Bin1 Ablation in Mammary Gland Delays Tissue Remodeling and Drives Cancer Progression

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Abstract

Genes that modify oncogenesis may influence dormancy versus progression in cancer, thereby affecting clinical outcomes. The Bin1 gene encodes a nucleocytosolic adapter protein that interacts with and suppresses the cell transforming activity of Myc. Bin1 is often attenuated in breast cancer but its ability to negatively modify oncogenesis or progression in this context has not been gauged directly. In this study, we investigated the effects of mammary gland–specific deletion of Bin1 on initiation and progression of breast cancer in mice. Bin1 loss delayed the outgrowth and involution of the glandular ductal network during pregnancy but had no effect on tumor susceptibility. In contrast, in mice where tumors were initiated by the ras-activating carcinogen 7,12-dimethylbenz(a)anthracene, Bin1 loss strongly accentuated the formation of poorly differentiated tumors characterized by increased proliferation, survival, and motility. This effect was specific as Bin1 loss did not accentuate progression of tumors initiated by an overexpressed mouse mammary tumor virus-c-myc transgene, which on its own produced poorly differentiated and aggressive tumors. These findings suggest that Bin1 loss cooperates with ras activation to drive progression, establishing a role for Bin1 as a negative modifier of oncogenicity and progression in breast cancer. [Cancer Res 2007;67(1):100–7]

Introduction

Breast cancer is currently the second leading cause of cancer-related death among women. In recent years, medical advances have improved detection at earlier stages, such that women with small localized breast tumors will tend to have a good prognosis after treatment. However, up to 20% of patients with ‘good prognosis’ will nevertheless relapse within 5 years with advanced disease. Conversely, patients considered to have a poorer prognosis are not necessarily fated to relapse with disease. Thus, the limited prognostic information available may cause some patients to be treated too aggressively, increasing therapy-related morbidity, and other patients to be treated too conservatively, increasing disease-related mortality. One way to help improve the management of breast cancer would be to use markers that can accurately predict disease course.

Modifier genes may offer usefulness in this regard given their effects on dormancy versus progression in the context of certain oncogenic pathways that drive neoplasia (1, 2). Alterations in the structure or regulation of a candidate modifier gene that correlates with progression status can offer one line of evidence for a marker. By evaluating alterations in an animal model, one can directly determine whether they are coincidental or causal to disease. To identify disease modifier genes, classic genetics can be used to map genes by “top-down” designs or reverse genetics can be used to assess candidates via “bottom-up” designs, with the understanding that a candidate will be phenotypically silent in the absence of relevant oncogenic lesions. In the present study, we used the latter approach to test the hypothesis that Bin1 acts as a negative modifier of breast cancer progression.

Bin1 encodes a nucleocytosolic BAR adapter protein that can interact with the c-Myc oncoprotein and inhibit its cell transforming activity (3–5). c-Myc is involved in the development of many human breast cancers where its overexpression has been associated with poor prognosis (6). At least 10 splice isoforms of Bin1 exist, with differences in the pattern of tissue distribution, subcellular localization, and protein interactions that indicate diverse functional roles (7–10). BAR adapter proteins include a signature fold termed the BAR domain that recognizes curved vesicular membranes (11). Although BAR adapter proteins have a canonical function in membrane dynamics (12), in certain family members that localize to the nucleus (e.g., including Bin1 and APPL proteins), a moonlighting function in transcriptional regulation has been suggested (4, 5, 13). Notably, only those Bin1 isoforms that are capable of localizing to the nucleus are capable of suppressing oncogenic transformation, facilitating cell suicide, and promoting immune escape of transformed cells in various model systems (3, 4, 14–20). Although attenuation of Bin1 by silencing or missplicing is a frequent event in many human cancers, including breast cancer (3, 16), the consequences of Bin1 loss on tumor progression have not been addressed directly in a preclinical model of disease. Therefore, we tested whether such losses were sufficient to drive initiation or progression of cancers in mice harboring mammary gland–specific deletions of Bin1.

Materials and Methods

Production of transgenic mouse strains. A Bin1-targeting plasmid with the structure shown in Fig. 1 was introduced by electroporation into embryonic stem (ES) cells derived from 129sv mice. Briefly, a neomycin resistance gene (neo) cassette flanked by wild-type (WT) loxP sites was inserted into a genomic targeting vector spanning introns 2 to 5 of the mouse

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Bin1 Inhibits Breast Tumor Progression

Bin1 allele were 5’-TGGAGTCTGCAACCTTCACCC-3’ (loxP1) and 5’GCTCATACACCTCCTGAAGACAC3’ (loxP2; Integrated DNA Technologies, Inc.) with expected sizes of 0.9, 1.07, and 0.31 kb for WT, flox, and recombined flox (loxΔ) alleles, respectively. Following a 4-min denaturation at 94°C, 35 cycles of PCR were done at 94°C for 20 s, 58°C for 1 min, and 72°C for 1 min with the addition of a 10-min final elongation step at 72°C. The primers and PCR conditions used to monitor the Bin1 KO allele have been described (22). The primers used to monitor the wap-cre gene were 5’-GGGCTTGCAGTAAAAACACTAC-3’ (Wap1) and 5’-GTGAACAGCATTGCTGACCT-3’ (Wap2) with allele-positive mice identified by a single 100-bp agarose gel band. PCR conditions for the wap-cre-gene were as follows: after a 4-min denaturation at 94°C, 40 cycles of PCR were done at 94°C for 30 s, 62°C for 1 min, and 72°C for 1 min with the addition of a 5-min final elongation step at 72°C. The primers used to monitor the MMTV-c-myc gene were 5’-CCCAAGGTTAAGTTTTTGG-3’ (Mycl) and 5’-GGGCAATAAGCAGGTTAAAACACT-3’ (Myc2) with allele-positive mice identified by a single 880-bp agarose gel band. PCR conditions for the MMTV-c-myc gene were as follows: after a 3-min denaturation at 96°C, 35 cycles of PCR were done at 94°C for 30 s, 58°C for 1 min, and 72°C for 1 min with the addition of a 5-min final elongation step at 72°C.

Mammary gland carcinogenesis. After one round of pregnancy, female mice received s.c. implants in the intrascapular area of two compressed pellets of 20 mg medroxyprogesterone acetate (Hormone Pellet Press). Three weeks later, we gave the first of four weekly doses of 50 mg/kg 7,12-dimethylbenz(a)anthracene (DMBA; Sigma, St. Louis, MO), given p.o. in cottonseed oil, with the three subsequent doses delivered 1, 3, and 4 weeks after the first dose. On this regimen, we observed mammary tumors to appear with a frequency of ~100% with an average latency of 112 days, not significantly longer than the 99 days reported for CD2F1 (BALB/CXDBA/2) mice (23).

Cell biology. Murine mammary epithelial cells (MMEC) explanted from breast tumors were cultured in DMEM containing 10% fetal bovine serum (FBS; HyClone) and antibiotics. Cells were passaged multiple times at a 1:4 passage ratio to rid explanted tissue of contaminating fibroblasts and other cells. Western blot and immunofluorescence analyses with E-cadherin and β-catenin antibodies (see below) were done to confirm the epithelial nature of MMEC cultures established in this manner. Cell proliferation assays were done by seeding 1 × 10⁴ cells in 100-mm dishes and harvesting at various times later for counting by trypan blue exclusion (24). For serum deprivation, cells were treated the day after seeding them into culture for 24 h with DMEM containing 0.1% FBS. For anchorage-independent growth, 1 × 10⁴ cells were seeded in soft agar, and colonies were documented as described previously (24). For flow cytometry, cells were harvested, washed once with PBS, fixed in 70% ethanol, stained with propidium iodide, and analyzed on a FACScan device (Becton Dickinson). For motility assays, cells were treated the day after seeding them into culture for 24 h with DMEM containing 0.1% FBS. For anchorage-independent growth, 1 × 10⁴ cells were seeded in 100 μL droplet in individual wells of a six-well plate and incubated for 16 h. When cells reached confluence within the droplet, its center was scratched, 2 mL DMEM plus 10% FBS was added to the well, and motility was documented at 48 h by photomicrography.

Western blot analysis. Cells were harvested by washing thrice in PBS before lysis in 1× radioimmunoprecipitation assay buffer [1× PBS containing 1% NP40, 0.5% sodium-deoxycholate, 0.1% SDS, 10 μg/mL phenylmethylsulfonyl fluoride] with 10 μL/mL Protease Inhibitor Set II and III (Calbiochem). Protein was quantitated by Bradford assay and 50 μg protein per sample was analyzed by SDS-PAGE. Gels were processed by standard Western blotting methods using the Bin1 antibody 2F11 (ammonium sulfate supernatant, 1:200 dilution) and horseradish peroxidase (HRP)–conjugated goat anti-mouse secondary antibody (1:2,000 dilution; Cell Signaling). For actin, a primary anti-actin goat polyclonal antibody was used (1:500 dilution; Santa Cruz Chemicals) and HRP-conjugated rabbit anti-goat secondary antibody (1:5,000 dilution; Southern Biotechnology Associates). For cell adhesion proteins, primary antibodies

Figure 1. Tissue-specific deletion of Bin1 in murine mammary gland. A, Bin1 flox targeting construct. White boxes, exons. Colored arrows, the WT (solid) and variant mutant (hatched) loxP sites. thin arrows, location of PCR primers with the size of the predicted amplification products given in bp. The structure of the tri-lox-targeting plasmid is noted along with the structure of the desired floxed or floxΔ alleles generated by Cre-mediated recombination in ES cells in vitro or in vivo, respectively. The position of the T-C mutation was introduced into the variant loxP site. B, mammary gland-specific recombination in wap-cre-/+flox mice. Genomic DNA isolated from various tissues was subjected to PCR using the Bin1 primers presented above (blue arrows). Conversion to the floxΔ allele occurred specifically only in the mammary glands of parous female mice. C, kinetics of wap-cre–mediated recombination. Generation of the floxΔ allele is apparent during and after pregnancy and weaning.

Bin1 gene (21). Three ES cell lines with the desired homologous recombination event were infected with a recombinant Cre adenovirus and subcloned to identify cell colonies that had selectively lost the neo marker, leaving intact the desired floxed exon 3 segment. These correctly targeted ES cell lines were microinjected into C57BL/6j blastocysts, and chimeric animals with germ-line transmission of the floxed Bin1 allele were generated. Bin1 is haplosufficient for viability (22). Therefore, to establish the most efficient system for producing Bin1-expressing or nonexpressing cells by a single Cre-mediated excision event, we crossed the ‘floxed’ allele (loxP) onto a strain with the ‘straight’ knockout (KO) allele (22). Cre recombinase was introduced by interbreeding with B6129-Tg(wap-cre)11738 Mam/j(wap-cre mice) (The Jackson Laboratory). Activated c-myc was introduced by interbreeding with mouse mammary tumor virus (MMTV)-c-myc FVB-N mice (Charles River Laboratories) under a license from DuPont Medical Products (Wilmington, DE).

Genotype analysis. PCR was used to genotype mice as follows. Mouse tissue samples were digested overnight at 60°C in lysis buffer [50 mmol/L Tris-HCl (pH 8.0), 1 mmol/L EDTA, 100 mmol/L NaCl, 1% SDS, 30 μg/mL protease K]. DNA-containing supernatant was diluted 1:50 in 10 mmol/L Tris-Cl (pH 8.0) and 2 μL of diluted supernatant were used for PCR in a final volume of 20 μL in a PTC-2000 Peltier Thermal Cycler (MJ Research). Amplification products were separated by electrophoresis on 2% agarose DNA (Fisher) as a molecular size marker. The primers used to monitor the

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used included anti-E-cadherin (1:1,000 dilution; clone 36, Transduction Laboratories), anti-β-catenin (1:1,000 dilution; clone 5H10, Zymed), anti-N-cadherin (1:1,000 dilution; clone 3B9, Zymed), anti-vimentin (1:1,000 dilution; clone Vim13.2, Sigma), and goat anti-mouse secondary antibody (1:1,000 dilution; Southern Biotechnology Associates). Detection was done routinely using a commercial kit (enhanced chemiluminescence-Western blot, Amersham).

**Zymogram analysis.** Gelatinase activity in protein extracts from established MMEC tumor cell lines was monitored as described.5

**Orthotopic tumor formation assay.** Cells (1 × 10^6) were suspended in 200 μL DMEM and injected orthotopically into the mammary fat pads of syngeneic F1 offspring from FVB-N and C57BL/6j breeders (The Jackson Laboratory) or immunocompromised CD-1 nude (CrlCD-1-nuBR) mice (Charles River Laboratories). When tumors reached ~20 mm in diameter, mice were euthanized and tumor weight(s) and volume(s) were calculated via caliper (volume = width^2 × length × 0.52).

**Results**

**Targeted deletion of Bin1 delays remodeling and involution of the mammary gland.** To determine whether Bin1 attenuation could directly affect initiation or progression of breast cancer, we embarked on an investigation of the consequences of deleting Bin1 in the mammary gland of the mouse. Previous work established that homozygous deletion causes perinatal lethality (22); therefore, for this work, we generated a conditional mutant using Cre-lox technology. The design is presented in Fig. 1 along with results confirming the desired in vivo operation of the ‘floxed’ allele in the mammary gland. In the ‘tri-lox’ scheme used, deletion of exon 3 leads to exon 2 to 4 splicing, producing an out-of-frame stop codon in exon 4 that abolishes protein expression from all alternately splice isoforms of Bin1 RNA. In one variation of the standard design, a mutant loxP site incorporating a T→C mutation was included, such that exon 3 was flanked on its 5’ side by a WT loxP site and on its 3’ side by the mutated loxP site (Fig. 1A). This variation conferred a selective advantage to Cre-mediated excision of the neo cassette in vitro, without compromising the subsequent ability of Cre to delete the floxed target sequence in vivo.5 Chimeric mice generated from targeted ES cell transfecants were interbred with transgenic mice to produce strains that included the WT Bin1 allele (+), floxed KO allele (flo), and ‘straight’ KO allele (22) along with a breast-specific wap-c-re transgene and, in some experiments, a MMTV-c-myc transgene. In animals carrying the wap-c-re gene, loxP-mediated recombination in females was induced by parity because the wap acidic protein (wap) promoter is activated in mammary epithelial cells during pregnancy. Bin1 is haplosufficient for survival (22), so the breeding scheme compared mice with +/flo or KO/flo genotypes to compare the effects of functional ablation. As expected, mice with a wap-creKO/flo genotype exhibited tissue-specific conversion of the floxed allele to the desired ‘floxA’ allele in genomic DNA isolated from mammary gland from late pregnancy through weaning (Fig. 1B and C). To simplify nomenclature, in the text that follows, we refer to mice with a wap-cre/+ or wap-creKO/flo genotype as Bin1+mam or Bin1Δmam mice, respectively, indicating the retention of one functional allele or the loss of both alleles in the mammary gland. In work to be reported elsewhere,6 we confirmed that the floxA allele is functionally inactivated based on its ability to phenocopy a ‘straight’ KO allele with regard to myocardial hypertrophy and perinatal lethality (22). These experiments confirmed that the model system operated as required to investigate the effect of Bin1 ablation on remodeling and tumorigenesis in the mammary gland.

To evaluate whether Bin1 loss affected mammary gland remodeling induced by pregnancy, female mice were set up for timed pregnancies by monitoring for vaginal plugs. After parturition, litter sizes were normalized to five pups and nursing was continued 1 week to ensure full lactation before pups were removed to induce mammary gland involution. Mammary gland tissues were isolated for analysis from virgins (control) or at 18.5 days post coitum (dpc), 7.5 days post partum (dpp; full lactation), 10.5 dpp (early involution), 17.5 dpp (late involution), and 27.5 dpp (full regression). A delay in the kinetics of ductolobular development was apparent at 18.5 dpc, at which time Bin1Δmam mice showed significantly less glandular remodeling than Bin1+mam mice (Fig. 2). However, during lactation at 7.5 dpp, this defect had resolved, such that no deficiencies were apparent in nursing and pups showed no signs of malnutrition. During glandular involution, a delay in remodeling again became apparent, such that ductolobular regression was achieved with somewhat slower kinetics in Bin1Δmam mice. We concluded that Bin1 was non-essential for formation of a fully functional lactating mammary gland but that it was needed to optimally support the rapid kinetics of ductolobular remodeling in the gland during pregnancy and weaning.

**Bin1 attenuation drives progression of ras-dependent mammary carcinomas.** We evaluated Bin1 as a classic suppressor or negative modifier gene in breast cancer by investigating whether its deletion was sufficient (a) to increase the incidence of mammary tumor formation, in the manner of an inactivated suppressor, or (b) only to increase the progression of mammary tumors initiated by primary oncogenic lesions, in the manner of an inactivated negative modifier.

To evaluate Bin1 as a suppressor gene, we compared the effect of tissue-specific ablation in three cohorts of Bin1+mam and Bin1Δmam female mice carried out as nonparous animals (virgin), uniparous animals (one round of pregnancy), or multiparous animals (seven rounds of pregnancy). After birth, litters were normalized to five pups, nursed 10 days, and then removed. By 2 years of age, both strains of mice developed mammary gland tumors with the same low frequency (Table 1). No differences were seen between uniparous and multiparous groups, which were combined as parous. Although the tumors that arose in the cohort of Bin1Δmam mice were relatively more poorly differentiated, the similarly low incidence observed argued against the notion that Bin1 functioned as a classic breast tumor suppressor gene.

To evaluate Bin1 as a negative modifier gene, we compared the effect of its tissue-specific ablation in tumors initiated by the carcinogen DMBA, which acts through ras activation (23), a MMTV-c-myc transgene, or both. We chose these well-established models of mammary carcinoma based on the evidence that Bin1 can suppress neoplastic transformation of primary cells by c-myc+ras (3, 4, 19). In the initial trials, Bin1+mam and Bin1Δmam female mice were treated with DMBA and monitored for tumor formation. Both cohorts displayed similar rates of tumor latency, multiplicity, and lung metastasis (Table 1). However, whereas Bin1+mam mice developed well-differentiated tumors, characterized by high tubule formation, low mitotic indices, and limited nuclear pleomorphism, Bin1Δmam mice developed poorly...
differentiated tumors, characterized by low tubule formation, high mitotic indices, and high degrees of nuclear pleomorphism (Table 1). Nuclear pleomorphism was particularly increased by Bin1 loss (Fig. 3). Additionally, Bin1Δmam mice displayed a relative increase in lymphocyte infiltration compared with Bin1+mam mice (57% versus 20% of tumors; A.P.S., data not shown). In parallel experiments done in a mosaic model, we saw a similar pattern of development of more poorly differentiated mammary tumors in Bin1Δmam mosaic mice. Together, these observations suggested that Bin1 functioned as a negative modifier to restrict the progression of tumors initiated by activation of the ras pathway.

An interesting feature of the mammary tumor-bearing Bin1Δmam cohort was that it displayed a coincident elevation in uterine endometritis and ovarian granulosa cell tumors, implying either haploinsufficiency or a non–cell autonomous effect of Bin1 loss in the uterus and ovary. DMBA treatment is known to cause such lesions in addition to mammary tumors (e.g., ref. 25) but not at the relatively higher penetrance observed in mice from the Bin1Δmam cohort. The transcriptional activity of the wap promoter is restricted to brain and the mammary gland during the lactational stage of pregnancy (26), and consistent with this pattern of expression, we did not detect recombination of the Bin1 floxed allele in the ovary or uterus of Bin1Δmam mice.

Table 1. Bin1 loss drives progression of DMBA-induced mouse mammary carcinomas

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Regimen</th>
<th>No. mice w/ tumors (%)</th>
<th>Tumors per mouse</th>
<th>Latency (d)*</th>
<th>Grade c T,N,M, (sum)</th>
<th>Differentiation status</th>
<th>Lung metastasis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bin1+</td>
<td>Nonparous</td>
<td>0/6 (0)</td>
<td>0</td>
<td>NA</td>
<td>ND</td>
<td>NA</td>
<td>0/0 (0)</td>
</tr>
<tr>
<td>Bin1+mam</td>
<td>Parous</td>
<td>1/19 (5)</td>
<td>1</td>
<td>NA</td>
<td>ND</td>
<td>WD</td>
<td>0/1 (0)</td>
</tr>
<tr>
<td>Bin1Δmam</td>
<td>Parous</td>
<td>2/24 (8)</td>
<td>1</td>
<td>NA</td>
<td>ND</td>
<td>PD</td>
<td>0/2 (0)</td>
</tr>
<tr>
<td>Bin1+mam</td>
<td>DMBA</td>
<td>8/8 (100)</td>
<td>2.3 ± 1.3</td>
<td>128 ± 47</td>
<td>1.6,1.5,2.3 (5.4)</td>
<td>WD</td>
<td>4/8 (50)</td>
</tr>
<tr>
<td>Bin1Δmam</td>
<td>DMBA</td>
<td>14/14 (100)</td>
<td>2.2 ± 1.4</td>
<td>155 ± 56</td>
<td>2.3,2.5,2.9 (7.8)</td>
<td>PD</td>
<td>7/14 (50)</td>
</tr>
</tbody>
</table>

NOTE: Nonparous and parous mice not treated with DMBA were monitored for their full life span for breast tumor formation. In these groups, the small number of tumors that arose was all seen in elderly animals of >1 year of age. Uniparous animals treated with DMBA exhibited similar latencies for mammary tumor formation regardless of genotype that were not significantly longer than 99 days reported in CD2F1 mice (23). All DMBA-treated animals were carefully examined at necropsy for lung metastases, other neoplasms, and other pathologic lesions in major organs (see text), with any suspected lesions confirmed by histologic analysis.

Abbreviations: NA, not applicable; ND, not determined; WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated.

*Number of days after last DMBA treatment.

Grade according to the Nottingham modification of the Bloom-Richardson system (three variables): T (tubule formation), 1-3; N (nuclear pleomorphism—nuclear variation in size and shape), 1-3; M (mitotic count—mitoses), 1-3.
Nevertheless, there was an increased incidence of DMBA-induced ovarian granulosa cell tumors in Bin1Δmam mice (43%) relative to Bin1+mam mice (13%), all of whom also had mammary tumors at diagnosis (Supplementary Figure S1; Supplementary Table S1). In the uterus, a similar incidence of cystic hyperplasia was observed but only the Bin1Δmam cohort displayed endometritis (Supplementary Figure S2; Supplementary Table S1). These observations corroborated the concept of Bin1 as a negative modifier of lesions produced by DMBA treatment, due to either haploinsufficiency or a non–cell autonomous mechanism of action in the ovary and uterus.

In contrast to the above observations, we found that Bin1 deletion had little effect when tumor formation was initiated by a c-myc transgene. In female MMTV-c-myc mice carried out under multiparous conditions to activate transgene expression, mammary adenocarcinomas develop at a frequency approaching 100% with a latency of 7 to 10 months (27). In multiparous c-Myc;Bin1-mam and c-Myc;Bin1Δmam females, we observed the development of similar moderate to poorly differentiated mammary adenocarcinomas, with similar latencies, high tumor grades, and robust metastatic propensities (Supplementary Table S2). These tumors were characterized by large round cells with histologic evidence of an abundance of infiltrating macrophages and apoptotic cells (data not shown). When these mice were treated with DMBA, we observed the development of similar poorly differentiated mammary carcinomas. However, c-Myc;Bin1Δmam mice also developed aggressive lymphomas that appeared in some animals before mammary carcinomas had formed (Supplementary Table S2). As above, this observation suggested either haploinsufficiency or a cell nonautonomous modifier effect on DMBA-induced tumors (28) in cooperation with MMTV-c-myc (the expression of which is leaky in lymphoid cells). Taken together, these findings argue that the effects of Bin1 loss were selective insofar as cooperation was only observed and apoptotic cells (data not shown). When these mice were processed for H&E staining of primary tumors confirmed common expression of E-cadherin and β-catenin, but not of vimentin, which is expressed strongly in mammary myoepithelial cells and fibroblasts (Fig. 4C). Immunohistochemical staining of primary tumors confirmed common expression of E-cadherin and β-catenin (data not shown), consistent with the likelihood that the established tumor cell populations are indeed epithelial in character. N-cadherin was also expressed in these cell lines; however, because there was no correlation to Bin1 status, this mesenchymal marker was interpreted as a general feature of DMBA-induced breast carcinogenesis in the mouse (Fig. 4C).

Bin1Δmam cells displayed a 3–4 times higher rate of in vitro proliferation under anchorage-dependent conditions (Fig. 4D). Under conditions of anchorage-independent growth in soft agar culture, only Bin1Δmam cells displayed detectable colony formation activity in parallel with their more aggressive growth character (data not shown). Bin1Δmam cells also exhibited severalfold greater resistance to apoptosis elicited by serum deprivation (Fig. 4E), extending evidence of a proapoptotic role for Bin1 in neoplastic cells (14–16, 18, 19). Lastly, Bin1Δmam cells displayed an increased motility in monolayer culture associated with increased gelatinase activity attributable to activated matrix metalloproteinase (MMP-9; Fig. 4F and G). Taken together, these results strengthened the evidence that Bin1 acts in the guise of a negative modifier in cancer.

We compared the ability of Bin1Δmam and Bin1+mam MMECs to form orthotopic tumors in syngeneic immunocompetent mice and immunocompromised nude mice, based on an earlier demonstration that Bin1 loss could promote immune escape of myc-ras-transformed keratinocytes (20). Unfortunately, none of the MMEC populations established from DMBA-induced mammary tumors could form tumors in syngeneic FVB/N × BL/6 F1 female mice, preventing us from exploring this issue further. In contrast, after orthotopic injection of 10⁷ cells into the fat pads of immunocompromised female nude mice, Bin1Δmam cells formed tumors efficiently, whereas Bin1+mam cells formed mainly indolent nodules up to 5 months after seeding (Fig. 4H). The more profound in vivo growth differences observed could not be fully explained by the in vitro differences documented, suggesting that Bin1 loss may provide an additional undefined benefit in vivo.

Nude mice retain natural killer (NK) cell and some B-cell immune functions, but we observed a similar pattern of tumor growth in severe combined immunodeficient (SCID) and SCID/beige murine hosts, which completely lack T/B and T/B/NK cell functions, respectively (data not shown). In summary, we concluded that Bin1 acts to limit the progression of DMBA mammary tumors at several intrinsic levels, including by negatively modifying the proliferation, survival, and motility of tumor cells.

**Discussion**

This study provides evidence that Bin1 functions as a negative modifier or antiproliferation gene during breast tumorigenesis. In the parous gland, Bin1 was dispensable for function, based on normal patterns of nursing and development in newborn pups. However, histologic analysis revealed a requirement for the rapidly
of the kinetics of ductolobular remodeling that occurs during pregnancy. Given an involvement of the yeast homologues of Bin1 in stress signaling (25, 29), the significance of its role in remodeling might depend on stresses in the natural environment that could limit milk production. In DMBA-induced tumors, we observed effects of Bin1 status on the activity of the MMP-9, which can contribute to remodeling of the mammary gland during pregnancy (30). However, because dysregulation of MMP-9 has significantly more pronounced effects on the normal mammary gland than that produced by Bin1 loss, we do not favor the interpretation that the phenotype produced by Bin1 loss relates to MMP-9 dysregulation.

We observed no long-term effects of Bin1 deletion on cancer incidence in virgin or parous animals, indicating that this gene does not function as a classic tumor suppressor in the mammary gland. In contrast, when mammary tumors were initiated by DMBA in Bin1-deficient mice, we found that high-grade carcinomas emerged that exhibited increased proliferation, survival, and motility relative to tumors induced in control mice expressing Bin1. Interestingly, we noted a coincident increase of ovarian tumors or lymphomas in Bin1 Dm mice, which reflected either haploinsufficiency or a non–cell autonomous mechanism of action in these settings. Although the underlying mechanisms of these effects were undefined, they provided further corroboration of the concept of Bin1 as a negative modifier in breast cancer.

In previous work in transformed mouse keratinocyte and fibroblast models, we observed that Bin1 loss strongly affected the capacity for immune escape with less effect on proliferation and survival (19, 20). In particular, in the transformed keratinocyte model, we had identified a role for indoleamine 2,3-dioxygenase (IDO) in mediating immune escape (20). Unfortunately, we could not evaluate effects of Bin1 loss on IDO-mediated immune escape in the DMBA mammary carcinogenesis model because none of the tumor MMEC populations had the ability to form grafts in immunocompetent hosts. In any case, other evidence suggests that in breast cancer, the mechanism of immune escape based on IDO elevation may be more relevant in the peripheral immune cells in tumor-draining lymph nodes than in the tumor cells themselves (31), the latter of which do not tend to overexpress IDO like other solid tumors (32). Therefore, breast models may not be especially pathophysiologically germane to evaluate how Bin1 attenuation in tumor cells affects immune escape. In assessing cell-intrinsic qualities, differences in the effects of Bin1 on proliferation and
survival in the keratinocyte and fibroblast models may reflect their in vitro generation, where strong selections for survival and proliferation are imposed (perhaps defeating the benefits of losing a negative modifier). In contrast, the findings from the in vivo–generated breast model reported here corroborate the findings of a large number of reports showing the ability of Bin1 to limit cell proliferation and survival (3, 4, 14–19, 33, 34). In this study, we also observed increased motility and elevated MMP-9 activity in MMEC tumor cell populations lacking Bin1, a finding that we have since corroborated in the transformed keratinocyte and fibroblast models characterized previously.7 Further assessment of the mechanism of MMP-9 dysregulation as well as the effects of Bin1 loss on proliferation and survival is currently being conducted in a mosaic model where direct in vivo evaluations in other tissues are possible.

It is apparent that the effects of Bin1 loss in the mammary gland were selective because of the specific cooperation of Bin1 loss in driving progression with ras activated by DMBA but not c-myc overexpressed from the MMTV promoter. These data imply that the functional effects of Bin1 loss and myc overexpression must overlap to some extent because of the ability of either Bin1 loss or myc activation to cooperate with ras activation to drive breast tumor progression (present study; ref. 35). Bin1 loss obviously does not fully phenocopy myc activation. Thus, along with evidence that nuclear Bin1 proteins functionally interact with c-Myc protein (3–5), a logical inference is that Bin1 acts to limit a subset of myc functions that are selectively important to progression in cooperation with ras. In this context, it is interesting to note that although maintaining the expression of myc throughout the cell cycle is sufficient to prevent cell cycle exit and to drive tumorigenesis, many human cancers not only deregulate myc but overexpress it (36). Following the implication that myc overexpression may benefit tumor progression, our data support a model where Bin1 loss partly or fully phenocopies such benefits in cooperation with ras activation (Fig. 5).

In considering models that incorporate a relationship between these genes, we note that recent genetic studies of myc in Drosophila seem to relate vesicle trafficking processes to a facet of Myc function that drives cell competition in tissues (37). Using the imaginal disc as a model system, a function for myc in driving cell competition was defined that could be fully phenocopied by overexpression of the small GTPase Rab5 (37), a well-characterized regulator of vesicle trafficking. Specifically, it was shown that high-Myc cells and high-Rab cells could compete equally for internalization of limiting growth factors in a tissue (37, 38), surviving at the cost of low-Myc or low-Rab cells that lost the competition and perished (37). The genetic complementarity these findings suggest that the cell competition function of Myc may rely on trafficking events of the type that involve Rab5 and Bin1 (12), which biochemically interact through Bin3, a Rab5 guanine nucleotide exchange factor that binds Bin1 on early endosomes (39). In future work, the conditional mutant mouse and pathologic footing developed here will provide a solid foundation to address questions about the precise mechanism(s) by which Bin1 loss facilitates tumor progression.

One clinical implication of our findings is that losses of nuclear Bin1 may predict poor prognosis of breast cancers when c-Myc is not overexpressed but Ras signaling is deregulated, for example, due to deregulation of an upstream growth factor receptor. Although some fraction of breast carcinomas overexpress c-Myc, signaling poor prognosis (40–44), and immunohistochemical losses of Bin1 that seem to occur more frequently (16, 45) may be useful in the larger number of cases where c-Myc is not overexpressed. The findings of this study prompt an examination of Bin1 in retrospective or prospective studies where its potential usefulness as a progression marker can be further evaluated (46).

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References


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7 J.B. DuHadaway, unpublished observation.
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