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ORIGINAL ARTICLE

Selective roles of E2Fs for ErbB2- and Myc-mediated mammary tumorigenesis

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Previous studies have demonstrated that cyclin D1, an upstream regulator of the Rb/E2F pathway, is an essential component of the ErbB2/Ras (but not the Wnt/Myc) oncogenic pathway in the mammary epithelium. However, the role of specific E2fs for ErbB2/Ras-mediated mammary tumorigenesis remains unknown. Here, we show that in the majority of mouse and human primary mammary carcinomas with *ErbB2/HER2* overexpression, *E2f3a* is up-regulated, raising the possibility that E2F3a is a critical effector of the ErbB2 oncogenic signaling pathway in the mammary gland. We examined the consequence of ablating individual *E2fs* in mice on ErbB2-triggered mammary tumorigenesis in comparison to a comparable Myc-driven mammary tumor model. We found that loss of *E2f1* or *E2f3* led to a significant delay in tumor onset in both oncogenic models, whereas loss of *E2f2* accelerated mammary tumorigenesis driven by Myc-overexpression. Furthermore, southern blot analysis of final tumors derived from conditionally deleted $E2f3^{-/loxP}$ mammary glands revealed that there is a selection against $E2f3^{-/-}$ cells from developing mammary carcinomas, and that such selection pressure is higher in the presence of *ErbB2* activation than in the presence of *Myc* activation. Taken together, our data suggest oncogenic activities of E2F1 and E2F3 in ErbB2- or Myc-triggered mammary tumorigenesis, and a tumor suppressor role of E2F2 in Myc-mediated mammary tumorigenesis.

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INTRODUCTION

Uncontrolled cell growth is one of the hallmarks of cancer, and typically involves genetic or epigenetic alterations to genes that directly regulate the cell cycle and cellular proliferation.[1] Previous studies have led to the delineation of a pathway controlling the progression of cells out of quiescence, through G_1 and into S phase that involves the activation of cyclin-dependent kinases, phosphorylation and inactivation of the retinoblastoma tumor suppressor (*Rb*) and the subsequent release of E2F transcription factors.[2–4] It is now evident, from studies using both *in vitro* cell culture systems and *in vivo* mouse models, that the tumor suppressor function of Rb is largely mediated through its interaction with members of the E2F family and its regulation of E2F-dependent transcriptional activation or repression.[5,6]

The mammalian E2F family of transcription factors consists of eight known genes (E2F1-8) encoding nine E2F proteins, with the *E2f3* locus encoding two distinct isoforms, E2F3a and 3b.[7–10] Based on their structure and function, E2Fs can be divided into two broad groups. The first group, consisting of E2F1, E2F2 and E2F3a, is collectively called activators since their primary function is to activate genes that are required for entry of cells into S phase. The remaining E2Fs form the repressor group, whose primary function is to repress genes in quiescent or terminally differentiated cells. Early studies using mouse embryonic fibroblasts (MEFs) suggest that the E2F activator subclass is critical for normal cellular proliferation since overexpression of any of the three *E2f* activators is sufficient to induce quiescent cells to enter the cell cycle. Using MEFs lacking the entire E2F activator subclass, we previously showed that E2F activators are essential for normal cellular proliferation.[11] In addition, we also demonstrated that E2F1, E2F2 and E2F3 are required for aberrant cell growth under oncogenic insults since loss of the three E2Fs prevents *Myc* and *Ras* oncogene-induced cellular transformation in primary MEFs,[12] suggesting that E2F1-3 are also required for tumor initiation and/or progression *in vivo*.

Considering the central role of the Rb/E2F pathway in the control of cell cycle, it is not surprising to find genetic alterations in this pathway in essentially all human malignancies.[13] Moreover, targeted deletion of *Rb* in mice leads to hyperplasia and carcinomas,[14–18] further supporting an important role of Rb in tumor suppression. Alterations of E2F activators may also contribute to aberrant cell growth and cancer development through either overexpression/amplification or disruption of their association with Rb. Overexpression of *E2F1* is associated with several types of human cancers.[19,20] More recently, it has been found that *E2F3* is up-regulated in 67% of prostate cancers, and patients with *E2F3* overexpression have poorer overall survival and reduced cause-specific survival.[21] Consistent with an important

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role of E2F3 in human cancer development, *E2F3* is either up-regulated or amplified in several other cancer types.[22–27] In mice, forced expression of *E2f1*, *E2f2* or *E2f3* leads to hyperplasia or neoplasia.[28–32] However, loss-of-function studies in mice do not always support an oncogenic role of E2Fs. For example, deletion of *E2f3* in $Rb^{+/-}$ mice reduces the incidence of pituitary tumors but enhances the metastasis of thyroid tumors.[33] In addition, loss of *E2f2* accelerates Myc-induced lymphomagenesis while the role of *E2f1* in this process remains controversial.[34–36] Furthermore, inactivation of *E2f1* or *E2f2* enhanced Myc-induced skin tumorigenesis.[37,38] Nonetheless, collectively these data suggest that E2F activators not only play important roles in regulating normal cellular proliferation but also contribute to aberrant cell growth and cancer development.

Early studies using MEFs have established a functional link between the Myc or ErbB2/Ras pathway and the Rb/E2F pathway, as Myc or Ras elicits mitogenic signals that activate the cyclin/cyclin-dependent kinases complexes, leading to the release of E2F activities that promote cell growth.[39] In addition, in MEFs the ability of Myc to induce proliferation or apoptosis is dependent on specific E2F activities.[40] The importance of E2Fs in mediating Myc or Ras signaling is further highlighted by the recent finding that *E2f1-3* are essential for Myc/Ras-induced cellular transformation.[12] Finally, recent studies using *in vivo* mouse models demonstrated that E2f1 and E2f2 mediate Myc-induced lymphomogenesis as loss of *E2f1* delays Myc-induced T cell lymphomas,[34] whereas loss of *E2f2* accelerates Myc-induced T cell and B cell lymphomas.[35,36]

To understand the *in vivo* role of E2F1, E2F2 and E2F3 in regulating oncogene-induced mammary tumorigenesis, we sought to determine whether loss of *E2f1*, *E2f2* or *E2f3* in mice impacts mammary tumorigenesis triggered by the mammary epithelium-specific overexpression of *ErbB2/HER2/Neu* or *Myc*, two oncogenes that are over-xpressed in up to 30% of human breast cancer patients. Here, we show that deletion of *E2f1*, *E2f2* or *E2f3* have differential effects on the development of ErbB2- and Myc-induced mammary tumors, raising the possibility that E2Fs, particularly E2F3, are important downstream effectors in Myc- and ErbB2-mediated oncogenic signaling in mammary glands.

RESULTS

E2F activators are up-regulated in *ErbB2*-overexpressing human primary mammary carcinomas

Considering that ErbB2- or Ras-mediated mammary tumorigenesis in mice is strictly dependent on Cyclin D1,[41] which has been shown to activate *E2f* activators through phosphorylation and inactivation of *Rb*, we decided to assess whether E2F activators are relevant factors in human breast cancers. To this end, we collected a set of 12 human primary mammary carcinomas and measured mRNA transcript levels of *E2Fs* by qPCR. Mammary tissues from six tissue reduction patients with normal breast pathology were used as controls. Among the 12 mammary tumor samples, four had elevated levels (20–80-fold) of *HER2* transcripts (Figure 1a). Importantly, tumor samples with *HER2* overexpression also had much higher levels of *E2F* activators, *E2F1*, *E2F2* and *E2F3a* than both normal controls and tumor samples without *HER2* overexpression. Furthermore, there were marginal increases in *E2F3b* transcripts in tumor samples with *HER2* overexpression than in the controls, but substantially lower levels of *E2F3b* transcripts in tumor samples without *HER2* overexpression.

Upregulation of *E2fs* in ErbB2-induced and Myc-induced mouse mammary carcinomas

We next asked whether *E2f1*, *E2f2* and *E2f3a* are also preferentially up-regulated in mouse mammary carcinomas with *ErbB2* overexpression. To this end, we collected a set of 10 primary mammary carcinomas from *MMTV-ErbB2* transgenic mice that carry the *ErbB2* oncogene driven by the *MMTV* promoter,[42] and that had gone through two pregnancy/lactation cycles. We then used qPCR to evaluate expression levels of various *E2fs* compared to those of normal mammary glands from wild-type littermate control mice. In parallel, we also carried out the same analysis with a set of nine primary mammary carcinomas from *Wap-Myc* mice where the *Myc* oncogene is driven by the *Wap* (*Whey Acidic Protein*) promoter.[43] As shown in Figure 1b, while there was a moderate increase ($\sim$2–3-fold) of *E2f3a* and a marginal increase of *E2f2* in *Wap-Myc* tumors compared to normal controls, there was essentially no change in expression levels of *E2f1* or *E2f3b*. Importantly, in almost all *MMTV-ErbB2* tumors, there were substantial increases ($\sim$15-fold) in *E2f3a* expression, and moderate increases ($\sim$5-fold) in *E2f1* expression.

E2F3 is not required for normal mammary gland development

The data presented above suggest that E2F activators are important mediators of mammary carcinomas with *ErbB2* overexpression in both mice and humans. Since *E2f3a* exhibited the largest upregulation in those mammary carcinomas, we initially focused on understanding how inactivation of *E2f3* impacts ErbB2- or Myc-induced mammary carcinomas in mice. We first determined whether E2F3 is required for normal mouse mammary gland development by using a *Wap-Cre* transgene[44] to delete an *E2f3* conditional knockout allele ($E2f3^{loxP}$)[11] specifically in mammary epithelial cells. Consistent with previous reports,[44] X-gal staining on whole mount mammary glands from the $Rosa26^{+/LSL-lacZ}$ reporter mice[45] confirmed that *Cre* expression under the control of the *Wap* promoter is restricted to the mammary epithelium (Figure 2a). However, *Wap-Cre*-mediated recombination and subsequent activation of the *lacZ* gene was incomplete as evidenced by the partial, patchy blue staining in mammary epithelial ducts of day 16.5 pregnant females.

To determine whether *Wap-Cre*-mediated deletion of *E2f3* in mammary epithelial cells affects mammary gland development, we examined the mammary gland structures of monoparous female mice with either 16.5 days of pregnancy or one day of lactation. As shown in Figures 2b and c, carmine-stained whole mount mammary glands of $Wap\text{-}Cre;E2f3^{-/loxP}$ mice displayed similar lobuloalveolar structures and ductal branching patterns as those of control $E2f3^{-/loxP}$ mice. Furthermore, analysis of H&E-stained sections of mammary glands did not show obvious differences between $Wap\text{-}Cre;E2f3^{-/loxP}$ mice and control mice (Figures 2b and c). These data suggest that *Wap-Cre*-mediated deletion of *E2f3* in mammary epithelial cells did not affect the mammary gland development in both pregnant females and lactating females. However, the patchy staining shown in Figure 2a suggests incomplete deletion of *E2f3* in mammary epithelium of $Wap\text{-}Cre;E2f3^{-/loxP}$ mice. Therefore, our data do not completely rule out the possibility that E2F3 is required for the normal mammary gland development. However, considering that *E2f3* germline knockout female mice in a mixed genetic background are fertile and are capable of nursing pups, it is unlikely that E2F3 is required for normal mammary gland development. It is important to note that *E2f1* or *E2f2* knockout female mice are also fertile and can nurse their pups normally. Therefore, neither E2F1 nor E2F2 is likely required for normal mammary gland development.

Inactivation of *E2f1*, *E2f2* or *E2f3* alters Myc- and ErbB2-mediated mammary tumorigenesis

To understand the *in vivo* roles of individual E2Fs in Myc- or ErbB2-mediated mammary tumorigenesis, we determined whether inactivation of *E2f1*, *E2f2* or *E2f3* affects Myc- or ErbB2-induced mammary carcinomas. To this end, we interbred $E2f1^{+/-}$, $E2f2^{+/-}$ or $E2f3^{-/loxP}$ mice with one of the two parents containing either oncogenic allele to generate cohorts of mice

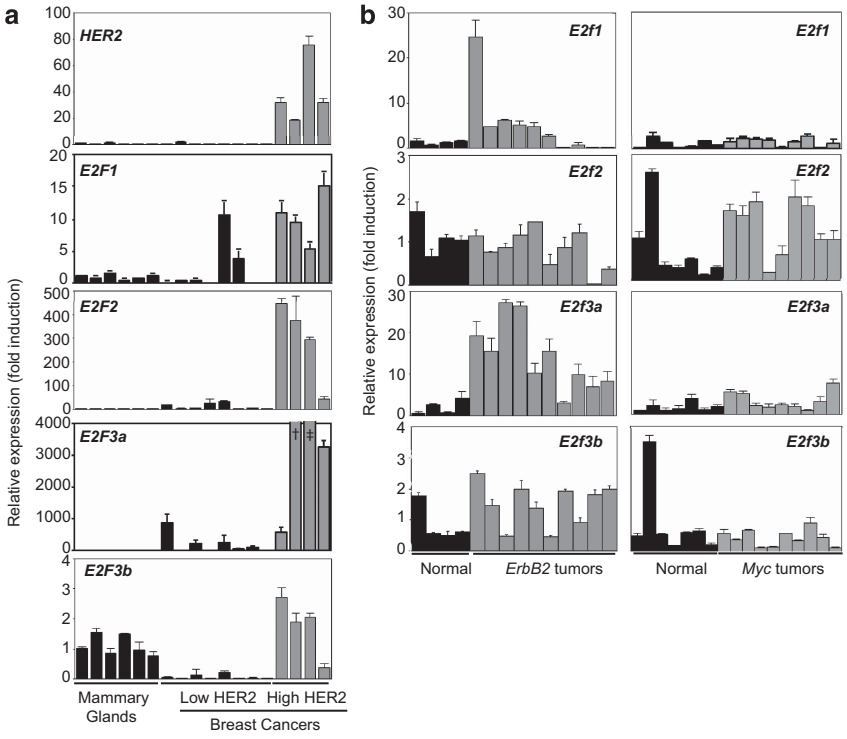


Figure 1. Expression levels of various *E2fs* in both mouse and human mammary tumors as well as mammary gland controls. (**a**) Real-time quantitative RT–PCR analysis of *E2F1*, *E2F2*, *E2F3a* and *E2F3b* for human breast tumors with high or low levels of *HER2* (*ErbB2*). Normal mammary glands from tissue reduction patients were used as controls. (**b**) Real-time quantitative RT–PCR analysis of *E2f1*, *E2f2*, *E2f3a* and *E2f3b* for mouse mammary tumors with *ErbB2* or *Myc* overexpression, compared to normal mammary gland controls. In all graphs, expression levels of *Gapdh* were used as a normalization control, and the average expression levels of the normal mammary gland control group were normalized to one. Each bar and error bar represent mean ± s.d. from triplicates except for Figure1a, *E2f3a* panel, mean ± s.d. = 92 041 ± 24 967 for † and = 22 879 ± 7986 for ‡.

overexpressing either oncogene and with or without *E2f1*, *E2f2* or *E2f3*. We monitored mammary tumor onset in all cohorts of mice by manual palpation bi-weekly, and evaluated tumor onset by using standard Kaplan–Meier tumor-free curves as well as t_{50}, or the number of days required for 50% of the mice to develop tumors. Tumor-free curves revealed no significant difference in tumor initiation among *E2f* wild-type (including $E2f3^{+/loxP}$ and $E2f3^{loxP/loxP}$) mice generated from intercrosses of $E2f1^{+/-}$, $E2f2^{+/-}$ or $E2f3^{-/loxP}$ mice overexpressing *ErbB2* or *Myc* ($P = 0.598$ and 0.117 for *ErbB2*- and *Myc*-overexpressing tumor mice, respectively). Therefore, for tumor initiation analyses we pooled all *E2f* wild-type mice (labeled as $+/+$ or $E2f^{+/+}$) overexpressing either oncogene. As shown in Figures 3a and b, genetic deletion of individual *E2f*s had various impacts on mammary carcinogenesis. Specifically, in mice with *ErbB2* overexpression, while deletion of *E2f2* had no significant impact on tumor initiation ($P = 0.261$), loss of *E2f1* or *E2f3* led to significant delay in tumor onsets ($P < 0.001$ in both cases), with t_{50} being increased by 34 days and 29 days, respectively. It is interesting to note that *MMTV-ErbB2;E2f1*$^{+/-}$ mice also had a significant delay in tumor onset compared to *MMTV-ErbB2* mice ($P < 0.001$), with t_{50} being increased by 14 days. Consistent with the important roles of E2F1 and E2F3 in mediating oncogene-triggered mammary tumorigenesis, loss of *E2f1* or *E2f3* also led to significant delays in tumor onset in mice with *Myc* overexpression ($P = 0.004$ and 0.003, respectively), with t_{50} being increased by 10 days and 21 days, respectively (Figures 3a and b). On the other hand, loss of *E2f2* in *Myc*-overexpressing mice led to a significant acceleration of tumor onset ($P < 0.001$), with t_{50} being reduced by 9 days. Consistent with specific roles of E2F1-3 on oncogene-triggered mammary tumorigenesis, *MMTV-ErbB2*; *Wap-Cre;E2f3*$^{-/loxP}$ mice had a significantly lower average number of tumors compared to *MMTV-ErbB2* mice ($P < 0.001$), and *Wap-Myc;E2f2*$^{-/-}$ mice had a significantly higher average number of tumors compared to *Wap-Myc* mice ($P < 0.01$) (Figure 3c). In addition, *MMTV-ErbB2;E2f1*$^{-/-}$ mice, *MMTV-ErbB2;E2f2*$^{-/-}$ mice and *Wap-Myc;Wap-Cre;E2f3*$^{-/loxP}$ mice all had moderate but statistically non-significant decreases in average numbers of tumors than control mice (Figure 3c).

To determine whether loss of various *E2fs* also affects tumor growth, we measured tumor volumes once a week for six consecutive weeks. As shown in Figure 3d, loss of individual *E2fs* did not affect the growth of *ErbB2*-overexpressing mammary tumors as tumors deficient in *E2f1*, *E2f2* or *E2f3* grew similarly to those with wild-type *E2fs*. Interestingly, although loss of *E2f1* or *E2f2* did not affect tumor growth in mice with *Myc*-overexpression either, tumors from *Wap-Myc;Wap-Cre;E2f3*$^{-/loxP}$ mice grew significantly slower than those from *Wap-Myc* mice ($P < 0.001$) (Figure 3d).

Most mammary tumors from *Wap-Cre;E2f3*$^{-/loxP}$ mice retained the $E2f3^{loxP}$ allele

As shown in Figures 3a and b, although there was only a marginal difference on tumor initiation between *MMTV-ErbB2;Wap-Cre;E2f3*$^{+/loxP}$ mice and the control mice (Figure 4a, $P = 0.047$; t_{50} of 177 days vs 171 days), there was a significant delay in tumor onset in *MMTV-ErbB2;E2f3*$^{-/loxP}$mice than in the control mice (Figure 4a, $P < 0.001$; t_{50} of 190 days vs 171 days). One potential explanation on the difference of tumor onset between the two types of *E2f3* heterozygous mice (*MMTV-ErbB2;Wap-Cre;E2f3*$^{+/loxP}$ mice vs *MMTV-ErbB2;E2f3*$^{-/loxP}$ mice) is that while all cells in *MMTV-ErbB2;E2f3*$^{-/loxP}$ mice were heterozygous for *E2f3*, a significant number of mammary epithelial cells in *MMTV-ErbB2;Wap-Cre;E2f3*$^{+/loxP}$ tumor mice retained the functional

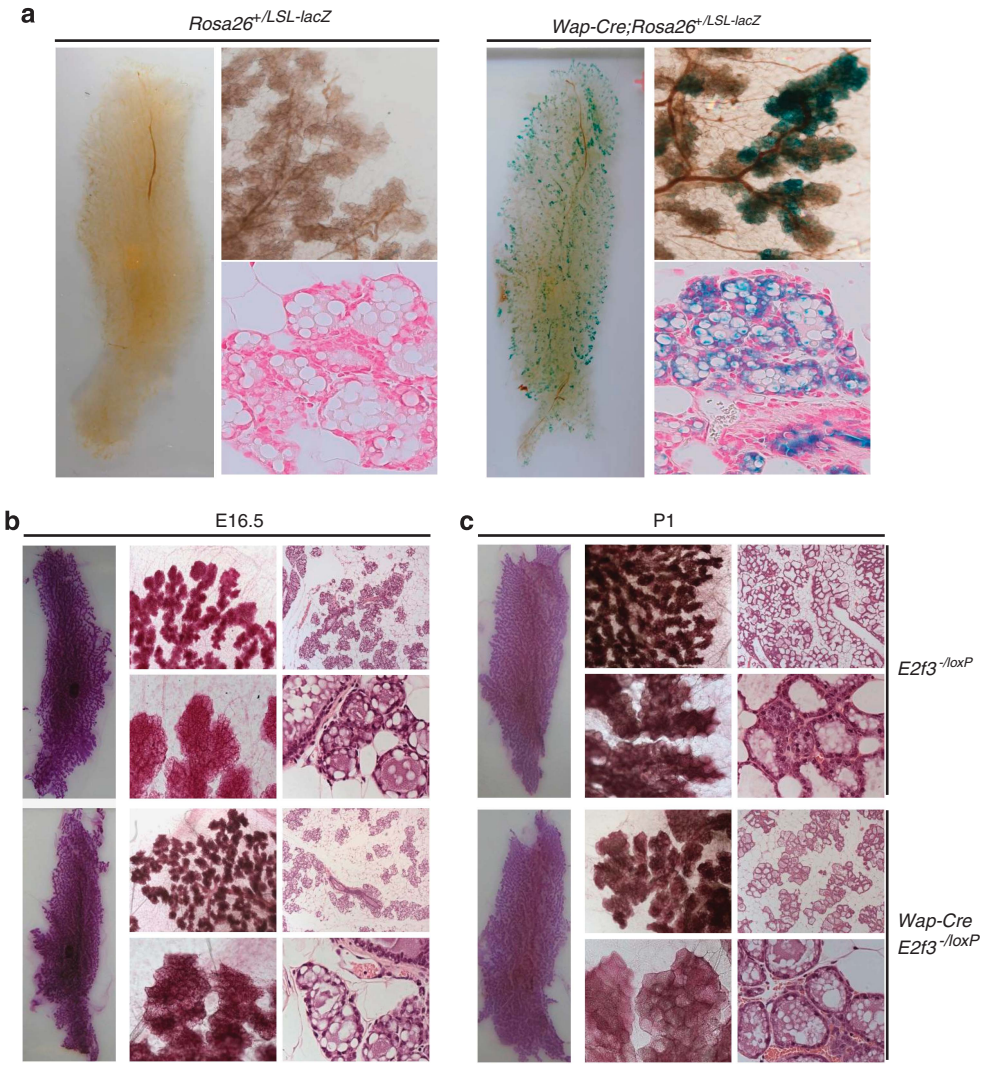


Figure 2. Mammary gland development of wild-type mice and mice deficient in *E2f3*. (**a**) X-gal staining of mammary glands from 16.5 days of pregnant mice with the indicated genotypes. For each genotype, left panel: whole mount; top right panel: higher magnification (5X) of the whole mounts; bottom right panel: paraffin-embedded sections counter-stained with eosin (40X). (**b** and **c**) Mammary gland morphology of 16.5 days of pregnant (**b**) or 1 day of lactating (**c**) mice with indicated genotypes. For each genotype, left panel: whole mount Carmine staining; middle panel: high magnification of whole mounts (top: 2.5X; bottom: 10X); right panel: Hemotoxylin- and eosin-stained paraffin embedded sections (top: 5X; bottom: 40X).

$E2f3^{loxP}$ allele due to incomplete deletion of the $E2f3^{loxP}$ allele as indicated in Figure 2a. In this case, we would expect that many tumors derived from *MMTV-ErbB2;Wap-Cre;*$E2f3^{-/loxP}$ mice and *Wap-Myc;Wap-Cre;*$E2f3^{-/loxP}$ mice were actually not deleted for *E2f3*. To test these possibilities, we first assessed the percentage of tumors derived from *Wap-Cre* mice with *Myc* or *ErbB2* overexpression and with sufficient Wap-Cre-mediated recombination that most likely has no impact on tumorigenesis. To this end, we generated cohorts of *MMTV-ErbB2;Wap-Cre;*$Rosa26^{+/LSL-lacZ}$ mice and *Wap-Myc;Wap-Cre;*$Rosa26^{+/LSL-lacZ}$ mice. We allowed these mice to go through two rounds of pregnancies/lactations, and analyzed tumors derived from those mice by whole mount X-gal staining. We quantified the numbers of 'blue' tumors and 'white' tumors and estimated that the deletion efficiency in *MMTV-ErbB2;Wap-Cre;*$Rosa26^{+/LSL-lacZ}$ mice and *Wap-Myc;Wap-Cre;*$Rosa26^{+/LSL-lacZ}$ mice were 42.1% and 44%, respectively (Figure 4d), suggesting that *Wap-Cre* was sufficiently expressed in about 40% of the mammary epithelial cells with either oncogene overexpression. These data also suggest that the majority of tumors from *MMTV-ErbB2;Wap-Cre;*$E2f3^{-/loxP}$ mice and *Wap-Myc;Wap-Cre;*$E2f3^{-/loxP}$ mice did not sufficiently express *Cre*, thereby retaining the functional $E2f3^{loxP}$ allele.

To determine the percentage of tumors that retained the $E2f3^{loxP}$ allele, we used southern blot to analyze tumors from *MMTV-ErbB2;Wap-Cre;*$E2f3^{-/loxP}$ mice and *Wap-Myc;Wap-Cre;*$E2f3^{-/loxP}$ mice. As shown in Figures 4c and d, in tumors derived from *MMTV-ErbB2;Wap-Cre;*$E2f3^{+/loxP}$ mice and *Wap-Myc;Wap-Cre;*$E2f3^{+/loxP}$ mice, the deletion efficiencies of the $E2f3^{loxP}$ allele were 31.6% and 32.5%, respectively. These numbers are about 25% lower than the deletion efficiencies of the stop cassette in the $Rosa26^{LSL-lacZ}$ reporter allele in similar settings (Figure 4d). More importantly, *E2f3* deletion efficiencies were further reduced to 22.5% in tumors derived from *Wap-Myc;Wap-Cre;*$E2f3^{-/loxP}$ mice, and to 12.1% in tumors derived from *MMTV-ErbB2;Wap-Cre;*$E2Ff3^{-/loxP}$ mice (Figure 4d). Taken together, these data strongly suggest that there is a selection pressure against $E2f3^{-/-}$ mammary epithelial cells to form tumors in both oncogenic models, and such pressure is even higher in tumors with *ErbB2* overexpression than in those with *Myc* overexpression. Considering that the majority of tumors (~88% for *MMTV-ErbB2;*

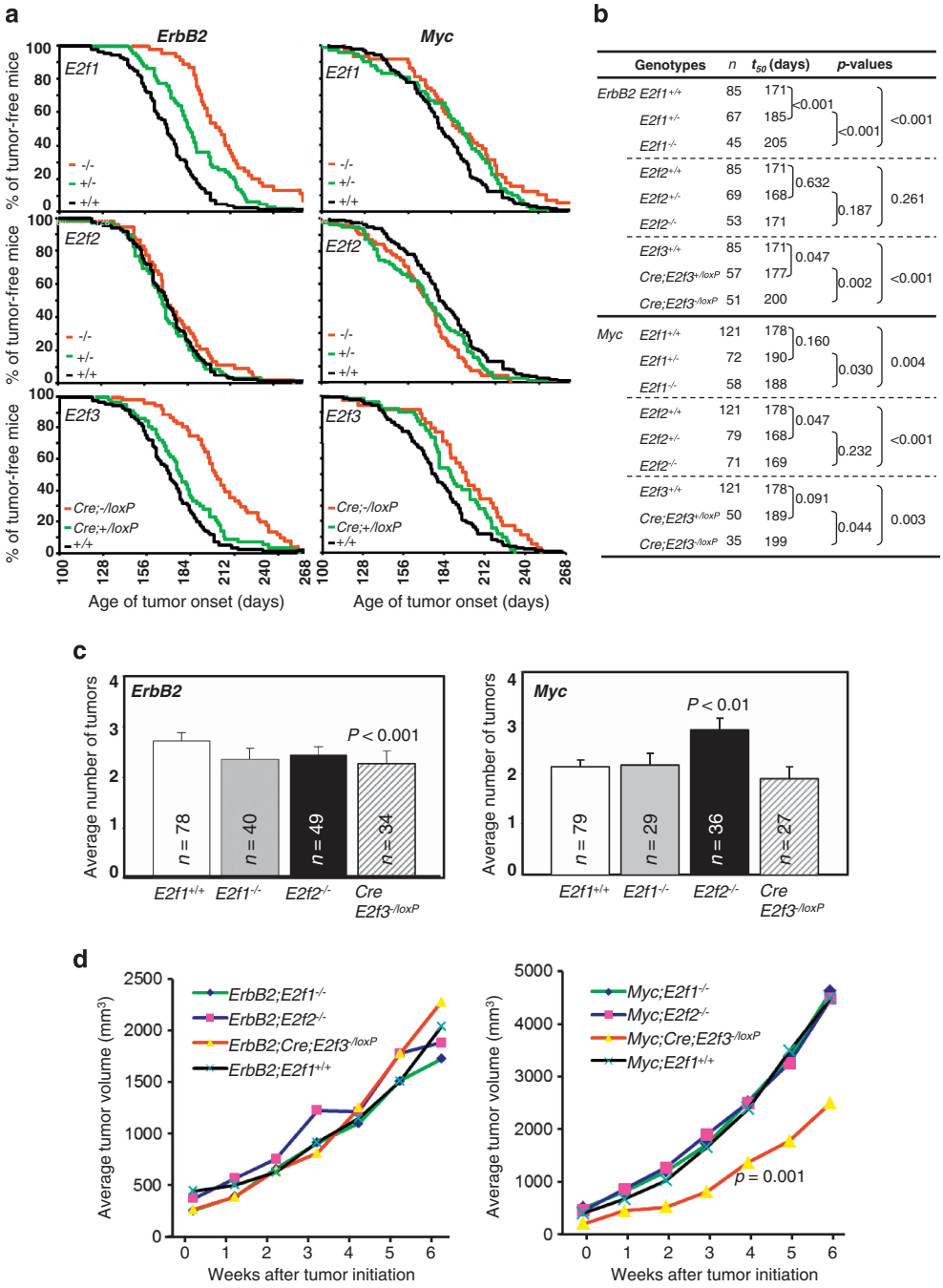
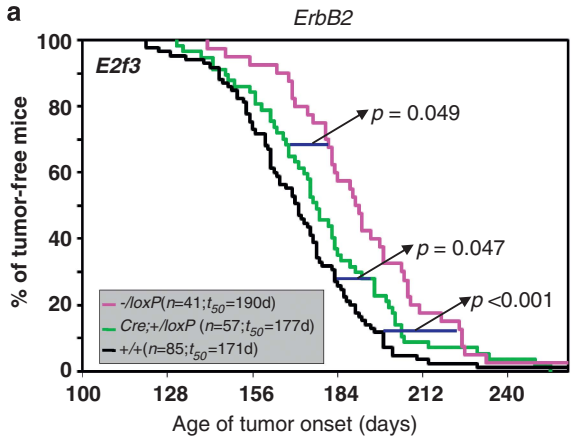


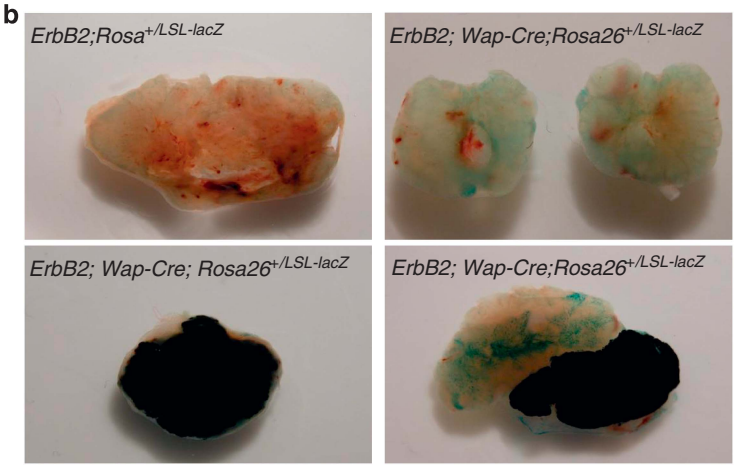
Figure 3. Effects of loss of *E2f1*, *E2f2* or *E2f3* on the development of mammary tumors induced by the overexpression of *ErbB2* or *Myc*. (**a**) Kaplan–Meier tumor-free curves of mice with indicated genotypes. (**b**) Statistical analysis of tumor incidence among mice with various genotypes. *n*: number of mice analyzed; t_{50}: the number of days required for 50% of the mice to develop mammary tumors; *Cre*: *Wap-Cre*. (**c**) Average numbers of tumors per mouse at six weeks after the first tumor was detected. *n*: number of mice analyzed. *p*: significant *P*-values between the indicated genotypic group and *E2f* wild-type group. (**d**) Average tumor volume per tumor throughout the six weeks after the first tumor was detected. Numbers of mice analyzed are identical to those in **c**. *p*: significant *P*-values between the indicated genotypic group and *E2f* wild-type group.

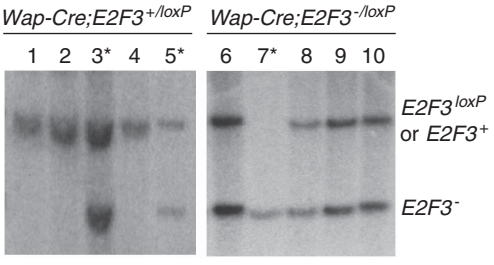
Wap-Cre;E2f3$^{+/loxP}$ mice and ~77% for *Wap-Myc;Wap-Cre;E2f3*$^{+/loxP}$ mice) are not deleted for *E2f3*, the delay in tumor onset in those mice depicted in Figure 3a was most likely substantially underestimated.

Deletion of *E2f3* leads to a marginal reduction of proliferation in pre-tumor state mammary epithelial cells

The tumor-free curves presented in Figure 3a, together with the qPCR data showing that *E2f1* and *E2f3a* are preferentially up-regulated in mammary carcinomas with *ErbB2* or *Myc* overexpression, raise the possibility that E2F1 and E2F3 are critical downstream effectors of the ErbB2-Ras-Cyclin D1 oncogenic signaling axis in the mammary epithelium. Considering that E2Fs are intimately involved in controlling cellular proliferation,[11,39,46] a biological process that often becomes aberrant during tumorigenesis, we investigated whether loss of individual *E2f*s affects cellular proliferation of mammary epithelium with oncogene-expression. Since loss of *E2f1* and *E2f3* led to the







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Genotypes	N*	%**
Myc;Rosa26$^{+/LSL\text{-}lacZ}$	25	44.0
Myc;E2F3$^{+/loxP}$	40	32.5
Myc;E2F3$^{-/loxP}$	71	**22.5**
ErbB2;Rosa26$^{+/LSL\text{-}lacZ}$	330	42.1
ErbB2;E2F3$^{+/loxP}$	76	31.6
ErbB2;E2F3$^{-/loxP}$	66	**12.1**

* Numbers of tumors quantified.
**: Percentage of recombination.

Figure 4. Selection against *E2f3*$^{-/-}$ cells from developing mammary carcinomas. (**a**) Kaplan–Meier tumor-free curves of mice with indicated genotypes. *n*: number of mice analyzed; t_{50}: the number of days (d) required for 50% of the mice to develop mammary tumors; *Cre*: *Wap-Cre*; *p*: *P*-values between indicated genotypic groups. (**b**) Whole mount X-gal staining of mammary tumors from mice with indicated genotypes. Under this system, a complete 'blue' tumor (bottom left) would indicate that in tumor cells *Wap-Cre*-mediated deletion of the stop cassette activates the *lacZ* transgene, a 'white' tumor (top right) with similar staining as a control tumor (top left) would indicate that *Wap-Cre* activity is not sufficient to delete the stop cassette and a mixed tumor (bottom right) would indicate tumors consisting of both *Wap-Cre* active cells and *Wap-Cre* inactive cells. (**c**) Southern blot analysis of individual mammary tumors derived from mice with indicated genotypes using a probe specific for the *E2f3* locus. *: tumors with sufficient *Cre* expression in mammary epithelial cells to convert the *E2f3*^{loxP} allele into an *E2f3* knockout allele. (**d**) Percentage (%) of *Cre*-mediated recombination for *Rosa26*$^{+/LSL-lacZ}$ allele and *E2f3*^{loxP} allele estimated by X-gal staining (**b**) and southern blot analysis (**c**), respectively.

most significant delays in tumor onset in *MMTV-ErbB2* mice, we focused on determining whether loss of *E2f1* or *E2f3* in pre-tumor state *MMTV-ErbB2* mice affects proliferation of mammary epithelial cells from mice that have gone through two pregnancy/lactation cycles. As shown in Figure 5a, while the percentages of Ki67-positive cells were very similar between *MMTV-ErbB2* mice and *MMTV-ErbB2;E2f1*$^{-/-}$ mice, there was a marginally significant decrease in percentages of Ki67-positive cells in *MMTV-ErbB2;Wap-Cre;E2f3*$^{-/loxP}$ mice than in ErbB2 mice (3.6% vs 6.5%; $P = 0.045$). Importantly, there was no significant difference in percentages of Ki67-positive cells between tumors with wild-type *E2fs* and those deficient in *E2f1*, *E2f2* or *E2f3* (Figures 5b and c). The fact that *E2f3*$^{-/-}$ tumors had similar numbers of Ki67-positive cells as observed in the control or *E2f3* heterozygous tumors suggests that either *E2f3* is dispensable for ErbB2- and Myc-induced tumor cells to proliferate or *E2f3*$^{-/-}$ tumor cells bypass the requirement of *E2f3* for maintaining normal levels of proliferation through compensational mutations.

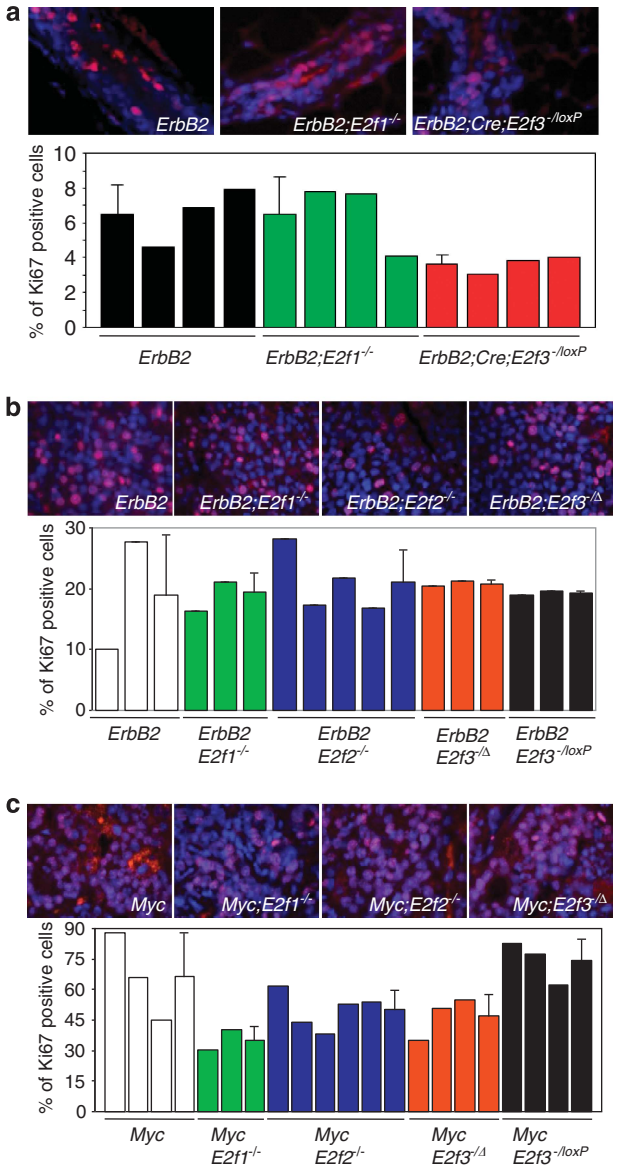

Figure 5. Proliferation status of mammary glands and mammary tumors. Immunofluorescence staining of mammary glands (**a**) or mammary tumors (**b** and **c**) from mice with indicated genotypes with Ki67-specific antibodies (red), counter-stained with DAPI (blue). Graphs with error bars show average percentage of Ki67-positive cells for all samples from respective genotypic groups. *E2f3*$^{-/\Delta}$: tumors from *E2f3*$^{-/loxP}$ mice with *Cre*-mediated *E2f3* deletion being confirmed by southern blot analysis as shown in Figure 4c.

Characterization of mammary tumors deficient in various *E2fs*

To investigate whether deletion of various *E2fs* affects the biology of mammary tumors with either oncogene overexpression, we first compared the histology of tumors deficient in *E2f1*, *E2f2* or *E2f3* to those with wild-type *E2fs*. As reported before, mouse mammary carcinomas resulted from *ErbB2* overexpression were solid tumors, while tumors resulted from *Myc* overexpression were more glandular (Figure 6a). However, careful examination of H&E-stained sections of primary tumors revealed no major microscopic changes in tumors deficient in various *E2fs* (Figure 6a and data not shown), suggesting that deletion of individual *E2fs* did not change the biological behavior of the mammary tumors.

Previous studies demonstrated substantial functional redundancies among various E2F activators[11] or repressors[47] for cellular proliferation and/or mouse development. To determine whether mammary tumors deficient in individual *E2fs* experience any compensational upregulation of other members of *E2f* activators, we used qPCR to measure expression levels of various *E2f* genes in tumors deficient in *E2f1*, *E2f2* or *E2f3*. As shown in Figure 6b, there was no obvious compensational up-regulation of any *E2f* activators in tumors deficient in *E2f1*, *E2f2*, or *E2f3* compared to tumors with wild-type *E2fs*, suggesting that mammary tumors in mice deficient in individual *E2fs* arose without compensational up-regulation of other *E2f* activators. Interestingly, de-regulation of some repressor *E2fs* was observed in tumors lacking certain E2Fs (Supplementary Figure 1).

We previously showed that E2F1-3 control E2F target gene activation and cellular proliferation through a p53-dependent negative feedback loop.[48] To determine whether mammary tumors deficient in various *E2fs* contain *p53* mutations, we sequenced the coding region of the *p53* gene from *E2f3*$^{-/\Delta}$ tumors with *Myc* or *ErbB2* overexpression compared to tumors with wild-type or floxed *E2f3* alleles. No mutation was found in *E2f3*$^{-/\Delta}$ tumors compared to control tumors, suggesting that the *p53* locus is most likely unaltered in mammary tumors deficient in *E2f3*.

DISCUSSION

Previous studies have demonstrated that cyclin D1 is an essential component of the ErbB2/Ras (but not the Wnt/Myc) oncogenic pathway in the mouse mammary epithelium.[41] However, the role of specific E2Fs in ErbB2/Ras- or Wnt/Myc-mediated mammary tumorigenesis remains largely unknown. In this study, we found that *E2f3a* is up-regulated in the majority of mouse and human primary mammary carcinomas with *ErbB2* or *Myc* overexpression (Figure 1), raising the possibility that E2F3a is a critical downstream effector of the ErbB2- or Myc-oncogenic pathway. Indeed, loss of *E2f3* (and *E2f1* to a lesser extent) leads to significant delays in mammary tumor onset in both oncogenic models (Figures 3a and b). Importantly, southern blot analysis of the final tumors revealed a strong selection against *E2f3*$^{-/-}$ cells from developing mammary tumors as the majority of the tumors ($\sim$77% for Myc mice and $\sim$88% for ErbB2 mice) retained a functional *E2f3*^{loxP} allele (Figure 4); Wap-Cre-mediated deletion efficiency for a *Rosa26-LSL-lacZ* reporter allele is about 40% in both oncogenic models, whereas the *E2f3* deletion efficiency is reduced to $\sim$23% in tumors with *Myc*-overexpression, and is further reduced to about 12% in tumors with *ErbB2* overexpression (Figure 4). Taken together, these data strongly suggest that E2F3 is a critical mediator for the ErbB2- and Myc-triggered mammary carcinogenesis. Since the *E2f3* locus encodes E2F3a and E2F3b isoforms and since the *E2f3* knockout mouse line used in the present study lacks both isoforms, it remains to be determined whether E2F3a or E2F3b or both play important roles in mediating ErbB2- or Myc-triggered mammary tumorigenesis. Considering that E2F3a is believed to be an activator E2F, whereas E2F3b a repressor E2f, and that *E2f3a* is up-regulated in the majority of mouse and human primary mammary carcinomas with *ErbB2* or *Myc* overexpression, we postulate that E2F3a is the primary isoform in modulating oncogene-induced mammary tumorigenesis. It is interesting to note that loss of *E2f2* in mice with *Myc* overexpression accelerates mammary tumor development, consistent with its tumor suppressor role that was recently demonstrated in Myc-induced T-cell lymphomagenesis[35] and epithelial tumorigenesis.[37] The fact that deletion of *E2f2* in mice accelerates Myc-induced mammary tumorigenesis but does not affect ErbB2-mediated mammary tumorigenesis suggests that the tumor suppression role of the *E2f2* locus is most likely dependent on *Myc* oncogene activation.

It has been well documented that E2F activators are critical for normal cellular proliferation.[11,46] Several recent studies using *in vivo* mouse models also implicated E2F activators in mediating

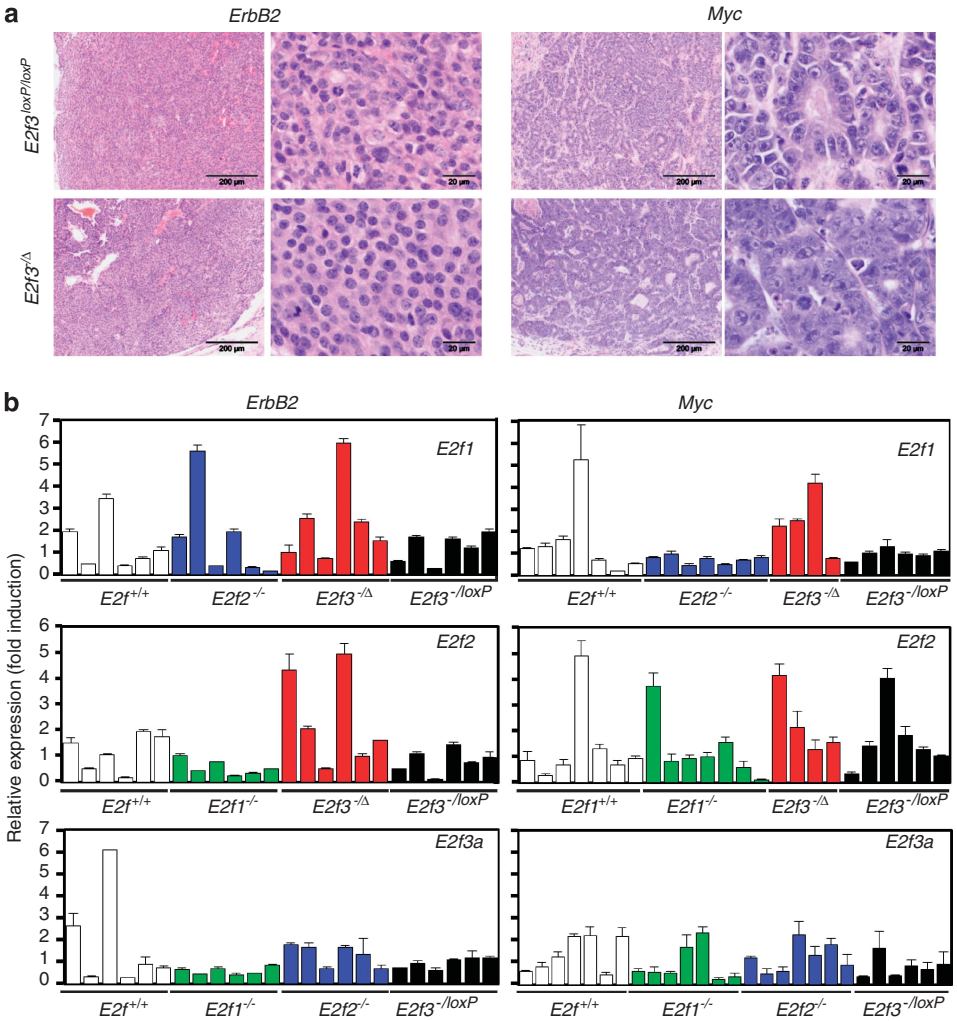

Figure 6. Characterization of final mammary tumors. (**a**) Hematoxylin- and eosin-staining of paraffin-embedded sections of mammary tumors with indicated genotypes. *E2f3* $^{-/\Delta}$: tumors from *E2f3* $^{-/loxP}$ mice with Cre-mediated *E2f3* deletion being confirmed by southern blot analysis as shown in Figure 4c. (**b**) Real-time quantitative RT–PCR analysis of various *E2f* genes for mouse mammary tumors with indicated genotypes. In all graphs, expression levels of *Gapdh* were used as a normalization control, and the average expression levels of tumors derived from mice with wild-type *E2f* were normalized to one. Only statistically significant *P*-values were shown.

oncogene-induced tumorigenesis. For example, while loss of *E2f1* impairs the ability of Myc to induce T cell lymphomas,[34] loss of *E2f2* accelerates Myc-induced lymphomagenesis.[35,36] In the present study, we showed that E2F activators are important downstream effectors in mediating ErbB2- and Myc-induced mammary tumorigenesis, since loss of *E2f1* or *E2f3* led to significant delays in tumor onset in both oncogenic models, and loss of *E2f2* accelerated tumor onset in the *Wap-Myc* model. While our data supports a tumor suppressor role of *E2f2* in the context of *Myc* activation that was recently postulated in several mouse tumor models,[35–37] it is seemingly inconsistent with a recent report suggesting a tumor promoting role of *E2f2* in a *MMTV-Myc*-driven mammary tumor model.[49] In addition, in the *MMTV-Myc*-driven mammary tumor model, E2F1 seems to play a tumor suppressor role as loss of *E2f1* accelerated mammary tumorigenesis.[49] These discrepancies may be explained by different promoters that drive the expression of the *Myc* oncogene (i.e., *MMTV* vs *Wap*), different physiological states of the mice (i.e., non-parous mice vs mice undergoing two rounds of pregnancies), and different genetic backgrounds. It is important to note that despite their differences, both the *Wap-Myc* model and the *MMTV-Myc* model support a tumor promoting role of E2F3. These data are consistent with recent findings of elevated levels of *E2F3* in various human cancers.[21–25] The emerging evidence from both *in vivo* mouse models and human clinical samples raises the possibility that E2F3 or its downstream targets are potential candidates for targeted therapies of cancer.

Breast cancer represents the highest estimated new cancer cases and the second leading cause of cancer mortality in woman in the United States.[50] Unfortunately, breast cancer therapy has been hampered by limited knowledge on the molecular bases of the disease. Although activation/overexpression of *ERBB2* or *MYC* has been found in up to 30% of breast cancer patients, the critical effector(s) induced by either oncogene remains unknown. In the present studies we identified E2F3 (and E2F1 to a lesser extent) as an important downstream effectors in mediating ErbB2- and Myc-triggered mammary tumorigenesis, and E2F2 as a tumor suppressor in mediating Myc-triggered mammary tumorigenesis. Future studies to delineate the mechanistic link between E2Fs and the *Myc* and *ErbB2* oncogenes in mouse mammary tumor development would allow us to link pathway deregulation with potential therapeutic strategies for breast cancer. Such studies may provide critical insights in developing customized molecular therapies and/or combinatorial therapies of cancers based on individual patients' defined genetic alterations of particular signaling pathways or molecules.

MATERIALS AND METHODS

Mice and PCR-based genotyping

MMTV-Neu mice[42] and *Wap-Myc* mice[43] were obtained on a FVB background, whereas *Wap-Cre*, *Rosa26-LSL-lacZ*, *E2f1*$^{-/-}$, *E2f2*$^{-/-}$ and *E2f3*$^{-/loxP}$ mice were backcrossed to a FVB background for five or six generations to minimize the heterogeneity arose from mixed genetic backgrounds. All mice for tumor studies had gone through two pregnancy/lactation cycles to maximize the *Wap-Cre* expression. Genotypes of mice were determined by standard PCR of genomic DNA isolated from mouse tails. All mice were maintained in a barrier facility in accordance with standards established by the Institutional Animal Care and Use Committee at the Ohio State University. Mice at end points were killed by CO_2 asphyxiation. Mammary glands or mammary tumors were either snap-frozen for molecular analysis, or fixed in 10% formalin for histological and immunocytochemical analyses.

Whole mounts, histology and immunofluorescence staining

Mammary glands number 4 or number 9 were dissected and mounted onto glass slides. After being fixed at 4 °C overnight in a Carnoy's fixative, the whole mount glands were stained overnight with carmine following a standard protocol. For histological analysis, 5 μm paraffin-embedded sections were stained with hematoxylin and eosin (H&E) (Sigma-Aldrich, St Louis, MO, USA). To assess *lacZ* expression patterns, we stained whole mount glands/tumors or 5 μm paraffin-embedded sections with X-gal using standard X-gal staining protocols. For immunofluorescence staining, 5 μm sections were de-paraffinized in xylene, sequentially rehydrated and rinsed in de-ionized water. Antigen retrieval was performed using a DAKO Target Retrieval solution. Slides were incubated with primary antibodies against Ki67 (BD Biosciences, San Jose, CA, USA, 1:100 dilution) and a fluorescence-conjugated secondary antibody (Alexa 488 from Molecular Probes, Eugene, OR, USA, 1:500 dilution) and counterstained with DAPI.

Southern blot analysis

Genomic DNA isolated from mammary tumors was digested with *EcoRV*. Southern blot was carried out as previously described.[11]

Real-time quantitative RT–PCR (qPCR)

Frozen mammary tissues were used for total RNA isolation using TRIzol (Life Technologies, Grand Island, NY, USA). Human patient samples were obtained and processed in accordance with the Institutional Review Board approval at the Ohio State University. Reverse transcription of 2–5 μg of total RNA was performed to generate cDNA using Superscript III reverse transcriptase (Invitrogen, Grand Island, NY, USA). qPCR was carried out in triplicates using a SYBR-Green-based system (BioRad, Hercules, CA, USA) and a BioRad iCycler. Data were analyzed using the ΔCt method, and were normalized by the expression levels of *Gapdh*.

Statistical analysis

Pairwise comparisons on Kaplan–Meier tumor-free curves were performed using the Log-rank test. For each comparison, we used a Bonferonni-adjusted alpha level to determine statistical significance. To assess tumor growth, we used an electronic caliper to measure palpable tumors once a week for six weeks after tumor onset. Tumor volumes were estimated by calculating $0.4 \times L \times S^2$ where L was the longest dimension and S was the shortest dimension. Tumor growth was analyzed by two way analysis of variance (ANOVA) for repeated measures over the course of the 6-week measurements and then by unpaired *t*-test to compare values at six weeks. The average number of palpable tumors was determined at six weeks following the onset of the first palpable tumor of each mouse and was tested for statistical significance by unpaired *t*-test. All other pairwise statistical analyses were done using unpaired *t*-test.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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