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pRb Inactivation in Mammary Cells Reveals Common Mechanisms for Tumor Initiation and Progression in Divergent Epithelia

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Retinoblastoma 1 (pRb) and the related pocket proteins, retinoblastoma-like 1 (p107) and retinoblastoma-like 2 (p130) (pRb, collectively), play a pivotal role in regulating eukaryotic cell cycle progression, apoptosis, and terminal differentiation. While aberrations in the pRb-signaling pathway are common in human cancers, the consequence of pRb loss in the mammary gland has not been directly assayed in vivo. We reported previously that inactivating these critical cell cycle regulators in divergent cell types, either brain epithelium or astrocytes, abrogates the cell cycle restriction point, leading to increased cell proliferation and apoptosis, and predisposing to cancer. Here we report that mouse mammary epithelium is similar in its requirements for pRb function; Rb inactivation by T121, a fragment of SV40 T antigen that binds to and inactivates pRb proteins, increases proliferation and apoptosis. Mammary adenocarcinomas form within 16 mo. Most apoptosis is regulated by p53, which has no impact on proliferation, and heterozygosity for a p53 null allele significantly shortens tumor latency. Most tumors in p53 heterozygous mice undergo loss of the wild-type p53 allele. We show that the mechanism of p53 loss of heterozygosity is not simply the consequence of Chromosome 11 aneuploidy and further that chromosomal instability subsequent to p53 loss is minimal. The mechanisms for pRb and p53 tumor suppression in the epithelia of two distinct tissues, mammary gland and brain, are indistinguishable. Further, this study has produced a highly penetrant breast cancer model based on aberrations commonly observed in the human disease.

Introduction

Aberrant retinoblastoma 1 (pRb) pathway activity, resulting from defects in pRb itself, cyclin-dependent kinase inhibitor 2A (p16INK4a), cyclin D1 (CCND1), or cyclin-dependent kinase 4 (CDK4), is observed in the majority of human sporadic cancers (Marshall 1991; Weinberg 1995; Sherr 1996; Ortega et al. 2002). This pathway is commonly altered early in cancer development, indicating an ability to predispose cells to tumorigenesis. However, whether the mechanism(s) is similar among cell types is not known. Examination of pRb inactivation in specific cell types in vivo has been technically challenging due to the apparent functional compensation or redundancy among pRb, retinoblastoma-like 1 (p107), and retinoblastoma-like 2 (p130) in many cell types of the mouse (Luo et al. 1998; Robanus-Maandag et al. 1998; Dannenberg et al. 2000; Sage et al. 2000). Thus, genetic inactivation of the Rb gene alone, either by conditional deletion (Marino et al. 2000) or by the generation of chimeric mice harboring pRb-deficient cells (Maandag et al. 1994; Williams et al. 1994) yields only medulloblastomas, pituitary, and thyroid tumors. We have begun to systematically examine the role of retinoblastoma protein family (pRb) inactivation in multiple cell types of the mouse by dominant expression of T121, a truncation mutant of simian virus 40 (SV40) T antigen that inactivates all three pRb-related proteins (DeCaprio et al. 1989; Dyson et al. 1989; Ewen et al. 1989; Stubdal et al. 1997; Sullivan et al. 2000). In this report we determine the role of pRb inactivation in mammary adenocarcinoma predisposition, establish a role for p53 inactivation in subsequent mammary adenocarcinoma progression, and, together with our previous studies, provide a comprehensive comparison of these mechanisms in distinct epithelial lineages.

pRb plays a critical role in eukaryotic cell cycle progression, when cells exit G0 or G1 and enter S phase, thereby acting as a crucial negative regulator of cellular proliferation and neoplasia (Sherr and McCormick 2002). In quiescent or early G1-phase cells, pRb is hypophosphorylated and associates with specific members of the E2F transcription factor family, converting them to active transcriptional repressors (Hamel et al. 1992; Weintraub et al. 1992). Gene repression is...
also mediated by pRb and p130 recruitment of histone deacetylase to promote formation of inhibitory nucleosomes (Brehm et al. 1998; Luo et al. 1998; Magnaghi-Jaulin et al. 1998). The many proteins found in association with pRb suggest other regulatory mechanisms may also be involved (Morris and Dyson 2001), although the biological potential for most of these interactions remains yet unproven. Cell cycle progression from G to S phase occurs when complexes of D-type cyclins/CDK4/CDK6 phosphorylate pRb, thereby derepressing E2Fs to direct transcriptions of DNA-replication machinery and nucleotide biosynthesis genes (Dyson 1998).

Like most human solid tumors, breast cancers harbor frequent alterations in the pRb pathway, including CCND1 overexpression in 45% (Buckley et al. 1993), p16INK4A loss in 49% (Geradts and Wilson 1996), and pRb loss in 6% of breast tumors (Geradts and Wilson 1996). In the Rb-deficient mouse mammary gland, p107 and/or p130 may play overlapping or compensatory roles, as they do during embryonic development, given that pRb is dispensable for normal mammary development and mammary tumor suppression, pRb-deficient embryonic stem cells participate in normal mammary gland formation in chimeric mice (Maandag et al. 1994), and donor pRb−/− mammary precursor cells transplanted into wild-type mice can populate a normal mammary gland without evidence of neoplasia, even after multiple pregnancies (Robinson et al. 2001).

The interplay between pRb signaling and the tumor protein p53 pathway is also critical to the understanding of breast cancer biology. Since the pRb pathway is defective in a majority of human tumors and the p53 gene is mutated in about half of them, including approximately a fifth of sporadic breast cancers (Nigro et al. 1989; Greenblatt et al. 1994), these aberrations often coexist. Whether loss of these tumor suppressor pathways collaborate in tumorigenesis is also cell type-specific. In a brain epithelial tumor model, we previously demonstrated that, in the absence of pRb function, inactivation of p53 significantly decreases apoptosis and accelerates tumor growth in vivo (Symonds et al. 1994). However, in astrocytic brain tumors induced by pRb inactivation, tumor progression is not accelerated by reduced p53 activity; rather, the phosphatase and tensin homolog (PTEN) regulates the apoptosis, and reduction in its function accelerates tumor growth (Xiao et al. 2002).

In this report, we extend our analysis of pRb function in vivo and examine the consequence of pRb loss specifically in mammary epithelium. These studies serve not only to provide insight into the cell specificity of tumor suppression mechanisms, but also to model the stepwise evolution of breast adenocarcinomas that harbor defects in this pathway.

**Results**

**Generation of Mice with Inducible pRb Deficiency in Mammary Cells**

Seven founder mice were generated in which the T121 gene was regulated by the whey acidic protein (WAP) transcriptional signals (Figure 1; see Materials and Methods). Of these, two founder animals died spontaneously of unknown causes, while the transgenic progeny of the third line died prematurely, also of unknown cause (Figure 2A). The extent to which the transgene contributed to these deaths was not investigated further; however, ectopic transgene expression was detected in several tissues (data not shown). Characterization of female mice of the four remaining lines is the focus of this report.

**T121 Is Expressed in Lactating Mammary**

Western immunoblotting analyses of mammary gland extracts demonstrated that this tissue expresses T121 protein at the expected size in all four lines (Figure 2B). T121 expression in lines 1 and 2 was only revealed following immunoprecipitation using an anti-T-antigen antibody prior to Western blot analysis, indicating lower levels of T121 (right panel in Figure 2B). A survey of select tissues showed that detectable expression was restricted to the mammary gland in lines 1–3, while expression was more widespread in the higher expressing line 4 (data not shown) and included brain and kidney expression. As expected, T121 expression was induced by lactation with highest levels observed 5 d postpartum (Figure 2C). Southern blot analyses indicate that mice in line 3, which was used as a representative line for extensive characterization, harbor approximately ten copies of the transgene at a single insertion site (data not shown).

**Impact of Rb Inactivation in Mammary Epithelium**

Representative histological analysis of lactating mammary glands (day 1) from single-pregnancy females of the line 2 founder (F0) and a line 3 F1 mouse shows that the impact of Rb perturbation is severalfold. Compared to an age- and parity-matched control tissue, the normal architecture of the lactating mammary tissue is disturbed. In contrast to normal tissue where acini consist of a single layer of secretory epithelia with milk-filled lumen (Figure 3A), transgenic animals have a lower density of acini (Figure 3K), consistent with atrophy, and are often atypical (Figure 3I). T121-positive mammary epithelial cells were associated with abnormalities (Figure 3B, 3F, and 3J). The line 2 F0 animal was mosaic for...
T121 protein expression with distinct regions of expressing and nonexpressing cells (Figure 3F), whereas T121 expression in the line 3 animal was in secretory epithelium distributed throughout the gland (Figure 3J). Increased proliferation, indicated by proliferating cell nuclear antigen (PCNA) staining, was also observed in transgenic mammary glands (Figure 3C, 3G, and 3K), concomitant with increased levels of apoptosis assayed by TUNEL staining (Figure 3D, 3H, and 3L).

Quantification of T121 expression and apoptosis revealed higher protein expression levels (see Figure 2B) correlate with higher percentages of apoptotic cells (Figure 4A). Consistent with a model for cell-autonomous functioning of T121, the pattern of abnormalities of morphology, proliferation, and apoptosis in the mosaic animal mimicked the regionalized T121 expression pattern, and conversely, where T121 protein was absent, the tissue appeared normal.

Figure 2. Expression of T121 Protein in WAP-T121 Mice and a Summary of Gross Phenotypes

As expected, each line showed mammary-specific expression following lactation induction, while line 4 showed more widespread expression, with protein detected in brain and kidney. Mice from the higher-expressing lines 3 and 4 failed to nurse because of lactation defects. Mammary glands of adult female mice from all four lines showed elevated proliferation and apoptosis. Glands from lines 1 and 2 were hyperplastic, while glands from lines 3 and 4 were atrophic. Lines 3 and 4 later developed carcinomas and other neoplasms. T121 protein was detected by Western blot analysis in lactating mammary glands of animals from all four lines (B), although the lower-expressing lines 1 and 2 required immunoprecipitation with anti-T-antigen antibody prior to Western blot analysis (right panel in [B]). Brain tumor extract (see Materials and Methods) was used for a positive control, and nontransgenic mammary tissue extract was used for a negative control. A timecourse analysis of T121 expression (C) shows lactation-induced expression peaking at 5 d postpartum.

Abbreviations: Adeno-Ca, adenocarcinoma; AP, elevated apoptosis in mammary gland; At, atrophy; dpc, postcoital; FTN, failure to nurse; Hyp, hyperplastic acini; MG, mammary gland; MIN, mammary epithelia neoplasia; ND, not determined; nt, nontransgenic; pp, postpartum; Pr, elevated proliferation in mammary gland; pw, post-weaning.

Footnotes: *Mosaic founder animal. †At earlier stages, development defects attributed to atrophy, while MIN and adenocarcinoma were observed at terminal stages. Approximately half of progeny died of unknown cause.

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Figure 3. Mammary-Specific Inactivation of the pRb Pathway Induces Extensive Abnormalities

Histologic comparisons of nontransgenic (A–D), mosaic (E–H), and transgenic (I–L) lactating mammary glands reveals that T121 expression results in increased proliferation and apoptosis. Hemotoxylin and eosin staining shows acini of the normal lactating gland are composed of a single layer of secretory epithelial cells (A) with milk-filled lumens. Consistent with atrophy, transgenic animals have a lower density of acini demonstrated by the presence of lipid-filled adipocytes (asterisk in [K]). Acini composed of T121-expressing cells are atypical. Many are collapsed and composed of tall columnar epithelia of large hyperchromatic cells with papillary tufting (arrows in [I]). Transgene-expressing cells have large pleomorphic nuclei (open arrows in [K]) as compared to nuclei of nonexpressing cells (arrows in [G]). Staining for T121 expression (blue in [B]–[J]) demonstrates increased apoptosis in transgenic animals (H and L); moreover, the regionalized apoptosis in the mosaic gland (H) strongly suggests that transgene expression and not precocious involution is the cause. All samples are from primiparous females on lactation day 1.

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Role of p53 in Apoptosis

To investigate the impact of germline loss of p53 on apoptosis levels in Rb-deficient mammary glands, we mated line 3 animals to p53 null mice to generate transgenic and nontransgenic females of distinct p53 genotypes (+/+, +/-, -/-). Transgene expression was induced by a single pregnancy, and mammary glands were examined on lactation day 1. As expected, nontransgenic mammary glands showed no appreciable apoptosis regardless of p53 status (Figure 4B). Decreasing levels of p53 activity correlate with lower levels of apoptosis in transgenic mammary glands (B). The mean percentage of apoptotic cells in p53 wild-type transgenic glands was 21%; in p53 heterozygous animals, 9%; and in p53 null animals, 5% (B), indicating that 75% of the apoptosis is p53-dependent. Apoptosis levels are further reduced to 2% in terminal stage tumors (B, Tumors). The percentage of PCNA staining cells remains unchanged in p53 heterozygous or nullizygous animals (C), indicating that reduction of p53 activity levels had no significant impact on cell proliferation. Samples were derived from primiparous animals on lactation day 1, except as indicated as tumor samples (B). Transgenic animals in (B) and (C) were from line 3.

Figure 4. Reduced p53 Activity Decreases Apoptosis but Does Not Increase Proliferation

Representative apoptosis levels of each mouse line correlate with T121 expression as indicated by the percentage of TUNEL positive cells (A). Decreasing levels of p53 activity correlate with lower levels of apoptosis in transgenic mammary glands (B). The mean percentage of apoptotic cells in p53 wild-type transgenic glands was 21%; in p53 heterozygous animals, 9%; and in p53 null animals, 5% (B), indicating that 75% of the apoptosis is p53-dependent. Apoptosis levels are further reduced to 2% in terminal stage tumors (B, Tumors). The percentage of PCNA staining cells remains unchanged in p53 heterozygous or nullizygous animals (C), indicating that reduction of p53 activity levels had no significant impact on cell proliferation. Samples were derived from primiparous animals on lactation day 1, except as indicated as tumor samples (B). Transgenic animals in (B) and (C) were from line 3.

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Role of p53 in Proliferation

In two other transgenic mouse models of breast cancer, where tumors were initiated by activated Harvey rat sarcoma viral oncogene homolog (v-Ha-ras) (Hundley et al. 1997) or wingless-related murine mammary tumor virus (MMTV) integration site 1 (Wnt-1) (Donehower et al. 1995), inactivation of p53 did not result in a reduction of apoptosis; rather, loss of p53 was associated with increased proliferation of the mammary epithelium. To determine whether p53 inactivation also impacted mammary cell proliferation induced by Rb<sub>i</sub> inactivation, glands from primiparous lactating (day 1) mice were assessed for the expression of nuclear PCNA. Unlike the tumors initiated by activated Ras or Wnt-1, p53 heterozygosity or nullizygosity had no significant impact on the level of cell proliferation (Figure 4C). This experiment indicates that p53 can have distinct mechanisms of action depending on the nature of the initiating lesion.

pRb Inactivation Predisposes to Tumorigenesis

All females from higher-expressing lines (lines 3 and 4) failed to nurse pups because of lactation defects and developed mammary tumors after multiple pregnancies. Because line 4 mice expressed T<sub>121</sub> in nonmammary tissues, further characterization focused on line 3. For this line, the median time following initial transgene induction until a palpable tumor appeared was 10 mo, and within 16 mo, all mice developed palpable tumors (Figure 5A). Interestingly, latency in this line on a BALB/cJ background (see Materials and Methods) was reduced to a median time of 8.5 mo (p = 0.0077; Figure 5A) indicating the presence of modifier alleles. The condensed timeframe for tumor development in this strain will also be valuable for future preclinical studies using this model. However, all further studies in the current report were carried out on the original B6D2F1 background.

The median onset for mammary tumors in line 4 was 14 mo (n = 3; data not shown), which indicates that the transgene and not its insertion caused tumorigenesis. With two exceptions, line 3 WAP-T<sub>121</sub> mice, regardless of p53 status, developed a single palpable tumor (87% of p53<sup>+/−</sup>, n = 15; 78% of p53<sup>−/−</sup>, n = 9). A single mouse with either two or three palpable tumors was also observed in both p53<sup>+/+</sup> and p53<sup>+/−</sup> backgrounds. At least one additional nonpalpable tumor was visible during necropsy in approximately one-third of all tumor-bearing mice. While the two lower-expressing lines, lines 1 and 2, were able to nurse pups and appeared grossly normal, both had hyperplastic lobular alveoli associated with increased levels of proliferation and apoptosis. However, females from low-expressing lines did not develop adenocarcinomas after at least four pregnancies and 20 mo of age (line 1, n = 2; line 2, n = 6) (data not shown).

Most terminal stage tumors in either wild-type or p53<sup>+/−</sup> backgrounds were adenocarcinomas (Figure 6A, 6B, and 6E); however, we also observed four pilar tumors (Figure 6C and 6E) and one spindle cell carcinoma (Figure 6D and 6E). Terminal-stage mammary adenocarcinomas resembled poorly to moderately differentiated invasive ductal adenocarcinoma in humans. Morphologically, we designate these tumors as mixed solid and glandular carcinomas with necrosis and fibrosis. Poorly differentiated solid tumors (Figure 6A) are composed of nests of epithelial cells with large pleomorphic nuclei and delicate chromatin patterns with inverted nucleocytoplasmic ratios, while glandular tumors (Figure 6B) are composed of irregular glands with varying degrees of differentiation. While most animals had a single tumor mass, the adenocarcinomas were multifocal, with solid tumors consisting of subclones of distinct expansile masses, and with only two exceptions, glandular tumors were coincident with solid tumors. The adenocarcinomas were malignant, infiltrating dense, fibrous connective tissue, and were accompanied by strong peripheral immune response (Figure 6A).

Mammary Tumor Onset and Growth Are Accelerated by p53 Reduction

Among line 3 animals, the median time following initial transgene induction until a palpable tumor appeared was 10 mo, and within 16 mo, all mice developed palpable tumors (red line in [A]). In p53<sup>−/−</sup> transgenic animals (blue line in [A]), mammary tumors were detected significantly earlier (p < 0.0003) with a median onset of 6 mo. Among mice with BALB/cJ background (black line in [A], median mammary tumor latency (8.5 mo) was significantly shorter (p = 0.0077) compared to mice of the hybrid BDF1 background strain and indistinguishable (p = 0.2466) from WAP-T<sub>121</sub>;p53<sup>−/−</sup> mice. Once palpable, WAP-T<sub>121</sub>;p53<sup>−/−</sup> tumors grew faster than the p53 wild-type counterparts (B). The average growth rates for p53<sup>−/−</sup> (black solid) and p53<sup>−/−</sup> (dashed) are indicated. DOI: 10.1371/journal.pbio.0020022.g005
tumor acceleration. To assess whether this was the case or whether there was selective pressure for p53 inactivation during tumor progression, real-time PCR analysis was employed to determine the status of the wild-type p53 allele in WAP-T121; p53<sup>f<sup>−</sup></sup> tumors. Of ten tumors, eight showed loss of the wild-type p53 allele (Table 1), indicating that the apoptosis reduction observed in WAP-T121; p53<sup>−/−</sup> mammary epithelium was not sufficient for tumor progression. Significant selective pressure favored cells that had completely inactivated p53, indicating that tumor progression requires further reduction of apoptotic activity and/or that p53 loss contributes to tumor progression through additional mechanisms that confer selective advantage. Assessment of apoptosis levels in terminal tumors showed apoptosis levels were indeed reduced in comparison to preneoplastic tissue (see Figure 4B).

### Comparative Genomic Hybridization Reveals Recurrent Chromosomal Imbalances in Tumors, but Limited Chromosomal Instability

Among the multiple mechanisms of tumor suppression attributed to p53, a common hypothesis is that p53 prevents genetic instability. Indeed, studies using other mouse models indicate loss of p53 function in tumors often correlates with chromosomal instability. These include other breast cancer models such as Wnt-1 p53<sup>−/−</sup> (Donehower et al. 1995) and MMTV-ras p53<sup>−/−</sup> (Hundley et al. 1997) and p53<sup>−/−</sup> thymic lymphomas and sarcomas (Venkatachalam et al. 1998). In marked contrast, our study of p53 deficiency in an evolving brain epithelial tumor showed that tumorigenesis progresses without chromosomal instability, indicating p53 loss contributes via alternative mechanisms (Lu et al. 2001). To determine whether this difference was due to cell-type specificity, differences in initiating mechanisms, or differences in experimental approaches, we analyzed the genome of mammary WAP-T121; p53<sup>−/−</sup> tumors. We employed two methods of comparative genomic hybridization (CGH): chromosome-based CGH (cCGH) (Panel I in Figure 7) (Kallioniemi et al. 1992) and microarray CGH (aCGH) (Panel II in Figure 7) (Solinas-Toldo et al. 1997; Pinkel et al. 1998).

Twelve mammary tumors were assayed by CGH: ten by cCGH, eight by aCGH, and six by both procedures. Both assays revealed limited genomic imbalances (Figure 7), yet only a single tumor showed loss of Chromosome 11 (which harbors p53). Among samples tested by both methods, there is strong concordance among large chromosomal changes, encompassing multiple cytological bands to whole chromosome lengths. For example, there is apparent whole chromosome changes. For example, there is apparent whole chromosome duplications of Chromosomes 6 and 15 in tumor C and of Chromosomes 8 and 18 in tumor H, monosomy of Chromosome 10 in tumor J, and loss of X Chromosome in tumor K. However, some lengths were more difficult, mainly due to the challenge of reconciling cytological and physical maps. Furthermore, technical limitations may account for real differences between the two assays: small imbalances detected by one to several bacterial artificial chromosome (BAC) clones are irresolvable by cCGH; on the other hand, the relatively low density of BAC clones may not adequately sample smaller regions detected by cCGH. Nevertheless, on average, about five imbalances per tumor were detected by cCGH. This

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**Figure 6. Tumor Morphologies**

Hematoxylin and eosin staining of WAP-T121 (C and D) and WAP-T121 p53<sup>−/−</sup> (A and B) (also representative of WAP-T121) tumor sections shows that terminal stage adenocarcinomas have varied morphologies. Poorly differentiated solid tumors were comprised of nests (A) or cords of epithelial cells (Tu) that infiltrate a fibrous stroma and were accompanied by necrosis (arrow) and strong immune response (arrowheads). Moderately differentiated glandular tumors (B) consisted of irregular, disorganized glands. In animals of wild-type p53 background, four pilar tumors (C), distinguished by swirls of laminar acellular keratin (arrow), and a single spindle cell carcinoma (D) were also observed. For comparison, a lactating gland from a wild-type animal is shown in Figure 3A. The percentage of animals displaying each of the phenotypes is summarized in (G). Since many tumors shared multiple morphologies, the sum exceeds 100%.

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**Table 1. Tumor Morphologies**

| Tumor Type          | Adenocarcinoma | Pilar Tumor | Spindle Cell Carcinoma |
|---------------------|---------------|------------|------------------------|
| TgWapT121 (n=10)    | 50%           | 40%        | 10%                    |
| TgWapT121:p53<sup>−</sup> (n=17) | 94%           | 29%        | 0%                     |

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**Selective Pressure for p53 Inactivation during Adenocarcinoma Development**

Since apoptosis was significantly reduced in WAP-T121; p53<sup>−/−</sup> mammary tissue compared with that of WAP-T121; p53<sup>−/−</sup> mice, it was possible that p53 heterozygosity was sufficient for...
Table 1. p53 LOH among the Majority of p53<sup>+/−</sup> Tumors

| Tissue<sup>a</sup> | Genotype | 2−(Average)ΔΔCt b | Number of Wild-Type p53 Alleles |
|------------------|----------|------------------|-------------------------------|
| Muscle           | Wild-type| 1.00             | 2                             |
| Muscle           | Wild-type| 0.51             | 1.75                          |
| Muscle           | Wild-type| 0.39             | 1.5                           |
| Muscle           | Wild-type| 0.32             | 1.25                          |
| Tumor 1          | TgWAP-T121;p53<sup>+/−</sup> | 0.31             | 1                             |
| Tumor 2          | TgWAP-T121;p53<sup>+/−</sup> | 0.73             | 2                             |
| Tumor 3          | TgWAP-T121;p53<sup>+/−</sup> | 0.90             | 2                             |
| Tumor 4          | TgWAP-T121;p53<sup>+/−</sup> | 0.91             | 2                             |
| Spleen           | TgWAP-T121;p53<sup>+/−</sup> | 0.22             | 1                             |
| Tumor 5          | TgWAP-T121;p53<sup>+/−</sup> | 0.34             | 1                             |
| Tumor 6          | TgWAP-T121;p53<sup>+/−</sup> | 0.25             | 1                             |
| Tumor 7          | TgWAP-T121;p53<sup>+/−</sup> | 0.12             | 0                             |
| Tumor 8          | TgWAP-T121;p53<sup>+/−</sup> | 0.08             | 0                             |
| Tumor 9          | TgWAP-T121;p53<sup>+/−</sup> | 0.13             | 0                             |
| Tumor 10         | TgWAP-T121;p53<sup>+/−</sup> | 0.10             | 0                             |
| Tumor 11         | TgWAP-T121;p53<sup>+/−</sup> | 0.07             | 0                             |
| Tumor 12         | TgWAP-T121;p53<sup>+/−</sup> | 0.04             | 0                             |
| Tumor 13         | TgWAP-T121;p53<sup>+/−</sup> | 0.16             | 0                             |
| Tumor 14         | TgWAP-T121;p53<sup>+/−</sup> | 0.15             | 0                             |

Real-time PCR was performed in duplicate to determine the status of the wild-type p53 alleles in the mammary tumors or tissues as indicated. Analysis of standard samples indicates that copy numbers of 2, 1, and 0 are indicated by 2−ΔΔCt values of greater than or equal to 0.7, 0.2–0.7, and less than 0.2, respectively (Lu et al. 2001). Of ten WAP-T<sub>121;p53</sub><sup>+/−</sup> tumors, eight show LOH of p53 gene, while all three WAP-T<sub>121;p53</sub><sup>−/−</sup> tumors retained both p53 alleles. Abbreviation: Tg, transgenic.

*Alleles were derived from line 3 animals, except tumor 1, which was derived from a line 4 animal.

<sup>a</sup>ΔΔCt = [sample C<sub>t</sub> (p53) − sample C<sub>t</sub> (β-actin)] − [p53<sup>+/+</sup> control Ct (p53) − p53<sup>+/+</sup> control C<sub>t</sub> (β-actin)]. C<sub>t</sub> = the number of cycles required to reach a threshold value, which is set within the exponential phase of the logarithmic scale amplification plot.

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Discussion

Common Mechanisms for Tumor Progression in Epithelial Cells of Distinct Origin

Here we report that loss of pRb family function in mammary epithelium predisposes to malignant adenocarcinoma. Using a single transgenic allele, we have thus far inactivated the pRb pathway in several cell types in the mouse: brain choroid plexus epithelium, astrocytes, and mammary epithelium. In each case, despite the marked differences among these divergent cell types, pRb inactivation causes a similar response, initially evoking increased proliferation and apoptosis and, ultimately, predisposing to tumorigenesis (Chen et al. 1992; Saenz-Robles et al. 1994; Symonds et al. 1994; Xiao et al. 2002).

Not surprisingly, the long latency of mammary adenocarcinomas indicates that additional events are required for tumor progression. We show that mammary epithelium is similar to brain epithelium (Symonds et al. 1994; Lu et al. 2001) in its requirement for p53 activity in the apoptotic response to aberrant proliferation caused by pRb<sub>a</sub> inactivation. Previous models using wild-type large T antigen (Li et al. 1996b; Husler et al. 1998; Green et al. 2000; Schulze-Garg et al. 2000) are unable to address the relative contribution of pRb and p53, since T antigen also binds and inactivates p53. As in brain epithelium, we show here that when the mammary tumor phenotype is initiated by pRb<sub>α</sub> inactivation, most of the apoptosis is mediated through p53. Furthermore, as in brain epithelium, heterozygosity for a null p53 allele significantly shortens tumor latency (discussed further below). Importantly, the Rb<sub>α</sub> deficiency-induced apoptotic response and inhibition of tumor progression are not universally dependent on p53. In astrocytes, we recently showed that PTEN, and not p53, modulates these same responses to Rb<sub>α</sub> inactivation. In contrast to the p53-dependent apoptosis of mammalian epithelial cells in response to pRb<sub>α</sub> deficiency, apoptosis associated with normal mammary involution subsequent to lactation does not require p53 (Li et al. 1996a). Thus, the “wiring” of the apoptotic response within this cell type is not global, but rather depends on the signal.

Although loss of p53-dependent apoptosis accounts for the acceleration of mammary tumorigenesis in WAP-T<sub>121;p53</sub>−/<sup>−</sup> mice, in models expressing either activated v-Ha-ras (Hundley et al. 1997) or Wnt-1 (Jones et al. 1997), earlier tumor formation in p53 heterozygous and homozygous null mice is accounted for by increased proliferation rather than attenuated apoptosis. An important caveat to this comparison is that the latter studies compared apoptosis in terminal tumors in which loss of apoptosis might have been selected regardless of initial p53 status, leaving open the possibility that tumor...
growth rates in these models reflect the combined effects of increased proliferation as well as reduced apoptosis. Nevertheless, there is a clear difference in WAP-T121 mammary gland in that, unlike the Ras and Wnt-1 models, proliferation levels do not depend on p53 status. Taken together, these observations indicate that the specific cellular response to an oncogenic stimulus depends on the nature of the initial insult. Given that the pRb pathway is directly disrupted in T121-expressing cells, this could be explained if these other initiating events evoke p53-dependent growth arrest which, in part, functions upstream of pRb.

High Selective Pressure for p53 Inactivation in the Transition to Aggressive Mammary Adenocarcinoma

Most of the apoptosis induced by pRb deficiency in both mammary (75%) and brain (85%) epithelia is p53-dependent as determined by comparing p53+/− and p53−/− tissue. However, while p53 heterozygosity had no impact on the level of apoptosis in the brain epithelium, in the mammary gland the level was reduced by half in p53+/− tissue. Given that apoptosis is the basis for selective inactivation of p53 in the brain tumor model (Lu et al. 2001; X. Lu and T. Van Dyke, unpublished data), it was possible that the pressure was relieved or reduced in WAP-T121;p53+/− mice. However, aggressive adenocarcinoma growth was accelerated with 100% penetrance, and 80% of these tumors underwent selective loss of the wild-type p53 allele, just as in the brain tumor model (Lu et al. 2001). This result indicates that tumor progression requires more than a simple reduction in the level of apoptosis; it follows that p53 may contribute to tumor suppression by multiple mechanisms.

While both mammary and brain carcinomas show high rates of p53 loss of heterozygosity (LOH), the mechanism of loss may be distinct. Chromosome loss clearly explains p53 LOH in the brain carcinoma model (Lu et al. 2001) where nearly all tumors (greater than 90%) are monosomic for Chromosome 11, whereas only a single mammary tumor analyzed by CGH showed Chromosome 11 loss. Alternative mechanisms that may explain p53 LOH in the mammary tumors include somatic recombination or chromosomal
Reduplication following mitotic nondisjunction. Whether these alternative routes of LOH represent bona fide tissue-specific phenomena or are due to relatively small sample sizes will require further analyses. Interestingly, most mammary tumors derived from Brca1-deficient mice lost p53; however, regions distal to p53 were amplified (Weaver et al. 2002). Thus, it is possible that mammary tumor promoting factor(s) is located on distal Chromosome 11, selecting against loss.

**Limited Chromosomal Instability in the Absence of p53**

Genomic instability is a hallmark of most human solid tumors, and a widely held view is that p53 represses instability to suppress tumorigenesis, although evidence for this activity has been mostly correlative. Contrary to this model, we demonstrated previously that in the absence of p53 activity in brain epithelia, tumors progress without chromosomal instability; except for Chromosome 11 loss, in a p53<sup>−/−</sup> background these carcinomas are diploid (Lu et al. 2001). Here we show that mammary tumors similarly harbor limited genome-wide alterations. While the number of aberrations within the mammary tumors is small, it is intriguing that some changes are recurrent, suggesting that their accrual is causal in tumorigenesis. T<sub>121</sub>-induced mammary carcinomas harbor more genomic imbalances than brain tumors (approximately five versus approximately one). One explanation for this observation is that, because the brain is a vital organ, animals succumb to their illness when the brain tumor is at a relatively earlier stage at which fewer changes have accumulated. However, chromosome content of choroid plexus tumors passed further in xenografts remained stable (X. Lu and T. Van Dyke, unpublished data). The converse experiment, analyses of early mammary tumors subsequent to p53 loss, will be required to determine the kinetics of chromosomal changes in this tissue.

**Pocket Protein Redundancy**

Chimera and tissue-grafting experiments with pRb-deficient cells indicate the absence of pRb alone is not sufficient for abnormal mammary development or tumor formation (Maandag et al. 1994; Robinson et al. 2001). Yet mammary-directed overexpression of CCND1, an upstream regulator of pRb, leads to mammary adenocarcinoma (Wang et al. 1994). Given other recent studies indicating the possibility for compensation of pRb function by p107 and/or p130, accounts for the difference. An EcoRI fragment containing the first 121 amino acids of the SV40 T antigen (see Figure 1) that encodes a J domain and a pRb-binding domain, which together are sufficient to cause transformation by inactivating the pRb proteins (DeCaprio et al. 1989; Dyson et al. 1989). Importantly, in contrast to other wild-type T antigen constructs encoding the entire SV40 early region (Husler et al. 1998; Green et al. 2000; Schulze-Garg et al. 2000), small T antigen expression is absent due to a deletion that removes the splice acceptor site. The importance of this is demonstrated by the recent observation that small T antigen alone is sufficient for tumorigenesis in the mammary gland (Goetz et al. 2001). Furthermore, p53 and EP300 (E1A-binding protein p300), which map to the carboxyl half of T antigen, are also abolished, thus permitting assessment of pRb inactivation without the confounding effects of altering additional suppressor pathways. An EcoRI fragment containing the full transgene (see Figure 1) at a concentration of 4 ng/μl was injected into fertilized eggs harvested from B6D2F1 (Jackson Laboratory, Bar Harbor, Maine, United States) mice as described previously (Yan et al. 1990). Transgenic mice were derived and characterized of transgenic mice. The 2.4 kb WAP promoter region was isolated from a WAP-TGFα construct (a gift from David Lee, University of North Carolina at Chapel Hill, United States [Sandgren et al. 1995]) and was cloned upstream of a 2.4 kb KpnI–SalI fragment of the d1137T plasmid (Chen et al. 1992). We targeted T<sub>121</sub> expression to mammary gland using the WAP promoter, which is induced late in pregnancy and expressed during lactation (Pittius et al. 1988) (see Figure 1). T<sub>121</sub> contains the T121 model, the gland undergoes normal development and then is subsequently subjected to acute pRb pathway loss. We presume that this scenario more closely mimics the situation of spontaneous somatic loss in adult human breast. The test of this alternative hypothesis awaits analyses of tissue-specific inactivation of pRb and the paralogous pocket proteins using conditional alleles.

**A Model for Mammary Tumorigenesis Initiated by Targeting the pRb Pathway**

The WAP-T<sub>121</sub> model is a significant addition to the current repertoire of preclinical mammary tumor models exploring the role of pRb pathway in tumorigenesis. Despite the prevalence of pRb pathway defects in human sporadic cancers, mice harboring germline mutations of p16<sup>−/−</sup>NK4a do not develop mammary cancer (Krimpenfort et al. 2001; Sharpless et al. 2001). In addition, mammary-directed expression of CCND1 is only mildly oncogenic (Wang et al. 1994), and as mentioned above, inactivation of pRb alone is not sufficient for tumorigenesis. Although the WAP promoter was a convenient means of directing mammary-specific expression for an initial assessment this model, it also presents the major shortcoming to this model in that expression of T<sub>121</sub> is located to lactogenic hormone activity, as in most existing murine mammary tumor models. Future improvements aim to direct expression of T<sub>121</sub> through hormone-independent methods. Finally, the advantage over wild-type T antigen models is that WAP-T<sub>121</sub> uncouples the simultaneous inactivation of pRb and p53 and permits an assessment of the relative contributions of the individual oncogenic pathways. Testing the combinatorial effects of Rb loss and other breast cancer mutations (e.g., Brca1 and Brca2), along with the further characterization of WAP-T<sub>121</sub> tumors, should help provide additional insights into human breast cancer biology.

**Materials and Methods**

Derivation and characterization of transgenic mice. The 2.4 kb WAP promoter region was isolated from a WAP-TGFα construct (a gift from David Lee, University of North Carolina at Chapel Hill, United States [Sandgren et al. 1995]) and was cloned upstream of a 2.4 kb KpnI–SalI fragment of the d1137T plasmid (Chen et al. 1992). We targeted T<sub>121</sub> expression to mammary gland using the WAP promoter, which is induced late in pregnancy and expressed during lactation (Pittius et al. 1988) (see Figure 1). T<sub>121</sub> contains the first 121 amino acids of the SV40 T antigen (see Figure 1) that encodes a J domain and a pRb-binding domain, which together are sufficient to cause transformation by inactivating the pRb proteins (DeCaprio et al. 1989; Dyson et al. 1989). Importantly, in contrast to other wild-type T antigen constructs encoding the entire SV40 early region (Husler et al. 1998; Green et al. 2000; Schulze-Garg et al. 2000), small T antigen expression is absent due to a deletion that removes the splice acceptor site. The importance of this is demonstrated by the recent observation that small T antigen alone is sufficient for tumorigenesis in the mammary gland (Goetz et al. 2001). Furthermore, p53 and EP300 (E1A-binding protein p300), which map to the carboxyl half of T antigen, are also abolished, thus permitting assessment of pRb inactivation without the confounding effects of altering additional suppressor pathways. An EcoRI fragment containing the full transgene (see Figure 1) at a concentration of 4 ng/μl was injected into fertilized eggs harvested from B6D2F1 (Jackson Laboratory, Bar Harbor, Maine, United States) mice as described previously (Yan et al. 1990). Transgenic mice were identified by PCR amplification of a 160 bp fragment using primers 5′-GAACCTTTGCAGCTAATGGACC-3′ and 5′-GACATCCAAGAAGCTCCTAAGAG-3′ with toe-derived genomic DNA as template. Cycling profile was as follows: 94°C, 2 min; 35 cycles of 94°C, 20 s; 62°C, 45 s; 72°C, 45 s; and final incubation at 72°C, 2 min. TgWAP-T<sub>121</sub> mouse lines were maintained by crossing to nontransgenic B6D2F1.
mice (Jackson Laboratory) and therefore are designated as B6D2-Tg(WAP-T121) Tvd. To study the effect of background differences, WAP- T22 +/+ males were backcrossed to BALB/cJ (Jackson Laboratory) female mice. To increase sample size, tumor onset analysis for BALB/c background mice combined data for N6 (n = 6), N7 (n = 1), and N9 (n = 4) generation mice. For tumor induction, female mice, unless noted otherwise, were housed with male mice to maximize the number of pregnancies, because WAP promoter activity is lactation-dependent (Pittius et al. 1988).

To study the effect of p53 mutation on mammary tumorigenesis in WAP-T22 mice, male WAP-T22 mice were mated to p53−/− females (p53−/− Jax/Jax, Jackson Laboratory). p53 genotypes were determined by PCR using two reactions (Low et al. 1993), one that amplifies the neo insertions site (neomycin primer: 5′-TTCCTGGTGCATTA- CGGTATC-3′; p53 primer: 5′-TATATCAAGAGGCGGCT-3′; 325 bp product) and a second that amplifies the endogenous p53 allele (substituting 5′-ACAGCGTGTGACACTTAT-3′ for the neo primer, 475 bp product). Cycling parameters were the same as the above WAP-T22 reaction. We performed the cross WAP-T22×p53−/−×WAP- T121×p53−/−, and transgenic female mice that were p53−/−, p53−/+, or p53+ were used for analyses while nontransgenic littermates served as controls.

Western immunoblotting analysis. Protein expression levels were assayed as previously described (Symonds et al. 1993). Fresh or flash-frozen tissue samples were homogenized in lysis buffer (50 mM Tris [pH 7.4], 150 mM NaCl, and 1% NP-40). Protein was determined by a Polytron® homogenizer (Kinematica, Littau-Lucerne, Switzerland). Total protein (10 μg) was electrophoresed through a 15% polyacrylamide denaturing gel and then transferred to nitrocellulose membrane (15 V, 30 min). Alternatively, for low-expressing lines, immunoprecipitation was performed prior to electrophoresis as previously described (Symonds et al. 1991). The filter was preincubated in 3% bovine serum albumin, followed by incubation with primary antibody against SV40 T antigen (Pab119 at a dilution of 1:5,000; Harlow et al. 1981). The filter was then washed, followed by incubation at room temperature with horseradish peroxidase-conjugated goat anti-mouse IgG (Amersham Biosciences, Little Chalfont, United Kingdom). The enhanced chemiluminescence method (Amersham Biosciences) was used for autoradiography.

Histopathology and immunohistochemistry. Mammary tissue and tumor samples were dissected from WAP-T22 transgenic or age- and parity-matched B6D2F1 animals. A portion of each tumor was flash-frozen in liquid nitrogen and the remaining tissue was fixed in 10% phosphate buffered formalin, embedded in paraffin, cut to a 5-μm thickness, and stained with hematoxylin and eosin or immunostained using the Vector ABC system (Vector Laboratories, Burlingame, California, United States) for histopathological examination. Apoptosis levels were evaluated by TUNEL assay (Gavrieli et al. 1992) essentially as described in Symonds et al. (1994).

Real-time PCR. Quantitative real-time PCR analysis was performed using a TaqMan approach on DNA derived from terminal tumors to determine the status of the wild-type p53 allele as previously described (Lu et al. 2001). The primers for the p53 allele were 5′-ATGGCCATCTCAAGAAGTACAG-3′ and 5′-ATGCGAG- CAGGGCTCATG-3′. The sequence of the p53 probe was 5′-ACAAGCCTGAGCCAGGGCT-3′. Each 25-μl reaction mixture contained 50 ng of DNA template, 18 nM p53 primers, 80 nM p53TATA control primers, 8 nM probe, and 12.5 μl of TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, California, United States) containing AmpliTaq Gold polymerase, deoxyribonucleoside triphosphates, and PCR buffer. The cycling conditions were 50°C for 2 min and 95°C for 10 min for 1 cycle, and 95°C for 15 s and 60°C for 1 min for 40 cycles. The reactions were performed using an ABI 7700 Sequence Detection system (Applied Biosystems), and the data analyzed using Sequence Detector 1.7 (Applied Biosystems) and standard protocols (http://www.appliedbiosystems.com). The copy number of each sample was determined by calculating ΔCt based on the formula ΔCt = [sample Ct(p53) − sample Ct(p53TATA)] × 10^[(p53TATA control Ct) × n]/C, where C is the number of cycles required to reach a threshold based on linear amplification. Analyses of standard samples (L. Chin, Harvard University, Cambridge, Massachusetts, United States, personal communication) indicate copy numbers of 2, 1, and 0 are indicated by 2^−ΔCt values of greater than 0.6, 0.15−0.6, and less than 0.15, respectively. Standard samples analyzed along with experimental samples confirmed the accuracy of these assignments.

Statistical analyses. Kaplan–Meier survival analysis was used to determine median tumor latencies (StatsDirect, Cambridge, United Kingdom), and the Log-Rank (Peto, StatsDirect) test was performed to evaluate statistical significance. The equivalence of tumor morphology distributions was tested using the Fisher–Flematon–Halton’s exact test.

CGH. Genomic DNA was extracted from end-stage tumors (1 cm in diameter) or tails using a DNeasy genomic tip (Qiagen, Valencia, California, United States) and further purified by proteinase K digestion followed by phenolchloroform extraction, ethanol precipitation, and resuspension in sterile H2O. cCGH was performed as described in Kallioniemi et al. (1992), Donehower et al. (1995), and Lu et al. (2001). aCGH was performed as described in Snijders et al. (1998), C12K (Kavac, www.ncbi.nlm.nih.gov/entrez/query.fcgi, SWAP-T121×p53−/−, or p53+ were used for analyses while nontransgenic littermates served as controls.

Supporting Information

Accession Numbers. The accession numbers for the genes and gene products discussed in this paper are Brca1 (LocusLink ID 12189), Ha-ras (LocusLink ID 30865), DCC (LocusLink ID 1021), c-myc (LocusLink ID 17869), cyclin D1 (LocusLink ID 595), cyclin E (LocusLink ID 859), H2B (P003516), HER2 (LocusLink ID 13866), histone acetyltransferase (LocusLink ID 3065), p100 (LocusLink ID 1029), p300 (LocusLink ID 7157), p107 (LocusLink ID 2053), p130 (LocusLink ID 3082), p300 (LocusLink ID 2035), PCNA (LocusLink ID 18538), pRb (LocusLink ID 5925), PTEN (LocusLink ID 5728), v-He-ras (LocusLink ID 3265), WAP (LocusLink ID 22573), and Wnt-1 (LocusLink ID 22408).

These databases may be found at www.ncbi.nlm.nih.gov/LocusLink/ (LocusLink), and www.ebi.ac.uk/Integrating (Integrating) Systems.

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Author contributions. KS, HW, and TVD conceived and designed the experiments. KS, HW, and LL performed the experiments. KS, HW, RDC, and TVD analyzed the data. DP and DA contributed reagents/materials/analysis tools. KS and TVD wrote the paper.
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