Loss of the 14-3-3σ Tumor Suppressor Is a Critical Event in ErbB2-Mediated Tumor Progression

Chen Ling1,2, Vi-Minh-Tri Su1,2, Dongmei Zuo1,2, and William J. Muller1,2,3

ABSTRACT

14-3-3σ is a putative tumor suppressor involved in cell-cycle progression and epithelial polarity. We demonstrate that loss of one or both copies of the conditional 14-3-3σ allele results in accelerated mammary and salivary tumorigenesis in mice expressing an activated erbB2 oncogene under the endogenous erbB2 promoter. Significantly, the majority of tumors bearing a single conditional 14-3-3σ allele lose expression of the remaining 14-3-3σ allele, which is associated with epigenetic methylation of the 14-3-3σ locus. In addition to accelerated tumor onset, in a mouse mammary tumor virus-driven ErbB2 tumor model, loss of 14-3-3σ results in enhanced metastatic phenotype that is correlated with loss of cellular junctions. Taken together, these results provide compelling evidence that 14-3-3σ is a potent tumor suppressor involved in ErbB2-driven breast cancer initiation and metastasis.

SIGNIFICANCE: 14-3-3σ has been identified as a normal mammary epithelial cell marker frequently downregulated during neoplastic development. Consistent with its potential role as a tumor suppressor, we demonstrate that targeted disruption of 14-3-3σ in a number of epithelial tissues can profoundly impact both the initiation and metastatic phases of ErbB2-mediated tumor progression through modulation of a number of distinct signaling networks. Cancer Discovery; 2(1); 68–81. © 2011 AACR.
INTRODUCTION

Breast cancer is a prevalent and poorly understood disease thought to arise through the accumulation of numerous genetic alterations that confer growth and survival advantages to tumor cells. One genetic alteration associated with poor outcome in patients with breast cancer is amplification and overexpression of the erbB2 proto-oncogene, a member of the epidermal growth factor receptor (EGFR) transmembrane receptor family (1–3). There is direct evidence supporting a role for EGFR family members and their various ligands in mammary tumorigenesis derived from observations made with transgenic mice. For instance, mammary epithelial expression of the EGFR or its ligand TGF-α in transgenic mice results in the frequent induction of mammary adenocarcinomas (4–7). Mammary epithelial-specific expression of activated erbB2 results in the rapid induction of metastatic multifocal mammary tumors (8–12). Although mammary epithelial expression of the erbB2 proto-oncogene is capable of efficiently inducing multifocal mammary tumors, no comparable activating mutations have been detected in human erbB2 (13). It is likely that the primary mechanism by which ErbB2 induces mammary tumorigenesis in human breast cancer is through elevated expression of the wild-type receptor.

To more closely mimic events involved in ErbB2-induced mammary tumor progression, we derived transgenic mice that carry a Cre-inducible activated erbB2 under the transcriptional control of the endogenous erbB2 promoter (herein referred to as the ErbB2 knock-in mouse model [ErbB2KI] strain) (14). In contrast to the rapid tumor progression observed in the mouse mammary tumor virus (MMTV)-activated erbB2 strains, focal mammary tumors arose only after an extended latency period in these strains. Tumor progression in these strains was further associated with a dramatic elevation of both ErbB2 protein and transcript levels. Remarkably, the elevated expression of erbB2 was further correlated with the selective genomic amplification of the activated erbB2 allele. Thus, as in human breast cancers, amplification of erbB2 appears to be a critical event in mammary tumor progression in this unique transgenic mouse model.

The similarity of the ErbB2KI mouse model to ErbB2-initiated human breast cancer is further highlighted by the observation that tumorigenesis in this model is associated with a number of other chromosomal alterations, including centrosome abnormalities and recurrent deletions of...
chromosome 4 (15). Refined CGH/BAC analyses revealed that in addition to the erbB2 amplicon, there was a frequent loss of a chromosomal region spanning the 14-3-3σ tumor suppressor locus (16, 17). The region of loss on mouse chromosome 4 is syntenic with human chromosome 1p35-36, which is also frequently lost in human sporadic breast cancer (18, 19). Inspection of candidate genes in this region identified the p53-responsive gene 14-3-3σ. Interestingly, 14-3-3σ has been identified as a normal mammary epithelial cell marker downregulated during neoplastic development (20). Consistent with its potential role as a tumor suppressor, it has also been demonstrated that a large number of primary breast cancers fail to express 14-3-3σ (21, 22) and that it is a potent inhibitor of cell-cycle progression after induction of the p53 tumor suppressor (23, 24). It has also been demonstrated that all human ErbB2-overexpressing breast cancer cell lines have no or reduced 14-3-3σ expression (25). Further molecular analyses suggested that selection for loss of 14-3-3σ may in part be related to its ability to sequester the EGR2/CITED1 transcription factor complex in the cytoplasm and thus interfere with its capacity to transactivate the endogenous erbB2 promoter (16). Taken together, these data argue that, in addition to sequestration of key translation and cell-cycle components, 14-3-3σ can directly modulate the transcription of ErbB2 through relocalization of key transcription factors involved in its transcriptional activation.

In addition to these critical roles, there is an increasing body of evidence indicating a role for 14-3-3σ in the regulation of epithelial polarity. A radiation-induced mouse mutant strain known as Repeated-epilation (Er) exhibits a severe skin polarization defect resulting from the expression of a truncated form of 14-3-3σ (26, 27). Consistent with these initial observations, targeted disruption of 14-3-3σ in mouse mammary gland results in a dramatic loss of epithelial polarity resulting from the absence of 14-3-3σ-dependent relocalization of the Par3 polarity complex to the apical surface (28). However, despite the severe disruption of mammary epithelial polarity observed in these strains, loss of 14-3-3σ in mammary epithelium was not sufficient to induce tumors within the observation period of 1 year.

Given the documented roles of 14-3-3σ in the regulation of epithelial polarity, ErbB2 expression, and ErbB2 tumor progression (16, 17, 28), we evaluated whether loss of 14-3-3σ in the mammary epithelium would impact ErbB2-driven tumor induction. To directly assess whether 14-3-3σ acts as a tumor suppressor in vivo, we assessed whether mammary epithelial disruption of 14-3-3σ could impact mammary tumor progression in the ErbB2KI mouse strain. We interbred the ErbB2KI strain with conditional 14-3-3σ strains and MMTV/Cre transgenic mice. One advantage of this genetic approach is that inactivation of 14-3-3σ by Cre recombinase is coupled to the simultaneous Cre-mediated activation of ErbB2. Thus, every mammary epithelial cell expressing the activated erbB2 allele will lack a functional 14-3-3σ. The results revealed that female ErbB2KI mice heterozygous or homozygous for the conditional 14-3-3σ allele developed mammary and salivary tumors at a much earlier age than the parental ErbB2KI strain. Interestingly, the induction of salivary tumors in 14-3-3σ-deficient animals represents a novel tumor type because salivary gland tumors were rarely observed in the parental ErbB2KI strain.

A critical feature of tumor suppressors is that cells that have lost one tumor suppressor allele frequently lose expression of the remaining allele during transition to the neoplastic state. Accordingly, a majority of ErbB2-induced tumors from mice heterozygous for the 14-3-3σ conditional allele lose expression of the remaining wild-type 14-3-3σ allele. Remarkably, like human breast cancers (22), the loss of 14-3-3σ expression is correlated with methylation of the 14-3-3σ locus. The 14-3-3σ-deficient tumors exhibit a corresponding increase in the expression of both ErbB2 transcript and protein, confirming the role of 14-3-3σ as a potent regulator of erbB2 promoter activity. Given the potential role of 14-3-3σ in regulating epithelial polarity and epithelial migration, we also evaluated whether targeted disruption of 14-3-3σ could impact the metastatic properties of ErbB2 tumors by crossing it to a separate mouse strain expressing both activated ErbB2 and Cre recombinase under the constitutive MMTV promoter (herein referred to as the MMTV-NDL-ires-Cre mouse model [NIC] strain) (29). Loss of 14-3-3σ was associated with a significant increase in metastatic properties and was further correlated with loss of cellular junction complexes and elevated mitogen-activated protein kinase (MAPK) activity. Elevated MAPK activity was associated with relocalization of c-Raf to the plasma membrane of tumor cells. Taken together, these genetic and biochemical observations strongly argue that 14-3-3σ is a critical tumor suppressor that is involved in both initiation and progression of ErbB2-induced mammary tumorigenesis.

RESULTS

Loss of 14-3-3σ Results in Both Accelerated ErbB2 Mammary Tumor Development and Altered Tumor Tropism

Previous studies with the ErbB2KI model indicated that one important genomic alteration involved in tumor progression is the loss of a chromosomal region spanning the 14-3-3σ locus that is correlated with loss of 14-3-3σ expression (15, 17). To directly assess whether the loss of 14-3-3σ is a critical event in ErbB2-driven tumor progression, we intercrossed mice bearing the conditional 14-3-3σ allele to ErbB2KI mice to generate cohorts of ErbB2KI female mice with one or two conditional 14-3-3σ alleles. These animals, together with wild-type ErbB2KI mice, were monitored for mammary tumor formation. Loss of one or both 14-3-3σ alleles resulted in a dramatic acceleration of focal mammary gland tumor onset (T50, 176 days and T50, 132 days, respectively) in contrast to wild-type ErbB2KI mice that developed focal mammary tumors only after a long latency period (T50, 396 days) (Fig. 1A). As expected, ErbB2KI tumors derived from female mice bearing either one or both 14-3-3σ conditional alleles exhibited efficient Cre-mediated recombination of the conditional 14-3-3σ alleles (Supplementary Fig. S1A and B). Histological analyses of the mammary tumors revealed that although 14-3-3σ-deficient tumors exhibited typical adenocarcinoma features as found in the parental ErbB2KI strain, they possessed a higher degree of collagen-rich stroma (Supplementary Fig. S1C). Given that the average tumor...
onset in the parental ErbB2KI strain is over 396 days of age (14), these observations suggest that loss of 14-3-3σ results in a significant acceleration of ErbB2-driven mammary tumor progression.

One of the characteristic features of tumor suppressors is that in the absence of one tumor suppressor allele, the expression of the remaining allele is suppressed during tumor progression (30). To evaluate whether ErbB2KI tumors...
heterozygous for the conditional 14-3-3σ allele suppressed the expression of wild-type 14-3-3σ, we performed immunoblot analyses on tumor lysates from the different genotypic combinations with a 14-3-3σ-specific antibody. Of the 10 tumors heterozygous for 14-3-3σ allele, 8 lost expression of the remaining wild-type allele (Fig. 1B). Further examination determined that tumors that lost expression of 14-3-3σ expressed high levels of ErbB2, whereas 2 tumors that retained expression of the remaining wild-type 14-3-3σ allele exhibited very low expression of ErbB2 (Fig. 1B, tumor numbers 5558 and 7926). Studies on another subset of these ErbB2KI mammary tumors revealed that elevated expression of ErbB2 protein in 14-3-3σ-deficient tumors was the result of elevated levels of ErbB2 transcript relative to the 14-3-3σ-proficient tumors, which expressed very low levels of ErbB2 transcript (Supplementary Fig. S1D, tumor number 5558). In the parental ErbB2KI strain, elevated ErbB2 expression is correlated with the selective amplification of the activated ErbB2KI allele (14). To ascertain whether the elevated levels of ErbB2 transcript observed in the 14-3-3σ-deficient tumors were the result of an increase in the copy number of erbb2 gene, Southern blot analyses were performed on these tumors with erbb2-specific probes. Unlike parental ErbB2KI tumors that exhibited high-level amplification of the ErbB2KI allele, the 14-3-3σ–deficient tumors possessed elevated ErbB2 transcript in the absence of ErbB2 amplification (Supplementary Fig. S1E). Taken together, these observations argue that loss of 14-3-3σ can directly modulate ErbB2 transcript levels independent of erbb2 copy number.

To evaluate whether loss of 14-3-3σ could impact ErbB2-coupled signaling, we measured the activation status of the Akt and MAPK signaling pathways (Fig. 1B). After quantification, a statistically significant increase in the levels of phospho-MAPK/total MAPK was observed (Fig. 1C) but no significant difference in the phosphor-Akt/total Akt levels (Supplementary Fig. S2A) in those tumors carrying the conditional 14-3-3σ alleles. To assess whether the observed increase in MAPK activity impacted the proliferative or apoptotic capacity of the tumors, we measured the levels of Ki67 and TUNEL-positive cells in both 14-3-3σ–deficient ErbB2KI and parental ErbB2KI tumors. Tumors bearing the deleted conditional alleles exhibited an elevated proliferative capacity compared with tumors derived from the parental ErbB2KI strain, whereas the apoptotic potency was not affected (Supplementary Fig. S1E–G). Taken together, these observations support the hypothesis that 14-3-3σ acts as a critical tumor suppressor in ErbB2-induced tumor progression.

In addition to the impact on mammary tumor progression, both male and female ErbB2KI mice carrying the conditional 14-3-3σ allele rapidly developed multifocal salivary gland tumors with 100% penetrance (Supplementary Fig. S3A). By contrast, only one of the 26 mice harboring the parental ErbB2KI alleles developed salivary gland tumors. Like the mammary tumors induced by the disruption of conditional 14-3-3σ allele, salivary tumors heterozygous for the conditional 14-3-3σ allele had no or little 14-3-3σ expression with a corresponding elevated expression of ErbB2 and activation of MAPK activity (Supplementary Fig. S3B and C), whereas the phosphor-Akt levels were comparable (Supplementary Fig. S2B). The observation that these mice develop salivary tumors in addition to mammary tumors indicates that loss of 14-3-3σ in the ErbB2K strain can alter tumor site tropism.

**Loss of 14-3-3σ Expression Is Associated with Methylation of the 14-3-3σ Locus**

One of the striking features of mammary tumors heterozygous for the conditional 14-3-3σ allele is that many of the tumors fail to express the remaining wild-type allele. In human breast cancer, loss of 14-3-3σ expression is frequently associated with epigenetic silencing of 14-3-3σ allele by CpG island methylation (22). To explore whether the loss of 14-3-3σ expression observed in tumors retaining one conditional 14-3-3σ allele occurs by the same mechanism, we performed sodium bisulfite DNA sequencing (SBDS) and methylation-specific PCR (MSP) analyses on genomic DNA derived from appropriate tumors (22). Genomic DNA extracted from the epithelial portion of mammary tumors and adjacent mammary epithelium was subjected to SBDS and MSP analyses. ErbB2KI tumors lacking expression of the remaining 14-3-3σ allele contained substantial regions of hypermethylation within the coding region of the conditional allele (Fig. 2). By contrast, genomic DNA spanning the 14-3-3σ locus from tumors retaining 14-3-3σ expression or from normal mammary epithelium was comparatively undermethylated (Fig. 2). Interestingly, genomic DNA derived from adjacent mammary epithelium of tumor-bearing animals with one conditional 14-3-3σ allele also exhibited robust methylation within the other 14-3-3σ locus. This indicates that epigenetic silencing of the 14-3-3σ gene may be a relatively early step in mammary tumor progression.

**Loss of 14-3-3σ in MMTV-Driven ErbB2 Models Is Associated with an Enhanced Metastatic Phenotype**

One confounding issue concerning the role of 14-3-3σ in ErbB2-induced mammary tumor progression is that 14-3-3σ is involved in cellular processes that act directly on ErbB2 transcription and epithelial polarity. To discern between ErbB2 transcription-dependent and -independent functions of 14-3-3σ, we interbred the conditional 14-3-3σ mice to NIC mice, which coexpress activated ErbB2 and Cre recombinase in the mammary epithelium under the transcriptional control of the MMTV promoter (29). In this strain, expression of activated erbB2 is directly coupled to the Cre-mediated excision of any LOXPI flanked conditional allele, in this case 14-3-3σ. Cohorts of virgin female mice were then monitored for mammary tumor development. Like the 14-3-3σ–deficient ErbB2KI model, loss of both 14-3-3σ conditional alleles resulted in a significant acceleration of mammary tumor onset (Fig. 3A). However, unlike ErbB2KI mice, we did not observe any morphologic difference between mammary tumors from 14-3-3σ-proficient and 14-3-3σ–deficient NIC mice (Supplementary Fig. S4A–C), nor did any salivary gland tumors develop in these mice. These observations suggest that these phenotypes may reflect tissue-specific differences between the endogenous erbB2 and MMTV promoters. Examination of ErbB2-coupled signaling pathways in these 14-3-3σ–deficient tumors indicated that, like the 14-3-3σ–deficient
ErbB2<sup>KI</sup> tumor model, they possessed elevated MAPK phosphorylation that was further correlated with elevated ErbB2 protein levels (Fig. 3B and C), and there was little difference in Akt phosphorylation (Supplementary Fig. S2C). Like the ErbB2<sup>KI</sup> model, the elevated MAPK activity was also associated with an increase in the proliferative capacity of tumor cells with little impact on their apoptotic status (Supplementary Fig. S4D–G). One possible explanation for the observed elevation of MAPK activity is that upstream c-Raf kinase is constitutively active. Indeed, previous studies have suggested that c-Raf can be sequestered by 14-3-3<sub>ζ</sub> in a complex (31, 32). To determine whether subcellular localization of c-Raf was altered in 14-3-3<sub>ζ</sub>-deficient NIC tumor cell lines, we performed immunofluorescence analyses with a c-Raf-specific antibody. By contrast to primary cytoplasmic staining of c-Raf observed in the 14-3-3<sub>ζ</sub>-proficient NIC mammary tumor cell line, the 14-3-3<sub>ζ</sub>-deficient cells exhibited a moderate elevation of the c-Raf levels and a distinct membrane localization of c-Raf (Fig. 4A and B). In addition, re-expression of 14-3-3<sub>ζ</sub> in the established 14-3-3<sub>ζ</sub>-deficient tumor cells could restore the cytoplasmic localization of c-Raf, although overexpressing 14-3-3<sub>ζ</sub> seemingly had a similar effect (Fig. 4C and D). These observations argue that elevated MAPK activity is correlated with relocation of the c-Raf kinase to the plasma membrane.

Given the documented role of 14-3-3<sub>ζ</sub> in the regulation of epithelial polarity, migration, and invasion (28), we also evaluated whether disruption of 14-3-3<sub>ζ</sub> could impact the metastatic potential of these tumor cells. To evaluate this possibility, we subjected serial sections of lungs derived from these tumor-bearing mice to histologic analyses for the presence of metastatic lesions. A significantly greater percentage of the 14-3-3<sub>ζ</sub>-deficient animals exhibited a metastatic phenotype (Fig. 5A). Consistent with observations, the 14-3-3<sub>ζ</sub>-deficient animals also carried a significantly greater number of lung lesions (Fig. 5B). The difference in metastatic capacity was not the result of differences in tumor burden because both control and 14-3-3<sub>ζ</sub>-deficient mice possessed equivalent tumor masses (Fig. 5C).

To examine whether the enhanced metastatic capacity of these 14-3-3<sub>ζ</sub>-deficient tumors cells was the result of enhanced migration and invasive properties of the primary tumor cells, we measured the migratory and invasive properties using transwell assays. Consistent with the in vivo metastatic data, primary tumor cells derived from the 14-3-3<sub>ζ</sub>-deficient animals possessed elevated migratory and invasive properties compared with their wild-type counterparts (Supplementary Fig. S5A and B). Finally, to ascertain whether loss of 14-3-3<sub>ζ</sub> impacted the capacity of the tumor cells to colonize the metastatic site, we performed tail vein injections with equal numbers of 14-3-3<sub>ζ</sub>-deficient and proficient primary tumor cells into athymic mice and enumerated the number of metastatic lesions. The results showed that, in addition to the increase in migratory and invasive potential of the tumor cells, loss of 14-3-3<sub>ζ</sub> contributes to a significant increase in the capacity of tumor cells to colonize the metastatic site (Supplementary Fig. S5C). Taken together, these observations argue that the enhanced metastatic
phenotype exhibited by 14-3-3σ-deficient cells involves both an increase of the invasive and colonization potential of these tumor cells.

**Loss of 14-3-3σ Results in Dissolution of Adherens and Tight Junctional Complexes in ErbB2 Tumor Cells**

Our previous studies with primary and established epithelial cells has revealed that downregulation of 14-3-3σ results in loss of both adherens and tight junctional complexes resulting from the inability of the Par3 complex to be properly recruited to the apical plasma membrane (28).

To investigate whether the enhanced metastatic properties of 14-3-3σ-deficient tumor cells involved a similar dissolution of cellular junctions, independent primary cell lines from 14-3-3σ-proficient and 14-3-3σ-deficient NIC tumors were established, which retained the molecular signatures of their respective tumors (Fig. 4A). We stained these primary cells with antibody reagents directed against tight junctional protein component ZO-1 or adherens junctional protein E-cadherin. Comparison of 2-dimensional cultures of tumor epithelium revealed that, in contrast to wild-type NIC tumors that still retained both tight and adherens junctions, the 14-3-3σ-deficient tumor epithelium drastically lost these junctional complexes (Fig. 6A).

A similar defect in junctional complex formation was also observed when these tumor cells were cultivated in 3 dimensions on a matrigel matrix (Fig. 6B and C), and these junctional defects were not the result of protein level changes of ZO-1 or E-cadherin (Fig. 6D). Like the primary mammary epithelial cell, the loss of these junctional complexes was further correlated with mislocalization of the Par3 complex (Supplementary Fig. S6).

Finally, to evaluate that the observed effects of 14-3-3σ deletion on migration, invasion, and junctional complexes were a direct result of 14-3-3σ, we re-expressed 14-3-3σ in these 14-3-3σ-deficient NIC tumor cells (Fig. 4C) and examined the effects on these cellular properties. In contrast to empty vector or 14-3-3σ-expressing cells, restoration of 14-3-3σ resulted in a strong reduction in migratory and invasive behavior.
properties of these ErbB2-expressing tumors cells (Fig. 7A and B) that was further correlated with restoration of adhesion and tight junctional complexes in both 2-dimensional and 3-dimensional cultures (Fig. 7C and D) without altering the expression levels of junctional proteins ZO-1 and E-cadherin (Fig. 7E). However, these cellular junction alteration coincided with restoration of the membrane localization of the Par3 complex (Supplementary Fig. S7). These observations argue that the invasive phenotype observed in 14-3-3σ-deficient cells reflects its critical role in maintaining these epithelial polarity complexes.

**DISCUSSION**

**Loss of 14-3-3σ Is a Critical Event in ErbB2 Mammary Tumor Induction**

There is an increasing body of evidence implicating 14-3-3σ as a critical tumor suppressor in ErbB2 tumor progression. We demonstrate that targeted disruption of 14-3-3σ in two independent ErbB2 tumor models can significantly accelerate both the initiation and the metastatic phases of ErbB2 tumor induction. We first show that mammary epithelial ablation of 14-3-3σ in a mouse model expressing an activated version of ErbB2 under the endogenous erbB2 promoter results in a dramatic acceleration in mammary tumor onset and the induction of salivary tumors (Fig. 1A; Supplementary Fig. S3A). Consistent with these observations, disruption of 14-3-3σ in a MMTV-driven erbB2 model also results in accelerated tumor development (Fig. 3A) associated with a significant enhancement of the metastatic phenotype (Fig. 5) resulting from disruption of cellular adhesion complexes (Fig. 6). These observations provide compelling evidence that 14-3-3σ is a major tumor suppressor involved in both the initiation and metastatic phases of ErbB2-induced tumor development.

One remarkable feature concerning the disruption of 14-3-3σ in the ErbB2 model is that removal of a single...
copy of conditional 14-3-3σ allele is sufficient to dramatically accelerate tumor onset. Eighty percent of mammary tumors carrying a single 14-3-3σ allele lose expression of the remaining allele correlated with extensive methylation (Figs. 1B and 2). Indeed, loss of 14-3-3σ expression in human breast cancer is also associated with its hypermethylation (22) suggesting that epigenetic silencing of 14-3-3σ is a common mechanism in both mouse and human tumor progression. Significantly, loss of 14-3-3σ function is also correlated with an increase in both ErbB2 protein and transcript levels similar to the parental ErbB2<sup>ki</sup> strain (Fig. 1B; Supplementary Fig. S1D). However, in contrast to the parental ErbB2<sup>ki</sup> strain, this increase in ErbB2 expression occurs in the absence of erbB2 amplification (Supplementary Fig. S1E), suggesting that early loss of 14-3-3σ can directly impact ErbB2-dependent transcription. Conversely, two tumors that retained expression of 14-3-3σ exhibited very low levels of ErbB2 (Fig. 1B). One likely explanation for these observations is that 14-3-3σ negatively regulates transcription factors involved in regulating ErbB2 expression. Consistent with this concept, one previous study has demonstrated that 14-3-3σ is involved in sequestration of the EGR2 transcription factor that directly activates the erbB2 promoter (16). Given that the expression of the activated erbB2 allele is driven by the endogenous erbB2 promoter in the ErbB2<sup>ki</sup> model, there is strong selective pressure for the loss of 14-3-3σ expression.

Another important observation that arose in the analyses of these strains is that loss of 14-3-3σ was also associated with rapid occurrence of multifocal salivary gland tumors (Supplementary Fig. S3A). This unexpected shift in tumor tropism suggests that the salivary gland is particularly sensitized to a combination of activated erbB2 being driven from its endogenous promoter and inactivation of 14-3-3σ. Previous studies with MMTV/Ras mice have shown that MMTV is particularly active in parotid salivary glands and that this can lead to development of parotid tumors (33). In this regard, it is interesting to note that a certain proportion of human salivary gland tumors express elevated levels of ErbB2 (34–37). Conversely, 14-3-3σ is downregulated through hypermethylation in certain salivary gland tumors (38). Another study demonstrated that a mutant polyomavirus unable to sequester 14-3-3 proteins failed to develop salivary tumors, which were characteristic of the wild-type polyomavirus (39). Taken together, these observations argue that loss of 14-3-3σ is a critical event in salivary gland tumor progression.

The observation that the majority of ErbB2<sup>ki</sup> tumors heterozygous for the conditional 14-3-3σ allele silence the remaining wild-type allele through methylation provides compelling evidence that there is strong selective pressure for abrogation of 14-3-3σ function in mammary tumor progression. In fact, a large proportion of human breast cancers lose expression of 14-3-3σ through methylation of the 14-3-3σ locus (22). In addition to epigenetic silencing, there is also evidence to suggest that loss of 14-3-3σ can occur through posttranslational mechanisms. For example, the estrogen-regulated E3 ubiquitin ligase E6<sub>p</sub> can specifically downregulate 14-3-3σ and promote breast cancer growth through proteolytic degradation (40). The emerging role of 14-3-3σ as a potent tumor suppressor in many cancers is further supported by the identification of the methylation-dependent silencing of 14-3-3σ in prostate, melanoma, vulval, and salivary tumors (41). Collectively, these observations implicate 14-3-3σ as a major tumor suppressor in a number of human malignancies.
Loss of 14-3-3σ Plays a Critical Role in the Metastatic Phase of ErbB2 Tumor Progression

In addition to its effects on the initiation phase of mammary tumor growth, we demonstrate that mammary epithelial disruption of 14-3-3σ in the NIC strain can have a profound effect on the metastatic phase of breast cancer progression (Fig. 5). We also identified an increased metastatic rate in the ErbB2KI models (Supplementary Fig. S3D and E). Our results generated from NIC tumor cell lines show that loss of 14-3-3σ can significantly increase the metastatic potential of ErbB2-driven tumor cells by enhancing their migratory, invasive, and colonization potential (Supplementary Fig. S5), an effect correlated with the loss of both adherens and tight junctional complexes (Fig. 6). Suggestively, re-expression of 14-3-3σ can completely reverse these metastatic properties by restoration of these junctional proteins (Fig. 7). These effects are not the result of altering the expression levels of these proteins (Figs. 6D and 7E) or through changing the subcellular localization of β-catenin (Supplementary Fig. S8). However, the profound impact of 14-3-3σ on epithelial polarity can be partly explained by its ability to recruit the polarity complex protein Par3 to the apical surface (Supplementary Fig. S6 and S7). In contrast, the closely related 14-3-3ζ protein has the opposite effect on cell migration, invasion, and metastasis (28, 42). Given that 14-3-3ζ is also known to interact with Par3 (43), the opposing effects of these two closely related 14-3-3 isoforms reflects their differential capacity to relocalize 14-3-3 to apical and basal compartments within the cell. Consistent with this view, mammary epithelial cells that have lost 14-3-3σ fail to polarize (28).

The dramatic and reversible effects of 14-3-3σ on epithelial polarity are consistent with observations made with the Er mouse mutant that harbors a truncated version of 14-3-3σ. Mice heterozygous for this mutant form of 14-3-3σ possess severely disrupted epithelial stratification in the skin (26, 27). The concept that genes involved in regulating polarity can impact on tumorigenesis is supported by several recent observations. For example, ErbB2-induced loss of polarity is thought to be the result of its capacity to interact with the Par6–aPKC complex (44). Moreover, it has recently been demonstrated that disruption of the scribble polarity gene plays an important role in c-Myc mammary tumor progression (45). Given the profound impact of loss of 14-3-3σ on a number of polarized epithelial models (28), the potent effects of disruption of 14-3-3σ on ErbB2 tumor progression reflects in part its critical role in epithelial polarity. This in combination
with the effects of 14-3-3σ on ErbB2 transcription (16) and MAPK inactivation (Fig. 4) accounts for a strong selection for 14-3-3σ silencing during breast cancer progression. In addition to breast cancer, loss of 14-3-3σ expression has been noted in a large number of different epithelial-derived cancers (46). Whether loss of 14-3-3σ expression plays a comparable role in progression of these prevalent cancer types remains to be addressed.
METHODS

Plasmid Constructs

14-3-3-δ cDNA and HA-tagged 14-3-3δ cDNA were cloned into the BglII/Hpal sites of pMSCVhygro (Clontech). Primer sequences used were: SfnF: 5’-CCGGATCCAGGAGAGCCAGTCTGATC-3’; SfnR: 5’-CCGATATCTCGACGCGGCTGGTTCC-3’; ZetaF: 5’-GAAGA TCTCGACGCGGTGATCATGCAC-3’; ZetaR: 5’-CCGATATCGGCAAG AAAATGGGAAGG-3’.

Cell Lines

Primary mammary/tumor epithelial cells were obtained from mouse mammary glands/tumors. Mammary tissues were harvested and processed with McIlWain Tissue Chopper and disassociated in Dulbecco minimal essential medium (DMEM) with 2.4 mg/mL Collagenase B (Roche) and 2.4 mg/mL Dispase II (Roche) at 37°C for 3 hours. Disassociated cells were washed with PBS/EDTA (1 mM), spun down at 800 rpm for 3 minutes, resuspended in DMEM with 2% fetal bovine serum (Wisent) with MEGS supplements (Cascade Biologies), and plated on culture dishes (NUNC). 14-3-3δ and 14-3-3δ expressing stable cell lines were generated by retroviral infection of established primary tumor cell lines followed by 300 μg/mL Hygromycin B (Multicell) selection for 7 days. All stable primary cell lines were generated in 2011 and authenticated by PCR and immunoblotting soon after establishment.

Sodium Bisulfite DNA Sequencing

DNA was extracted from mouse primary mammary/tumor epithelial cells and subjected to sodium bisulfite modification using EpiTect Bisulfite Kit (Qiagen). Bisulfite-converted DNA was amplified using primers that cover the majority of the exon of the 14-3-3δ gene, which generated a 529-bp PCR product: SfnSBF: 5’-ccggagTTTTAGGAAGGTTGTTGT-3’; SfnSBR: 5’-ggagTCCTAATATCCAACTC-3’.

Conditions for PCR were as follows: one cycle at 95°C for 5 minutes, 35 cycles at 95°C for 45 seconds, 60°C for 45 seconds and 72°C for 60 seconds, and one cycle at 72°C for 4 minutes. The product was cloned into the EcoRI/HindIII sites of pcDNA3 (Invitrogen), and six clones for each sample were purified using a QIAprep Spin Miniprep Kit (Qiagen) and subsequently sequenced by McGill Genome Quebec Innovation Centre.

Methylation-Specific PCR

Fifty nanograms of sodium bisulfite-treated DNA was analyzed by MSP by using a primer set that covered CG dinucleotide numbers 3, 4, 13, and 14. Primers specific for methylated DNA: MethF: 5’-GAGCTGTCGTAAGAAAAGGCG 3’; MethR: 5’-CCCGATCTCTTTTACCTCG 3’; UmerHF: 5’-AAAGGTTGTGTTGAAAAGGGTTG 3’; UmerHR: 5’-TCCCAATACCTTTCACCTCA 3’. MSP yielded a 177- to 180-bp PCR product. Conditions for MSP were as follows: one cycle of 95°C for 5 minutes, 30 cycles of 95°C for 45 seconds, 64°C for 30 seconds, 72°C for 30 seconds, and one cycle of 72°C for 4 minutes.

Three-Dimensional Matrigel Cell Culture

Eight-well Chamber slides (NUNC) were used. For each well, 100 μL Matrigel (BD Biosciences) was plated and solidified at 37°C for 30 minutes. Later, 1,500 monodispersed cells mixed in 300 μL DMEM with 2% fetal bovine serum, 2% Matrigel, and MEGS supplements were seeded on top. Medium was changed every 3 days. Cells were stained after 10 days of culture.

Immunofluorescence

Cells were fixed at room temperature with 2% paraformaldehyde in phosphate-buffered saline for 15 minutes, permeabilized in 0.5% Triton X-100 in phosphate-buffered saline for 10 minutes, followed by washes of 100 mmol/L glycine in phosphate-buffered saline. Blocking was performed with immunofluorescence buffer (phosphate-buffered saline, 0.1% bovine serum albumin, 0.2% Triton X-100 0.05% Tween-20) plus 2% bovine serum albumin for 1 hour followed by 1-hour incubation at room temperature with the following primary antibodies diluted in immunofluorescence buffer: Par3 (1:100; Millipore); 14-3-3δ (1:100; Millipore); E-cadherin (1:200; BD Transduction Laboratories); and ZO-1 (1:200; Zymed). Cells were washed in immunofluorescence buffer and incubated in a humidified chamber with the appropriate Alexa-fluor conjugated secondary antibodies (1:1000; Molecular Probes) diluted in immunofluorescence buffer for 45 minutes at room temperature. The nuclei were counterstained with 4’,6-diamidino-2-phenylindole (Jackson Laboratories) for 5 minutes.

Immunoblotting

For immunoblotting, membranes were blocked in Tris-buffered saline with 3% skim milk, and incubated overnight with primary antibodies in 3% skim milk: 14-3-3δ (1:2000, Millipore); β-actin (1:5000, Sigma); ErbB2 (1:1000, Santa Cruz); Erk1/2 (1:1000, Cell Signaling); phospho-Erk1/2 (1:1000, Cell Signaling); Akt (1:1000; Cell Signaling); phospho-Akt (1:1000; Cell Signaling); HA.11 (1:2000; Covance); and e-Raf (1:1000, Santa Cruz). Later, membranes were treated by 1 hour incubation at room temperature with horseradish peroxidase-conjugated mouse, rabbit, and goat secondary antibodies (1:5000 in Tris-buffered saline/3% milk; Jackson Laboratories). Blots were developed with enhanced chemiluminescence (Amersham). For quantitative analysis of immunoblots, fluorophore-conjugated mouse and rabbit secondary antibodies (1:10,000; LI-COR Biosciences) were used, and fluorescent detection and quantification were done with the Odyssey imaging system (LI-COR Biosciences) according to the manufacturer’s manual.

Confocal Imaging

Confocal imaging was performed using an Axiovert 200M microscope (Carl Zeiss Microimaging) with ×63 and ×100/1.4 plan-APOCHROMAT objectives equipped with a confocal microscope system (LSM 510 Meta confocal microscope; Carl Zeiss Microimaging). Image analysis was carried out using the LSM 5 image browser (Emipix Imaging).

Boyden Chamber Migration/Invasion Assay

For the migration assay, 24-well plates were used. Monodispersed cells that were 1 × 10⁵ from each cell line were suspended in DMEM and gently applied onto the top of a Boyden chamber (Falcon), whose bottom part was submerged in complete medium. After 37°C incubation for 24 hours, cells passing through the membrane were fixed with 10% formalin, stained with 20% crystal violet, and viewed using a bright-field microscope. For invasion assay, each Boyden chamber membrane was precoated with 5% Matrigel, allowed to solidify at 37°C for 30 minutes, and treated as described previously for the migration assay.

Histology Analysis

Tumor and lung tissues were harvested from tumor-bearing mice at the end point. Tissues were fixed overnight in 10% neutral buffered formalin, paraffin-embedded, and sectioned at 4 μm in the Histology Core Facility of McGill Goodman Cancer Center. Tumor sections were stained with hematoxylin and eosin or underwent Masson’s trichrome staining. For pulmonary metastases analysis, 5 even sections from a total depth of 50 μm of each lung sample were obtained and hematoxylin and eosin-stained. Lung lesions were counted through microscopic analysis.
Statistical Analysis
Statistically significant differences were determined using the Student t test. Differences were considered significant if P < 0.05.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest have been disclosed.

Acknowledgments
We are grateful to Dr. Graeme Hodgson for providing important reagents and advice.

Grant Support
C. Ling received a scholarship from the Department of Defense Breast Cancer Research Program (W81XWH-06-1-0700). All authors were supported by grants from the Canadian Institute of Health Research (CIHR MOP-10594) and the Terry Fox Foundation (NCIC 020002).

Received July 28, 2011; revised October 25, 2011; accepted November 8, 2011; published OnlineFirst November 29, 2011.

REFERENCES
The Role of 14-3-3σ in ErbB2-Induced Tumor Progression

Loss of the 14-3-3σ Tumor Suppressor Is a Critical Event in ErbB2-Mediated Tumor Progression

Chen Ling, Vi-Minh-Tri Su, Dongmei Zuo, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/2159-8290.CD-11-0189

Supplementary Material
Access the most recent supplemental material at:
http://cancerdiscovery.aacrjournals.org/content/suppl/2011/11/11/2159-8290.CD-11-0189.DC1

Cited articles
This article cites 46 articles, 19 of which you can access for free at:
http://cancerdiscovery.aacrjournals.org/content/2/1/68.full#ref-list-1

Citing articles
This article has been cited by 4 HighWire-hosted articles. Access the articles at:
http://cancerdiscovery.aacrjournals.org/content/2/1/68.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.