14-3-3ζ-/- mammary tumors (**Figure 6B**, **6C**), consistent with our previous findings in human mammary epithelial cells [12]. p53 was reported to transcriptionally upregulate the pro-apoptotic protein, Bax [24] and inhibit the transcription of the pro-angiogenic protein VEGF [25]. Increased Bax and

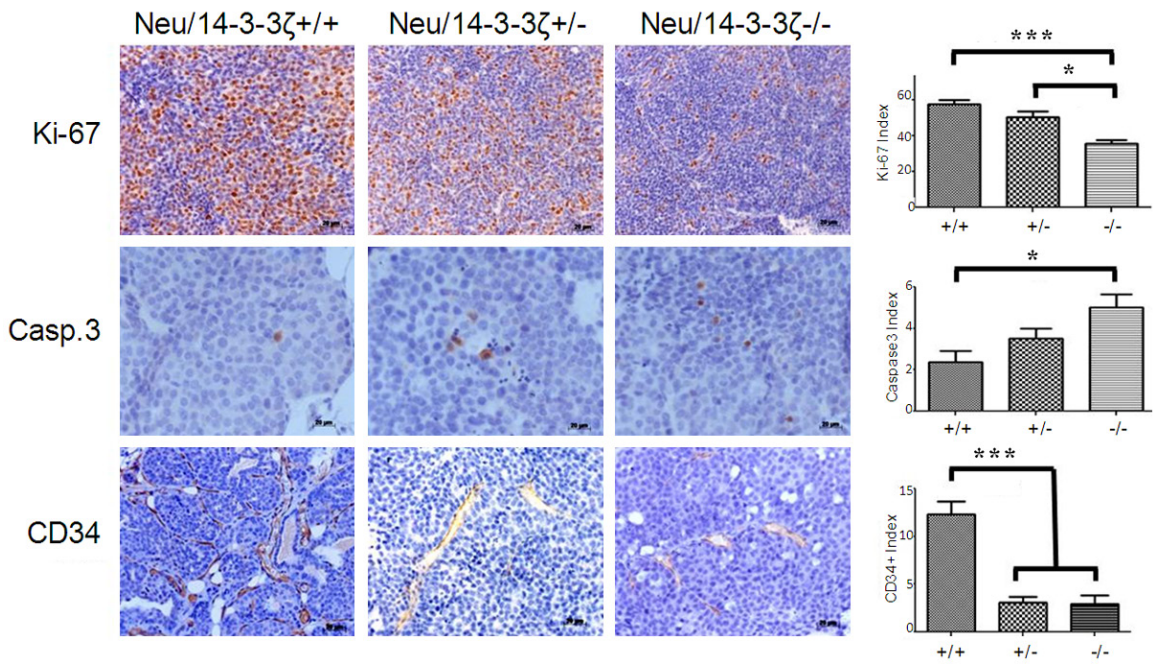

Figure 5. 14-3-3ζ expression positively regulates proliferation and angiogenesis and negatively regulates apoptosis in Neu induced mammary tumors. Left: Representative IHC staining of Ki67, Caspase 3 and CD34 on Neu-induced mammary tumors of different 14-3-3ζ genotypes as indicated. Right: The quantification of the IHC stainings. ***, **, * indicate *P* value < 0.001, 0.01 and 0.05 respectively. Length of the scale bar (represents 50 μM) is indicated in each panel.

reduced VEGF were detected in the mammary tumors of 14-3-3ζ-/- mice (**Figure 6B**, **6C**). Notably, the PyMT expression level was partly reduced in the mammary tumors of 14-3-3ζ-/- mice (**Figure 6B**), as 14-3-3ζ mediates PyMT protein stabilization via binding to phosphoserine 257 on PyMT [26]. However, Neu expression in the 14-3-3ζ-/- tumors was comparable to that in 14-3-3ζ+/+ mice (**Figure 6C**). Thus 14-3-3ζ-loss led to significantly attenuated Ras/Raf/Erk and PI3K/Akt signaling, elevated p53 and Bax levels, and reduced VEGF expression. These changes effectively led to reduced proliferation, increased apoptosis, and reduced angiogenesis in the mammary tumors of both PyMT/14-3-3ζ-/- and Neu/14-3-3ζ-/- mice (**Figure 7**), resulting in inhibition of tumorigenesis and metastasis.

Discussion

We have previously shown that ~40% of breast cancers from patients exhibit 14-3-3ζ overexpression, predicting a poor clinical outcome [11]. More in-depth analysis showed that 14-3-3ζ overexpression is initiated at the atypical ductal hyperplasia stage suggesting that 14-3-3ζ overexpression is an early event in breast cancer onset [12]. In non-transformed mammary epithelial cells overexpression of 14-3-3ζ lead to hyper-activation of the PI-3K/Akt pathway promoting the phosphorylation and translocation of the MDM2 E3 ligase promoting enhanced degradation of the tumor suppressor p53 [12]. Overexpression of 14-3-3ζ in non-transformed mammary epithelial cells expressing the Erbb2 oncogene lead to hyperactivation of the TGF-β/Smad pathway and promoted epithelial to mesenchymal transition [14]. Additionally histological analysis of tissue samples from breast cancer patients showed that patients with overexpression of both Erbb2 and 14-3-3ζ had an increased risk of progression to metastatic disease and death [14]. Recently, we reported that 14-3-3ζ acts as a molecular switch turning TGF-β's function from a tumor suppressor to metastasis promoter in breast cancer by contextual changes of Smad partners from p53 to Gli2 [15]. Additionally overexpression of 14-3-3ζ in MMTV-Neu transgenic mice resulted in accelerated mammary tumor onset and metastasis [18]. All of these studies focused on overexpression of 14-3-3ζ but provided no evidence to show that targeted dele-

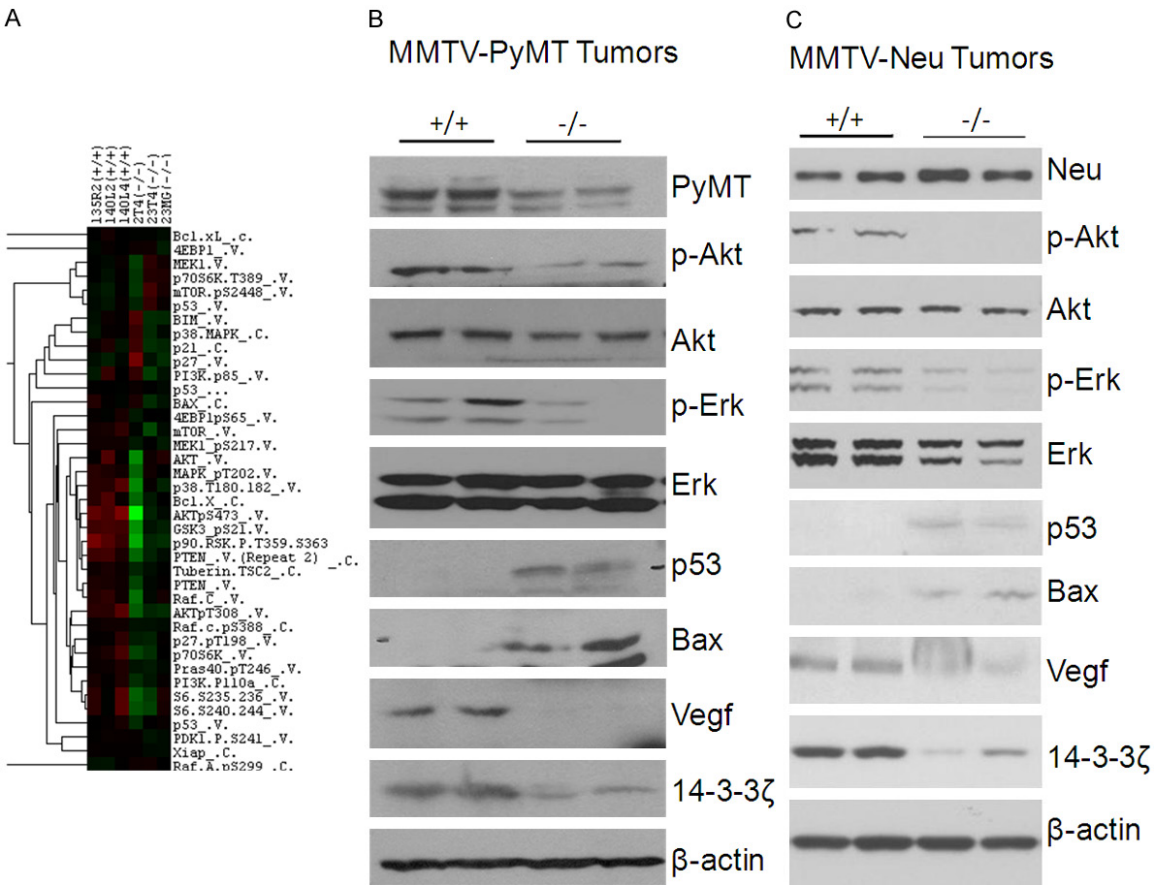

Figure 6. Loss of 14-3-3ζ inhibited Ras/Raf/Erk and PI-3K/Akt signaling pathways. A. Heat map of the RPPA analysis in mammary gland tumors. Samples were clustered into 14-3-3ζ wild-type (+/+) and homozygous mutant (-/-) groups using Cluster 3.0 software. B. Analysis of PyMT, p-Akt (Ser 473), Akt, phosphor-Erk1/2 (Thr202/Tyr204), Erk1/2, p53, Bax, VEGF, 14-3-3ζ and β-actin by western blotting in tumor protein lysates from PyMT/14-3-3ζ+/+ and PyMT/14-3-3ζ-/- mice. C. Analysis of Neu, p-Akt (Ser 473), Akt, phosphor-Erk1/2 (Thr202/Tyr204), Erk1/2, p53, Bax, VEGF, 14-3-3ζ and β-actin by western blotting in tumor protein lysates from PyMT/14-3-3ζ+/+ and PyMT/14-3-3ζ-/- mice.

tion of 14-3-3ζ can delay mammary tumor onset and metastasis.

Here, we generated a 14-3-3ζ-/- model to empower exploration of *in vivo* functions of 14-3-3ζ, and enable identification of *in vivo* targets downstream of 14-3-3ζ for development of novel therapeutics to benefit patients with 14-3-3ζ overexpressing tumors. Our data demonstrated that loss of 14-3-3ζ impeded PyMT and ErbB2/Neu oncogene-induced mammary tumor formation and metastasis. Loss of 14-3-3ζ in PyMT and Neu driven mammary tumors significantly inhibited Raf/Mek/Erk pathway, VEGF expression, and tumor angiogenesis. The similar findings in these two distinct mammary tumor models suggest that 14-3-3ζ plays an essential role in regulating oncogene-induced mammary tumor formation. As 14-3-3ζ is overexpressed in a large proportion of patients' breast cancers, development of a 14-3-3ζ inhibitor could benefit these patients. It is important to note that loss of 14-3-3ζ did not completely ablate tumor formation induced by PyMT and ErbB2/Neu, suggesting that targeting 14-3-3ζ alone may not be sufficient. This is true for many targeted therapies currently in clinical use (e.g. Herceptin).

14-3-3ζ has been reported to play a role in promoting resistance to cancer therapeutics. In ER+ breast cancers, tamoxifen therapy promotes the attenuation of miR-451 leading to an enhanced expression of 14-3-3ζ which enhances the transcriptional activity of FOXM1 and ultimately leads to endocrine resistance [27,

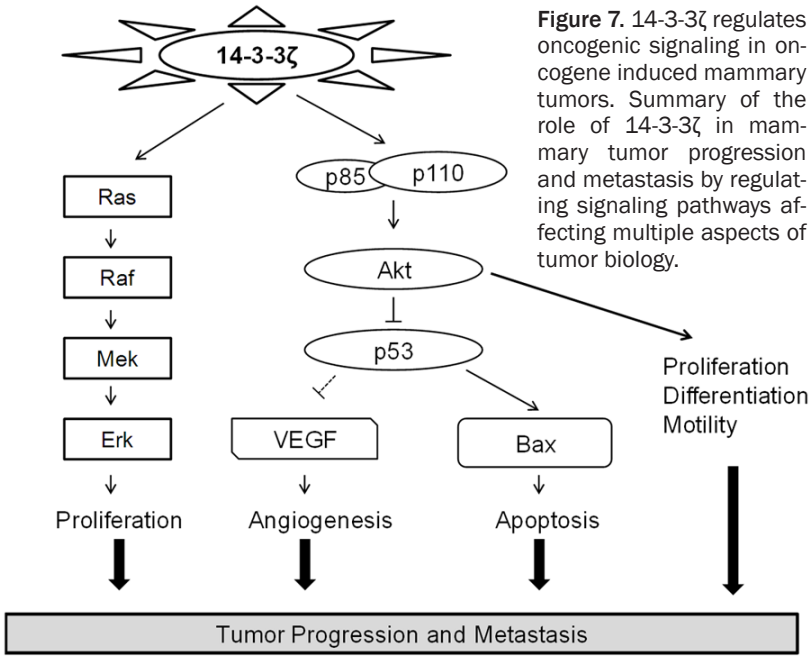

Figure 7. 14-3-3ζ regulates oncogenic signaling in oncogene induced mammary tumors. Summary of the role of 14-3-3ζ in mammary tumor progression and metastasis by regulating signaling pathways affecting multiple aspects of tumor biology.

28]. Overexpression of 14-3-3ζ in hepatocellular carcinoma confers resistance to the chemotherapeutic *cis*-diamminedichloridoplatinum (CDDP) and also promotes resistance to radiotherapy [29, 30]. In diffuse large B cell lymphoma, enhanced expression of 14-3-3ζ promotes resistance to an anthracycline-based chemotherapeutic therapy. Additionally in lung cancer upregulation of 14-3-3ζ may play a role in resistance to cisplatin [31]. Thus multiple studies have shown that overexpression of 14-3-3ζ can confer resistance to multiple oncogenic therapies. These results combined with our data providing evidence for a mammary tumor onset and metastasis promoting activity for 14-3-3ζ provide a strong rationale for developing targeted therapeutic attenuating the activity and/or expression of 14-3-3ζ.

As 14-3-3ζ is a signaling molecule with no kinase activity there is no clinically approved therapeutics to target 14-3-3ζ. As 14-3-3ζ is a member of the highly conserved and ubiquitously expressed 14-3-3 family proteins which includes the tumor suppressor 14-3-3σ [32] it will be important to develop a highly specific inhibitor for 14-3-3ζ. Results from this study suggest that depletion of 14-3-3ζ in combination with chemotherapy or targeted therapy may provide a strong therapeutic benefit with minimal side-effects. Currently, a peptide inhibitor difopein has been reported to bind to 14-3-3 proteins with high affinity preventing their interaction with their target proteins [33]. *In vitro* and xenograft experiments with glioblastoma cell lines have provided promising results showing that difopein treatment can induce apoptosis and suppress tumor growth in mice [34]. These encouraging results along with our 14-3-3ζ knockout mice studies suggest an emerging need to develop therapeutics targeting the oncogenic activity of 14-3-3ζ and enhancing sensitivity to oncogenic therapies.

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Disclosure of conflict of interest

None.

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