Breast cancer is a highly heterogeneous disease, with several different subtypes being characterized by distinct histology, gene expression patterns, and genetic alterations (1–4). Markers analysis revealed the existence of human epidermal growth factor receptor 2–positive (HER-2/NEU+) and estrogen receptor–positive (ER+) breast tumors as well as triple-negative tumors (TNs), which do not express hormone receptors or HER-2/NEU (5). Likewise, microarray profiling identified luminal-A–like, luminal-B–like, HER2/NEU+, and basallike breast carcinomas (2, 6–8). More recently, basallike breast carcinoma were recognized as a subgroup of TN that also include metaplastic and claudin-low subtypes (9, 10, 11–14). The metaplastic and claudin-low tumors exhibit epithelial-to-mesenchymal transition (EMT). The EMT-type tumors and a subset of the basal-like tumors, but not luminal-B–like tumors, expressed mutant forms of the tumor suppressor p53. Accordingly, targeted deletion of both Rb and p53 in stem/bipotent progenitors led to histologically uniform, aggressive, EMT-type tumors. Reintroduction of Rb into these tumor cells suppressed growth in vitro and tumor formation in vivo. These results establish a causal role for Rb loss in breast cancer in mice and demonstrate that cooperating oncogenic events, such as mutations in p53, dictate tumor subtype after Rb inactivation.

Introduction
Breast cancer is a highly heterogeneous disease representing tumors with distinct histology, gene expression patterns, and genetic alterations (1–4). Markers analysis revealed the existence of human epidermal growth factor receptor 2–positive (HER-2/NEU+) and estrogen receptor–positive (ER+) breast tumors as well as triple-negative tumors (TNs), which do not express hormone receptors or HER-2/NEU (5). Likewise, microarray profiling identified luminal-A–like, luminal-B–like, HER2/NEU+, and basallike breast carcinomas (2, 6–8). More recently, basallike breast carcinoma were recognized as a subgroup of TN that also include metaplastic and claudin-low subtypes (9, 10, 11–14). The metaplastic and claudin-low tumors exhibit epithelial-to-mesenchymal transition (EMT) and a cancer stem cell expression signature (9, 10, 14, 15). Although the basal-like subtype responds to chemotherapy, no effective treatment is available for metaplastic and claudin-low tumors; metastatic disease is virtually untreatable.

The tumor suppressors breast cancer 1 (Brca1), p53, phosphatase and tensin homolog (Pten), and retinoblastoma 1 (RB1) are commonly lost in familial and sporadic forms of TN (16–19). RB1 gene rearrangement was reported in approximately 10% of primary breast carcinomas of undefined subtype and in approximately 20–25% of breast cancer cell lines, including MDA-MB436, MDA-MB468, and BT549, which are derived from TNs (20–27). Microarray analysis revealed that RB1 transcripts are downregulated in about 70% of basallike breast tumors with concomitant induction of the CDK4/6 inhibitor p16Ink4a, a target for transcriptional repression by the RB1 protein, pRb (28). Low RB1 gene expression and loss of heterozygosity (LOH) at the RB1 locus were subsequently identified at high frequency in luminal-B–like and basal-like/TNs (29). In accordance, a recent study has demonstrated loss of pRb expression coupled with high expression of p16Ink4a and p53, presumably a stabilized mutant form, in most basal-like/TNs (30).

pRb regulates cell growth and differentiation by modulating the activity of transcription factors such as E2F family members (31, 32). Among E2F-responsive genes are factors required for cell cycle progression and apoptotic cell death (33). Apoptosis downstream of pRb is often, but not always, mediated by the tumor suppressor p53 (34, 35). Accordingly, pRb and p53 are commonly lost together in cancer; various DNA viruses harbor oncoproteins, such as SV40 large T antigen (SV40 Tag), that transform host cells by sequestering pRb and its relatives p107 and p130 as well as p53 (36, 37). Much insight into Rb function was gained through analysis of mutant mice. Most Rb+/– heterozygote mice die at approximately 11 months of age with a wasting disease caused by pituitary tumors, whereas Rb−/− embryos die at midgestation (38). Rb loss in many tissues leads to ectopic cell proliferation, apoptosis, and incomplete differentiation (38). Mammary placodes from Rb−/− embryos develop...
op normally when transplanted into recipient mammary glands (39). In contrast, mammmary gland–specific transgenic expression of SV40 Tag, which binds the pRb protein family (pRb, p107, and p105), which is coexpressed with Rb in the mammary gland, by concurrent inactivation of Rb (45), yielding WAP-Cre:ROSA26 females exhibited lactation defects, leading to impaired development and death of their offspring (Figure 1A). Newborns from these WAP-Cre:Rbebre; p107−/− females suckled, but contained very little milk in their stomachs, and their growth retardation and morbidity could be rescued by transferring to foster mothers (data not shown). Consistent with this defect, lobuloalveoli of lactating mammary glands from WAP-Cre:Rbebre; p107−/− females were less dense compared with those from controls (Figure 1, B and C) and contained adipocytes, which are normally absent at late pregnancy and lactation, as well as many involuting lobuloalveoli (Figure 1, D–F). Whole-mount analysis of 10 mammary glands from 1- to 2-year-old nulliparous and multiparous WAP-Cre:Rbebre; p107−/− mice failed to detect any microscopic tumors, and of more than 40 WAP-Cre:Rbebre; p107−/− females that survived for up to 2 years, only 1 developed a tumor (solid adenocarcinoma; data not shown).

**Targeted inactivation of Rb in mammary epithelium via MMTV-Cre induces squamous metaplasia and acinar hyperplasia.** To delete Rb during ductal morphogenesis we used a mouse mammary tumor virus–Cre (MMTV-Cre) deleter, MMTV-CreNLST (44). As with WAP-Cre, ductal morphogenesis, lobuloalveologenesis, involution, and lactation proceeded apparently normally in MMTV-CreNLST:Rbebre and MMTV-CreNLST:Rbebre; p107−/− females (data not shown). However, at 10–14 months of age, mammary glands from 16 of 20 multiparous Rb mutant (Rbebre) females were found to contain micronodules (Figure 2, A and B). None of 10 control mammary glands contained such lesions (Figure 2, C and D). Histopathology of nodules from 2 MMTV-Cre:Rbebre; p107−/−, 4 MMTV-Cre:Rbebre; p107−/−, and 2 MMTV-Cre:Rbebre; p107−/− mice revealed that each represented acinar hyperplasia with keratinizing squamous metaplasia (Figure 2E).

The hyperplastic lesions (n = 8) exhibited high levels of apoptosis and cell proliferation, as determined by in situ TUNEL and immunohistochemistry (IHC) for proliferating cell nuclear antigen (PCNA), respectively (Figure 2, F and G). The lesions expressed cyclin-dependent kinases (CDKs), a marker of mammary myoepithelium and epidermis, and the epidermis-specific differentiation marker CK6 (Figure 2, H and I). Similar results were obtained regardless of p107 status. Thus, inactivation of Rb or Rb plus p107 via MMTV-CreNLST induced focal lesions characterized by hyperplastic growth and apoptosis with transdifferentiation into epidermal-like structures.

MMTV-CreNLST and WAP-Cre delete Rbebre in different cells within the mammary stem cell hierarchy. X-gal staining of mammary glands from WAP-Cre:ROSA26 females revealed high level of recombination during pregnancy and lactation (Figure 3A and ref. 51). In contrast, MMTV-CreNLST:ROSA26 mice exhibited high levels of recombination during pregnancy and lactation as well as during ductal morphogenesis and alveologenesis (Figure 3B).

Next, we sought to identify the cells within the mammary stem cell (MSC) hierarchy in which Rb is deleted by WAP-Cre versus MMTV-Cre. MSCs, luminal progenitors, and myoepithelial progenitors were identified on the basis of expression of the cell surface markers CD24 (52) and either β1-integrin (CD29) or α6-integrin (CD49f; refs. 53, 54). A typical FACS profile of lin-
eage-depleted (Lin-) mammary epithelial cells sorted for CD24 and CD49f expression is shown in Figure 3C. The presumptive MSCs or mammary repopulating units (MRU; CD24+CD49f+), myoepithelial progenitors (MYO; CD49f+), and colony-forming luminal progenitor cells (CFC; CD24+), as defined by Stingl et al. (54), are indicated. To test their differentiation potential, sorted cells were seeded onto collagen-1–coated plates, induced to differentiate for 3 days, and immunostained with the luminal marker CK18, the stem/myoepithelial marker CK14, and the myoepithelial marker SMA. The CD24–CD49f+ fraction differentiated into mostly CK14+CK18+ double-positive cells and was negative of the CK14+CK18+ cells is yet to be defined, these results are consistent with the assignment of CD24–CD49f+ cells as myoepithelial progenitors, CD24+CD49f– cells as luminal progenitors, and CD24+CD49f+ cells as MSCs or bipotent common progenitors.

To determine in which cells $Rb^{fl/fl}$ was excised, 6 mammary glands each (nos. 3, 4, and 5) from 4 multiparous MMTV-Cre^{NLST}; $Rb^{fl/fl};p107^{+/–}$ or multiparous WAP-Cre:$Rb^{fl/fl};p107^{+/–}$ mice approximately 6 months old were pooled (24 mammary glands), Lin– mammary epithelial cells were sorted as in Figure 3C, and DNA was extracted from each fraction. Cre-mediated recombination was then assessed by PCR with primers flanking exon 19, which can amplify 235-bp WT, 283-bp nonrecombined $Rb^+$, or 260-bp recombined $Rb^{fl/fl}$ alleles (Figure 3D and ref. 45). In WAP-Cre: $Rb^{fl/fl};p107^{+/–}$ mammary epithelium, the recombined $Rb^{fl/fl}$ allele was reproducibly observed only in the CD24+ luminal fraction, not in the CD24+CD49f+, CD49f-, or CD24+CD49f+ fractions (Figure 3E). Densitometry analysis revealed that the percentage of $Rb^+$ relative to $Rb^{fl/fl}$ was 15% in CD24+ cells, but undetectable in other cell fractions (Figure 3F). In contrast, in MMTV-Cre^{NLST}; $Rb^{fl/fl};p107^{+/–}$ mammary glands, recombination was observed primarily in the CD24+CD49f+ MSC/bipotent cell fraction, with 41% of the cells containing the recombined allele relative to $Rb^+$ (Figure 3, E and F). Recombined $Rb^{fl/fl}$ was also observed in CD24+ and CD24+CD49f+ fractions upon loading more DNA and after a second round of PCR amplification, respectively (Figure 3, E and F, and Supplemental Figure 1, A and B; supplemental material available online with this article; doi:10.1172/JCI41490DS1). Together, these results indicated that MMTV-Cre and WAP-Cre delete $Rb$ in different cells within the MSC hierarchy, and this may underlie the different phenotypes of MMTV-Cre:$Rb^{fl/fl};p107^{+/–}$ and WAP-Cre:$Rb^{fl/fl};p107^{+/–}$ mice.

Deletion of $Rb$ in mammary epithelium via MMTV-Cre, but not WAP-Cre, expands MSCs/bipotent cells. To assess the effect of $Rb$ deletion on the CD24+CD49f+ MSC/bipotent cell population, we compared the percentage of CD24+CD49f+ cells in MMTV-Cre:$Rb^{fl/fl};p107^{+/–}$ (n = 5) or WAP-Cre:$Rb^{fl/fl};p107^{+/–}$ (n = 5) mice relative to control littermates. The proportion of CD24+CD49f+ cells in WAP-Cre:$Rb^{fl/fl};p107^{+/–}$ relative to control animals was nearly identical, at a ratio of 0.98 (SD, 0.22; P = 0.44; Figure 3G). In contrast, the percentage of CD24+CD49f+ cells in MMTV-Cre:$Rb^{fl/fl};p107^{+/–}$ mice was on average 2.32-fold higher than controls (SD, 0.94; P = 0.034), ranging from 1.44- to 3.5-fold (Figure 3G). In addition, we seeded Lin– epithelial cells from multiparous MMTV-Cre:$Rb^{fl/fl};p107^{+/–}$, WAP-Cre:$Rb^{fl/fl};p107^{+/–}$, and control mammary glands into ultra-low attachment plates and measured the number of primary mammospheres. The number of mammospheres in MMTV-Cre:$Rb^{fl/fl};p107^{+/–}$ and control mammary glands from 8 independent mutant mice. Shown are TUNEL analysis for apoptosis (F) and IHC for PCNA (G), CK5 (H), and CK6 (I). Arrows point to representative stained cells. Original magnification, ×8 (A); ×10 (C); ×50 (B and D); ×100 (E); ×400 (F–I).
Figure 3
Differential effects of MMTV-Cre and WAP-Cre on Rb deletion and stem/bipotent cell compartments. (A) Whole-mount X-gal staining of a lactating WAP-Cre:ROSA26 gland. (B) X-gal staining of developing endbuds and ducts during ductal morphogenesis (left, cross section; center, transverse section), and lobuloalveoli during lactation (right, whole-mount) in MMTV-CreNLST:ROSA26 mice. (C) CD24-CD49f FACS profile of multiparous Lin- normal mammary epithelium and gating used for cell sorting, and immunofluorescent staining for SMA, CK14, and CK18 on the indicated cell populations. DP, CD24+CD49f+ double positive. (D) Schematic representation of floxed Rb exon 19 and primers used to detect WT Rb, Rbfl, and recombined RbΔfl alleles. (E) PCR analysis of DNA extracted from pooled, sorted Lin- mammary epithelium from multiparous WAP-Cre:Rbfl/fl; p107−/− (n = 5) or MMTV-Cre:Rbfl/fl; p107−/− (n = 4) mice. Cells were gated as in C. Blot at bottom right shows overexposure after loading twice as much DNA, revealing low-level recombination in CD24+ cells. (F) Densitometry analysis of RbΔfl and Rbfl PCR fragments from E. DN, double negative. (G) Percentage of Lin-CD24−CD49f− cells in multiparous WAP-Cre:Rbfl/fl; p107−/− (n = 5) and MMTV-Cre:Rbfl/fl; p107−/− (n = 5) mice relative to control. *P = 0.034, 1-tailed paired Student’s t test. Original magnification, ×63 (A); ×100 (B, left); ×400 (B, center, and C); ×50 (B, right).
MMTV-Cre:Rb\textsuperscript{fl/fl} and MMTV-Cre:Rb\textsuperscript{fl/fl}:p107\textsuperscript{−/−} mice develop transplantable mammary tumors. (A) Dissected MMTV-Cre:Rb\textsuperscript{fl/fl}:p107\textsuperscript{−/−} female with 2 large tumors in mammary gland nos. 4 and 5 (arrows). (B) Tumor-free curve for a cohort of 22 Rb\textsuperscript{fl/fl} mice (2 MMTV-Cre:Rb\textsuperscript{fl/fl}:p107\textsuperscript{−/−}, 12 MMTV-Cre:Rb\textsuperscript{fl/fl}:p107\textsuperscript{−/−} and 8 MMTV-Cre:Rb\textsuperscript{fl/fl}:p107\textsuperscript{−/−}) and 20 control (without MMTV-Cre) littersmates. (C) Complex PCR analysis with the 3 primers. Lane 1, Rb\textsuperscript{fl} mouse tail; lane 2, Rb\textsuperscript{fl} mouse tail; lane 3, MMTV-Cre\textsuperscript{NLST}; Rb\textsuperscript{fl}:p107\textsuperscript{−/−} mammary tumor; lane 4, MMTV-Cre\textsuperscript{G}:Rb\textsuperscript{fl}:p107\textsuperscript{−/−} heterozygote mammary tumor, demonstrating LOH at the Rb locus. Lanes were run on the same gel but were noncontiguous (white line). (D–G) H&E staining of representative Rb\textsuperscript{fl} tumors and pulmonary metastasis. (D) Tumor 2, adenosquamous carcinoma. (E) Tumor 4, solid adenocarcinoma with comedo patterns. (F) Tumor 3, pleomorphic adenocarcinoma with cystic changes. (G) Pulmonary metastasis in MMTV-Cre:Rb\textsuperscript{fl/fl}:p107\textsuperscript{−/−} mutant shown in F. (H) Flow cytometry analysis of Lin− control mammary epithelium (SD, 1.53; P < 0.05; Figure 4, H–J). To test for cell-autonomous growth, we assessed the ability of Rb\textsuperscript{fl} tumor cells to form spheres in nonadherent cultures and xenografts in vivo. After seeding Lin− tumor cells on nonadherent plates in defined medium containing EGF and FGF (56–58), 8 of 11 tumors gave evidence of autonomous growth. Note glandular tumors with histology similar to the original tumor. Inset: low-magnification image of the pulmonary metastasis (right; normal lung tissue on left). (I) Revealed LOH at the Rb locus (Figure 4C, lane 4). In contrast, PCR analysis revealed LOH at the Rb locus (Figure 4C, lane 4). In contrast, PCR analysis revealed LOH at the Rb locus (Figure 4C, lane 4). In contrast, PCR analysis revealed LOH at the Rb locus (Figure 4C, lane 4). In contrast, PCR analysis revealed LOH at the Rb locus (Figure 4C, lane 4).
### Table 1: Characteristics of RbΔfl mammary tumors

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Histology/IHC and cluster analysis were performed on different regions of each tumor and therefore may not be directly comparable. AC, adenocarcinoma; SM, squamous metaplasia/keratoacanthoma; ker, keratinized area; str, stroma; ND, not determined.

RbΔfl mammary tumors expressed basal cytokeratins, but not ERα. To begin to classify the RbΔfl tumors into subtypes, we stained histological sections for the myoepithelial/basal cell markers CK5 and CK14 as well as ERα. Of 12 RbΔfl tumors, 8 expressed CK5 in tumor epithelium or squamous areas (Figure 5, A–C, and Table 1). We also observed that 2 tumors were negative for CK5 in the epithelium compartment, but positive in keratinized areas (Figure 5D). CK14 was expressed at different levels in all tumors except for tumors 4 and 10 (Figure 5, E–H, and Table 1). Importantly, with the exception of the papillary adenomyoepithelioma (tumor 11), all RbΔfl tumors were negative for, or expressed very low levels of (tumor 4), ERα (Figure 5, I–L, and Table 1). Consistent with its role in epidermal transdifferentiation (59), we observed variable levels of β-catenin expression at the cell membrane in nodular areas of tumors (Figure 5, N–P), but almost exclusive nuclear staining in cells surrounding keratinized areas in adenosquamous tumors (Figure 5M, arrows).
Figure 5
Most RbΔfl mammary tumors express variable levels of basal cytokeratins, but not ERα. Representative IHC of tumors 2, 4, 9, and 11 for CK5 (A–D), CK14 (E–H), ERα (I–L), and β-catenin (M–P). Inset in D shows CK5 expression in squamous area. Arrows in M mark cells surrounding keratinized area (Ker) with nuclear localized β-catenin. Original magnification, ×400 (A–P and D, inset).

Array CGH analysis revealed a tendency toward more gains and/or losses in the Rb-deficient Tag/TNTs (tumors 3 and 4) compared with the 7 other Rb tumors (Supplemental Figure 4A and data not shown). Major losses and gains were found in chromosome 15 in tumors 3 and 4, but not in tumors 1, 5, 6, and 9 (Supplemental Figure 4B) or in tumors 2, 10, or 11 (data not shown). This region in chromosome 15 showed conservation of synteny with human chromosomes 5p13 and 8q22, which frequently undergo chromosomal gains in human breast cancer (60). Thus, these RbΔfl tumors could be divided into 2 major groups: T121/luminal-B–like (nos. 1, 5, 6, and 9) and basal-like (nos. 2, 3, 4, and 11). The basal-like group could be further divided into 2 subgroups: Tag-like (nos. 3 and 4) and DMBA-like (nos. 2 and 11).

Tag/basal-like, but not T121/luminal-B–like, RbΔfl mammary tumors express a human RB1-LOH proliferation gene signature. Analysis of human breast tumors with LOH at the RB1 locus led to the generation of a RB1-specific proliferation signature (29). We created an average expression value for the human RB1-LOH signature and compared it with the levels of expression in RbΔfl mammary tumors. The top 2 samples were the Rb-deficient Tag/basal-like tumors 3 and 4, with values of 0.37 and 0.47, respectively. The average value for T121/luminal-B–like tumors (nos. 1, 5, 6, and 9) was 0.13 (0.04, 0.22, 0.09, and 0.17, respectively). Thus, the RbΔfl tumors that share the human RB1-LOH proliferation signature are tumors 3 and 4, which cluster with Tag/basal-like carcinoma.

Tag/basal-like RbΔfl tumors express high levels of p53. To begin to understand the basis for tumor heterogeneity downstream of Rb, we investigated the status of p53. Remarkably, Tag/basal-like RbΔfl tumors exhibited high, near-uniform expression of nuclear p53, whereas DMBA-like RbΔfl tumors expressed nuclear p53 in a small fraction of the cells (Figure 7A, top, and Supplemental Figure 5). In contrast, RbΔfl luminal-B or NEU+ tumors did not express detectable levels of p53 (Figure 7A, bottom, and Supplemental Figure 5). Analysis of additional RbΔfl tumors identified 3 more with high expression of p53: a solid adenocarcinoma (tumor 48; Supplemental Figure 5), and 2 spindle-cell/EMT tumors (tumors 18 and 30; Figure 8, A and B, and see below).

The RbΔfl basal-like tumors exhibited higher rates of cell proliferation and similar levels of apoptosis compared with the luminal-like tumors (Figure 7, B and C), which suggested that they contained mutant p53 (see below). In addition, IHC analysis for p16INK4a identified small pockets of positive cells in RbΔfl basal-like tumors, but not luminal-B–like tumors (Figure 7D). The relatively low IHC signal was likely related to the low sensitivity of anti-mouse p16INK4a antibodies. Indeed, at the RNA level, p16INK4a was highly expressed in the RbΔfl basal-like tumors, but not other tumors (Figure 6G). This elevated p16INK4a expression is consistent with its high expression in RB1-negative human basal-like breast cancer (28–30).

RbΔfl spindle cell tumors express mutant p53 and markers of EMT. As noted above, tumors 18 and 30 exhibited spindle-cell morphology (Figure 8, A and B). These tumors expressed high levels of p53 as well as the EMT-associated markers N-cadherin and desmin (Figure 8B, data not shown, and ref. 61). They readily grew as monolayer cultures and were highly aggressive; transplantation of 1,000 monolayer cells into the mammary glands of recipient mice gave rise to large tumors within 2–3 weeks that retained the same histology and marker expression as the parental tumors (Figure 8B). Consistent with their mesenchymal characteristics, flow cytometry analysis revealed that these tumors lost the CD24+ luminal marker, exhibiting a high CD49f+ to CD24+ ratio (Figure 8D). EMT tumors with spindle-cell morphology were previously described in several mouse models of breast cancer, including some rare mammary tumors with null mutations in p53 (62). In humans, EMT tumors in breast cancer have been recognized within the larger TNT group that includes claudin-low and metaplastic breast cancer (9–14). Thus, some RbΔfl mammary tumors express nuclear p53, are highly aggressive, and exhibit spindle-cell/EMT morphology.
Figure 6
Rb\textsuperscript{Δfl} mammary tumors cluster with DMBA and Tag-induced basal-like or T\textsubscript{121} induced luminal-B–like breast tumors. (A) Overview of expression of 866 reference genes in tumors from Rb\textsuperscript{Δfl} and 13 mouse models of breast cancer. Colored regions at left correspond to the regions shown in B–F. (B) Selected genes representing the luminal gene cluster. (C) CK5 basal-like gene cluster. (D) CK14 basal-like gene cluster. (E) Proliferation-associated gene cluster. (F) EMT gene cluster. (G) Subset of proliferation-associated genes. Asterisks at the top mark the 9 Rb\textsuperscript{Δfl} tumors; tumor models that clustered with Rb\textsuperscript{Δfl} tumors are highlighted at the bottom. DMBA, 7,12-dimethylbenz[a]-anthracene.
Tag/basal-like and EMT RbΔfl tumors contain p53 mutations. To determine whether high p53 expression in these tumors was caused by mutations in p53, which stabilizes the protein (63, 64), we sequenced exons 2–9 of p53 in tumors 3, 4, 18, and 30. We identified missense mutations K129M, H165R, C138R, and C138R, respectively, in the DNA-binding domain of these tumors (Figure 9). These amino acids were conserved in human p53 (K132, H168, C141, and C141, respectively) and are frequently mutated in human cancer (65, 66). Tumors 3 and 4 only harbored the mutant allele, whereas tumors 18 and 30 contained both the WT and mutant alleles, which suggests that C138R acts as dominant negative.

Combined deletion of Rb and p53 in mammary epithelium via MMTV-Cre induces aggressive spindle cell/EMT tumors. The above results suggest that p53 status dictates whether the focal hyperplastic lesions in MMTV-Cre;RbΔfl/mice develop into luminal-B or basal-like/EMT tumors. We predicted that combined deletions of Rb and p53 would lead to uniform basal-like or EMT tumors at the expense of luminal-B. To test this notion, we crossed the MMTV-Cre;RbΔfl/ mice with floxed p53 (p53fl/fl) mice (67). Mammary-specific deletion of p53fl/fl leads to ERα-positive and -negative tumors with a latency of more than 11 months (68). MMTV-Cre;RbΔfl/p53fl/fl double-mutant mice were viable and fertile, yet nearly all developed lethal lymphomas within 2–6 months. To circumvent this, we purified Lin− mammary epithelial cells from 2 independent MMTV-Cre;RbΔfl/p53Δfl mice and transplanted 10,000 cells each into the mammary glands of 4- to 5-week-old NOD/SCID mice. Tumors developed with an average latency of 126 days (6 tumors in 12 injections; 50%) and 58 days (10 tumors in 24 injections; 42%). Given that the mammary epithelium was derived from 76- and 119-day-old mice, the total latency for tumor formation was 202 and 177 days, an average of 190 days (i.e., about 6.5 months). PCR analysis confirmed deletion of Rb and p53 (Figure 10A). Analysis of 10 independent tumors revealed similar mesenchymal histology, characterized by spindle-shaped cells with abundant pink cytoplasm (Figure 10B). Some regions contained epithelial components surrounded by mesenchymal cells (Figure 10C), consistent with EMT. Indeed, expression of CK8 (Figure 10E) was restricted to epithelial cells within tumors, whereas mesenchymal areas expressed the EMT markers desmin and N-cadherin (Figure 10, F and G) and were indistinguishable from the RbΔfl/p53Δfl mesenchymal tumors derived from MMTV-Cre;RbΔfl/mice (Figure 8).

Reintroduction of Rb into aggressive RbΔfl/p53Δfl EMT tumor cells suppresses their growth. Many tumors are dependent on (i.e., addicted to) the continuous expression of oncogenes that drive them (reviewed in refs. 69, 70). Although introduction of constitutively activated, unphosphorylatable pRb alleles often suppress cell proliferation, WT pRb seems to inhibit only certain cells, likely with low levels of cyclins (71–75). To test whether growth of RbΔfl/p53Δfl tumor cells is dependent on the continuous absence of pRb, we transduced WT Rb via a retrovirus vector that also encodes GFP. Expression of pRb was confirmed by immunofluorescent staining (data not shown). GFP+ cells transduced with Rb (pMXIE-GFP-Rb) or vector alone (pMXIE-GFP) were sorted, and 500 GFP+ cells were seeded onto 96-well plates. After 1 week, MTT assays revealed approximately 5-fold inhibition of cell growth in Rb-transduced cultures relative to controls (Figure 11A). The GFP-sorted cells were also transplanted into mammary glands of 4- to 5-week-old NOD/SCID mice. Mice transplanted with 1,000 control tumor cells (empty vector) developed large tumors within 2–3 weeks (12 of 12; Figure 11B). In contrast, mice transplanted with Rb-GFP+ tumor cells did not develop tumors (0 of 12; Figure 11C). Thus, despite their aggressive nature, the RbΔfl/p53Δfl mammary tumors were addicted to continual absence of Rb and readily inhibited when reconstituted with this tumor suppressor.

Discussion
We describe the consequences of Rb deletion in the mammary epithelium and delineate several stages in tumor initiation and progression leading to distinct breast cancer subtypes (Figure 10H). First, loss of Rb in stem/bipotent progenitor cells expands this cell population. As these cells attempt to exit the cell cycle and differentiate into single-lineage (luminal or myoepithelial) progenitors, they undergo apoptotic death, which restricts their growth. Second, in cooperation with a limited number of oncogenic events (M2), some RbΔfl epithelial cells continue to proliferate to form focal acinar hyperplasia with propensity to squamous transdifferentiation. These lesions exhibit high levels
of both cell proliferation and apoptosis. Third, additional mutations, which increase cell proliferation and/or inhibit apoptosis or differentiation, further transform these hyperplastic lesions into distinct tumor subtypes. We showed here that mutations in p53 induced basal-like or EMT tumors. We note that whereas the basal-like tumors were defined by microarray cluster analysis, the EMT tumors were identified by their spindle-cell morphology and expression of mesenchymal markers. Of interest, knockdown of RB1 in MCF7 breast cancer cells induces EMT (76). However, our results demonstrated that Rb loss also induced luminal-B-like as well as HER2-like tumors that did not express EMT markers. Thus, other mutations, which are yet to be defined, cooperate with Rb loss to induce luminal-B-like tumors or other subtypes. Remarkably, combined mutations in Rb and p53 led to uniform types of EMT tumors at the expense of all others, which suggests that the timing of tumor suppressor inactivation may also dictate tumor subtype.

Why does deletion of Rb in mammary epithelium lead to mammary tumors, whereas Rb−/− mammary epithelial cells from embryonic mammary gland can be transplanted into recipient mice do not (39)? We suggest the discrepancy may be due to dependency on pregnancy for tumor formation in Rb-deficient mammary epithelium. Indeed, we found that mammary tumors developed only in parous MMTV-Cre:RbΔfl/Δfl mice. In contrast, the transplanted embryonic Rb−/− mammary gland developed only in recipient mice. The effect of Rb deletion was critically dependent on the cell within the mammary epithelial hierarchy in which it was disrupted: deletion of Rb via WAP-Cre led to minor lactation defects but not tumor formation, whereas deletion via 2 different MMTV-Cre lines led to mammary tumors. We showed that MMTV-Cre induced efficient Rb deletion primarily in CD24+CD49f+ stem/bipotent progenitors, whereas WAP-Cre induced deletion in CD24− luminal progenitors. WAP-Cre is expressed in parity-identified multipotent alveolar stem cells (43, 77). Some low level of recombination within the stem cell/bipotent cell compartment may occur in WAP-Cre:RbΔfl/Δfl mammary glands, but this is masked and greatly underrepresented relative to CD24+ cells, in which WAP-Cre is most abundantly expressed (78). The fact that tumors arose in MMTV-Cre:RbΔfl/Δfl mice, but not WAP-Cre:RbΔfl/Δfl mice, indicates that even if some low level of Rb deletion occurs in parity-identified multipotent epithelial cells, these cells are inherently different or exposed to a different microenvironment that diminishes their susceptibility to neoplastic transformation compared with ductal stem/bipotent progenitors.
Given the high frequency with which Rb and p53 are lost in TNT/basal-like breast cancer, and our results demonstrating that mutations in p53 cooperated with Rb loss to induce aggressive EMT tumors, these tumor suppressor pathways may be ideal therapeutic targets for TNTs. Importantly, we showed that transduction of WT pRb completely abolished growth of RbΔfl/p53Δfl TNTs. Thus, these tumors were addicted to Rb loss; drugs that mimic pRb function (79), perhaps in combination with drugs that activate mutant p53 (80), may therefore suppress the growth these tumors. The mouse models described here as well as 2 other models of TNTs—combined deletion of Brca1 and p53 (81) and overexpression of the Met receptor tyrosine kinase (82)—provide invaluable preclinical platforms to identify and assess potential therapeutics for this aggressive breast cancer subtype (83).

Methods

Mouse genotyping and genetic analysis. All procedures were done in accordance with the current Canadian Animal Care Council guide for the care and use of laboratory animals and were approved by the Toronto General Research Institute Animal Research Committee. MMTV-Cre:Rbfl/fl, MMTV-Cre:Rbfl/fl:p107–/–, and WAP-Cre:Rbfl/fl:p107–/– mice were on mixed genetic backgrounds. Rbfl/fl:p107–/– mice were provided by R. Bremner (Toronto Western Hospital, Toronto, Ontario, Canada). MMTV-Cre was purchased from Jackson Laboratory. p53fl/fl mice were obtained from the NCI Mouse Repository. Genotyping was based on published primers and confirmed in tumor-bearing animals by second rounds of PCR reactions. Recombined Rb alleles were resolved on 3% NuSieve gel.

Whole mount and X-gal staining. Mammary glands were fixed in 4% PFA, dehydrated in 100% ethanol, and stained with 0.5% X-gal.

Figure 9
Identification of p53 mutations in Tag/basal-like and EMT RbΔfl tumors. (A) DNA sequencing reaction around K129 in control DNA (left) and tumor 3 (right). (B) Schematic representation of human p53 and relative location (asterisks) of mutations identified in mouse RbΔfl tumors: Tag/basal-like tumor 3, K129M (human, K132M); Tag/basal-like tumor 4, H165R (human, H168R); EMT tumors 18 and 30, C138R (human, C141R). TAD, N-terminal transactivation domain; PRR, proline-rich region; TET, tetramerization domain; CT, C terminus.

Figure 10
MMTV-Cre:RbΔfl:p53Δfl double mutant mice develop spindle cell/EMT tumors. (A) PCR analysis of tail from MMTV-Cre:RbΔfl:p53Δfl mice and tumor DNA for recombined Rb and p53 alleles or Cre. (B and C) Histology staining of Rb/p53 tumor showing spindle/mesenchymal and epithelial components (C). (D–G) Representative IHC staining for the indicated antibodies. e, epithelium. (H) Schematic model for tumor formation after Rb deletion in the mammary epithelium. See Discussion for details. Original magnification, ×400.
Sections were counterstained with methyl green. TUNEL used at 1:400 dilution, CK5 at 1:500 (catalog no. PRB-160P; Covance), and Rb (catalog no. D33; DAKO), and Rb (catalog no. 554136; BD) at 1:200 dilution. BD Biosciences—Pharmingen), and FACSCalibur flow cytometer or 555736; BD Biosciences—Pharmingen), CD24-FITC (catalog no. 553261; BD Biosciences—Pharmingen), and FACSCalibur flow cytometer or FACSAria-13 color Cell Sorter (BD) at 30 psi.

In vitro differentiation and immunocyto staining. Sorted cells were seeded onto collagen-1–coated coverslips (catalog no. 354089; VWR, BD Biosciences) in growth media (DMEM/F-12, 10% FBS, 5 μg/ml insulin, 1 μg/ml hydrocortisone, 5 ng/ml EGF, and penicillin/streptomycin) for 3 days. Differentiation was induced for 3 additional days in DMEM/F-12 containing 5 μg/ml insulin, 1 μg/ml hydrocortisone, 3 μg/ml prolactin, and penicillin/streptomycin. Cells were stained as described previously (58) using rabbit anti-SMA (1:200 dilution; catalog no. 600-531; Novus Biologiques), mouse anti-K18 (1:200 dilution; catalog no. RD1-PR061028; Fitzgerald), and rabbit anti-CX14 (1:200 dilution; catalog no. E6264; Panomics). Secondary antibodies were goat anti-rabbit Alexa Fluor 488 (green, 1:200 dilution; catalog no. A11008; Invitrogen) or goat anti-mouse Alexa Fluor 568 (red, 1:200 dilution; catalog no. A11004; Invitrogen). Nuclei were visualized with DAPI (catalog no. D9542; Sigma-Aldrich) and viewed under Zeiss Axiophot 2 fluorescence microscope.

**Tumor and mammosphere culture and transplantation.** Lin+ tumor cell suspensions were seeded onto ultra-low attachment plates (catalog no. 3471; Corning Costar) as described previously (58). For transplantation, primary tumorspheres were dissociated, resuspended in 10 μl media, mixed at a 1:1 ratio with 10 μl Matrigel (BD Bioscience) on ice, and injected into no. 4 mammary glands of immunocompromised Rag2−/− or NOD/SCID mice. Lin− mammary epithelial cells were seeded at 5,000 cells/well onto 96-well plates, and mammospheres were counted 2 weeks later.

**Retrovirus infection.** Plasmids pMXIE, pMXIE-WT-Rb, and pMXIE-RbΔK11 (provided by R. Bremner) as well as p-Help and p-CSVG were purified by endotoxin-free QIAGEN Maxi kit, transfected into Phoenix Eco packaging cells using TransIT-LT1 transfection reagent (product nos. MIR 2300, 2305, and 2306; Mirus Bio), and harvested after 40–68 hours. Rb/p53 tumor cells maintained in DMEM with 10% FBS were infected with viral supernatants at 1:10 dilution with 8 μg/ml polybrene. After 48 hours, cells were immunostained for Rb or sorted for GFP, and either replated in 96-well plates (500 cells/well) for MTT analysis or transplanted into mammary glands of NOD/SCID mice.

**Microarray analysis and array CGH analysis.** Tumors were snap-frozen in liquid nitrogen. RNA and DNA were purified from the same biopsies using RNaseasy and DNAeasy kits from Qiagen. DNA was labeled with Cy5 and used for microarray analysis as described previously (9). The dataset was deposited in the GEO database (http://www.ncbi.nlm.nih.gov/geo/; accession no. GSE14457). DNA was labeled according to the direct incorporation method of Agilent, hybridized to Agilent 244K CGH arrays (model G4415A), and scanned (model G2505B) at a resolution of 5 μm. Tail DNA from 4 Rb mutant female mice was used as reference.

**p53 sequencing.** Tumors 3 and 4 were sequenced from biopsies, tumor 18 from a derived cell line, and tumor 30 from both biopsy and a derived cell line. Primers were as follows: exons 2–4 reverse, 5′-CGACGATCCCAATCTCTTCTCTTACAG-3′; exons 2–4 reverse, 5′-CGGGAGTTCAAAAGGCTACAGGAAGACAC-3′; exons 5–6 forward, 5′-TAAGGATCCCTTTGTTGCCACTTGACCC-3′; exons 5–6 reverse, 5′-TTCGCAATTCTCTCAGGCTGAGTCACTGTCC-3′; exons 7–9 forward, 5′-TAAAGGATCCCTTTGTTGCCACTTGACCC-3′; exons 7–9 reverse, 5′-CCGCCCAATTCTCTTCAGGCTGAGTCACTGTCC-3′. PCR amplification reactions were purified on Qiagen PCR purification columns and subjected to high-quality capillary-based fluorescent sequencing on dual ABI 3730XL instruments at the Hospital for Sick Children TCAG Sequencing Facility. Identified mutations were verified by 2 additional PCR reactions/sequencing.

**Statistics.** Average across independent experiments is presented as mean of each experiment relative to control, which was set as 1 or 100%, and analyzed by 2-tailed Student’s t test (except 1-tailed analysis in Figure 3G). A P value of 0.05 or less was considered statistically significant.
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