Phosphorylation Regulates c-Myc's Oncogenic Activity in the Mammary Gland

Xiaoyan Wang1, Melissa Cunningham1, Xiaoli Zhang1, Sara Tokarz1, Bryan Laraway1, Megan Troxel2, and Rosalie C. Sears1

Abstract

Expression of the c-Myc oncoprotein is affected by conserved threonine 58 (T58) and serine 62 (S62) phosphorylation sites that help to regulate c-Myc protein stability, and altered ratios of T58 and S62 phosphorylation have been observed in human cancer. Here, we report the development of 3 unique c-myc knock-in mice that conditionally express either c-MycWT or the c-MycT58A or c-MycS62A phosphorylation mutant from the constitutively active ROSA26 locus in response to Cre recombinase to study the role of these phosphorylation sites in vivo. Using a mammary-specific Cre model, we found that expression of c-MycWT resulted in increased mammary gland density, but normal morphology and no tumors at the level expressed from the ROSA promoter. In contrast, c-MycT58A expression yielded enhanced mammary gland density, hyperplastic foci, cellular dysplasia, and mammary carcinoma, associated with increased genomic instability and suppressed apoptosis relative to c-MycWT. Alternatively, c-MycS62A expression reduced mammary gland density relative to control glands, and this was associated with increased genomic instability and normal apoptotic function. Our results indicate that specific activities of c-Myc are differentially affected by T58 and S62 phosphorylation. This model provides a robust platform to interrogate the role that these phosphorylation sites play in c-Myc function during development and tumorigenesis.

Introduction

The c-Myc oncoprotein is a pleiotropic transcription factor involved in controlling many cellular functions, including cell proliferation, cell growth, and cell differentiation, and pathways that regulate genome stability and cell death (1). c-Myc is constitutively highly expressed in most human tumors, and high expression of c-Myc in animal models can drive tumorigenesis (2–4). c-Myc-induced tumorigenesis is tempered by its activation of intrinsic tumor suppressor pathways involving engagement of cell cycle checkpoint and apoptotic cell death pathways (5). Activation of these intrinsic tumor suppressor pathways induces cell death in normal cells (6), but in tumor cells these fail-safe mechanisms are bypassed by secondary lesions that likely evolve from genomic instability associated with high c-Myc expression. Thus, to maintain normal cell function, c-Myc expression is tightly controlled at the level of transcription, mRNA stability, translation, and protein stability. In breast cancer, c-MYC gene amplification has been reported in approximately 16% of cases and increased mRNA expression in approximately 22% (7, 8). However, elevated expression of c-Myc protein is reported in approximately 70% of human breast tumors, arguing for a potentially critical role for posttranslational regulation of c-Myc expression (2, 9, 10).

Phosphorylation of c-Myc at conserved residues serine 62 (S62) and threonine 58 (T58) can regulate c-Myc protein stability in response to mitogen signaling (11). Phosphorylation of S62 by ERK or CDK kinases transiently increases c-Myc stability whereas phosphorylation of T58 by GSK3β triggers dephosphorylation of S62 by protein phosphatase 2A (PP2A), ubiquitination by the SCF-Fbw7 E3 ligase, and proteasomal degradation (12). The scaffold protein Axin1 coordinates c-Myc degradation through this pathway (13). Mutations in PP2A subunits, FBW7 and AXIN1 have been reported in many human cancers (14–16), suggesting that this c-Myc degradation pathway can be deregulated in cancer cells leading to altered levels of T58 and S62 phosphorylation and increased c-Myc stability.

In cell culture experiments, mutation of S62 to alanine (S62A) reduces c-Myc’s transforming activity whereas mutation of T58 to alanine (T58A) enhances c-Myc’s transforming activity, suggesting that phosphorylation changes at these sites can affect c-Myc function (17–20). Furthermore,
decreased T58 and increased S62 phosphorylation have been observed in human cancer cell lines associated with increased c-Myc protein stability (13, 21). This phosphorylation ratio is partially mimicked in the c-Myc<sup>T58A</sup> mutant, which has no T58 and constitutive high S62 phosphorylation (18, 22, 23).

To carefully address the role of T58 and S62 phosphorylation in c-Myc's activity in vivo, we have generated 3 conditional c-myc knock-in mice that express c-Myc<sup>WT</sup>, c-Myc<sup>T58A</sup>, or c-Myc<sup>S62A</sup> (which lacks phosphorylation at both sites due to their hierarchical nature) from the endogenous ROSA26 locus in response to Cre-mediated recombination. In this study, we used Wap-Cre to drive expression in the mammary gland as it provides an elegant system to interrogate multiple activities of c-Myc. In normal mammary development, c-Myc expression is important for both pregnancy-associated proliferation and apoptosis during involution (24, 25). High-level expression of c-Myc can also drive mammary gland tumorigenesis in transgenic mouse models (3, 26, 27). The extended mammary tumor latencies in these studies suggest that additional lesions are required for mammary tumorigenesis. Results from this study indicate that lesions affecting phosphorylation at T58 and S62 could contribute to tumorigenesis, as the phosphorylation status of these sites substantially affected c-Myc function and tumorigenic potential in the mammary gland.

Methods

Generating RFS-myc<sup>WT</sup>, RFS-myc<sup>T58A</sup>, and RFS-myc<sup>S62A</sup> mice

ROSA-Floxed-Stop (RFS)-myc<sup>WT</sup>, RFS-myc<sup>T58A</sup>, and RFS-myc<sup>S62A</sup> mice were generated using an established gene knock-in strategy (28; see Supplementary Material). Briefly, murine c-myc<sup>WT</sup>-HA or phosphorylation mutant c-myc<sup>T58A</sup>-HA or c-myc<sup>S62A</sup>-HA cDNAs (29) were cloned into targeting vectors and electroporated into 129 ES cells. Correctly targeted and sequence-confirmed ES clones were injected into C57BL/6 blastocysts to obtain chimeric mice. Germline transmission was obtained by crossing with C57BL/6 mice to establish homozygous knock-in RFS-myc<sup>WT</sup>, RFS-myc<sup>T58A</sup>, and RFS-myc<sup>S62A</sup> strains. RFS-myc<sup>WT</sup>-Ha<sup>T58A</sup> and RFS-myc<sup>S62A</sup> were crossed with Wap-Cre transgenic mice (C57BL/6), obtained from the National Cancer Institute (NCI) Mouse Models of Human Cancer Consortium (MMHCC).

Genotyping and detection of recombination

RFS-myc<sup>WT</sup>, RFS-myc<sup>T58A</sup>, and RFS-myc<sup>S62A</sup> knock-in mice and the presence of Wap-Cre were identified by PCR analysis using tail DNA and specific primer sets (see Supplementary Material). Cre-mediated recombination in RFS-myc<sup>WT</sup>, T58A, S62A/Wap-Cre mice was detected by PCR analysis and specified primers (see Supplementary Material).

RNA analysis

RNA was isolated from mammary glands using TRIzol reagent (Invitrogen) according to manufacturer's protocol. cDNA was prepared from isolated RNA and analyzed by PCR or quantitative PCR (see Supplementary Material).

Antibodies and Western blotting

Antibodies are listed in Supplementary Material. Mouse mammary gland samples were lysed by homogenizing in EBC buffer with protease and phosphatase inhibitors, and subject to Western analysis (see Supplementary Material).

Quantification and statistics

Western blots were visualized and quantified using LI-COR Odyssey Infrared software version 1.2, which is linear over 4 orders of magnitude. Statistical significance was determined by Student's t test.

Cycloheximide half-life

Primary mouse embryo fibroblasts (MEF) or mammary epithelial cells (MEC; see Supplementary Material; 1–2 passages) were infected with Ad-Cre for 48 hours and then starved in 0.1% FBS for another 48 hours. Cells were treated with cycloheximide and harvested at each time point (see Supplementary Material). HA-tagged WT, T58A, or S62A Myc was immunoprecipitated with HA antibody and Western blotted with Y69 Myc antibody.

Pathologic assessment

Mice were anesthetized with avertin, sacrificed, and tissues were fixed in 10% formalin, embedded in paraffin, and 5-μm thick sections were stained with Mayer's hematoxylin and eosin (H&E). Pathologic findings were classified according to the MMHCC NIH/NCI by Dr. Troxell (30).

Apoptosis assay

Apoptosis was detected by terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) staining using the Apo Tag kit (Chemicon International) and the percentage of apoptotic cells was determined in 10 random fields per slide.

Whole mount analysis of mammary gland

Mammary gland tissues were dissected, spread on glass slides, and fixed immediately with Carnoy's Fixative. Tissues were hydrated with serially diluted ethanol and stained with 0.6% carmine solution overnight at 4°C. Tissues were cleared of lipid with xylene and mounted with permount.

Chromosome spreads and centrosome staining

Primary mammmary epithelial cells, 80% confluent, were used for centrosome detection, and chromosome spreads after colcemid treatment (see Supplementary Material). Chromosome spreads were stained with Giemsa. Centrosomes were detected by immunofluorescence for pericentrin. G-banding was done by the OHSU Cytogenetics Laboratory.

Results

Generation of conditional RFS-myc<sup>WT</sup>, RFS-myc<sup>T58A</sup>, and RFS-myc<sup>S62A</sup> mice

To study the effects of altering c-Myc phosphorylation at T58 and S62 on c-Myc's activity in vivo, we utilized 2 phosphorylation mutants: c-Myc<sup>T58A</sup>, which lacks T58 and S62 phosphorylation. Cre-mediated recombination in RFS-myc<sup>WT</sup>, RFS-myc<sup>T58A</sup>, and RFS-myc<sup>S62A</sup> mice was detected by PCR analysis and specified primers (see Supplementary Material).
phosphorylation and has constitutively high S62 phosphorylation, as it is resistant to PP2A-mediated dephosphorylation (20); and c-Myc\(^{\text{S62A}}\), which lacks phosphorylation at both sites due to their hierarchical nature (Supplementary Fig. S1A). We used an established gene knock-in strategy to insert HA-tagged cDNAs, either murine wild-type c-myc\(^{\text{WT}}\), or phosphorylation site mutants c-myc\(^{\text{T58A}}\) or c-myc\(^{\text{S62A}}\), preceded by a transcription stop sequence flanked by lacP sites, into the constitutively active ROSA26 gene locus (Fig. 1A; Supplementary Fig. S1B). The ROSA26 locus ubiquitously drives gene expression in all embryonic and adult tissues; however, due to the transcription stop sequence, the knock-in c-myc genes will not be expressed unless Cre recombinase is present. We have established homozygous breeding pairs for each of these strains, termed Rosa-Floxed-Stop (RFS-\text{m}yc\(^{\text{WT}}\), RFS-\text{m}yc\(^{\text{T58A}}\), and RFS-\text{m}yc\(^{\text{S62A}}\) (Supplementary Fig. S1C). Primary MEFs from each strain were infected with Ad-Cre and nuclear expression of HA-tagged c-Myc was verified by immunofluorescence (Supplementary Fig. S2A). The mean half-life of c-Myc\(^{\text{WT}}\), c-Myc\(^{\text{T58A}}\) and c-Myc\(^{\text{S62A}}\) in the primary MEFs was 19.5, 63.0, and 44.5 minutes, respectively, under serum-starved conditions (Supplementary Fig. S2B). We did note variability in the half-life of the S62A mutant. Because we do not know what E3 ligase targets this mutant, the reason for this variability is currently unknown.

**Initial characterization of RFS-\text{m}yc\(^{\text{WT}}\)/Wap-Cre, RFS-\text{m}yc\(^{\text{T58A}}\)/Wap-Cre, and RFS-\text{m}yc\(^{\text{S62A}}\)/Wap-Cre mice**

To address the consequences of altering c-Myc T58 and S62 phosphorylation on c-Myc activity in the mammary gland, we crossed our 3 RFS \text{m}yc strains with WAP (whey acidic protein)-Cre transgenic mice, which express Cre in the secretory luminal and ductal epithelial cells of the mammary gland during late pregnancy and lactation (31), and experimental mice were subjected to 3 consecutive rounds of pregnancy/lactation. This resulted in approximately 50% Cre-mediated recombination in the mammary epithelial cells as detected by breeding with the R26R reporter mouse (Supplementary Fig. S1D). PCR analysis of genomic DNA from various tissues showed that the recombined allele was primarily detected in mammary gland from RFS-\text{m}yc mice with Wap-Cre, but not in control mice without Wap-Cre (Supplementary Fig. S1E). The advantage of this mouse model is that unlike other c-myc transgenic mouse models in which c-Myc is expressed at very high levels, the ROSA26 promoter is relatively weak and ubiquitously drives expression of only 2 copies of the knocked-in c-myc genes at near physiologic levels (Fig. 1B, Total-\text{m}yc, Pregnancy; ref.32). We observed partial suppression of the endogenous c-myc gene with expression of ectopic c-Myc\(^{\text{WT}}\), c-Myc\(^{\text{T58A}}\), and c-Myc\(^{\text{S62A}}\) in this system (data not shown), consistent with other reports (33, 34). As expected, expression of ectopic c-myc\(^{\text{WT}}\), c-myc\(^{\text{T58A}}\), and c-myc\(^{\text{S62A}}\) mRNA was equivalent and Cre-dependent (Fig. 1B, Ectopic-myc). Expression of ectopic c-Myc\(^{\text{T58A}}\) protein was on average higher than c-Myc\(^{\text{S62A}}\), which was generally higher than c-Myc\(^{\text{WT}}\) (Fig. 1B, HA IP/Myc WB, and Ectopic Myc graph). However, total c-Myc levels measured in the IP input were not distinguishable across all of the strains during pregnancy, when the epithelial cells are stimulated to proliferate and endogenous c-Myc protein expression is high (Fig. 1B, Input-Myc and Total Myc graph). This result highlights the physiologic expression level of this model. We also measured the half-life of the ROSA-driven c-Myc proteins in primary MECs under serum starvation conditions (Fig. 1C). c-Myc\(^{\text{T58A}}\) had the longest half-life, followed by c-Myc\(^{\text{S62A}}\) and c-Myc\(^{\text{WT}}\), consistent with their expression level (Fig. 1B), and consistent with the MEF data (Supplementary Fig. S2B), although differences in stability were not as pronounced as in the MEFs.

Although differences in expression of the ectopic Myc proteins was not dramatic, histologic examination of mammary glands from the RFS-\text{m}yc/Wap-Cre strains during the third pregnancy showed significant alterations in the numbers of lobule-alveolar units and mean alveolar area. Specifically, Myc\(^{\text{S62A}}\)-expressing glands showed significantly reduced numbers of alveoli compared with control, whereas Myc\(^{\text{T58A}}\) had the opposite effect, with substantially expanded alveolar areas and the presence of enlarged ducts containing secretion (Fig. 1D and graph). Analysis of proliferation by BrdU incorporation did not reveal any substantial differences between the strains during pregnancy or early parous stage (data not shown).

**Delayed and incomplete mammary gland involution with c-Myc\(^{\text{T58A}}\) expression**

c-Myc is a potent inducer of apoptosis under growth-limiting conditions. Involution of the mammary gland following weaning represents a period of intense apoptotic activity associated with tissue remodeling, and c-Myc expression has been shown to play an important role in this process (25). We examined the effects of expressing c-Myc\(^{\text{T58A}}\) and c-Myc\(^{\text{S62A}}\) compared with c-Myc\(^{\text{WT}}\) on involution. Histologic analysis of mammary glands 3 days postweaning showed clear signs of involution with shrunken alveoli and the reappearance of adipose cells in control, Myc\(^{\text{WT}}\)-, and Myc\(^{\text{S62A}}\)-expressing mice (Fig. 2A). In contrast, enlarged alveoli with prominent secretions predominated in Myc\(^{\text{T58A}}\) mammary glands. Analysis of apoptotic cells by TUNEL staining showed a dramatic reduction in apoptosis in Myc\(^{\text{T58A}}\) expressing mammary glands (Fig. 2B and C). This reduction in apoptosis and impaired involution likely contributed to the expanded lobule-alveolar areas, and the persistence of enlarged secretion-filled ducts in Myc\(^{\text{T58A}}\) mammary glands (Fig. 1D). In contrast, mammary glands expressing Myc\(^{\text{WT}}\) showed an increase in apoptosis compared with control, consistent with wild-type c-Myc’s known apoptotic activity (Fig. 2C). Thus, expression of c-Myc\(^{\text{T58A}}\), but not c-Myc\(^{\text{S62A}}\), inhibits the wave of apoptosis in the mammary gland following weaning.

Analysis of mammary glands at 3 days of involution for the expression of several proteins involved in apoptosis showed that proapoptotic Bim (both BimEL and BimML) is reduced in Myc\(^{\text{T58A}}\)-expressing mammary glands relative to the other strains (Fig. 2D and graphs). No significant change in expression of antiapoptotic Bcl2, or proapoptotic Bax or p53 was found across all 4 strains (data not shown). Together, these data show that c-Myc\(^{\text{T58A}}\) expression suppresses apoptosis in the mammary gland at least in part due to downregulation of
Figure 1. Generation and characterization of RFS-mycWT/WAP-Cre, RFS-mycT58A/WAP-Cre, and RFS-mycS62A/WAP-Cre mice. A, knock-in strategy for conditional expression of c-mycWT-HA, c-mycT58A-HA, or c-mycS62A-HA. Arrowheads, loxP sites. tpA is a transcription stop sequence. Inserted c-myc cDNAs have a C-terminal HA tag. Cre recombination activates expression of the inserted c-myc cDNA driven from the ROSA promoter. RFS, ROSA Floxed Stop; see Supplementary Fig. S1 for more detail. B, expression of ROSA-driven c-Myc. Top, RNA was isolated from mammary glands from the indicated control (ctrl: no Wap-Cre) or RFS-myc/WAP-Cre strains (WT, T58A, S62A) at the indicated stages: pregnancy 18 days, lactation 18 days, parous 2 months. HA-tagged ectopic and total c-myc mRNA is shown by RT-PCR. Bottom, lysates from mammary glands from 2 mice per indicated strain harvested at pregnancy day 17 were immunoprecipitated with anti-HA followed by Western blotting with anti-c-Myc. Input total c-Myc and actin are shown. Data are representative of 4 mice per genotype. Graphs, quantification of the actin-normalized expression of HA-tagged ectopic and total c-Myc from 3 mice per genotype/C6 SD. P values are given on graphs with significant differences. C, analysis of ectopic c-Myc half-life in primary MECs from the indicated strains. Mean half-life/C6 SD was calculated from 3 independent experiments. CHX, cycloheximide. D, expanded lobule-alveolar areas with c-MycT58A expression. The fourth glands from the indicated mice on day 17 of the third consecutive pregnancy were analyzed by H&E section staining. Arrowhead, enlarged duct. Scale bars, 50 μm. Data are representative of 4 mice per genotype. Mean alveolar area was analyzed by counting 10 areas each section and 3 mice per genotype by ImageJ and graphed C6 SD.
Bim proteins, which have been shown to play an important role in mammary gland apoptosis (35).

**Increased chromosomal instability and centrosome amplification in mammary epithelial cells with altered c-Myc phosphorylation**

Aberrant c-Myc expression has been associated with increased genomic instability both in cell culture and in vivo, which can contribute to the cancer phenotype (36). To examine whether deregulated c-Myc T58 and S62 phosphorylation can affect genomic stability, we analyzed and quantified chromosome numbers in mitotic spreads from first passage primary MECs harvested at 2 months parous. We found the highest rates of polyploidy in the Myc<sup>T58A</sup> MECs with a substantial number of cells showing greater than tetraploid karyotypes (Fig. 3A). In addition, G-banding analyses revealed a high degree of aneuploidy in cells expressing both Myc<sup>T58A</sup> and Myc<sup>S62A</sup>, with 9 of 20 cells analyzed for each showing abnormalities including nonclonal trisomies, translocations, chromosomal breakages, and various extra and missing chromosomes (Fig. 3B). In contrast, Myc<sup>WT</sup>-expressing cells showed only 4 of 20 cells with abnormal karyotypes compared with control cells with 2 of 20 abnormal cells (data not shown).

To examine potential molecular alterations that could contribute to chromosomal instability, we examined the expression of several genes important for the spindle assembly checkpoint (SAC) in 2-month parous mammary glands. Interestingly, we found that expression of Bub1b, a key player in this pathway, was substantially downregulated in Myc<sup>S62A</sup>-expressing mammary glands whereas it was substantially upregulated in Myc<sup>T58A</sup>-expressing glands (Fig. 3C). We also found that expression of Aurora kinase B, a chromosomal passenger serine/threonine protein kinase involved in the SAC, was dramatically upregulated with expression of Myc<sup>T58A</sup> whereas it was substantially downregulated with expression of Myc<sup>S62A</sup> (Fig. 3C), and Aurora A showed a similar trend, but less dramatic (data not shown). Because precise levels of these proteins are critical for proper SAC function (37–40), these results may partially explain the enhanced genomic instability with Myc<sup>T58A</sup> and Myc<sup>S62A</sup> expression.

Abnormalities in the SAC leading to failed cytokinesis can result in centrosome amplification, as can overexpression of Aurora kinases (38). Immunofluorescent staining for centrosomes revealed clear examples of abnormal numbers of centrosomes in Myc<sup>T58A</sup> MECs (Fig. 3D). Quantification revealed a high degree of centrosome amplification in Myc<sup>T58A</sup> MECs, compared with Myc<sup>S62A</sup> and Myc<sup>WT</sup>, which were still significantly higher than control cells (Fig. 3D, graph). Together, these results reveal a potential for c-Myc–associated genomic instability in our mouse model, and expression of c-Myc<sup>T58A</sup> and c-Myc<sup>S62A</sup> seem to exacerbate this in different ways.

**Altered mammary gland morphology with expression of the Myc phosphorylation mutants**

We assessed the phenotypic effects of expressing ROSA-driven c-Myc<sup>WT</sup>, c-Myc<sup>T58A</sup>, and c-Myc<sup>S62A</sup> in the mammary gland 2 and 5 months parous after the third pregnancy and found substantial differences in morphology at both time points (Fig. 4A; Supplementary Fig. S3). Expression of Myc<sup>WT</sup> showed a consistent increase in mammary ductal branching relative to control mammary glands, whereas Myc<sup>S62A</sup> showed a reduction, particularly when compared with Myc<sup>WT</sup>. In contrast, expression of Myc<sup>T58A</sup> resulted in dramatically increased mammary ductal branching and alveolar budding (Fig. 4A) and the appearance of hyperplastic foci resembling hyperplastic alveolar nodules by 5 months (Supplementary Fig. S3A; Fig. 5A). H&E sections revealed normal architecture in all strains except Myc<sup>T58A</sup>, in which expanded alveolar regions with increased stromal and epithelial cells were detected (Fig. 4B; Supplementary Fig. S3B). Mean alveolar area reflected the observed changes in density with expression of the 3 c-Myc isoforms (Fig. 4C). Further analyses of mammary gland sections from the Myc<sup>T58A</sup> mice at 5 to 8 months parous revealed hyperplastic foci (Fig. 5A, middle) with high proliferation (Fig. 5A, right), areas of dysplasia including atypical nuclei with multiple nucleoli (Fig. 5B, left), disorganized alveolar structures with hyperchromatic crowded nuclei, loss of polarity, and mitotic figures (Fig. 5B, middle), and abnormal foci with increased stromal cells, atypical nuclei, and immune cell infiltration including mast cells (Fig. 5B, right). Loss of polarity and alveolar disorganization was also apparent in costaining for the luminal epithelial marker Keratin8/18 (green) and myoepithelial marker Keratin14 (red), which normally form a single layer of supporting cells around the polarized luminal epithelial cells (Fig. 5C, left, arrow). Moreover, alveoli in Myc<sup>T58A</sup> glands showed downregulation of E-cadherin expression (red) compared with alveoli in Myc<sup>WT</sup> glands (Fig. 5C, middle versus right), suggesting loosened adhesion of intercellular junctions in the luminal cells. H&E sections and K8/18, K14 staining also revealed ductal hyperplasia (Fig. 5D).

**RFS-myc<sup>T58A</sup>/Wap-Cre mice develop mammary gland tumors**

To study potential effects of altering c-Myc phosphorylation on tumorigenesis, we followed cohorts of RFS-myc<sup>WT</sup>, T58A, and S62A/Wap-Cre and control RFS-myc (no Cre) female mice that had undergone 3 cycles of pregnancy (25–28 mice per genotype). Neither the control nor RFS-myc<sup>WT</sup>/Wap-Cre mice developed mammary gland tumors out to 2 years, although the latter expressed deregulated c-Myc. In contrast, RFS-myc<sup>T58A</sup>/Wap-Cre mice developed mammary adenocarcinomas between 7 and 13 months of age. These were poorly differentiated carcinomas of solid, cribriform, papillary, and/or adenosquamous architecture, many with necrosis (Fig. 6A and data not shown), and all with a high proliferation index (Fig. 6B and data not shown). Invasion was indicated by absence or disruption of myoepithelial smooth muscle actin (SMA), and K14 staining in most of the tumors (Fig. 6C and data not shown; ref. 41). Several of these were accompanied by in situ carcinoma at the periphery (data not shown). Moreover, one of these tumors metastasized to lung, liver, and spleen, and the...
Figure 2. Inhibition of mammary gland involution and apoptosis with c-Myc<sup>T58A</sup> expression. A, the fourth gland from the indicated mice was harvested after 12 days of lactation, 3 days postweaning after the third pregnancy, and analyzed by H&E section staining. Scale bars, 50 μm. Data are representative of 4 to 6 mice per genotype. B, c-Myc<sup>T58A</sup> expression inhibits apoptosis during involution. Mammary gland sections as in A were analyzed by TUNEL assay. Scale bars, 50 μm. C, TUNEL assays as in B were quantified to determine the percent apoptotic epithelial cells. Graph, 4 mice per strain ± SD. D, reduced expression of proapoptotic Bim in mammary glands expressing c-Myc<sup>T58A</sup>. Protein lysates from the indicated mice (2 per genotype) 3 days postweaning were Western blotted with the indicated antibodies and quantified. Data are representative of 4 mice per genotype. Average actin-normalized BimEL and BimML expression relative to control is graphed ± SD.
metastatic lesions also showed high rates of proliferation (Fig. 6D and data not shown). Of the 28 RFS-myc<sup>T58A</sup>/Wap-Cre mice, 5 mammary gland tumors developed in 4 mice (Table 1). Unfortunately, but of interest, the remainder of the RFS-myc<sup>T58A</sup>/Wap-Cre cohort succumbed to brain tumors with a median survival of approximately 10.5 months (46 weeks; Table 1). This corresponded with spurious expression of Wap-Cre in the newborn mouse brain (Supplementary Fig. S4) as previously reported (31). Choroid plexus and pituitary tumors were observed. Interestingly, RFS-myc<sup>S62A</sup>/Wap-Cre mice also developed the same spectrum of brain tumors with a median survival of 12.5 months (54 weeks; Table 1). Of the RFS-myc<sup>S62A</sup>/Wap-Cre and RFS-myc<sup>S62A</sup>/Wap-Cre mice with pituitary tumors, one each also developed a mammary tumor. Owing to potential confounding effects from prolactin in mice with pituitary hyperplasia or tumors, any mice exhibiting these features were excluded from data presented in this paper. Analysis of the 7 Myc<sup>T58A</sup> mice that succumbed to choroid plexus tumors without evidence of abnormal pituitary, showed that 80% had atypical mammary gland changes similar to those shown in Figure 5, suggesting that given more time, these mice may also have developed mammary gland tumors (data not shown). In contrast, Myc<sup>S62A</sup> mammary glands from mice succumbing to choroid plexus tumors only, displayed reduced density similar to that shown in Figure 4 and Supplementary Figure S3 (data not shown). Together, these results indicate that phosphorylation of c-Myc at T58 and
S62 has profound and differential affects on c-Myc's oncogenic potential in the mammary gland.

**Discussion**

Although c-MYC is a well-known oncogene, many questions remain about how deregulation of c-Myc leads to tumorigenesis. c-Myc is constitutively overexpressed in the majority of human tumors, and it is clear that the expression level of c-Myc helps to determine its oncogenic activity (32, 34). Specifically, deregulated low levels of c-Myc increase cell proliferation without engaging tumor suppressor pathways to induce apoptosis (32). Higher levels of c-Myc activate both proliferation and apoptosis (3, 32), and high levels of c-Myc are associated with genomic instability, which can contribute to cancer development (36). c-Myc expression level is in part controlled by phosphorylation at T58 and S62 that affect c-Myc stability. In this study, we investigated the consequences of altering T58 and S62 phosphorylation in vivo for mammary gland development and tumorigenesis. We found that the level of T58 and S62 phosphorylation can have dramatic effects on specific activities of c-Myc in the mammary gland. These results have important implications for the oncogenic function of c-Myc with altered phosphorylation that we find expressed in human breast cancer (Ref. 13; Sears lab, unpublished data).

**T58 and S62 phosphorylation affects c-Myc activity in the mammary gland**

Previous reports have indicated that mutation of T58 or S62 to alanine affects c-Myc–mediated cell transformation in tissue culture in which T58A Myc is more transforming and S62A less transforming compared with c-MycWT (18–20). In addition, a study using retrovirally transduced hematopoietic stem cells (HSCs) expressing c-MycWT or c-MycT58A showed more robust lymphomagenesis with c-MycT58A expression (42). Although these studies all point to a role for these phosphorylation sites in regulating c-Myc function, we have found that high expression of c-Myc, whether wild-type or phosphorylation mutant, can swamp out or dilute differences in their activity, making interpretation difficult (data not shown). In this regard, expressing c-MycWT, c-MycT58A, or c-MycS62A from the constitutive, but weak, ROSA promoter is an ideal system to study how these phosphorylation sites affect specific activities of c-Myc in vivo at physiologic levels both in development and for tumorigenesis. We focused on the mammary gland as it is a highly dynamic organ, which undergoes repeated cycles of proliferation and apoptosis, and c-Myc is involved in both of these processes (24, 25). We found that expression of c-MycT58A inhibited the apoptotic program of involution associated with reduced proapoptotic Bim expression, similar to MycT58A-expressing HSCs (42), whereas c-MycWT increased

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apoptosis and did not impair involution even though c-MycWT is expressed at lower protein levels in our system and thus should be less likely to induce apoptosis (32). Moreover, c-Myc S62A, which is expressed at intermediate levels between c-MycWT and c-MycT58A was also competent for apoptosis and involution. These results point to differential specific activities of c-Myc with altered phosphorylation. However, since expression of the different c-Myc proteins in this system is not equivalent due to differences in their stability (see Fig. 1), we cannot definitively distinguish quantitative versus qualitative effects.

We also found that changes in the phosphorylation of T58 and S62 affected genomic stability in primary MECs isolated from the mice. Although expression of c-MycWT showed some increase in genomic instability over control, expression of c-MycT58A substantially increased chromosomal instability associated with dramatically increased expression of SAC proteins, Bub1B and Aurora B, which are overexpressed in many human cancers associated with chromosomal instability (37, 38). Interestingly, the expression of c-MycS62A was also associated with significant chromosomal instability, particularly evident in the G-banding studies, but it had the opposite effect on the expression of Bub1B and Aurora B, which showed substantially reduced expression. Decreased expression of these proteins is also associated with aneuploidy, but generally results in an aging phenotype, growth arrest, and apoptosis (39, 40), which could contribute to the reduced mammary gland density observed in the MycS62A mice.

Figure 5. Preneoplastic changes with expression of c-MycT58A at 5 to 8 months parous. A, hyperplastic foci in RFS-mycT58A/WAP-Cre mammary glands 5 to 8 months parous after the third pregnancy. Whole mount, H&E section, and immunohistochemistry for BrdU labeling are shown. Arrow in whole mount, hyperplastic foci. In H&E, dark blue/purple represents calcifications in acinar lumens. B, areas of cellular dysplasia in MycT58A mammary glands. H&E sections from MycT58A mice as in A. Arrows (from left to right), atypical nuclei with multiple nucleoli, mitotic figure, and mast cell. C, alveolar disorganization and reduced adhesion in MycT58A mammary glands. Immunofluorescence costaining for luminal marker K8/K18 (green) and myoepithelial marker K14 (red) in mice as in A. Arrow, disorganized alveolar region. E-cadherin staining (red), DAPI stained nuclei (blue) in MycT58A (middle) and MycWT (right) mammary glands. D, ductal hyperplasia with c-MycT58A expression. H&E staining and immunofluorescence K8/K18 (green)/K14 (red) in mice as in A. Dilated ducts with proliferative epithelium (tufting) are shown. All whole mount and H&E images are from different MycT58A mice and are representative of 8 RFS-mycT58A/WAP-Cre mice 5 to 8 months parous. Immunofluorescence images are representative of 3 mice per genotype. All scale bars, 50 μm.
Analysis of mammary glands over time revealed that Myc\textsuperscript{WT}-expressing mammary glands had increased ductal branching, but maintained phenotypically normal alveoli, whereas Myc\textsuperscript{T58A}-expressing glands had increased ductal branching, and developed expanded and disorganized alveoli. We speculate that increased genomic instability in the face of reduced apoptotic potential in the Myc\textsuperscript{T58A} mammary glands contributes to their dysplastic phenotype. In contrast, Myc\textsuperscript{S62A} mammary glands generally showed reduced ductal branching with fewer alveoli. In this case, genomic instability in the face of competent activation of apoptotic programs could lead to elimination of aneuploid cells, potentially explaining the reduction in mammary gland density in the Myc\textsuperscript{S62A} glands. In addition, recent publications report that phosphorylation at S62 is important to inhibit Ras-induced cellular senescence and that reducing pS62 through elimination of Cdk2 increases c-Myc-driven senescence (43, 44). Increased cellular senescence in the Myc\textsuperscript{S62A} expressing glands could also contribute to their reduced density, and future experiments will address this. In addition, future ChIP-Seq experiments to identify global differences in DNA binding among the 3 isoforms of c-Myc in MECs from our model may provide insight into how the T58 and S62 phosphorylation sites affect c-Myc function.

<table>
<thead>
<tr>
<th>Mice with mammary tumor</th>
<th>WT/Cre (n = 27)</th>
<th>T58A/Cre (n = 28)</th>
<th>S62A/Cre (n = 28)</th>
<th>Control (n = 25)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice with brain tumor</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mean mammary tumor latency</td>
<td>38 weeks</td>
<td>24</td>
<td>28</td>
<td>46 weeks</td>
</tr>
</tbody>
</table>

NOTE: Cohorts of the indicated numbers of RFS-Myc\textsuperscript{WT}/Wap-Cre, RFS-Myc\textsuperscript{T58A}/Wap-Cre, and RFS-Myc\textsuperscript{S62A}/Wap-Cre or control (RFS-Myc\textsuperscript{WT}, T58A, or S62A no Cre) were maintained for 24 months or until moribund with tumor. Numbers of mice with the indicated tumor types along with mean latency are indicated.
Phosphorylation at T58 and S62 impacts c-Myc tumorigenic potential

Mice with mammary gland expression of c-MycT58A, but not c-MycWT or c-MycS62A, developed mammary carcinoma. In addition to increased genomic instability and suppressed apoptosis contributing to this, MycT58A glands showed disorganization of alveolar glandular architecture. Studies have shown that expression of c-Myc does not induce proliferation in polarized epithelial cells in three-dimensional culture, but disruption of polarity allows c-Myc driven proliferation (45); consistent with our observation of mitotic figures in dysplastic foci in MycT58A glands. We also observed that c-MycT58A—expressing mammary glands had increased STAT3 activation (data not shown), which is common in human breast cancer and contributes to the disruption of epithelial adhesion and polarity (46). Thus, disruption of alveolar architecture could allow for inappropriate proliferation with accompanying genomic instability, promoting tumorigenesis in MycT58A glands.

In contrast, although c-MycWT—expressing mammary glands showed increased density, they seemed morphologically normal, suggesting that the deregulated, but near physiologic expression of c-MycWT from the ROSA locus was able to achieve some sort of homeostatic, nontumorigenic balance. This provides a potentially important new model for examining collaborative interactions with c-Myc in vivo, which will be important to exploit in future studies. Interestingly, c-MycS62A—expressing mammary glands, which generally showed higher c-Myc protein expression compared with c-MycWT glands, had reduced density; nevertheless, expression of c-MycS62A was tumorigenic in the choroid plexus and pituitary. This finding is difficult to reconcile with both our data showing decreased mammary gland density with c-MycS62A expression and recent reports suggesting that decreased S62 phosphorylation may preferentially drive senescence (43). One possibility is that neural epithelial tissues are much less sensitive to drivers of apoptosis and senescence, and in this setting, the increased genomic instability with c-MycS62A is protumorigenic. We also cannot rule out the possibility that c-MycS62A expression in the mammary gland could eventually be tumorigenic, similar to the Bub1b−/− mice, which show increased genomic instability and aging phenotypes, with cancer predisposition (39).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


Phosphorylation Regulates c-Myc's Oncogenic Activity in the Mammary Gland

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