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Award Number: W81XWH-09-1-0073

TITLE:

HER2 Oncogene-Induced DNA Damage Response as a Barrier that must be overcome to form breast tumors in normal mammary epithelium

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REPORT DATE:

March 2010

TYPE OF REPORT: annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE (DD-MM-YYYY) 01-03-2010		2. REPORT TYPE ANNUAL		3. DATES COVERED (From - To) 1 Mar 2009 - 28 Feb 2010	
4. TITLE AND SUBTITLE HER2 Oncogene-Induced DNA Damage Response as a Barrier that must be overcome to form breast tumors in normal mammary epithelium				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-09-1-0073	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Yi Li				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Baylor College of Medicine Houston, TX 77030				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT approved for public release; distribution unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT Activated ErbB2 stimulates mammary cell proliferation in culture and in mice; yet it is not sufficient by itself to cause mammary tumors when tested in mice. Oncogene-induced over-proliferation (but not high proliferation associated with normal development) has been reported to induce a DNA damage response (DDR), leading to apoptosis and senescence, but it is not known whether this response may be what prevents breast cancer initiation in cells that have activated ErbB2 or other common breast cancer genes. We discovered that DDR signaling was strongly induced in preneoplastic lesions arising from individual mammary cells transduced in vivo by retroviruses expressing either PyMT or ErbB2. Thus, activation of an oncogene after normal tissue development causes a DDR. Furthermore, in this somatic ErbB2 tumor model, ATM, and thus DDR, is required for p53 stabilization, apoptosis, and senescence. In palpable tumors in this model, p53 stabilization and apoptosis are lost, but unexpectedly senescence remains in many tumor cells. Thus, ErbB2-induced aberrant mammary cell proliferation leads to an ATM-mediated DDR that activates apoptosis and senescence, and at least the former must be overcome to progress to malignancy.					
15. SUBJECT TERMS Breast cancer, initiation, DDR, mouse model, ErbB2					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 18	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

Table of Contents

	<u>Page</u>
Introduction.....	1
Body.....	1
Key Research Accomplishments.....	3
Reportable Outcomes.....	3
Conclusion.....	3
References.....	4
Appendices.....	4

BC085050: HER2 Oncogene-Induced DNA Damage Response as a Barrier that Must Be Overcome to Form Breast Tumors in Normal Mammary Epithelium

Yi Li, Ph.D.

INTRODUCTION

Activated ErbB2 (Neu/HER2) stimulates mammary cell proliferation in culture and in mice; yet it is not sufficient by itself to cause mammary tumors when tested in mice. Oncogene-induced over-proliferation (but not high proliferation associated with normal development) has been reported to induce a DNA damage response (DDR), leading to apoptosis and senescence, but it is not known whether this response may be what prevents breast cancer initiation in cells that have activated ErbB2 or other common breast cancer genes. Although four conventional transgenic oncogene models we examined failed to induce a DDR, PyMT introduced using our novel mouse model into isolated somatic mammary cells did induce a robust response. Since ErbB2 activates many of the same oncogenic pathways as PyMT, we hypothesize that ErbB2 can induce a DDR, leading to induction of p53, apoptosis, and senescence. This DDR activation blocks cancer initiation by ErbB2, so that secondary genetic/epigenetic alterations that compromise either the DDR itself or DDR effectors must be gained in order for ErbB2-activated cells to evolve into tumors.

BODY

Task 1. Demonstrate that ErbB2 induces a DDR in a somatic model of breast cancer, and that ablation of the DDR pathway greatly accelerates tumor induction by ErbB2. Months 1-24

1) Inject 30 6-week-old MMTV-tva transgenic mice with RCAS-ErbB2, harvest mammary glands after 2 (n=5) and 4 (n=5) weeks, examine the early precancerous lesions for γ H2AX and 53BP1, and perform TUNEL and SA β gal staining. Monitor the remaining 20 mice for tumors. Test these tumors for these markers as well. In addition, perform Western blotting on these tumors for phosphorylated proteins in the DDR pathway.

We have found that DDR markers, p53, apoptosis, and senescence are upregulated in early lesions. In tumors, p53 and apoptosis are down-regulated, but DDR markers and senescence remain. The results are now published as part of the attached manuscript (Reddy et al (2010) Proc Natl Acad Sci U S A 107: 3728-33).

2) Intraductally inject RCAS-ErbB2 virus into both MMTV-tva and ATM-/-MMTV-tva mice (n=35 for each group, age: 6 weeks). Stain early lesions from 5 mice for lack of DDR markers. Monitor the rest for tumors.

DDR markers, p53, apoptosis, and senescence are all downregulated in early lesions arising in ATM-null mice compared to the early lesions arising in intact mice. The results from this early lesion comparison are now published as part of the attached manuscript (Reddy et al (2010) Proc Natl Acad Sci U S A 107: 3728-33). The actual tumor latency is difficult and has been placed on hold: For the comparison to work, the infection rate has to be similar in the two lines of tva mice. However, it is very difficult to inject the ATM-null mice.

3) Intraductally inject RCAS-ErbB2 virus into both MMTV-tva and ARF-/-MMTV-tva mice (n=35 for each group, age: 6 weeks). Stain early lesions from 5 mice for DDR markers. Monitor the rest for tumors.

ARF knockout mice have been purchased and we are expanding the colony.

Task 2. Determine how a DDR in ErbB2-mutated breast cells is overcome in the progression to cancer. Months 12-36

1) Test whether p53 is downregulated at transcription or post-translational levels, using Q-PCR.

We have found that the p53 mRNA is made in tumors. The results are now published as part of the attached manuscript (Reddy et al (2010) Proc Natl Acad Sci U S A 107: 3728-33).

2) If p53 mRNA is absent, examine the p53 gene locus for evidence of deletion or mutation by Southern blotting and by direct sequencing. We will test for epigenetic silencing using methylation-specific PCR.

The message is present, so there is no need to further pursue this sub-aim.

3) If at post-translational levels, test whether p53 is prevented from accumulation using pulse chase and assaying for levels of Mdm2. If p53 is unstable, we will test whether the pathway above p53 is compromised in tumors so that it can no longer phosphorylate and stabilize the p53 protein. We will examine the levels of phosphorylated ATM, Chk2, and Chk1 in RCAS-ErbB2-induced tumors using Western blotting.

We have found that p53 protein is made but cannot be stabilized in RCAS-ErbB2-induced tumors. pATM is still made in tumors. The results are now published as part of the attached manuscript (Reddy et al (2010) Proc Natl Acad Sci U S A 107: 3728-33).

Staining for Chk2 and Chk1 is pending.

4) Determine how c-Myc is downregulated in tumors using the same approach described for p53.

pending

Task 3. Determine whether models of genomically unstable human breast cancer may be generated by targeting ErbB2 to somatic mammary epithelial cells.
Months 6-36

1) Examine the extent of genomic amplification and deletion in RCAS-ErbB2 tumors (n=20) that we have already generated using BAC array CGH.

We have performed the analysis on 10 tumors (fig 1). Genomic gains and losses were detected, but not extensively. The most notable one was the partial or complete loss of chromosome 4, as in the tumors arising in MMTV-Neu mice.

2) Use BAC array to test chromosomal amplifications and losses in 20 tumors that we will collect from ATM-/-/MMTV-tva mice infected by RCAS-ErbB2.

On hold since we have not generated tumors from these mice.

3) Create RCAS expressing shRNA against ATM, and co-inject this virus with RCAS-ErbB2 into 20 MMTV-tva transgenic mice. Test the resulting tumors for genomic instability as above.

Pending.

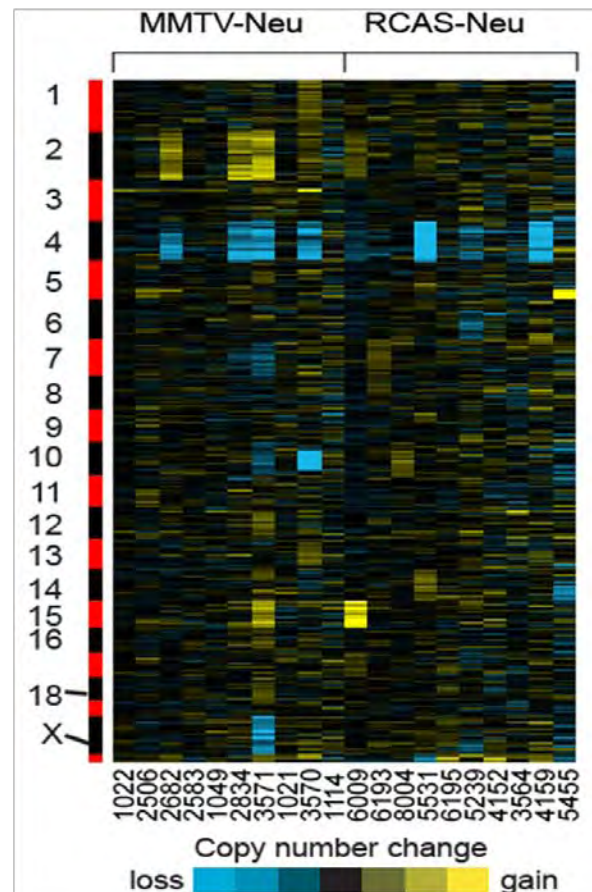


Figure 1. Heat map of BAC array analysis of tumors from MMTV-neu mice and RCAS-Neu infected mice. Tumor DNAs were hybridized with 100-kb titling probes. Profiles for each sample taken twice, once with cy5:cy3 and once with cy3:cy5. Within each tumor, for each BAC probe, average of the log ratios of the two profiles was used to plot

KEY RESEARCH ACCOMPLISHMENTS:

- Activation of ErbB2 after normal tissue development causes a DDR, uncovering an unexpected effect of ErbB2 activation previously known for its pro-survival roles.
- ATM, and thus DDR, is required for p53 stabilization, apoptosis, and senescence in ErbB2-activated mammary cells.
- DDR-induced apoptosis has to be overcome for early lesions to progress to malignancy, but senescence may remain in some of the tumor cells.

REPORTABLE OUTCOMES: Provide a list of reportable outcomes that have resulted from this research to include:

- Reddy et al (2010) Proc Natl Acad Sci U S A 107: 3728-33.
- 2010: 1st Place Platform Speaker Award to my graduate student Jay Reddy for his presentation of his work on DDR in breast cancer initiation, Annual Baylor-Rice University MSTP Symposium, Houston, TX
- 2009: American Association for Cancer Research (AACR) Scholar-in-Training Award to my graduate student Jay Reddy for his work on DDR in breast cancer initiation, and for him to attend the AACR-Advances in Breast Cancer Research Conference, San Diego, CA
- 2009: AACR Scholar-in-Training Award to my graduate student Jay Reddy for his work on DDR in breast cancer initiation, and for him to attend the AACR-Frontiers in Basic Cancer Research Conference, Boston, MA
- 2009: 2nd Place Platform Speaker Award to my graduate student Jay Reddy for his presentation of his work on DDR in breast cancer initiation, Lester and Sue Smith Breast Center 5th Annual Symposium at Baylor College of Medicine, Houston, TX

CONCLUSIONS:

p53, apoptosis, and senescence are frequently activated in preneoplastic lesions and are barriers to progression to malignancy. These barriers have been suggested to result from an ATM-mediated DNA damage response (DDR), which may follow oncogene-induced hyperproliferation and ensuing DNA replication stress. To elucidate the currently untested role of DDR in breast cancer initiation, we examined the effect of oncogene expression in several mouse models of breast cancer. We did not observe a detectable DDR in early hyperplastic lesions arising in transgenic mice expressing several different oncogenes. However, DDR signaling was strongly induced in preneoplastic lesions arising from individual mammary cells transduced in vivo by retroviruses expressing either PyMT or ErbB2. Thus, activation of an oncogene after normal tissue development causes a DDR. Furthermore, in this somatic ErbB2 tumor model, ATM, and thus DDR, is required for p53 stabilization, apoptosis, and senescence. In palpable tumors in this model, p53 stabilization and apoptosis are lost, but unexpectedly senescence remains in many tumor cells. Thus, this first mouse model that fully recapitulates both early DDR signaling and the eventual suppression of its endpoints in tumorigenesis provides compelling evidence that ErbB2-induced aberrant mammary cell proliferation leads to an ATM-mediated DDR that activates apoptosis and senescence, and at least the former must be overcome to progress to malignancy. This in vivo study also uncovers an unexpected effect of ErbB2 activation previously known for its pro-survival roles, and suggests that protection of the ATM-mediated DDR-p53 signaling pathway may be important in breast cancer prevention.

REFERENCES:

none

APPENDICES

Reddy et al (2010) Proc Natl Acad Sci U S A 107: 3728-33

Defining the ATM-mediated barrier to tumorigenesis in somatic mammary cells following ErbB2 activation

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Edited by Michael B. Kastan, St. Jude Children's Research Hospital, Memphis, TN, and accepted by the Editorial Board December 17, 2009 (received for review September 17, 2009)

p53, apoptosis, and senescence are frequently activated in preneoplastic lesions and are barriers to progression to malignancy. These barriers have been suggested to result from an ATM-mediated DNA damage response (DDR), which may follow oncogene-induced hyperproliferation and ensuing DNA replication stress. To elucidate the currently untested role of DDR in breast cancer initiation, we examined the effect of oncogene expression in several murine models of breast cancer. We did not observe a detectable DDR in early hyperplastic lesions arising in transgenic mice expressing several different oncogenes. However, DDR signaling was strongly induced in preneoplastic lesions arising from individual mammary cells transduced in vivo by retroviruses expressing either *PyMT* or *ErbB2*. Thus, activation of an oncogene after normal tissue development causes a DDR. Furthermore, in this somatic ErbB2 tumor model, ATM, and thus DDR, is required for p53 stabilization, apoptosis, and senescence. In palpable tumors in this model, p53 stabilization and apoptosis are lost, but unexpectedly senescence remains in many tumor cells. Thus, this murine model fully recapitulates early DDR signaling; the eventual suppression of its endpoints in tumorigenesis provides compelling evidence that ErbB2-induced aberrant mammary cell proliferation leads to an ATM-mediated DDR that activates apoptosis and senescence, and at least the former must be overcome to progress to malignancy. This in vivo study also uncovers an unexpected effect of ErbB2 activation previously known for its prosurvival roles, and suggests that protection of the ATM-mediated DDR-p53 signaling pathway may be important in breast cancer prevention.

oncogene | DNA damage response | p53 | apoptosis | senescence

Apoptosis and senescence are frequently found in precancerous lesions but are rarely detected in cancerous tissues (1). These cellular responses in mutated, precancerous cells have been suggested to be oncogene-activated barriers to tumorigenesis that prevent progression to malignancy. Accordingly, these barriers must be inactivated before cancer can arise (2). The p53 tumor suppressor, which is frequently associated with apoptosis and senescence, is a key player in halting progression to cancer and often inactivated in tumors (3). Activation of a few oncogenes, such as *c-Myc* and *Ras*, is known to cause apoptosis and/or senescence through the activation of ARF, leading to p53 accumulation (3). However, more recently it has been suggested that a DNA damage response (DDR) pathway follows oncogene-induced aberrant cell proliferation and is responsible for p53 stabilization, apoptosis, and senescence (4). This response is caused by DNA replication stress, replication fork collapse, and double strand breaks (DSBs) that may follow hyperproliferation, leading to the recruitment of the serine-threonine kinase ataxia-telangiectasia mutated (ATM) to the damaged chromosomal sites (4). ATM phosphorylates the histone variant H2AX (hereafter termed γ H2AX) and p53 binding protein 1 (53BP1), which are also recruited to DSBs (5, 6). ATM also directly phosphorylates p53 and indirectly regulates p53 phosphorylation by activating Chk2 and Chk1 (7). Components of this DDR signaling pathway are activated in preneoplastic lesions

but are mutated or inactivated in several cancers, including breast cancer, suggesting that the DDR must be overcome during the process of tumorigenesis (8, 9). In support of this, activation of *c-Myc* has been reported to induce DDR signaling and ATM-dependent apoptosis in skin and hematopoietic cells (10, 11). Activation of *Ras* and several other oncogenes have been found to induce DDR and ATM-dependent senescence in cultured cells (12–14). However, it has not been tested whether an oncogene-induced DDR is required for senescence observed in preneoplastic lesions in vivo. It is also not known whether the DDR plays a critical role in inducing apoptosis in the initiation of epithelial cancers in tissues besides the skin. Furthermore, in several tissues, oncogene activation either fails to induce a DDR or the resulting DDR fails to induce oncogenesis barriers. For example, *ATM* was reported to be dispensable for p53-dependent apoptosis in a murine model of choroid plexus tumorigenesis (15), and for oncogene-induced DDR induction, senescence, and p53-dependent tumor suppression in both *K-Ras*-driven lung carcinoma and chemically induced fibrosarcoma murine models (16).

In breast carcinogenesis, apoptosis and senescence were detected in preneoplastic breast lesions in rodent models of breast tumor formation induced by *Ras* or *ErbB2* (17, 18). However, it is not known whether DDR signaling is activated in the mammary gland at any point after oncogene activation and is responsible for the induction of apoptosis or senescence in premalignant lesions. The only potential supporting evidence thus far has been the increased levels of γ H2AX and phospho-Chk2 following ectopic expression of *Wnt-1* in cultured mammary epithelial cells (19) and promotion of p53 heterozygous or DMBA-initiated mammary tumorigenesis by *ATM* heterozygosity (20, 21).

In this study, we surveyed various murine models of breast cancer to gain insight into the possible role of DDR signaling in breast carcinogenesis. We found that the retrovirus RCAS-mediated expression of oncogenes in somatic mammary epithelial cells (22), which more closely mimics human cancer initiation than conventional models, induces a potent DDR that is required for p53 stabilization, apoptosis, and senescence. Furthermore, we found persistent senescence in fully developed tumors, directly challenging the long-standing assumption that both senescence and apoptosis must be inactivated before tumor formation.

Author contributions: J.P.R., J.M.R., L.A.D., and Y.L. designed research; J.P.R., S.P., W.B., and J.Z. performed research; S.H., Y.-C.N.D., K.P., and L.A.D. contributed new reagents/analytic tools; J.P.R., S.P., and Y.L. analyzed data; and J.P.R. and Y.L. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission. M.B.K. is a guest editor invited by the Editorial Board.

Freely available online through the PNAS open access option.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0910665107/DCSupplemental.

Results

Poor DDR Induction in Transgenic Mouse Models of Mammary Cancer.

We surveyed several murine models of mammary cancer for evidence of DDR induction. Mammary glands from female mice transgenic for *ErbB2* ($n = 4$), *c-Myc* ($n = 4$), *H-RasG12D* ($n = 3$), or *PyMT* (encoding the polyoma middle T antigen) ($n = 4$) developed early, precancerous lesions by the age of 7–9 weeks, which were harvested and stained for several DDR markers, γ H2AX, 53BP1, and p-S1981-ATM. We found no γ H2AX focal staining in MMTV-*PyMT* early lesions and only modest induction of γ H2AX foci in the lesions from MMTV-*ErbB2*, *-Myc*, and *-H-RasG12D* mice (Fig. 1). Furthermore, there was little 53BP1 and phospho-ATM focal staining in any of the transgenic lines examined (Fig. 1). In addition, we observed no p53 staining in the hyperplastic lesions of any of the transgenic mice examined (Fig. 1). These observations indicate that the DDR is poorly activated in the early lesions of germline transgenic mouse models of mammary cancer.

To test whether mammary cells in a transgenic mouse are still capable of mounting a DDR in response to classical stimuli, we irradiated three 8-week-old MMTV-*PyMT* mice with 6 Gy of ionizing radiation and assayed the mammary glands for DDR markers 30 min later. Although γ H2AX foci were detected, the intensity was much less as compared to irradiated, nontransgenic control mammary glands (Fig. S1). This reduction was observed both in relatively benign ducts and in the precancerous early lesions in the transgenic glands, indicating that the mammary epithelium in MMTV-*PyMT* mice possesses a weaker DDR as compared with wild-type mice. A similar pattern was observed when 53BP1 and p-S1981-ATM were examined by immunofluorescence (Fig. S1). Thus, the oncogene-expressing mammary epithelium in these germline transgenic models exhibits a partial defect in its ability to mount a DDR.

Somatic Activation of PyMT Oncogenic Signaling Induces a Robust DDR in Mouse Mammary Glands. Next, we sought to determine whether acute, somatic activation of an oncogene could engender a robust DDR response. First, we used the potent viral oncogene

PyMT to provide maximal oncogenic stress in somatic cells, because *PyMT* promotes hyperproliferation, neoplastic growth, and tumor formation at a rapid rate in mammary epithelial cells (23). We chose two different approaches to express *PyMT* in somatic mammary cells. One model is an inducible transgenic mouse line, in which *PyMT* expression is induced by administration of doxycycline to MMTV-*rtTA*/tet-O-*PyMT-IRES-Luc* bitransgenic mice (24). Another model is the RCAS-TVA system, in which mammary epithelial cells in MMTV-*tva* transgenic mice are infected via intraductal injection of the avian retrovirus RCAS carrying *PyMT* (22). One advantage of the RCAS-TVA system is that oncogenes can be introduced into relatively few mammary epithelial cells, which are then allowed to evolve into cancer in the context of a normal mammary gland, thus better recapitulating the etiology of human carcinogenesis than transgenic oncogene models (25, 26). Four MMTV-*tva* and three MMTV-*rtTA*/tet-O-*PyMT-IRES-Luc* mice were exposed to *PyMT* signaling by intraductal delivery of RCAS-*PyMT* or administration of doxycycline in the diet, respectively, for 1 week at 6 weeks of age to allow early hyperplasias to develop. Early lesions arising in RCAS-*PyMT* and MMTV-*rtTA*/tet-O-*PyMT-IRES-Luc* mammary cells displayed equivalent oncogene expression based on immunostaining for antibodies against *PyMT*, and similarly increased mitotic index measured by staining for phospho-histone H3 (Fig. S2A and B).

In these somatic models of oncogenic activation, significant, intense staining of γ H2AX foci in the lesions was observed as compared with that in the normal ducts (Fig. S2A). There was also marked induction of 53BP1 focal staining in the RCAS-*PyMT* early lesions; however, there was minimal 53BP1 staining in the MMTV-*rtTA*/tet-O-*PyMT-IRES-Luc* lesions (Fig. S2A). These results indicate that acute induction of *PyMT* induces a strong DDR in somatic cells, and that this DDR is most readily observed using the RCAS-TVA methodology.

To assess whether activated DDR resulted in stabilization of downstream effector molecules, we examined these early lesions for p53 accumulation using immunohistochemistry. We observed high levels of nuclear p53 staining in the RCAS-*PyMT* early lesions ($10.82 \pm 1.58\%$) and only moderate staining in the lesions generated in MMTV-*rtTA*/tet-O-*PyMT-IRES-Luc* mice ($2.62 \pm 0.21\%$, $P < 0.05$; Fig. S2A and C). To assess the functional activity of the observed p53 stabilization, we examined the level of apoptosis in the early lesions by TUNEL staining. Increased levels of apoptosis were observed in the RCAS-*PyMT* early lesions ($3.54 \pm 0.48\%$) with slightly reduced levels in the MMTV-*rtTA*/tet-O-*PyMT-IRES-Luc* early lesions ($2.17 \pm 0.38\%$, $P < 0.05$; Fig. S2A and D). These data suggest that somatically acquired *PyMT* signaling causes early mammary lesions with marked induction of a DDR that culminates in the stabilization of the effector protein p53, which presumably suppresses progression to cancer by initiating the apoptotic cascade. Once again, this is most easily observed in the RCAS model of acute oncogene induction.

To confirm that the DDR observed in RCAS-*PyMT* infected glands was not induced by viral infection per se, we injected five MMTV-*tva* mice with RCAS-*GFP* and then harvested the glands 4 days, 1 week, or 2 weeks later for analysis of DDR induction. GFP-positive cells detected at any time point did not exhibit any 53BP1 staining (Fig. S3), indicating that RCAS viral infection itself does not induce a DDR in the target cell, which is consistent with the reported lack of significant production of any viral proteins other than the product from the exogenous gene cloned into this vector (27).

A DDR Persists in RCAS-*PyMT* Induced Tumors. Next, we sought to examine the level of DDR in tumors generated in these two somatic models and, for comparison, in the tumors arising in MMTV-*PyMT* mice. Similar to the early lesions, there was intense focal staining of γ H2AX in the RCAS-*PyMT*-induced tumors ($n = 4$), whereas tumors from MMTV-*rtTA*/tet-O-*PyMT-IRES-Luc* mice ($n = 4$) kept on doxycycline had markedly less

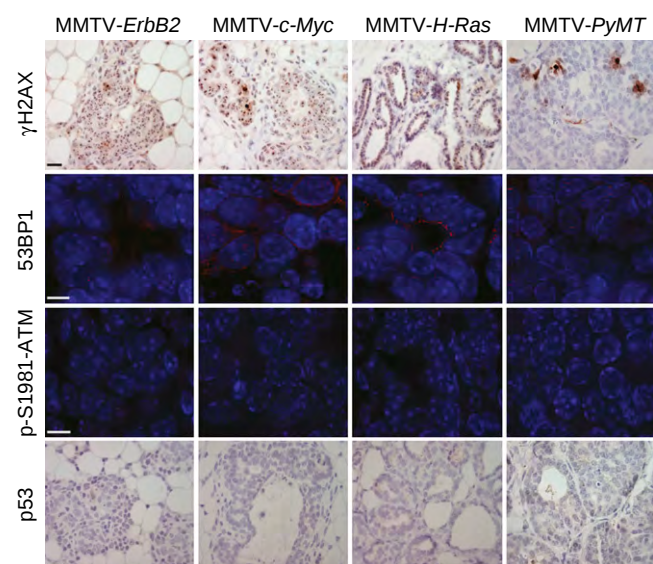


Fig. 1. Poor DDR induction in transgenic mouse models of mammary cancer. Early lesions in mammary glands from 7 to 9-week-old MMTV-*ErbB2* ($n = 4$), MMTV-*c-Myc* ($n = 4$), MMTV-*H-RasG12D* ($n = 3$), and MMTV-*PyMT* ($n = 4$) transgenic mice were stained for the proteins indicated, by immunofluorescence or immunohistochemistry. Brightfield images are 40 $\times$ with 20 μ m scale bar. Deconvolution immunofluorescent images are 100 $\times$ (53BP1) or 60 $\times$ (p-S1981-ATM) with 5- μ m scale bar.

(Fig. S44). Similar results were observed with respect to 53BP1 nuclear focal staining (Fig. S44). These results indicate that early DDR sensing remains intact and potent in these two somatic models of tumor induction, especially in the RCAS-TVA model. Tumors arising in MMTV-*PyMT* mice ($n = 3$) did not display dramatic staining of γ H2AX or 53BP1 (Fig. S44), consistent with the absence of these DDR markers in the early hyperplastic lesions that develop in this transgenic mouse line.

We then determined whether the DDR signaling cascade in these two somatic models still results in stabilized p53 and apoptosis. We observed abundant nuclear p53 ($14.53\% \pm 3.43\%$) and TUNEL staining ($3.83\% \pm 0.16\%$) in the RCAS-*PyMT* tumors (Fig. S4). In contrast, we observed significantly lower levels of p53 ($1.19 \pm 0.15\%$ and $0.55 \pm 0.34\%$) and TUNEL staining ($1.77 \pm 0.49\%$ and $1.30 \pm 0.21\%$) in the tumors from MMTV-*rtTA*/tet-*O-PyMT-IRES-Luc* mice kept on doxycycline and in the MMTV-*PyMT* tumors ($P < 0.01$ for both, univariate ANOVA; Fig. S4). Therefore, the above data suggest that in the RCAS-*PyMT* model DDR signaling remains fully intact at least in some of the tumor cells.

The observation of high levels of p53 and apoptosis in tumors generated from *PyMT*-activated somatic mammary cells is contrary to the hypothesis that the DDR must be inactivated for tumors to progress. Because *PyMT* is an extremely potent viral oncogene that can induce tumors with a median latency of approximately 2 weeks in the RCAS-TVA system (22), DDR inactivation may not be required for *PyMT*-induced tumorigenesis in the mouse mammary epithelium. It is possible that the high rate of cellular proliferation induced by *PyMT* may overcome the concurrent loss of cells to apoptosis induced by DDR signaling, leading to a rapid net expansion of transformed cells.

A Full DDR Signaling Cascade Is Activated in Response to Aberrant Proliferation Induced by Somatic Activation of *ErbB2*. *ErbB2*, which activates many of the same signaling pathways as *PyMT*, is encoded by a human proto-oncogene that has a well-documented role in breast carcinogenesis (28). Activated *ErbB2* in the RCAS-TVA system induces mammary tumors with a median latency of 4 months (22). We therefore asked whether RCAS-*ErbB2* could induce a DDR in hyperplastic lesions, and if so, whether this barrier is overcome in the evolution to cancer.

We injected four 12-week-old MMTV-*trA* mice with RCAS-*ErbB2* (tagged with an HA epitope) and harvested mammary glands 2 weeks later to examine the early precancerous lesions. *ErbB2*-induced early lesions exhibited focal staining of γ H2AX and 53BP1, indicating the presence of a DDR similar to that in RCAS-*PyMT*-induced early lesions (Fig. 2A). This was further confirmed by focal staining for p-S1981-ATM (Fig. 2A), in a pattern reminiscent of irradiated normal cells. In addition to activated DDR signaling, we also observed ARF induction in *ErbB2* early lesions (Fig. 2A). Both ATM and ARF regulate oncogene-activated tumor suppressor pathways that converge on p53. p53-positive nuclei were indeed observed in the *ErbB2*-induced early lesions ($6.02\% \pm 0.92\%$), and apoptotic cells were readily detected by TUNEL staining ($2.24\% \pm 0.4\%$), suggesting that p53 is functionally active (Fig. 2). Another endpoint to DDR induction and p53 activation in response to oncogenic stress is cellular senescence (29, 30). To determine whether acute *ErbB2*-induced DDR activation promotes senescence, we double-stained mammary lesions for senescence-associated β -galactosidase (SA- β -Gal) (31) and for the proliferation marker Ki67. As expected, senescent cells, which stained positively for SA- β -Gal but negatively for Ki67, were readily observed in $\approx 75\%$ of *ErbB2*-induced early lesions. This finding was further confirmed by immunostaining for two additional senescence markers, p16 and decoy receptor 2 (DcR2, Fig. 2A) (32, 33). These data collectively indicate that acute activation of the *ErbB2* oncogene induces hyperplasias that exhibit DDR signaling and ARF induction, leading to p53 stabilization, apoptosis, and senescence.

Fully Developed Mammary Tumors Induced by Somatic Activation of *ErbB2* Retain Upstream DDR Signaling and Senescence but Fail to Up-Regulate p53 or Apoptosis. Mammary tumors that eventually arose in MMTV-*trA* mice infected by RCAS-*ErbB2* also exhibited evidence of DDR signaling, with respect to focal nuclear staining of γ H2AX, 53BP1, and p-S1981-ATM, as well as ARF activation ($n = 4$; Fig. 2A). However, p53 staining was detected in only very few tumor cells ($0.96\% \pm 0.27\%$), and this correlated with a very low apoptotic index ($0.55 \pm 0.25\%$; Fig. 2). These observations strongly suggest that DDR and ARF functionality at the level of p53 is eventually compromised in *ErbB2*-induced mammary carcinogenesis, and that these alterations may be required for tumors to arise because of the subsequent loss of apoptosis.

However, to our surprise, positive staining for SA- β -Gal, p16, and DcR2 persisted in many tumor cells (20–40% of cells; Fig. 2A). This observation suggests that a p53-independent senescence cascade still remains in some of these tumor cells, although we cannot exclude the possibility that undetectable levels of p53 may still contribute to the senescence observed in these tumor cells.

The down-regulation of p53 was not due to lack of p53 transcription, as abundant amounts of the p53 transcript were detected in these tumors ($n = 5$) by qRT-PCR compared with the levels in normal or irradiated spleens (Fig. S5B). To test whether this reduced level of p53 resulted from failed stabilization of this very labile gene product, we examined whether p53 could be stabilized by classical DNA damage. We applied γ -radiation (6 Gy) and assayed the tumors ($n = 3$) 16 h later for p53 and its transcriptional targets *Mdm2* and *p21*. p53 was indeed phosphorylated and stabilized upon irradiation (Fig. S5A). Both *Mdm2* and *p21* were induced significantly compared with levels in nonirradiated tumors ($n = 5$, $P < 0.05$), and to higher levels than in irradiated spleen controls ($n = 3$; Fig. S5C and D). Therefore, in these RCAS-*ErbB2*-induced tumors, the p53 gene locus appears to be normal, and the product from its transcript can be stabilized and rendered functional after ionizing radiation. Thus, the observed down-regulation of p53 protein levels in RCAS-*ErbB2*-induced tumors likely stems from misregulation at the posttranslational level.

ATM-Ablated Mammary Cells Fail to Activate DDR Signaling, Apoptosis, or Senescence in Response to *ErbB2*-Induced Aberrant Proliferation. To directly test whether a DDR plays an essential role in activating p53, apoptosis, and senescence under oncogenic stress, we crossed MMTV-*trA* mice into an *ATM*-deficient mouse line (34), and injected 10–12 week-old MMTV-*trA*/*ATM*^{−/−} mice ($n = 3$) with RCAS-*ErbB2* to generate hyperplastic lesions. Heterozygous mice ($n = 3$) and wild-type littermate controls ($n = 3$) were also included. *ErbB2*-expressing early lesions developed in all genotypes (Fig. 3A). However, lesions in MMTV-*trA*/*ATM*^{−/−} mice displayed markedly reduced γ H2AX and 53BP1 focal staining, indicating diminished DDR signaling in the absence of *ATM* (Fig. 3A). As expected, ARF induction as a consequence of *ErbB2* oncogenic signaling was not affected by *ATM* status (Fig. 3A).

The *ATM*-null lesions exhibited markedly reduced p53 stabilization ($2.57\% \pm 0.92\%$) and apoptosis ($1.4\% \pm 0.3\%$) as compared to wild-type controls (p53: $9.79\% \pm 0.37\%$; TUNEL: $5.3\% \pm 0.3\%$; $P < 0.01$ for both, univariate ANOVA; Fig. 3). Furthermore, *ATM* ablation also resulted in loss of the senescence markers SA- β -Gal and p16 (Fig. 3A). These data suggest that ATM, and thus DDR signaling, are required for induction of p53, apoptosis, and senescence following *ErbB2* activation, and that ARF induction by itself is not sufficient to stabilize p53 or to induce apoptosis or senescence.

Discussion

DDR signaling is absent at the preneoplastic stage of four different germline transgenic models of breast cancer, and at least in the MMTV-*PyMT* transgenic model, a DDR cannot be robustly activated even by high dose ionizing radiation. In contrast, a potent

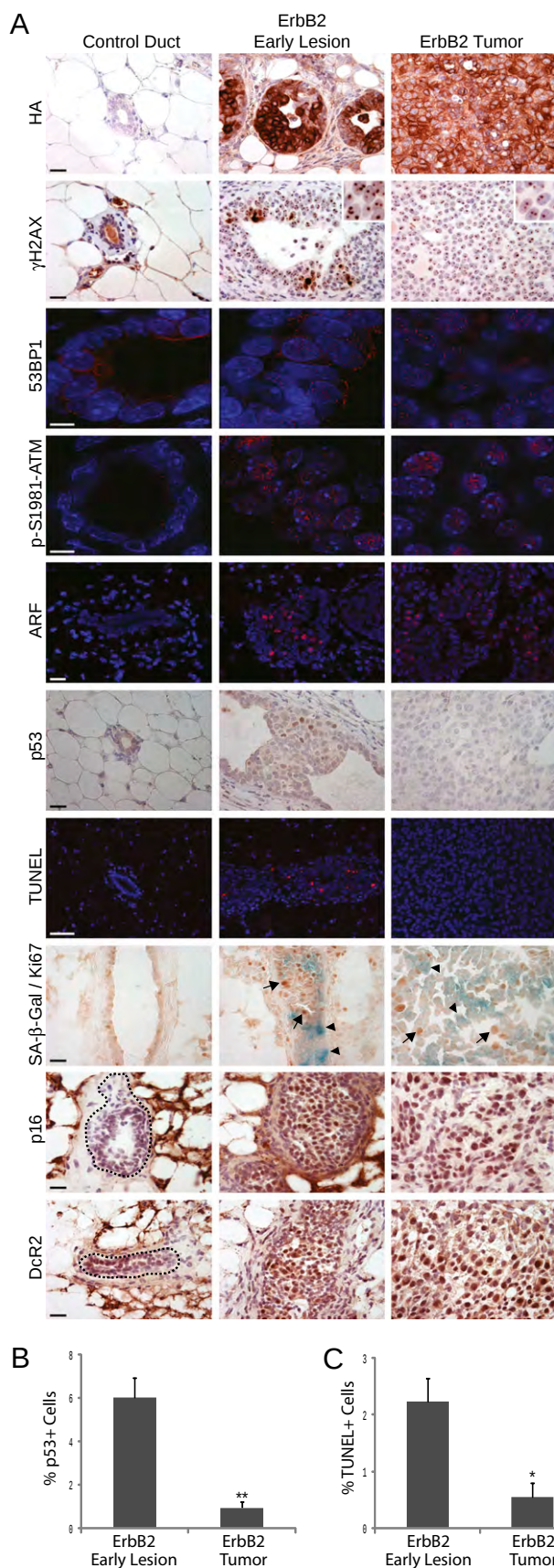


Fig. 2. Somatic activation of ErbB2 induces a DDR that becomes inactivated during tumor progression. (A) Normal mammary ducts and hyperplasias in RCAS-ErbB2-infected mammary glands from 14-week-old MMTV-tva mice ($n = 4$) and

DDR is present in early lesions and persists in tumors in somatic models. These data suggest that the DDR is affected by the developmental stage of the mammary gland that has suffered an oncogenic mutation. One potential explanation for the lack of DDR in these germline models is that oncogene expression during embryonic or prepubertal mammary gland development may have selected for DDR-insensitive cells to populate the mammary fat pad. Alternatively, mammary cells exposed to oncogenic mutations early in development may have adapted to these oncogenes by permanently downregulating the DDR pathway. In support of the latter hypothesis, epithelial cells in embryonic mammary glands have a different transformation response to oncogenic stimuli than cells in more mature mammary glands (35). Nonetheless, models that adopt a germline transgenic approach to oncogene expression appear to be unsuitable for investigating the significance of DDR signaling in human breast cancer initiation, which frequently exhibits DDR signaling. This potential deficiency with germline models may help explain why DDR-mediated anticancer barriers were not detected in some tissues (15).

Somatic introduction of an oncogene, particularly by the RCAS-TVA approach, more closely recapitulates human breast cancer initiation (25, 26). In the inducible PyMT model of breast cancer, a DDR is modestly activated (Fig. S2), whereas RCAS-mediated introduction of PyMT or activated ErbB2 leads to hyperplastic lesions that display components of a potent DDR including γ H2AX, 53BP1, and p-S1981-ATM (Fig. 2 and Fig. S2). In these ErbB2-induced early lesions, p53, apoptosis, and senescence are activated (Fig. 2). This is striking, considering that ErbB2 exhibits potent anti-apoptotic functions in numerous studies (36). In the resulting tumors, both p53 and apoptosis are down-regulated, as expected (Fig. 2). However, the p53 transcript remains induced and its gene product can still be stabilized after γ -irradiation (Fig. S5), presumably by signaling emanating from DSBs, indicating that the diminished p53 in ErbB2-induced tumors is likely caused by the destabilization of this labile protein or posttranslational misregulation. The p53 reduction is very likely responsible for the concurrent disappearance of apoptosis and, thus, for progression to cancer. Inactivated p53 promotes carcinogenesis in many tissues including the breast (37), and there is a strong correlation between p53 missense mutations and ErbB2 alterations in human breast cancers (38). Our data further illustrate the importance of p53 as part of the barrier to tumorigenesis and provide another explanation for frequent p53 alterations in breast cancer patients.

By inducing early lesions in ATM-null mice using RCAS-ErbB2, we provide compelling evidence that the ATM-mediated DDR signaling pathway is required for p53 stabilization and the erection of both apoptosis and senescence barriers after the activation of ErbB2 signaling. These data are consistent with observations of an important role for DDR in activating p53 and the apoptosis barrier in several other tissues (10, 11), and establish a direct causal relationship between DDR and senescence in vivo. Our observations also reveal a new dimension to ErbB2 signaling, as an overwhelming number of ErbB2 studies in cultured cells failed to detect senescence or apoptosis in ErbB2-activated cells. Because ErbB2 and its signaling pathways

mammary tumors from infected mice ($n = 4$) were stained for the proteins indicated and by TUNEL. Arrows indicate Ki67 cells (brown), whereas arrowheads indicate SA- β -Gal-positive cells (blue). Dashed lines, when present, outline normal ducts. Brightfield images are 40 $\times$ with 20- μ m scale bar. 53BP1 and p-S1981-ATM deconvolution immunofluorescent images are 100 $\times$ with 5- μ m scale bar. ARF and TUNEL immunofluorescent images are 40 $\times$ with 20 μ m scale bar. (B and C) Percentages of p53-positive nuclei (B) and TUNEL-positive cells (C) in early lesions ($n = 4$) and tumors ($n = 4$) arising in 12-week-old MMTV-tva mice injected with RCAS-ErbB2. Columns represent mean $\pm$ SEM. * $P = 0.01$ –0.05; ** $P = 0.001$ –0.01.

pression of apoptosis, while senescence remains in some of the tumor cells. These data provide further mechanistic insights into the frequent deactivation of DDR signaling and p53 in breast cancer, and suggest that protection of the DDR-p53 signaling pathway is important in breast cancer prevention.

Experimental Procedures

Transgenic Mice and Animal Care. MMTV-*tva* (22), MMTV-*PyMT* (56), MMTV-*ErbB2* (57), MMTV-*c-Myc* (58), MMTV-*H-RasG12D* (59), and MMTV-*rtTA/tet-O-PyMT-IRES-Luc* (24) mice have been reported. These mouse strains were maintained in an FVB genetic background. To generate MMTV-*tva/ATM*^{−/−} mice, MMTV-*tva* mice were crossed to *ATM*^{−/−} mice generously provided by Chengming Zhu (34). The resulting offspring had a mixed FVB/129Sv/C57B6 genetic background. All mouse lines were housed in pathogen-free housing in accordance with National Institutes of Health guidelines.

1. Halazonetis TD, Gorgoulis VG, Bartek J (2008) An oncogene-induced DNA damage model for cancer development. *Science* 319:1352–1355.
2. Venkitaraman AR (2005) Medicine: aborting the birth of cancer. *Nature* 434:829–830.
3. Lowe SW, Cepero E, Evan G (2004) Intrinsic tumour suppression. *Nature* 432:307–315.
4. Bartek J, Bartkova J, Lukas J (2007) DNA damage signalling guards against activated oncogenes and tumour progression. *Oncogene* 26:7773–7779.
5. Kastan MB (2008) DNA damage responses: Mechanisms and roles in human disease: 2007 G.H.A. Clowes Memorial Award Lecture. *Mol Cancer Res* 6:517–524.
6. Harper JW, Elledge SJ (2007) The DNA damage response: Ten years after. *Mol Cell* 28:739–745.
7. Kastan MB, Bartek J (2004) Cell-cycle checkpoints and cancer. *Nature* 432:316–323.
8. Bartkova J, et al. (2005) DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. *Nature* 434:864–870.
9. Gorgoulis VG, et al. (2005) Activation of the DNA damage checkpoint and genomic instability in human precancerous lesions. *Nature* 434:907–913.
10. Reimann M, et al. (2007) The Myc-evoked DNA damage response accounts for treatment resistance in primary lymphomas in vivo. *Blood* 110:2996–3004.
11. Pusapati RV, et al. (2006) ATM promotes apoptosis and suppresses tumorigenesis in response to Myc. *Proc Natl Acad Sci USA* 103:1446–1451.
12. Bartkova J, et al. (2006) Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints. *Nature* 444:633–637.
13. Di Micco R, et al. (2006) Oncogene-induced senescence is a DNA damage response triggered by DNA hyper-replication. *Nature* 444:638–642.
14. Mallette FA, Gaumont-Leclerc MF, Ferbeyre G (2007) The DNA damage signaling pathway is a critical mediator of oncogene-induced senescence. *Genes Dev* 21:43–48.
15. Liao MJ, Yin C, Barlow C, Wynshaw-Boris A, van Dyke T (1999) Atm is dispensable for p53 apoptosis and tumor suppression triggered by cell cycle dysfunction. *Mol Cell Biol* 19:3095–3102.
16. Efeyan A, et al. (2009) Limited role of murine ATM in oncogene-induced senescence and p53-dependent tumor suppression. *PLoS One*, 4:e5475, 1–11.
17. Woditschka S, Haag JD, Sullivan R, Gould MN (2009) A short-term rat mammary carcinogenesis model for the prevention of hormonally responsive and nonresponsive in situ carcinomas. *Cancer Prev Res* 2:153–160.
18. Sarkisian CJ, et al. (2007) Dose-dependent oncogene-induced senescence in vivo and its evasion during mammary tumorigenesis. *Nat Cell Biol* 9:493–505.
19. Ayyanan A, et al. (2006) Increased Wnt signaling triggers oncogenic conversion of human breast epithelial cells by a Notch-dependent mechanism. *Proc Natl Acad Sci USA* 103:3799–3804.
20. Umesako S, et al. (2005) Atm heterozygous deficiency enhances development of mammary carcinomas in p53 heterozygous knockout mice. *Breast Cancer Res* 7:R164–R170.
21. Lu S, et al. (2006) Atm-haploinsufficiency enhances susceptibility to carcinogen-induced mammary tumors. *Carcinogenesis* 27:848–855.
22. Du Z, et al. (2006) Introduction of oncogenes into mammary glands in vivo with an avian retroviral vector initiates and promotes carcinogenesis in mouse models. *Proc Natl Acad Sci USA* 103:17396–17401.
23. Marcotte R, Muller WJ (2008) Signal transduction in transgenic mouse models of human breast cancer—implications for human breast cancer. *J Mammary Gland Biol Neoplasia* 13:323–335.
24. Podsypanina K, et al. (2008) Seeding and propagation of untransformed mouse mammary cells in the lung. *Science* 321:1841–1844.
25. Du Z, Li Y (2007) RCAS-TVA in the mammary gland: An in vivo oncogene screen and a high fidelity model for breast transformation? *Cell Cycle* 6:823–826.
26. Reddy JP, Li Y (2009) The RCAS-TVA system for introduction of oncogenes into selected somatic mammary epithelial cells in vivo. *J Mammary Gland Biol Neoplasia* 14:405–409.
27. Hughes SH (2004) The RCAS vector system. *Folia Biol (Praha)* 50:107–119.
28. Ursini-Siegel J, Schade B, Cardiff RD, Muller WJ (2007) Insights from transgenic mouse models of ERBB2-induced breast cancer. *Nat Rev Cancer* 7:389–397.
29. Campisi J, d'Adda di Fagnaga F (2007) Cellular senescence: When bad things happen to good cells. *Nat Rev Mol Cell Biol* 8:729–740.
30. Di Micco R, Fumagalli M, d'Adda di Fagnaga F (2007) Breaking news: High-speed race ends in arrest—how oncogenes induce senescence. *Trends Cell Biol* 17:529–536.
31. Dimri GP, et al. (1995) A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proc Natl Acad Sci USA* 92:9363–9367.

Virus Preparation and Mammary Gland Delivery. RCAS-*PyMT*, RCAS-*ErbB2*, and RCAS-*GFP* viruses were prepared and injected as described previously (22).

Tissue Harvest and Analysis. Mammary glands and tumors were removed, fixed, and analyzed as described in *SI Experimental Procedures*. Detailed antibody information can be found in *SI Experimental Procedures*.

ACKNOWLEDGMENTS. We thank Drs. Pumin Zhang, Lei Li, Xin-Hua Feng, Mike Lewis, Powell Brown, Sue Hilsenbeck, and Gary Chamness for stimulating discussions and/or critical review of this manuscript, and the Pathology Core at the Breast Center for tissue processing. This work was supported in part by U.S. Army CDMRP Grants BC085050 (to Y.L.) and BC073703 (to Y.L.) and by National Institutes of Health Grants CA113869 (to Y.L.), CA100420 (to L.A.D.), and CA16303 (to J.M.R.). J.P.R. is supported by the Robert and Janice McNair Foundation and a CDMRP pre-doctoral fellowship (BC083190), Y.N.D. is supported by CA105492, K.P. is supported by CA118731, and S.P. was supported by BC050677.

32. Collado M, et al. (2005) Tumour biology: Senescence in premalignant tumours. *Nature* 436:642.
33. Collado M, Serrano M (2006) The power and the promise of oncogene-induced senescence markers. *Nat Rev Cancer* 6:472–476.
34. Borghesani PR, et al. (2000) Abnormal development of Purkinje cells and lymphocytes in Atm mutant mice. *Proc Natl Acad Sci USA* 97:3336–3341.
35. Andrechek ER, Hardy WR, Laing MA, Muller WJ (2004) Germ-line expression of an oncogenic erbB2 allele confers resistance to erbB2-induced mammary tumorigenesis. *Proc Natl Acad Sci USA* 101:4984–4989.
36. Hynes NE, Lane HA (2005) ERBB receptors and cancer: The complexity of targeted inhibitors. *Nat Rev Cancer* 5:341–354.
37. Donehower LA (1996) The p53-deficient mouse: A model for basic and applied cancer studies. *Semin Cancer Biol* 7:269–278.
38. Langerød A, et al. (2007) TP53 mutation status and gene expression profiles are powerful prognostic markers of breast cancer. *Breast Cancer Res*, 9:R30, 1–16.
39. Deng CX, Wang RH (2003) Roles of BRCA1 in DNA damage repair: A link between development and cancer. *Hum Mol Genet* 12(Spec No 1, Spec No 1):R113–R123.
40. Palmero I, Pantoja C, Serrano M (1998) p19ARF links the tumour suppressor p53 to Ras. *Nature* 395:125–126.
41. Zindy F, et al. (1998) Myc signaling via the ARF tumor suppressor regulates p53-dependent apoptosis and immortalization. *Genes Dev* 12:2424–2433.
42. Swarbrick A, Roy E, Allen T, Bishop JM (2008) Id1 cooperates with oncogenic Ras to induce metastatic mammary carcinoma by subversion of the cellular senescence response. *Proc Natl Acad Sci USA* 105:5402–5407.
43. D'Amico M, et al. (2003) The role of Ink4a/Arf in ErbB2 mammary gland tumorigenesis. *Cancer Res* 63:3395–3402.
44. Debies MT, et al. (2008) Tumor escape in a Wnt1-dependent mouse breast cancer model is enabled by p19Arf/p53 pathway lesions but not p16 Ink4a loss. *J Clin Invest* 118:51–63.
45. Michaloglou C, et al. (2005) BRAFE600-associated senescence-like cell cycle arrest of human naevi. *Nature* 436:720–724.
46. Chen Z, et al. (2005) Crucial role of p53-dependent cellular senescence in suppression of Pten-deficient tumorigenesis. *Nature* 436:725–730.
47. Braig M, et al. (2005) Oncogene-induced senescence as an initial barrier in lymphoma development. *Nature* 436:660–665.
48. Trost TM, et al. (2005) Premature senescence is a primary fail-safe mechanism of ERBB2-driven tumorigenesis in breast carcinoma cells. *Cancer Res* 65:840–849.
49. Visvader JE, Lindeman GJ (2008) Cancer stem cells in solid tumours: Accumulating evidence and unresolved questions. *Nat Rev Cancer* 8:755–768.
50. Rosen JM, Jordan CT (2009) The increasing complexity of the cancer stem cell paradigm. *Science* 324:1670–1673.
51. Aliouat-Denis CM, et al. (2005) p53-independent regulation of p21Waf1/Cip1 expression and senescence by Chk2. *Mol Cancer Res* 3:627–634.
52. Chen CR, et al. (2005) Dual induction of apoptosis and senescence in cancer cells by Chk2 activation: Checkpoint activation as a strategy against cancer. *Cancer Res* 65:6017–6021.
53. Serrano M, Lin AW, McCurrach ME, Beach D, Lowe SW (1997) Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell* 88:593–602.
54. Beauséjour CM, et al. (2003) Reversal of human cellular senescence: Roles of the p53 and p16 pathways. *EMBO J* 22:4212–4222.
55. Narita M, et al. (2003) Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence. *Cell* 113:703–716.
56. Guy CT, Cardiff RD, Muller WJ (1992) Induction of mammary tumors by expression of polyomavirus middle T oncogene: A transgenic mouse model for metastatic disease. *Mol Cell Biol* 12:954–961.
57. Guy CT, et al. (1992) Expression of the neu protooncogene in the mammary epithelium of transgenic mice induces metastatic disease. *Proc Natl Acad Sci USA* 89:10578–10582.
58. Leder A, Pattengale PK, Kuo A, Stewart TA, Leder P (1986) Consequences of widespread deregulation of the c-myc gene in transgenic mice: Multiple neoplasms and normal development. *Cell* 45:485–495.
59. Sinn E, et al. (1987) Coexpression of MMTV/Δ-H-ras and MMTV/c-myc genes in transgenic mice: Synergistic action of oncogenes in vivo. *Cell* 49:465–475.

Supporting Information

Reddy et al. 10.1073/pnas.0910665107

SI Experimental Procedures

Tissue Harvest and Analysis. Mammary glands and tumors were removed and either fixed in 10% buffered formalin overnight at 4°C, frozen in frozen section medium (Neg-50, Richard-Allan Scientific) on dry ice, or snap-frozen in liquid nitrogen. Formalin-fixed tissues were paraffin embedded, and 3- μ m sections were placed on slides for immunostaining. Sections (10 μ m) were generated from frozen tissue blocks and placed on slides. Immunohistochemistry and immunofluorescence were performed as described previously (1). Briefly, formalin-fixed paraffin sections were deparaffinized, rehydrated, and subjected to antigen retrieval in 10 mM sodium citrate, pH 6.0. Frozen sections were fixed in 4% paraformaldehyde and permeabilized in 0.1% Triton-X before staining. The manufacturer's protocol for the MOM, Vectastain Elite ABC Rabbit, or Rat kits (Vector Labs; cat no. PK-2200, PK-6101, PK-6104) was then followed. Western blotting was performed as described previously (2, 3).

Antibodies. Primary antibodies used in these experiments include rabbit antibodies against PyMT (Calbiochem, DP10L), phospho-S10 Histone H3 (Upstate, 06-570), p53 (Novocastra, CM5p), Ki67 (Novocastra, Ki67p), p-S15-p53 (Cell Signaling, 9284), β -actin (Cell Signaling, 4970), p16 (Santa Cruz, sc-1207), DcR2 (Stressgen, AAP-371), and 53BP1 (Bethyl, A300-273A); mouse monoclonal antibodies against GFP (BD Biosciences, 632281), HA (Covance, MMS-101P), γ H2AX (Upstate, 05-636), and p-S1981-ATM (Rockland, 200-301-500); and a rat ARF antibody (Abcam, ab26696).

TUNEL and SA- β -Gal Staining. For TUNEL staining, paraffin sections were treated identically as above but without antigen retrieval, and the manufacturer's protocol for the Apoptag Red *In Situ* TUNEL detection kit (Chemicon, S7165) was followed. SA- β -Gal staining was performed on 10- μ m mammary gland or mammary tumor cryosections either using a SA- β -Gal activity kit (Cell Signaling, 9860) or a method described previously (4, 5). For SA- β -Gal/Ki67 double-staining, standard immunohistochemistry for Ki67 was performed on frozen sections immediately on the completion of SA- β -Gal staining.

Microscopy. All brightfield images were captured at 40 $\times$ using a Leica DMLB microscope. Fluorescent images were either captured at 40 $\times$ with a ZeissAxioskop 2 plus microscope or at 63 $\times$ or 100 $\times$ with a Zeiss/Applied Precision SoftWoRx Image Restoration Microscope and processed using SoftWoRx deconvolution software.

Expression Analysis. RNA was extracted from mouse tissue using the RNeasy kit (Qiagen). The following probe sets were used for qRT-PCR analysis: *p53* (Mm01731287_m1), *Mdm2* (Mm01233136_m1), *p21* (Mm1303209_m1), and *18s* (Hs99999901_s1) (Applied Biosystems).

Statistical Analysis. All images shown are representative of at least 10 fields viewed over two stained sections per animal. Quantitation was performed using a minimum of three random fields per animal. All columns represent mean $\pm$ SEM. All statistical analyses were performed using Student's *t* test unless otherwise indicated.

1. Du Z, et al. (2006) Introduction of oncogenes into mammary glands in vivo with an avian retroviral vector initiates and promotes carcinogenesis in mouse models. *Proc Natl Acad Sci USA* 103:17396–17401.
2. Li Y, et al. (2001) Deficiency of Pten accelerates mammary oncogenesis in MMTV-Wnt-1 transgenic mice. *BioMed Central Mol Biol* 2:2.
3. Li Y, et al. (2003) Evidence that transgenes encoding components of the Wnt signaling pathway preferentially induce mammary cancers from progenitor cells. *Proc Natl Acad Sci USA* 100:15853–15858.

4. Dimri GP, et al. (1995) A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proc Natl Acad Sci USA* 92:9363–9367.
5. Bandyopadhyay D, Gatz C, Donehower LA, Medrano EE (2005) Analysis of cellular senescence in culture in vivo: The senescence-associated beta-galactosidase assay. *Curr Protoc Cell Biol* 27:18.9.1–18.9.9.

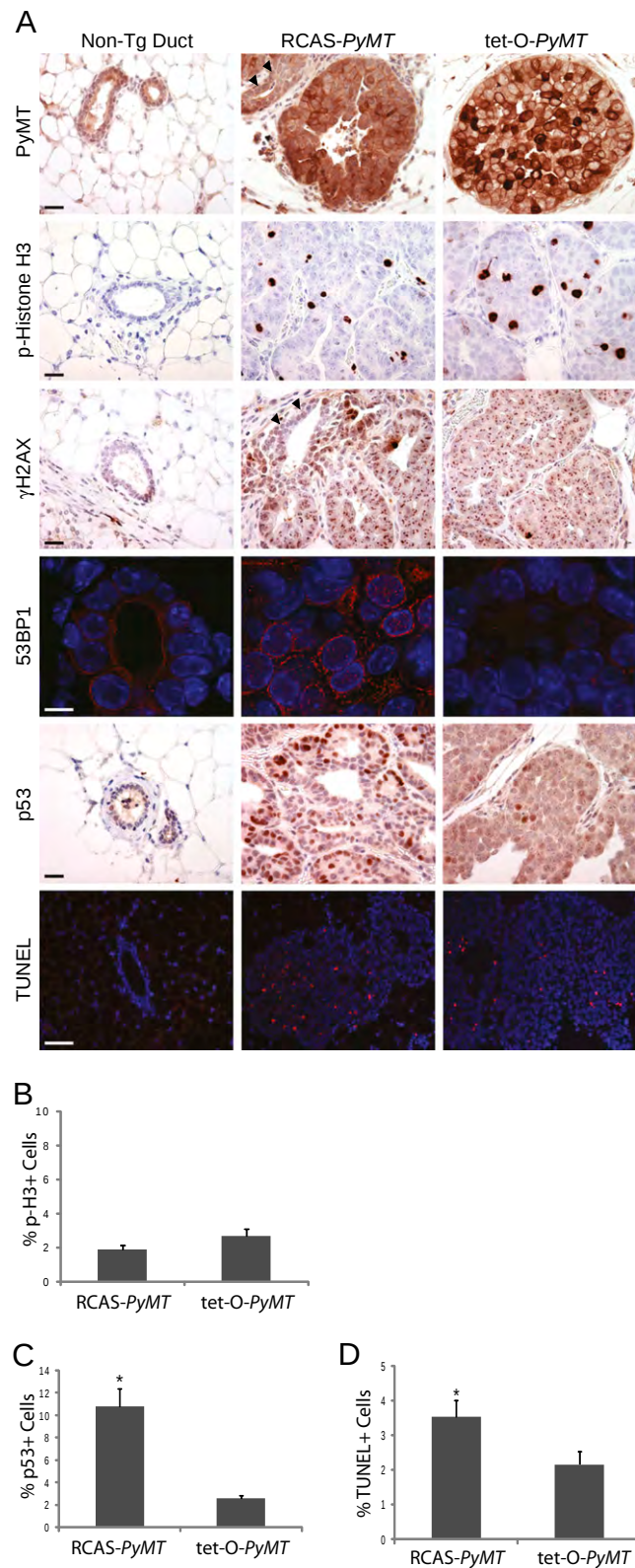


Fig. S2. Somatic activation of PyMT oncogenic signaling induces a robust DDR in mouse mammary glands. (A) RCAS-PyMT ($n = 4$) and MMTV-rtTA/tet-O-PyMT-IRES-Luc mammary hyperplasias ($n = 3$) as well as nontransgenic ducts from 7-week-old mice were stained by immunohistochemistry or immunofluorescence for the proteins indicated at the right or by TUNEL. Arrowheads indicate adjacent normal mammary epithelium included as a comparison with RCAS-PyMT early lesions. Brightfield images are 40 $\times$ with 20 μ m scale bar. 53BP1 deconvolution immunofluorescent images are 100 $\times$ with 5- μ m scale bar. TUNEL immunofluorescent images are 40 $\times$ with 20- μ m scale bar. (B–D) Percentages of phospho-histone H3-positive nuclei (B), p53-positive nuclei (C), and TUNEL-positive cells (D) in hyperplastic early lesions arising in 7-week-old RCAS-PyMT ($n = 4$) or MMTV-rtTA/tet-O-PyMT-IRES-Luc ($n = 3$) mice. Columns represent mean $\pm$ SEM. * $P = 0.01$ – 0.05 .

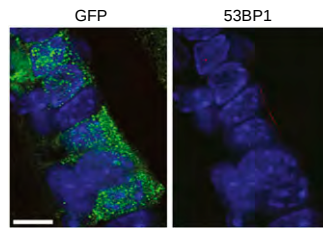


Fig. S3. RCAS-*GFP* viral infection does not induce a DDR. Representative 53BP1 and GFP co-immunofluorescence in RCAS-*GFP*–infected cells in mammary glands from 12-week-old MMTV-*tva* mice ($n = 5$). Deconvolution immunofluorescent images are 63 $\times$ with 5- μ m scale bar.

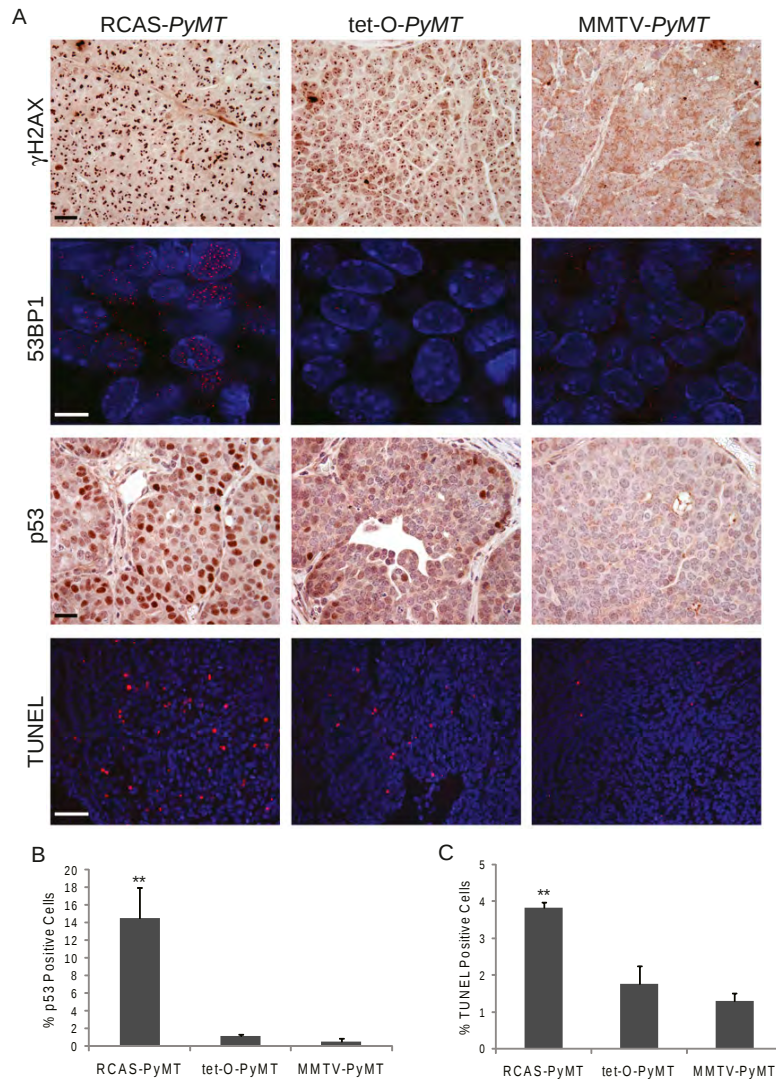


Fig. S4. A DDR persists in RCAS-PyMT-induced tumors. (A) Mammary tumors induced by RCAS-PyMT ($n = 4$), MMTV-*rtTA*/tet-O-PyMT-IRES-Luc ($n = 4$), or MMTV-PyMT ($n = 3$) were stained by immunohistochemistry or immunofluorescence for the proteins indicated or by TUNEL. Brightfield images are 40 $\times$ with 20- μ m scale bar. 53BP1 deconvolution immunofluorescent images are $\times 100$ with 5- μ m scale bar. TUNEL immunofluorescent images are 40 $\times$ with 20- μ m scale bar. (B and C) Percentages of p53-positive nuclei (B) or TUNEL-positive cells (C) in tumors induced by RCAS-PyMT ($n = 4$), tet-O-PyMT ($n = 3$), or MMTV-PyMT ($n = 3$). Columns represent mean $\pm$ SEM. Statistical analysis was performed using univariate ANOVA. *, $P = 0.01$ –0.05; **, $P = 0.001$ –0.01.

