Final Progress Report for Research Projects Funded by Health Research Grants

Instructions: Please complete all of the items as instructed. Do not delete instructions. Do not leave any items blank; responses must be provided for all items. If your response to an item is "None", please specify "None" as your response. "Not applicable" is not an acceptable response for any of the items. There is no limit to the length of your response to any question. Responses should be single-spaced, no smaller than 12-point type. The report **must be completed using MS Word**. Submitted reports must be Word documents; they should not be converted to pdf format. Questions? Contact Health Research Program staff at 717-783-2548.

1. **Grantee Institution:** The Trustees of the University of Pennsylvania

2. **Reporting Period (start and end date of grant award period):** 1/1/2009-12/31/2012

3. **Grant Contact Person (First Name, M.I., Last Name, Degrees):** Gearline R. Robinson-Hall, BSF

4. **Grant Contact Person's Telephone Number:** 215-746-6821

5. **Grant SAP Number:** 4100047654

6. **Project Number and Title of Research Project:** 5 - Understanding the Biology of Residual Neoplastic Disease

7. **Start and End Date of Research Project:** 1/1/2009-12/31/2012

8. **Name of Principal Investigator for the Research Project:** Lewis A. Chodosh, MD, PhD

9. **Research Project Expenses**.

   9(A) Please provide the total amount of health research grant funds spent on this project for the entire duration of the grant, including indirect costs and any interest earned that was spent:

   $ 410,807.38

   9(B) Provide the last names (include first initial if multiple individuals with the same last name are listed) of **all** persons who worked on this research project and were supported with health research funds. Include position titles (Principal Investigator, Graduate Assistant, Post-doctoral Fellow, etc.), percent of effort on project and total health research funds expended for the position. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name	Position Title	% of Effort on Project	Cost
Alvarez	Sr. Research Investigator	12%YR2	11,168.75
Belka	Director	14%YR2;16%YR4	27,482.33
Chen	Research Project Mgr	6%YR2;15%YR1	20,160.54
Chodosh	Professor	7%YR2	23,912.06
Feng	Research Associate	9%YR2	4,256.00
Notarfrancesco	Research Specialist	6%YR2	3,932.16
Pant	Data Analyst	10%YR2	6,985.38
Pan	Data Analyst, Sr.	8%YR2	6,370.06
Perez	Post-doctoral Researcher	5%YR2	1,997.16
Shields	Research Specialist	9%YR2	5,735.50
Smith	Research Project Mgr	16%YR2;13YR4%	24,009.16
Sterner	Research Project Mgr	15%YR2	10,355.07
Whitehouse	Research Specialist	16%YR2	6,038.13
Young	Research Specialist	10%YR2	5,305.53

9(C) Provide the names of **all** persons who worked on this research project, but who *were not* supported with health research funds. Include position titles (Research Assistant, Administrative Assistant, etc.) and percent of effort on project. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name	Position Title	% of Effort on Project
None		

9(D) Provide a list of **all** scientific equipment purchased as part of this research grant, a short description of the value (benefit) derived by the institution from this equipment, and the cost of the equipment.

Type of Scientific Equipment	Value Derived	Cost
None		

10. Co-funding of Research Project during Health Research Grant Award Period. Did this research project receive funding from any other source during the project period when it was supported by the health research grant?

Yes_____ No____X_____

If yes, please indicate the source and amount of other funds:

11. Leveraging of Additional Funds

11(A) As a result of the health research funds provided for this research project, were you

able to apply for and/or obtain funding from other sources to continue or expand the research?

Yes_____ No____X_____

If yes, please list the applications submitted (column A), the funding agency (National Institutes of Health—NIH, or other source in column B), the month and year when the application was submitted (column C), and the amount of funds requested (column D). If you have received a notice that the grant will be funded, please indicate the amount of funds to be awarded (column E). If the grant was not funded, insert "not funded" in column E.

Do not include funding from your own institution or from CURE (tobacco settlement funds). Do not include grants submitted prior to the start date of the grant as shown in Question 2. If you list grants submitted within 1-6 months of the start date of this grant, add a statement below the table indicating how the data/results from this project were used to secure that grant.

A. Title of research project on grant application	B. Funding agency (check those that apply)	C. Month and Year Submitted	D. Amount of funds requested:	E. Amount of funds to be awarded:
None	☐NIH ☐ Other federal (specify:_____) ☐ Nonfederal source (specify:_)		$	$

11(B) Are you planning to apply for additional funding in the future to continue or expand the research?

Yes___X_____ No_____

If yes, please describe your plans:

Grant applications will be submitted to the Department of Defense Breast Cancer Research Program as well as the Susan G. Komen for the Cure Foundation to support further work on this topic.

12. Future of Research Project. What are the future plans for this research project?

Follow-up studies are planned under the auspices of future projects.

13. New Investigator Training and Development. Did students participate in project supported internships or graduate or post-graduate training for at least one semester or one summer?

Yes___X_____ No_____

If yes, how many students? Please specify in the tables below:

	Undergraduate	Masters	Pre-doc	Post-doc
Male				
Female				1
Unknown				
Total				**1**

	Undergraduate	Masters	Pre-doc	Post-doc
Hispanic				1
Non-Hispanic				
Unknown				
Total				**1**

	Undergraduate	Masters	Pre-doc	Post-doc
White				1
Black				
Asian				
Other				
Unknown				
Total				**1**

14. Recruitment of Out-of–State Researchers. Did you bring researchers into Pennsylvania to carry out this research project?

Yes_____ No____X_____

If yes, please list the name and degree of each researcher and his/her previous affiliation:

15. Impact on Research Capacity and Quality. Did the health research project enhance the quality and/or capacity of research at your institution?

Yes_____ No___X_____

If yes, describe how improvements in infrastructure, the addition of new investigators, and other resources have led to more and better research.

16. Collaboration, business and community involvement.

16(A) Did the health research funds lead to collaboration with research partners outside of your institution (e.g., entire university, entire hospital system)?

Yes_____ No___X_____

If yes, please describe the collaborations:

16(B) Did the research project result in commercial development of any research products?

Yes_____ No___X_____

If yes, please describe commercial development activities that resulted from the research project:

16(C) Did the research lead to new involvement with the community?

Yes_____ No____X_____

If yes, please describe involvement with community groups that resulted from the research project:

17. Progress in Achieving Research Goals, Objectives and Aims.

List the project goals, objectives and specific aims (as contained in the grant agreement). Summarize the progress made in achieving these goals, objectives and aims for the period that the project was funded (i.e., from project start date through end date). Indicate whether or not each goal/objective/aim was achieved; if something was not achieved, note the reasons why. Describe the methods used. If changes were made to the research goals/objectives/aims, methods, design or timeline since the original grant application was submitted, please describe the changes. Provide detailed results of the project. Include evidence of the data that was generated and analyzed, and provide tables, graphs, and figures of the data. List published abstracts, poster presentations and scientific meeting presentations at the end of the summary of progress; peer-reviewed publications should be listed under item 20.

This response should be a DETAILED report of the methods and findings. It is not sufficient to state that the work was completed. Insufficient information may result in an unfavorable performance review, which may jeopardize future funding. If research findings are pending publication you must still include enough detail for the expert peer reviewers to evaluate the progress during the course of the project.

Health research grants funded under the Tobacco Settlement Act will be evaluated via a performance review by an expert panel of researchers and clinicians who will assess project work using this Final Progress Report, all project Annual Reports and the project's strategic plan. After the final performance review of each project is complete, approximately 12-16 months after the end of the grant, this Final Progress Report, as well as the Final Performance Review Report containing the comments of the expert review panel, and the grantee's written response to the Final Performance Review Report, will be posted on the CURE Web site.

There is no limit to the length of your response. Responses must be single-spaced below, no smaller than 12-point type. If you cut and paste text from a publication, be sure symbols print properly, e.g., the Greek symbol for alpha (α) and beta (ß) should not print as boxes (□) and include the appropriate citation(s). DO NOT DELETE THESE INSTRUCTIONS.

Understanding the Biology of Residual Neoplastic Disease - A cardinal feature of human breast cancers is the survival and persistence of residual neoplastic cells in a presumed quiescent state following the apparently successful treatment of the initial tumor. Ultimately, these residual cells re-emerge from their dormant state and resume growth, leading to cancer recurrence. Indeed, analyses of bone marrow samples show that disseminated cells are present in 20-40% of primary breast cancer patients without any clinical or histopathological signs of metastasis. As such, residual neoplastic disease is a major obstacle to the successful treatment of breast cancer and other human cancers. Accordingly, the purpose of this project is to investigate the biological properties of residual neoplastic cells, since these cells represent the reservoir from which tumor recurrences invariably arise.

Project Overview

A particular difficulty in understanding the mechanisms of breast cancer recurrence has been the challenge of identifying and isolating residual neoplastic cells in patients, and the lack of animal models that recapitulate this key feature of breast cancer progression. We have generated conditional transgenic mouse models for breast cancer and have obtained important preliminary evidence indicating that essentially all oncogene-induced mammary tumors that have regressed to a non-palpable state following oncogene down-regulation leave behind residual neoplastic cells. These represent a unique set of genetically engineered models for residual neoplastic disease in breast cancer induced by defined oncogenic pathways. In this project, we propose to use these models to define critical features of residual neoplastic disease relevant to developing improved therapeutic approaches to human cancers.

The specific aims of this project are: Aim I: Identify morphological lesions containing residual neoplastic cells; Aim II: Analyze the cellular components of residual neoplastic lesions; Aim III: Determine if autophagy is a survival mechanism for residual neoplastic cells. These aims will be accomplished by using doxycycline-inducible transgenic mouse models in which oncogenic pathways important in human cancer can be conditionally activated or inactivated. Following tumor development in these models, tumor regression can be induced by oncogene down-regulation in a manner that simulates the treatment of patients with molecularly targeted agents. The tumor cells that survive oncogene down-regulation will first be identified in the mammary glands of these animals by histopathological and cell marking approaches. The cellular components and cellular microenvironment of these residual neoplastic cells will then be defined by co-localization of cell-type specific markers. Finally, the role of autophagy in the survival of residual neoplastic cells will be determined by retroviral transduction approaches. Together, these approaches should permit clinically relevant insights into the biology of residual

neoplastic cells and should facilitate the development of more effective therapies targeted against this critical population of cells in patients.

Expected Research Outcomes and Benefits

Breast cancer is typically treated by resection of the primary tumor followed by treatment with some combination of radiation, chemotherapy, and adjuvant hormonal therapy. Depending upon a spectrum of factors including tumor size and grade, lymph node status, and histological characteristics, patients will either remain disease-free following this course of treatment, or else develop recurrent breast cancer—either locally or at distant sites in the body. While the pathways leading to the initial growth of primary breast cancers have been extensively studied, far less is known about the genes and processes that govern the ability of tumor cells to survive and recur following therapy. This paucity of information is due in large part to the lack of animal models suitable for studying this critically important process.

This research strategy should help to illuminate the biology of the critical population of tumor cells that survives molecularly targeted therapies and that ultimately give rise to tumor recurrence. This project will improve our understanding of breast cancer in several important ways. First, understanding the mechanisms by which tumors recur should aid in the diagnosis and clinical management of breast cancer. Furthermore, the tumor markers and pathways identified will represent potential targets for therapeutic intervention. Drugs targeting pathways that promote survival of residual tumor cells are most likely to extend the survival of patients with dormant, residual disease.

In summary, we have developed a novel set of genetically engineered mouse models to study tumor progression and have coupled these with a unique approach to study the biology of tumor cells that are important for recurrence. This research strategy should provide important insight into the biology of breast cancer recurrence, a process that receives scant attention and remains poorly understood, but that is critically important for the survival of patients with breast cancer.

Summary of Research

Residual lesions are observed in tumor bearing mammary gland wholemounts

The latency of primary tumor formation in MMTV-rtTA/TetO-neu (MTB/TAN) bitransgenic mice dosed on 2 mg/mL doxycycline is 48 days. We have reported transgene-independent tumor recurrence following doxycycline withdrawal and full regression occurring as early as 50 days and even longer than 200 days post-deinduction. After deinduction of the primary tumor and prior to spontaneous tumor recurrence, there is a period where the mammary tumor is fully regressed and the tumor is non-palpable. Wholemounts harvested at and beyond 28 days post-deinduction in tumor bearing mammary glands revealed a visible residual lesion that was not present in non-tumor bearing mammary glands (Fig. 1A). Residual lesions were distinctly visible in glands harvested as long as 112 days after full regression of the tumor.

Residual lesions are also prominent in carmine-stained tumor-bearing wholemount glands (Figs. 1B, 2A). Although the average latency of tumor induction is 48 days, glands that have been

induced with 2 mg/mL doxycycline for a period as long as 56 days show no evidence of a residual lesion in mice in which there was no tumor formation (Fig. 1C). Glands induced with doxycycline showed epithelial hyperplasia at early time points up to 15 days, but after that period glands appear normal until a focal tumor is identified. This further suggests that residual lesions are the result of focal tumor formation and not from doxycycline-induced epithelial hyperplasia.

To locate and identify residual tumor cells in lesions, we developed an orthotopic model. MTB/TAN primary tumors were harvested and the isolated neu-driven epithelial tumor cells labeled with a green fluorescent histone (H2B-GFP) binding protein. Recipient mice maintained on doxycycline were injected with 2x10^5 MTB/TAN cells in the #4 mammary gland. After the formation of tumors, recipient mice were deinduced and the glands examined for evidence of residual lesions. Tumor bearing glands were positive for residual lesions (Fig. 1D, 1E), which were absent in control PBS-injected glands (Fig. 1F). This further supports the conclusion that lesions are the residue from focal tumor formation and not from doxycycline induction or injury at the injection site.

Importantly, residual lesions appeared morphologically identical to the lesions in the inducible bitransgenic model. In addition, GFP-labeled cells were located solely within residuals lesion and were not observed outside of the lesion or in any other compartment of the gland (Fig. 1I). As such, this orthotopic model in which tumor cells are labeled with a nuclear H2B-GFP protein (Fig. 1G) enables the identification of tumor cells from the stromal and endogenous epithelial cells present in mammary glands bearing residual lesions (Fig. 1H).

Characterization of residual lesions

Hematoxylin and eosin (H&E) staining of residual lesions revealed an architecturally abnormal microenvironment populated by numerous cell types, including epithelial, mesenchymal, endothelial, and immune cells, surrounded by an abnormal stroma (Fig. 2B, 2D, 2E). Acinar structures were also observed on occasion (Fig. 2C), but generally the environment of the lesion was characterized by a tortuous and chaotic extracellular milieu surrounding the many cell types present.

Examination of tissue sections containing residual lesions at higher magnification stained by H&E or Masson's Trichrome revealed the heavy deposition of collagen fibers in the microenvironment of the lesion. (Fig. 2F). Collagen was present only in the stromal compartment of MTB/TAN primary tumors, whereas recurrent tumors expressed collagen throughout the tumor, reflecting an abundant stromal component.

Further investigation by immunofluorescence with antibodies specific for collagen type I versus collagen type IV revealed the collagen present in the lesion to be type I, which was uniformly distributed throughout the lesion (Fig. 2G). Type IV collagen was found at the boundaries of the residual lesion (Fig. 2H). Strikingly, however, type IV collagen was not found in contact with H2B-GFP-positive tumor cells, which were confined within the collagen type I-positive matrix.

Macrophages are abundant in residual neoplastic lesions

Collagen has been reported to be deposited by fibroblasts as well as leukocytes. Given the low number of Fsp-1-positive fibroblasts observed in residual lesions, as well as the presence of hemosiderin in many of the lesions, we considered the possibility that macrophages were present within residual lesions.

To identify macrophages, we performed immunohistochemistry on residual neoplastic lesions using the murine anti-macrophage antibody, F4/80. We first quantified macrophages as a percentage of total cells in normal mammary adipose tissue and stroma surrounding terminal end buds, where they have been reported to reside. We then compared the percent of macrophages present in primary recurrent tumors to the percent present in the residual lesion.

In the stroma of the nulliparous mammary gland, macrophages were found to represent 7.6% of total cells present, although this percentage increased to 9.7% at terminal end ducts (Fig. 3A). Macrophages are required for terminal end bud elongation during puberty. Within MTB/TAN primary tumors, macrophages constituted as few as 1% of total cells present within epithelial regions, however in the stromal compartment of the primary tumor this number increased to 17.3% (Fig. 3B). Recurrent tumors showed no such compartmentalization and the percent of F4/80-positive cells was 19.7% throughout the entire tumor (Fig. 3C). Remarkably, F4/80-positive cells represented 22% of the total cells present in residual lesions (Fig. 3D). This unexpected finding raises the possibility that the innate immune response may play a critical role in the mechanism of tumor recurrence.

Residual lesions are well-vascularized

To investigate the status of perfusion and vascularization of residual lesions, we performed a series of immunofluorescent studies using the antibody CD31(PECAM), which is specific for endothelial cells (Fig. 4). Both primary and recurrent tumors in intact bitransgenic and orthotopic models were found to be well-vascularized with only rare areas of necrosis evident in large tumors. Tumors were positive for CD31 expression throughout the lesion, consistent with the lack of necrosis observed in H&E stained sections of lesions. These findings suggest that angiogenesis may not be a rate-limiting step for tumor recurrence in our model system.

To characterize the properties of residual tumor cells present in the mammary glands of previously tumor-bearing mice, we developed an orthotopic model based upon neu-driven epithelial tumor cells labeled with a green fluorescent histone (H2B-GFP) binding protein. Recipient mice maintained on doxycycline were injected with 2x10^5 MMTV-rtTA/TetO-neu cells in the #4 mammary gland. After the formation of tumors, recipient mice were deinduced and the glands examined for evidence of residual lesions. Tumor bearing glands were positive for residual lesions, which were absent in control PBS-injected glands. Residual lesions appeared morphologically identical to the lesions in the intact inducible bitransgenic model previously described by our laboratory. In addition, GFP-labeled cells were located solely within residuals lesion and were not observed outside of the lesion or in any other compartment of the gland. This orthotopic model in which tumor cells are labeled with a nuclear H2B-GFP protein, which enables the identification of tumor cells from the stromal and endogenous

epithelial cells present in mammary glands bearing residual lesions, was used for the following experiments to characterize residual tumor cells.

Rare GFP-labeled tumor cells are positive for basal epithelial markers

In order to assess and characterize markers expressed by residual GFP-labeled tumor cells in the lesion, a series of immunofluorescence experiments were performed in the above orthotopic model. MTB/TAN primary tumor cells are predominantly positive for the luminal epithelial marker cytokeratin 8 (CK8). There are areas of the primary tumor in which the myoepithelial marker CK14 is also highly expressed, and some CK14-positive cells are also positive for CK8. Other areas of the primary tumor have rare single CK14 positive cells and these two expression patterns reflect the heterogeneity of MTB/TAN tumors. Recurrent tumors had very rare, low-expressing CK8 positive cells. CK14 was expressed at higher levels than CK8, but again, rare cells were positive. The residual lesion reflects the status of the recurrent tumor more than the primary tumor. There are a small number of CK8 light-positive cells in the residual lesion in the bitransgenic model, but in the orthotopic model no GFP positive cells have been observed as CK8 positive. In the residual lesion there are a higher number of CK14-positive cells and some individual cells, though not all, are also GFP-positive (Fig. 5A). Single, individual GFP positive cells are double-positive for CK5 (Fig. 5B), and a slightly smaller number are positive for CK6 (Fig. 5C).

GFP-labeled residual tumor cells are negative for mesenchymal and stromal markers

The morphology of MTB/TAN primary tumors is epithelial, but this changes to a mesenchymal morphology in recurrent tumors. Therefore, we investigated the following markers for mesenchymal cells. GFP positive cells are negative for PDGFR-alpha (CD140-alpha), although PDGFR-alpha is expressed primarily as a border around the residual cells in the lesion (Fig. 5D). The GFP positive cells are nestled in a region clearly negative for PDGFR-alpha. Nevertheless, GFP-positive tumor cells are seen to express PDGFR-alpha in the recurrent tumor.

In the residual lesion, immunoreactivity for alpha-smooth muscle actin (α-SMA) showed a pattern similar to primary tumors displaying an irregular structure and patterning. However, none of the GFP-positive cells have been observed to co-express α-SMA (Fig. 5E). Fsp-1 positive cells were present in the stromal regions of the primary tumor and were found uniformly throughout the recurrent tumor. In the orthotopic residual lesion, GFP-positive tumor cells do not express Fsp-1. Residual lesions consistently expressed abundant extracellular matrix proteins, including fibronectin and vimentin (Fig. 5F) and residual lesions could thereby be identified by the high and contrasting expression amongst the mammary stroma.

GFP-labeled tumor cells in the residual lesion are non-proliferating

To examine the proliferation status of the tumor cells in the residual lesion, we examined the lesion by immunoreactivity with the antibody Ki-67 (Fig. 6). In the orthotopic primary tumor, where as many as 60% of the cells are GFP positive, Ki-67 positivity accounts for up to 50% of the cells. However, only ~16% of GFP-positive cells were Ki67-positive. In the orthotopic

residual lesion, where GFP-positive cells can account for up to 37 % of the cells present, none of the residual tumor cells were positive for Ki-67 (Fig. 6).

To further investigate the cycling status of the residual cells over a longer time period, a 7-day BrdU label of the lesion after full regression of the primary tumor was performed. This revealed that, despite the longer labeling period, no GFP-positive cells were labeled with BrdU. As many as 6% of the surrounding cells in the mammary gland are labeled with BrdU which reflects normal turnover. Consequently, residual lesions could be identified by the lack of BrdU label in the area compared to the surrounding normal mammary gland stroma.

Residual neoplastic disease in intact deinduced MMTV-rtTA/TetO-Ras mice

Given that fully regressed MTB/TRAS tumors recur spontaneously in the same location of the original tumor, this raised the possibility that residual neoplastic cells remained following deinduction that serve as target cells for tumor recurrence that we could visualize in intact animal models. We therefore examined deinduced, fully regressed MTB/TRAS tumors histologically and immunocytochemically for evidence of residual neoplastic disease. We observed that mammary glands bearing tumors that had fully regressed in response to dox withdrawal, lacking any palpable outgrowths still contained microscopic lesions that may represent residual tumor cells that fail to be cleared upon deinduction (Fig. 7A). These cells retained luminal epithelial character as evident by staining for Cytokeratin 18 (Fig. 7A). Typically, there was a large increase in hemosidarin deposits present by histological staining (Fig. 7A). Changes in myoepithelial content were not evident based upon Ck14 staining (Fig. 7B). By contrast, non-tumor bearing glands harvested from these mice did not display any such lesions by histological staining or by Ck18 immunofluorescence (Fig. 7A)

As both primary and recurrent tumors overexpress the *Ras* transgene relative to MTB/TRAS hyperplastic epithelium (Fig. 7B), we questioned whether these residual lesions overexpressed oncogenic Ras, or whether overexpression of Ras was detectable exclusively in actively growing tumor cells. Bioluminescence *in vivo* suggested that *Ras* transgene levels were completely down-regulated several weeks after deinduction, but were re-activated at a point prior to tumor recurrence. Upon staining for total Ras protein at a time point near the median tumor recurrence latency, we found that these lesions expressed Ras at levels comparable to either primary or recurrent tumors, and significantly higher than surrounding tissue or non-tumor-bearing glands (Fig. 7B). However, despite this overexpression of Ras, the rates of proliferation within lesions were dramatically lower than in either primary or recurrent tumors, as shown by BrdU incorporation (Fig. 7B). This suggested transgene reactivation occurs prior to recurrence, but is insufficient to re-stimulate proliferation within residual disease lesions.

We next questioned whether direct re-activation of the *Ras* transgene could accelerate proliferation of residual lesions. We therefore re-induced mice bearing fully regressed tumors with 0.012 mg/mL dox for 48 hrs. Upon re-induction, we did observe an increase in proliferation in residual lesions containing high Ras expression, similar to levels present in recurrent tumors (Fig. 7B). This could indicate that other cells must re-activate the *Ras* transgene, beyond those observed in fully regressed, non-reinduced glands (Fig. 7B). It is noteworthy that levels of total Ras protein in residual lesions reinduced with doxycycline are

comparable to levels in deinduced residual lesions that have reactivated the *Ras* transgene, suggesting that the effects observed are not due to alterations in transgene expression levels in the residual neoplastic cells themselves (Fig. 7B).

Tumor cells within the MRL express mesenchymal markers

Minimal residual disease (MRD) in breast cancer patients is detected in bone marrow using markers specific to epithelial cells. While some clinical studies suggest that residual breast cancers in humans following chemotherapy display a mesenchymal phenotype, the fraction of tumor cells that undergo epithelial-mesenchymal transition (EMT) following therapy in clinical scenarios is difficult to measure. Additionally, whether residual disease from HER2/neu-driven tumors displays mesenchymal markers following targeted therapy remains unknown.

In order to address residual disease following targeted oncogene inhibition, we chose to investigate residual disease in a previously described model of HER2/neu-driven tumorigenesis, MTB/TAN bitransgenic mice. The proto-oncogene HER2/neu is amplified and overexpressed in up to 30% of human breast cancers, and multiple targeted therapies which inhibit HER2 signaling are clinically approved and in wide use. In MTB/TAN mice, doxycycline administration leads to hyperplasia, development of invasive adenocarcinomas and metastasis. Following doxycycline withdrawal, primary tumors in MTB/TAN mice regress and become non-palpable. Intriguingly, these tumors spontaneously recur with variable latency, suggesting a period of tumor dormancy.

We investigated the expression of epithelial and mesenchymal markers on H2B-eGFP labeled MTB/TAN tumor cells in both primary tumors and MRLs using immunofluorescence staining. Staining revealed that primary tumors expressed epithelial markers, including CK8, CD24 and CD49f. These markers were strikingly absent in the majority of tumor cells within the MRL. In the MRL, CK8 was detectable in less than 1/1000 cells, while CD24 and CD49f were detectable in fewer than half of all tumor cells. A panel of an additional ten epithelial markers revealed the absence of common luminal epithelial and myoepithelial markers in residual lesion tumor cells.

More than half of all tumor cells within the residual lesion expressed the mesenchymal markers FSP-1 and CD49e, though these markers were not expressed by cells within the primary tumor. Enrichment of mesenchymal markers within the residual lesion is consistent with clinical descriptions of mesenchymal cells within residual disease.

As tumor cell-stroma interactions may in part regulate dormancy, we investigated the composition of the extracellular matrix (ECM) within the residual lesion. The ligand for CD49e, fibronectin, was abundant within the residual lesion. Additionally, collagen type-I was abundant, but collagen type-IV was absent, reminiscent of ECM changes associated with scarring. However, fibroblasts within the MRL expressed PDGFRα, but not αSMA, indicating that they are not myofibroblasts found within scars and remain similar to fibroblasts normally associated with mammary ducts.

Tumor cells within minimal residual lesions are dormant and not senescent

Clinical data suggest that residual disease following therapy is dormant. Conclusive demonstration of dormancy is challenging to demonstrate in MRD in humans, however, as the

specific population of cells responsible for recurrence is unknown in breast cancer. Growth kinetics of spontaneous tumor recurrence in both MTB/TAN mice and nude mice with orthotopic MTB/TAN tumors suggest that a period of post-treatment dormancy precedes recurrence, analogous to that seen clinically. Therefore we hypothesized that the latency of tumor recurrence may be explained by dormancy within MRLs.

Consistent with a role for cellular dormancy in this model, the percentage of GFP+ cells expressing Ki67 in the MRL either 28 or 56 days after doxycycline was less than 0.1%, compared to over 10% in the primary tumor (Fig. 8A, D). As tumors can remain non-palpable for extended periods of time, we hypothesized that the extremely low rate of tumor cell proliferation within minimal residual lesions is balanced by a low rate of tumor cell apoptosis. Consistent with this, we identified a rare population of tumor cells within minimal residual lesions that expressed the apoptosis marker, cleaved caspase-3.

In light of the extremely low level of proliferation within MRLs, we implanted osmotic pumps loaded with BrdU into mice with MRLs to quantify the number of cells entering the cell cycle over a 2-week period. An average of 8% of the tumor cells within MRLs entered S phase over a 2-week period, compared with over 90% in the primary tumor (Fig. 8B, E). The ratio of Ki67+ cells in the MRL to the primary tumor (~1:100) is much lower than the ratio of 2-week BrdU uptake for the same populations (1:9). This suggests that the rare cells within MRLs that incorporate BrdU enter the cell cycle less frequently than cells in the primary tumor.

Both senescence and dormancy can explain cell cycle arrest in tumor cells, however senescent cells are incapable of re-entering the cell cycle. In order to functionally test whether tumor cells in the MRL are dormant and not senescent, we re-administered doxycycline for 3 days to MRLs, 28 days after initial doxycycline withdrawal. We found that the proliferation rate in the MRL following re-induction is identical to that seen in primary tumors, suggesting that tumor cells within the MRL were not senescent (Fig. 8C, F).

While this functionally demonstrates that many of the residual tumor cells are dormant, it is possible that a sub-population of MRL tumor cells are senescent. To ascertain whether any tumor cells within the MRL are senescent, we performed immunofluroescence for p16Ink4a, a marker for senescence and cell cycle arrest. No tumor cells within MRLs expressed p16, indicating that tumor cells within MRLs are not senescent (Fig. 8G)

<u>Minimal residual lesions are well-vascularized with functional vasculature and are not hypoxic</u>
Several models have indicated that insufficient angiogenesis is a major mechanism regulating dormancy both in primary tumor development and following oncogene downregulation. It is unknown whether post-treatment residual disease and tumor relapse are limited by angiogenesis.

We used immunofluorescence staining to determine whether MRLs may be growth-limited by a lack of vascularization. Surprisingly, MRLs were well-vascularized and contained many contiguous CD31+ blood vessels (Fig. 9A, B). In order to assay whether these represent functional vessels, we injected Lectin-Streptavidin-AF647 intravenously prior to sacrifice. Over 90% of CD31+ blood vessels were labeled with Lectin-Streptavidin-AF647, demonstrating that CD31+ vessels are well-perfused.

To determine whether diffusion of small molecules from blood vessels into the MRL is a limiting factor for nutrient supply to MRLs, we injected Lectin-Streptavidin-AF647 in combination with Hoechst 33342. Tissue sections from mice injected with Lectin-Streptavidin-AF647 in combination with Hoechst 33342 revealed that small molecules can diffuse from these blood vessels into tumor cells within the MRL (Fig. 9C). This suggests that tumor cells within MRLs are not hypoxic.

In order to directly test whether MRLs are hypoxic, pimonidazole was injected with Hoechst 33342 in order to directly assess whether MRLs are hypoxic. Pimonidazole forms covalent adducts in hypoxic microenrivonments (pO2<10mmHg), and is sufficiently sensitive to detect physiological hypoxia surrounding hepatic veins. Though pimonidazole was present within low-oxygen regions of the liver adjacent to venous vasculature (Fig. 9G, arrowhead), pimonidazole did not bind within the MRL in the same mouse, indicating that these MRLs are not hypoxic.

<u>MTB/TWNT and MTB/TOM tumors leave MRLs following tumor regression</u>
While up to 30% of human tumors overexpress HER2/neu, the oncogenes that drive other breast cancers are heterogeneous. The Wnt pathway is implicated in human breast cancer progression and signals through pathways distinct from the HER2/neu pathway. Additionally, the oncogene Myc is involved in numerous oncogenic processes and multiple human cancers, including breast cancer. To test the hypothesis that MRLs result from tumor regression due to targeted therapy against oncogenes other than HER2/neu, we investigated residual disease in doxycycline-dependent WNT1-driven and c-Myc-driven tumors. These models demonstrate spontaneous recurrence following doxycycline withdrawal, suggesting the presence of residual disease.

Consistent with the MTB/TAN model, glands from MTB/TWNT and MTB/TOM mice which previously bore tumors harbored foci that were absent from glands that did not bear tumors (Fig. 10A, B). As tumor cells within MRLs formed from the MTB/TWNT and MTB/TOM lines express the epithelial marker CK8, this marker was sufficient to specifically identify residual tumor cells within these intact models.

As was seen in the MTB/TAN model, tumor cells within the MTB/TWNT and MTB/TOM MRLs do not take up BrdU, suggesting they are dormant (Fig. 10D). Re-administration of doxycycline to previously tumor-bearing MTB/TOM mice indicated that previously tumor bearing glands harbored residual disease that was capable of re-initiating growth upon re-administration of doxycycline. MTB/TWNT residual cells are also not senescent, as they are able to begin proliferating upon re-administration of doxycycline (Fig. 10D, E, data not shown).

Finally, to determine whether vascularization is a common feature in MRLs from tumors driven by different oncogenes, we performed immunofluorescence staining for CD31. This revealed the presence of large blood vessels, indicating that these MRLs are also well-vascularized (Fig. 10C). Erythrocytes within blood vessels were visible on H&E, suggesting that functional vascularization is not a limiting factor for tumor dormancy in either of these models (Fig. 10B).

Aim III. Determine if autophagy is a survival mechanism in residual neoplastic cells.

The anti-HER2 targeted therapy trastuzumab has been reported to induce autophagy in breast

cancer cell lines *in vitro*. Therefore, we wished to determine whether HER2/neu pathway down-regulation induces autophagy *in vivo*. Primary tumor cells were generated from a primary mammary adenocarcinoma that arose in an *MTB/TAN* mouse and cultured in the presence of doxycycline to maintain HER2/neu levels. These cells were then subjected to acute doxycycline withdrawal to induce *HER2/neu* down-regulation. HER2/neu protein levels were rapidly down-regulated following doxycycline withdraw (Fig. 11a). A decrease in HER2/neu expression was accompanied by an acute increase in levels of LC3-II, the cleaved, lipidated form of rat microtubule-associated protein 1 light chain 3 (LC3) that serves as a marker for autophagy. Consistent with this, analysis of these cells by electron microscopy revealed an increase in the number of double-membraned autophagosomes per cell upon doxycycline withdrawal (Fig. 11b,c).

In addition to these steady-state methods of monitoring autophagy, we wanted to determine whether cytoplasmic contents sequestered by the autophagosome were reaching the lysosome and being degredaded in our primary *MTB/TAN* tumor cells. To test this, acute doxycycline withdrawal was combined with chloroquine treatment. Chloroquine (CQ) raises lysosomal pH and inhibits protein degradation within the autolysosome. Cells that have flux through the autophagic pathway show an additional increase in LC3-II levels when treated with chloroquine. The combination of *HER2-neu* down-regulation and chloroquine treatment resulted in an increase in LC3-II in primary *MTB/TAN* tumor cells compared to cells undergoing acute *HER2/neu* down-regulation alone (Fig. 11a).

These results suggest that acute *HER2/neu* down-regulation induces autophagy as well as flux through the autophagic pathway. To further confirm these results, we generated a *MTB/TAN* cell line that stably expressed the autophagy marker EGFP-LC3. Induction of autophagy induces the incorporation of cleaved, lapidated LC3-II into autophagosomes, which gives rise to a change in LC3 subcellular localization from diffusely cytoplasmic to a punctate distribution. Doxycycline withdrawal from EGFP-LC3 expressing primary tumor cells resulted in an increase in the number of EGFP-positive punctae per cell compared to cells grown in the presence of HER2/neu (Fig. 11d,e). Together, these results indicate that acute *HER2/neu* down-regulation triggers autophagy *in vitro*.

We next asked whether acute *HER2/neu* down-regulation induces autophagy *in vivo*. HER2/neu expression was deinduced in primary *MTB/TAN* mammary tumors by doxycycline withdrawal for 48 hours and p62 levels were analyzed by immunoblotting. p62 (also known as SQSTM1) recognizes ubiquitin-marked proteins and sequesters them for degradation through autophagy. As a consequence, p62 itself is degraded in cells undergoing autophagy. Consistent with our *in vitro* observations, acute *HER2/neu* down-regulation resulted in a reduction in total p62 protein levels compared to primary tumors (Fig. 12a). The *in vivo* induction of autophagy by oncogene down-regulation was further supported using primary tumors generated from *MTB/TAN* tumor cells expressing EGFP-LC3. These tumors exhibited an increase in the number of EGFP-positive punctae per tumor cell following acute *HER2/neu* down-regulation (Fig. 12b). These results suggest that autophagy is induced in actively growing primary tumor cells following acute *HER2/neu* down-regulation.

While autophagy has been shown to occur *in vitro* in actively growing tumor cells subjected to multiple cellular stresses, whether autophagy occurs in dormant tumor cells *in vivo* has not been established. We have demonstrated that a small number of primary *MTB/TAN* tumor cells survive *HER2/neu* down-regulation and persist in a dormant state within residual lesions within the mammary gland, where they remain competent to resume growth, resulting in recurrent tumors. Analysis of EGFP-LC3-labeled dormant tumor cells in residual lesions *in vivo* by fluorescence microscopy revealed a greater number of EGFP-positive punctae per cell compared to actively growing orthotopic primary tumors (Fig. 12b). Together, these observations suggest that autophagy is triggered in tumor cells *in vivo* following acute *HER2/neu* down-regulation and that dormant residual tumor cells undergo autophagy *in vivo*.

Our observations that autophagy occurs *in vivo*, is triggered by acute *HER2/neu* down-regulation in actively growing primary tumor cells, and persists in dormant mammary tumor cells, were equally consistent with models in which autophagy was either tumor suppressive or tumor promoting. To begin to distinguish between these possibilities, we treated *MTB/TAN* tumor-bearing mice with chloroquine. Chloroquine is an attractive approach to inhibiting autophagy *in vivo* since this drug has a favorable therapeutic index and has been used safely in millions of people worldwide for the prevention and treatment of malaria. Additionally, in this experimental context use of a therapeutic agent permits the temporally controlled inhibition of autophagy in existing primary tumors in mice, while avoiding complicating effects of autophagy inhibition during primary tumor development and growth. To determine the effect of chloroquine treatment on mammary tumor recurrence, female *nu/nu* mice maintained on doxycycline were injected with primary *MTB/TAN* tumor cells. Once primary tumors formed, tumor regression was induced by doxycycline withdrawal and *HER2/neu* down-regulation as previously described and, coincident with doxycycline withdrawal, daily treatment with chloroquine was initiated (Fig. 13a). Mice bearing fully regressed primary tumors were then monitored for recurrence. This revealed that daily chloroquine administration markedly delayed the onset of tumor recurrence in mice, with the mean latency for tumor recurrence increasing from 84 to 140 days (H.R. = 2.27 [1.45-6.72], $P = 0.007$; Fig. 13b). This findings suggested the possibility that autophagy promotes, rather than inhibits, mammary tumor recurrence.

To extend these results, we determined the effect on tumor recurrence of genetically inhibiting autophagy by knocking down the expression of *Atg5* or *Atg7*, each of which is required for autophagy. Atg5 and Atg7 are components of an ubiquitin-like conjugation system wherein the E1-like molecule Atg7 and the E2-like molecule Atg10 covalently link Atg5 to Atg12. Atg5-Atg12 then forms a complex with Atg16 that is necessary for the formation of the autophagosome. To determine if inhibiting autophagy genetically delays tumor recurrence, primary *MTB/TAN* tumor cells were generated that expressed shRNAs targeting either *Atg5* or *Atg7*. qRT-PCR and immunoblotting confirmed knockdown of *Atg5* and *Atg7* (Fig. 14a-d). These cells were then injected orthotopically into the mammary glands of *nu/nu* mice on doxycycline, as were cells transduced with a vector control. As above, once primary tumors reached a target size, doxycycline was withdrawn to induce *HER2/neu* down-regulation, which resulted in the regression of the tumors to a non-palpable state. Mice were then monitored for recurrence. This analysis revealed that genetic inhibition of autophagy by either *Atg5* or *Atg7* knockdown dramatically impaired tumor recurrence (H.R. = 5.35 [1.72-16.62], $P = 0.004$; H.R.= 7.56 [2.33-24.58], $P < 0.001$; Fig. 14e). These results were consistent with results

obtained from the pharmacological inhibition of autophagy by chloroquine, and further suggest that autophagy in this context is tumor promoting insofar as it is required for the recurrence of HER2/neu-induced tumors.

Our results suggest that autophagy is a pro-tumorigenic process that is required for breast cancer recurrence as inhibition of autophagy significantly delays the onset of recurrence in mice. This is contradictory to the multiple lines of evidence that suggest autophagy is a tumor suppressive process in primary breast cancer. In particular, we show that the tumor suppressor Beclin 1 actually promotes HER2/neu-induced mammary tumor recurrence. These context-specific functions of autophagy highlight the differing mechanisms, and therefore potential therapeutic targets, of recurrent cancer compared to primary cancer.

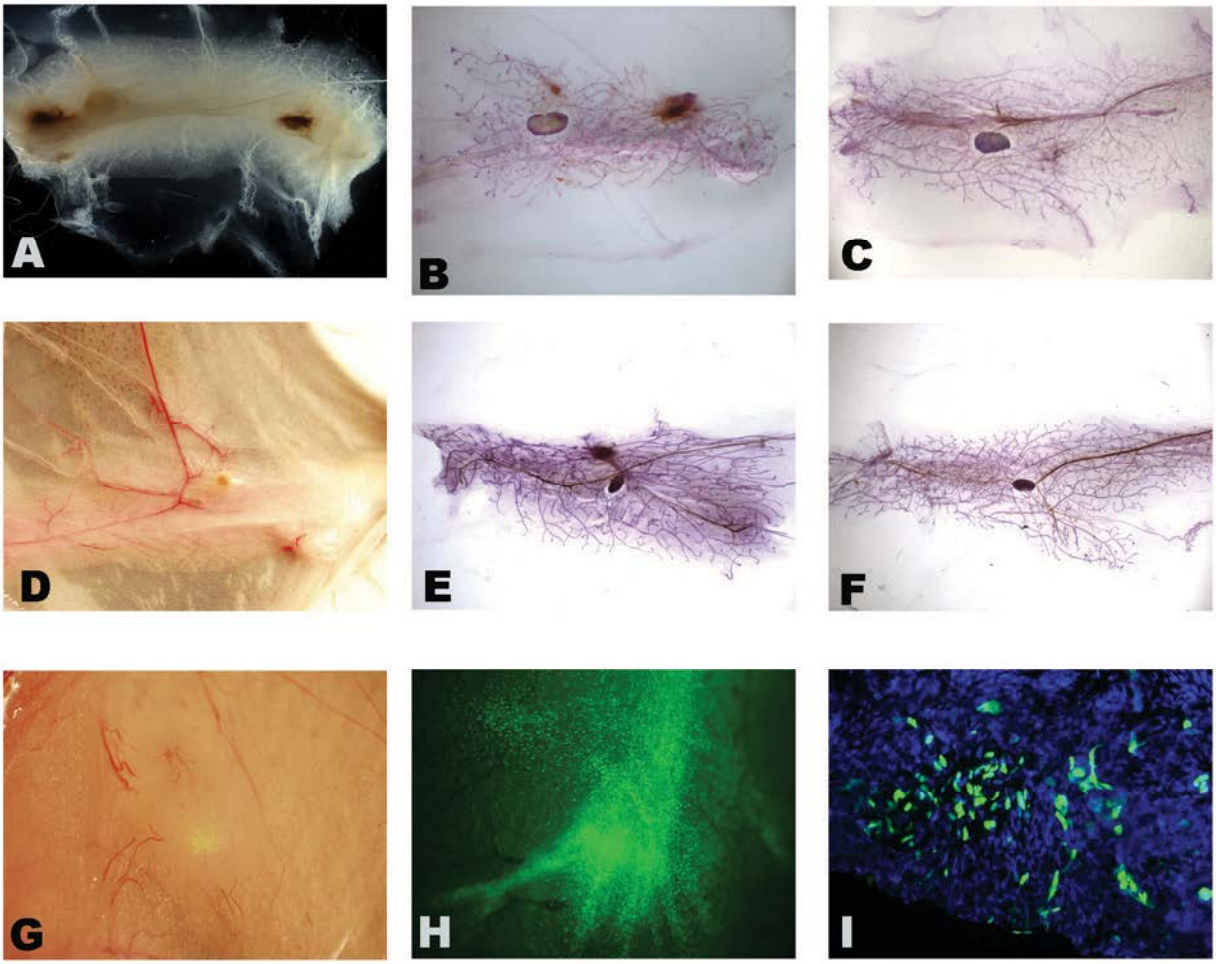

Figure 1: Identification of residual neoplastic lesions and residual tumors cells in mammary glands following HER2/neu down-regulation in mice bearing H2B-GFP-labeled primary orthotopic tumors.

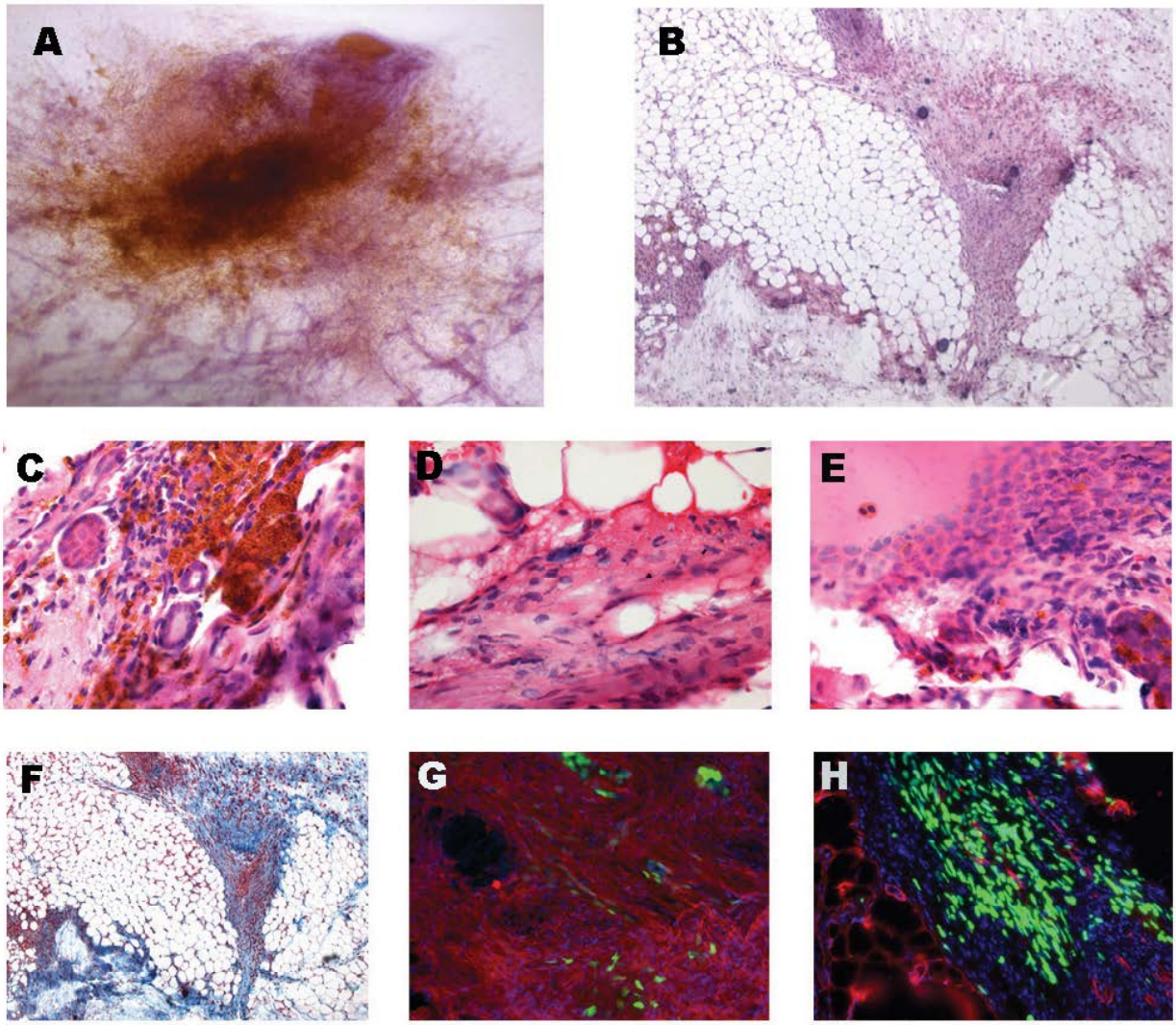

Figure 2: Histological characterization of residual neoplastic lesions.

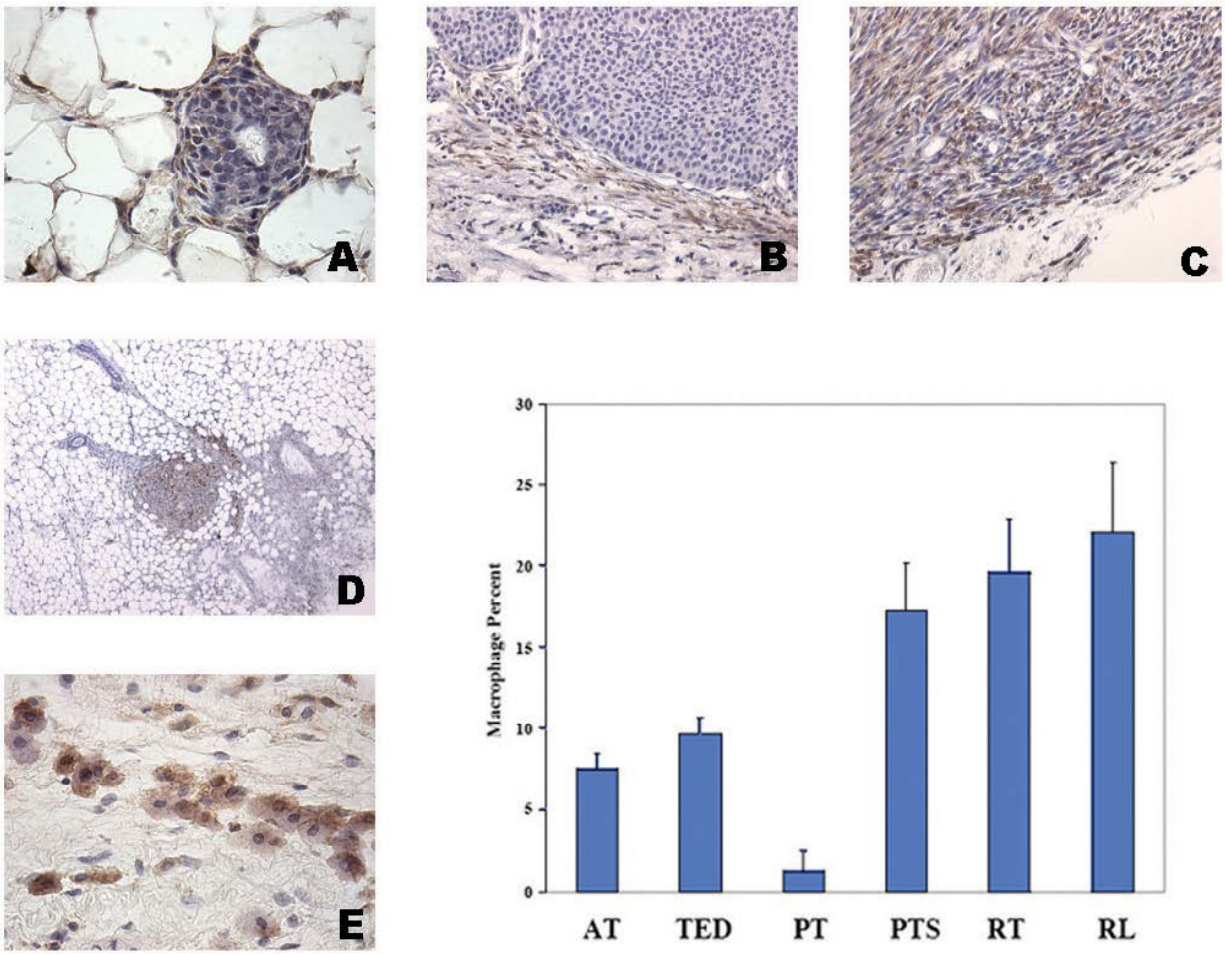

Figure 3: Immunohistochemical demonstration of abundant macrophages in residual neoplastic lesions.

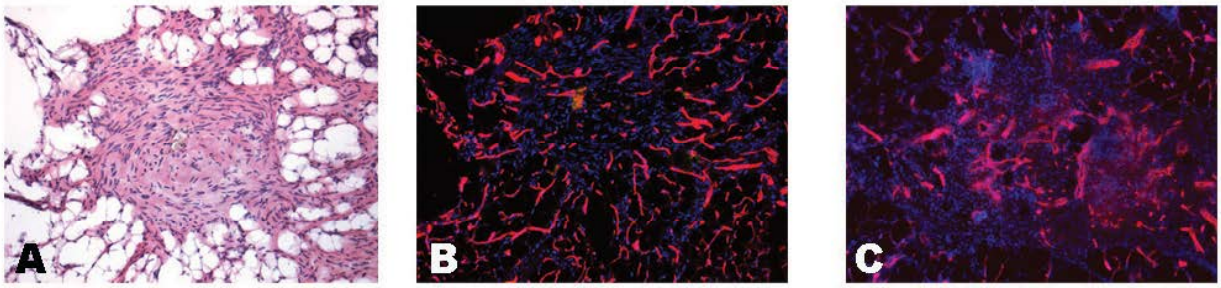

Figure 4: CD31 staining reveals that residual neoplastic lesions are well-vascularized.

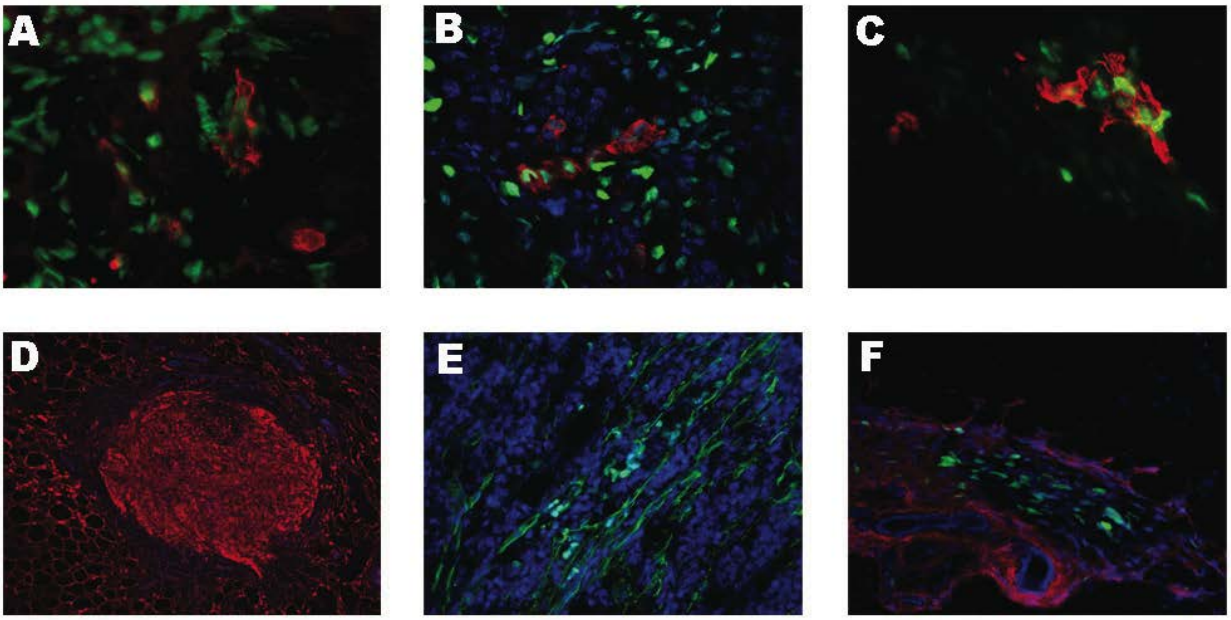

Figure 5 – Immunofluorescence analysis of epithelial and stromal markers in residual lesion. (A) Occasional residual tumor cells (green) express CK14 (red); (B) Occasional residual tumor cells (green) express CK5 (red); (C) Occasional residual tumor cells (green) express CK6 (red); (D) PDGFR-α expression (red) is located around lesion and GFP positive cells are found in center of lesion which is negative for PDGFR-α 200X. (E) α-SMA (green) in residual lesion infiltrates and runs through residual lesion but GFP-positive cells are negative for α-SMA 400X. (F) Residual lesion can be identified by dense fibronectin expression (red). 100X

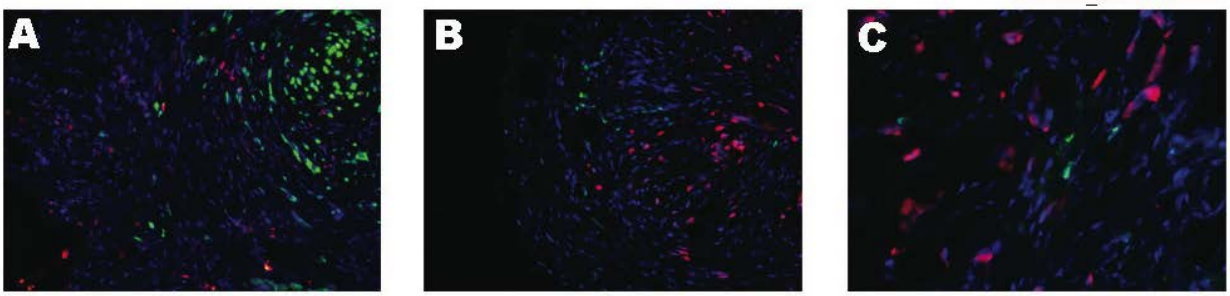

Figure 6 – GFP labeled residual cells are negative for proliferation markers. (A) BrdU labeled cells (red) are predominantly found outside the lesion 200X. (B-C) GFP-labeled cells do not costain with BrdU label (red) 100X.

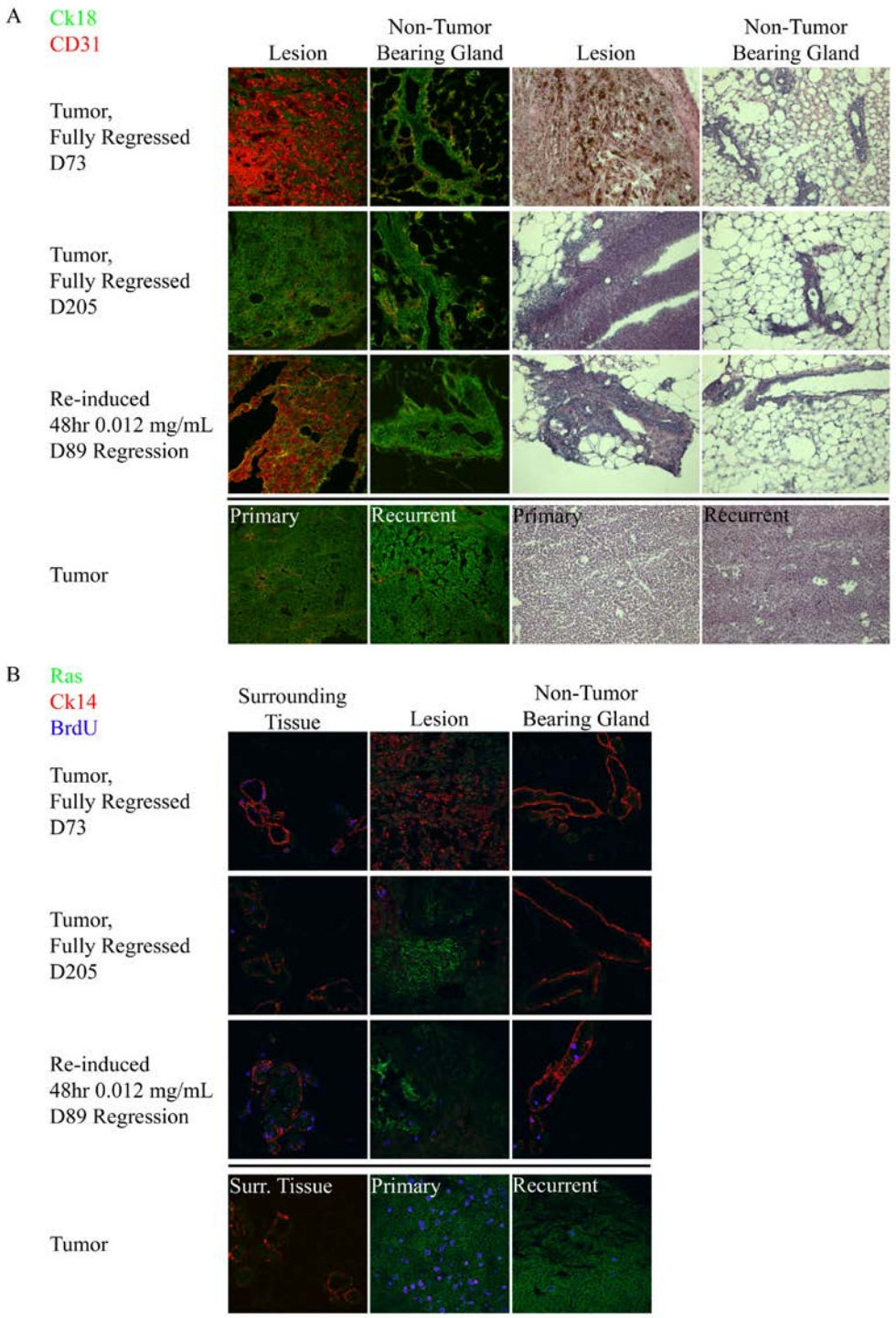

Figure 7 – Residual neoplastic disease in MTB/TRAS mice. (A) Cytokeratin 18 and hematoxylin and eosin staining (10 mm frozen sections) of fully regressed MTB/TRAS tumors deinduced or reinduced with 0.012 mg/mL dox for the indicated times. (B) Immunofluorescent staining for total Ras, Cytokeratin 14, and BrdU in MTB/TRAS mammary glands. Both residual disease lesions and surrounding areas of tumor-bearing glands are pictured.

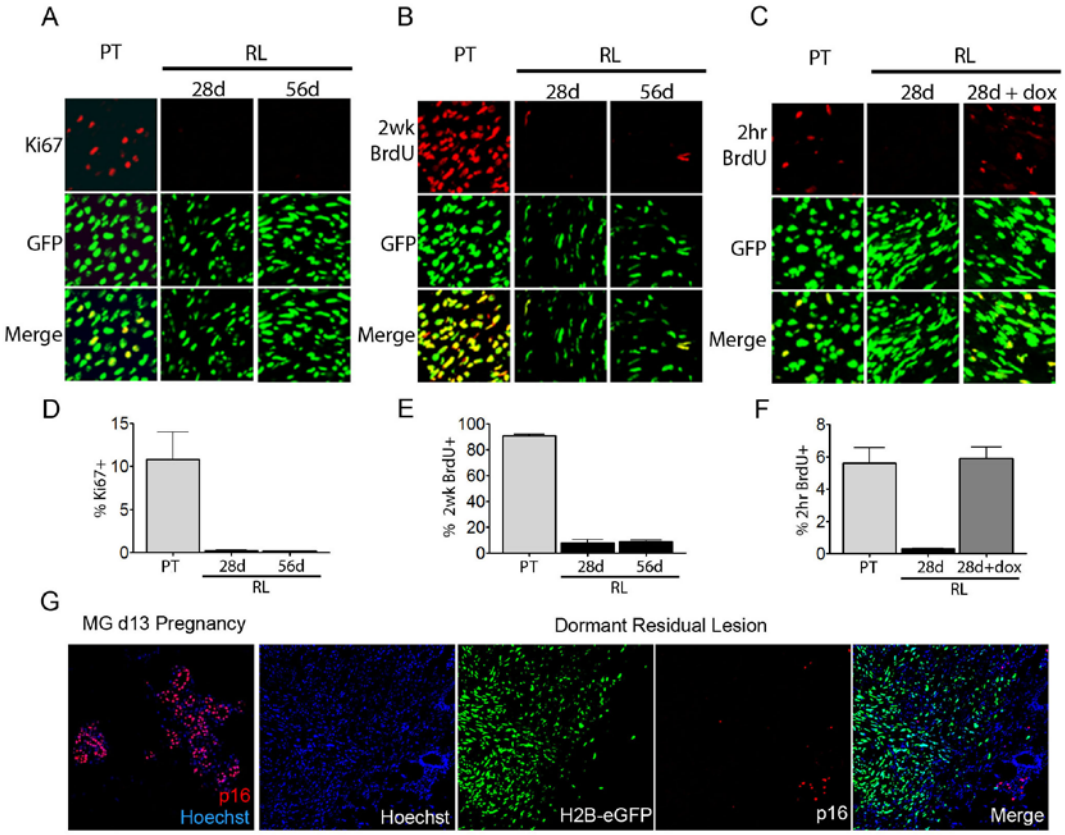

Figure 8. Tumor cells within minimal residual lesions are dormant and not senescent.

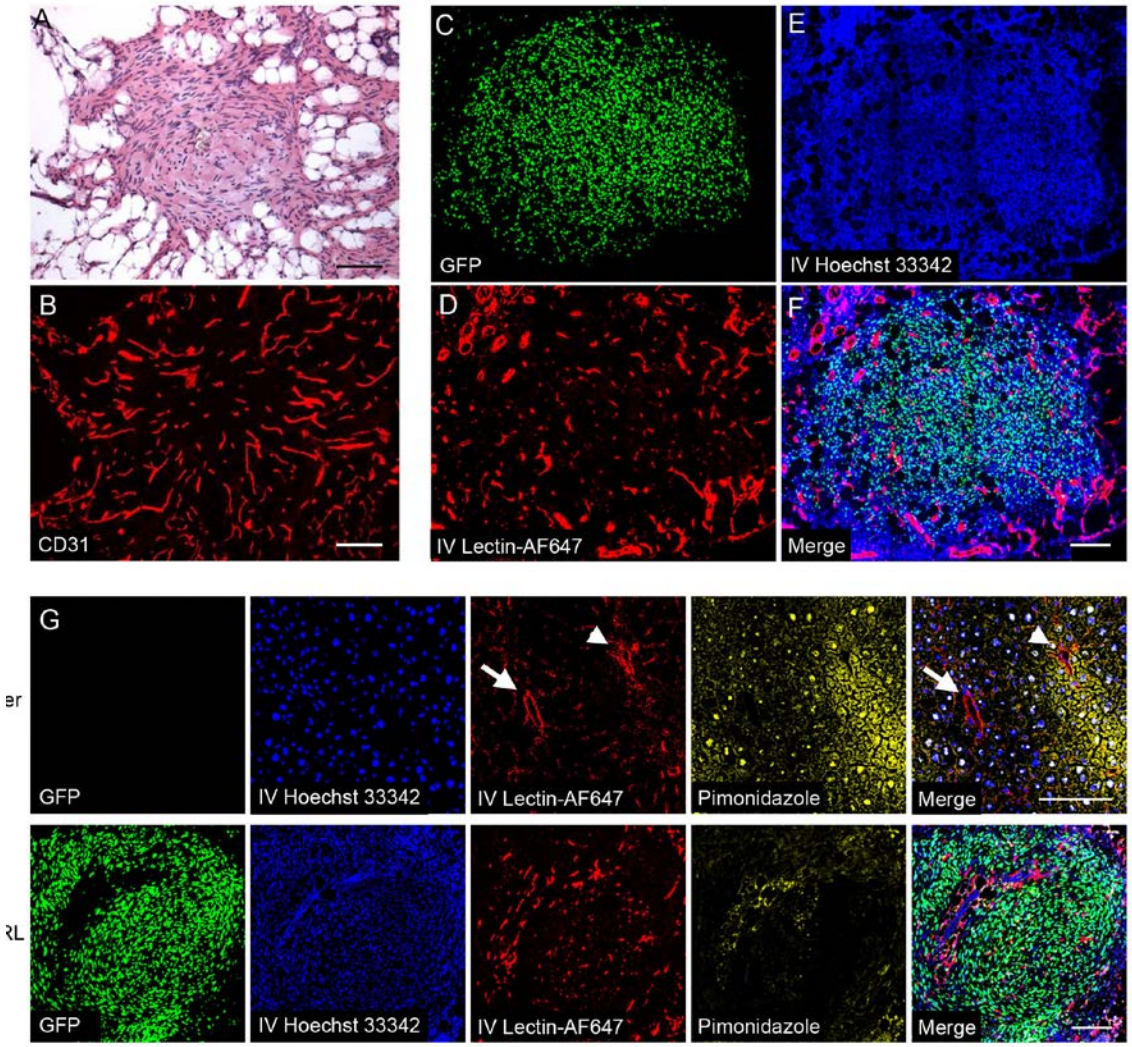

Figure 9. Minimal Residual Lesions are well-vascularized and not hypoxic.

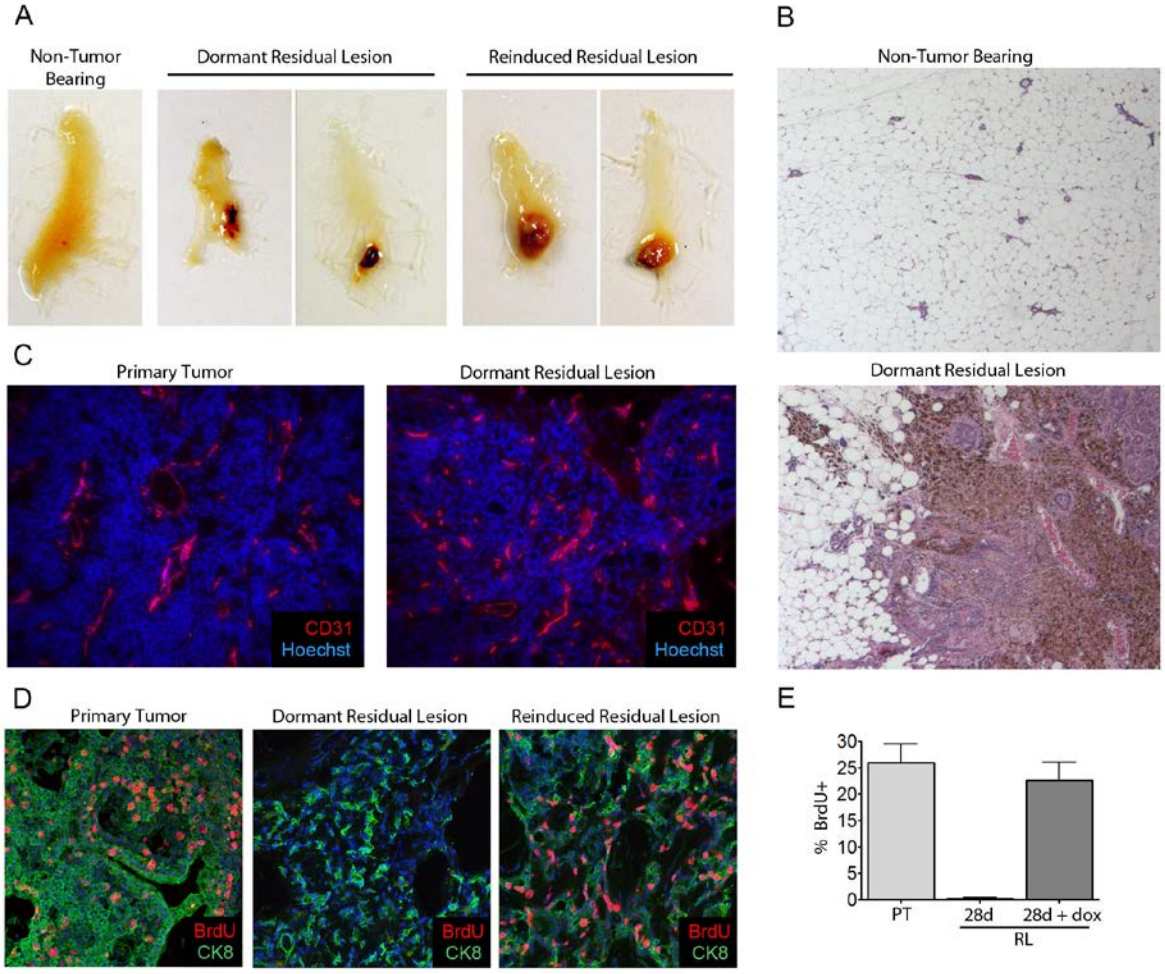

Figure 10. Minimal Residual Lesions in MTB/TWNT mice exhibit cellular dormancy and functional vasculature.

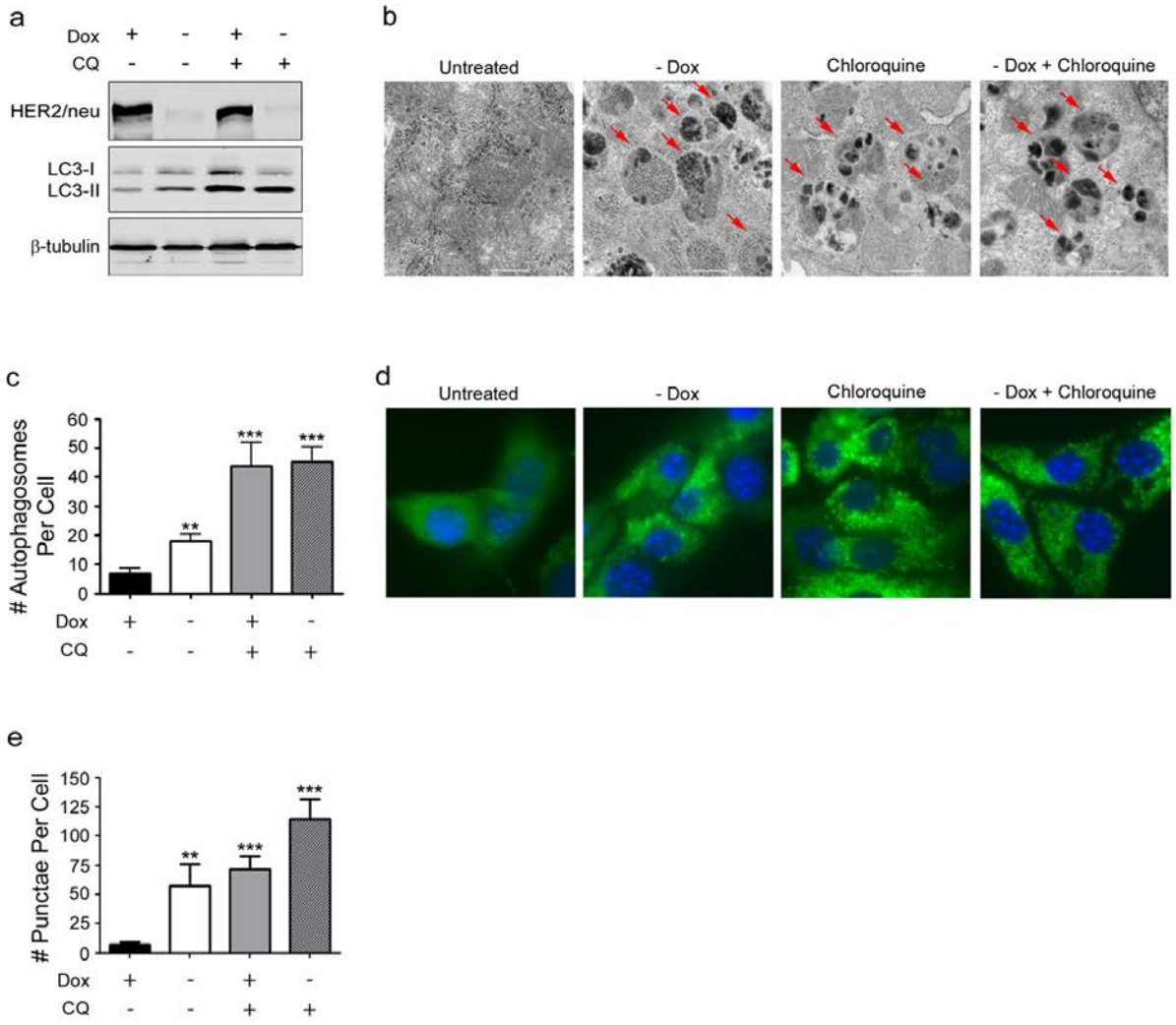

Figure 11 *HER2/neu* down-regulation induces autophagy in primary tumor cells *in vitro*. (a-e) *MTB/TAN* primary tumor cells subjected to doxycycline withdrawal and/or 50 μM chloroquine treatment for 24 h. (a) HER2/neu levels and conversion of LC3-I to LC3-II determined by western blotting. β-tubulin is shown as a loading control. (b) Representative images of double-membraned autophagosomes (arrows) visualized by electron microscopy. Bar = 500nm. (c) Quantification of autophagosomes per cell in (b). (d) Representative images of subcellular localization of EGFP-LC3 visualized by fluorescence microscopy. Original magnification, x400. (e) Quantification of average EGFP-LC3 punctae per cell in (d). Data represent mean ± s.e.m. $^{**}P < 0.001$, $^{***}P < 0.0001$.

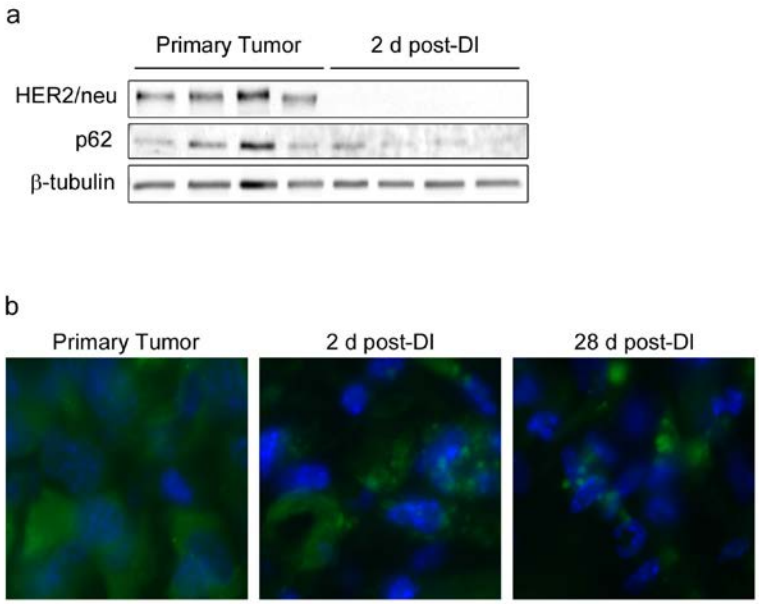

Figure 12 *HER2/neu* down-regulation induces autophagy in primary and dormant tumor cells *in vivo*. (a) HER2/neu and p62 levels evaluated by western blotting in orthotopic *MTB/TAN* primary tumors in the presence of doxycycline or following doxycycline withdrawal for 2 d. (b) Representative images of subcellular localization of EGFP-LC3 in *MTB/TAN* primary orthotopic tumors or orthotopic tumors subjected to 2 d or 28 d of doxycycline withdrawal. Original magnification, x400.

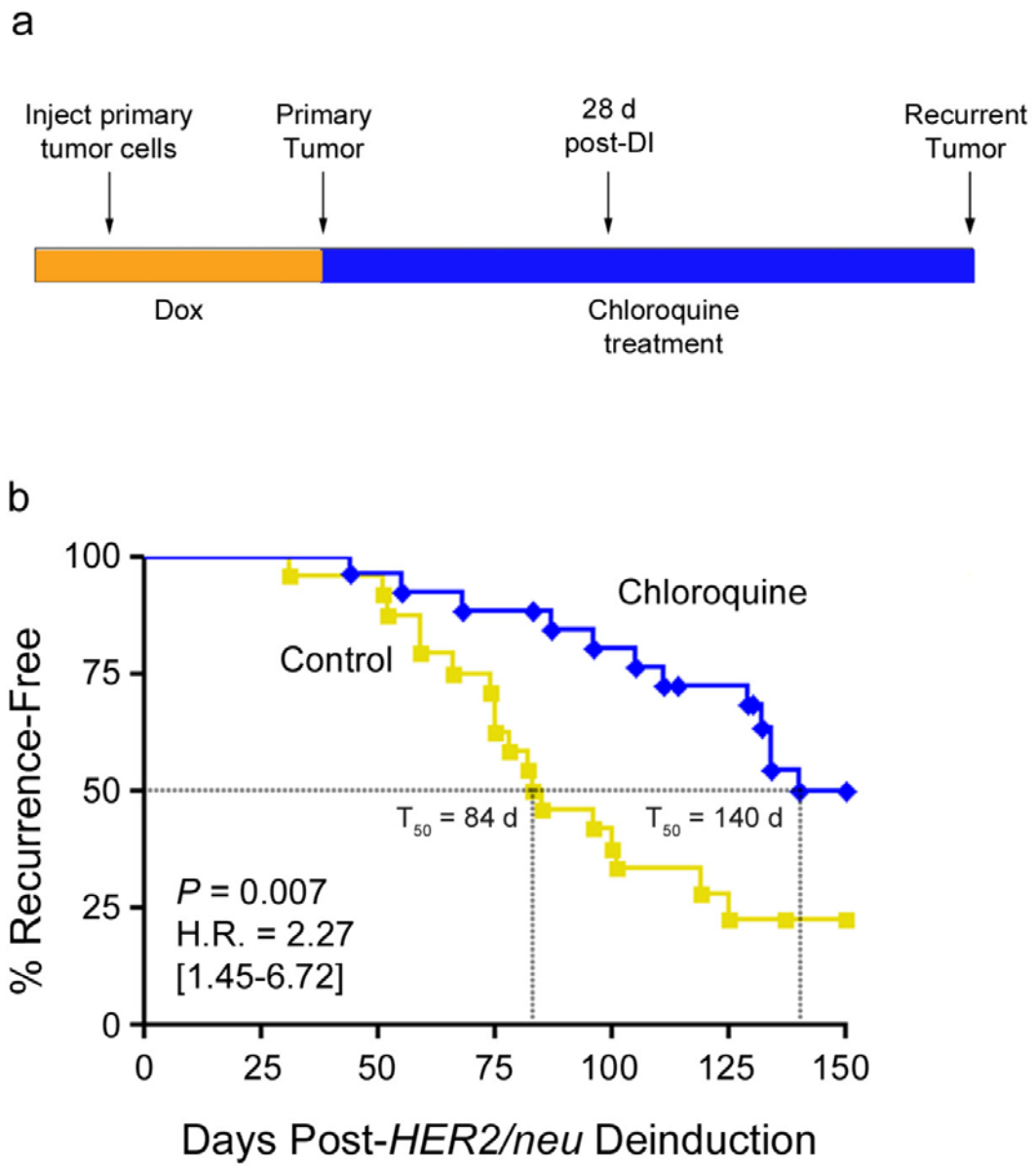

Figure 13. Chloroquine treatment delays recurrence of HER2/neu-induced tumors. (a) Schematic of orthotopic recurrence model and timing of chloroquine treatment. (b) Recurrence-free survival of female *nu/nu* mice harboring *MTB/TAN* orthotopic primary tumors induced to regress by doxycycline withdrawal and treated with vehicle (n = 24) or 60 mg/kg/d chloroquine (n = 26) as described in (a). Mean recurrence latencies indicated.

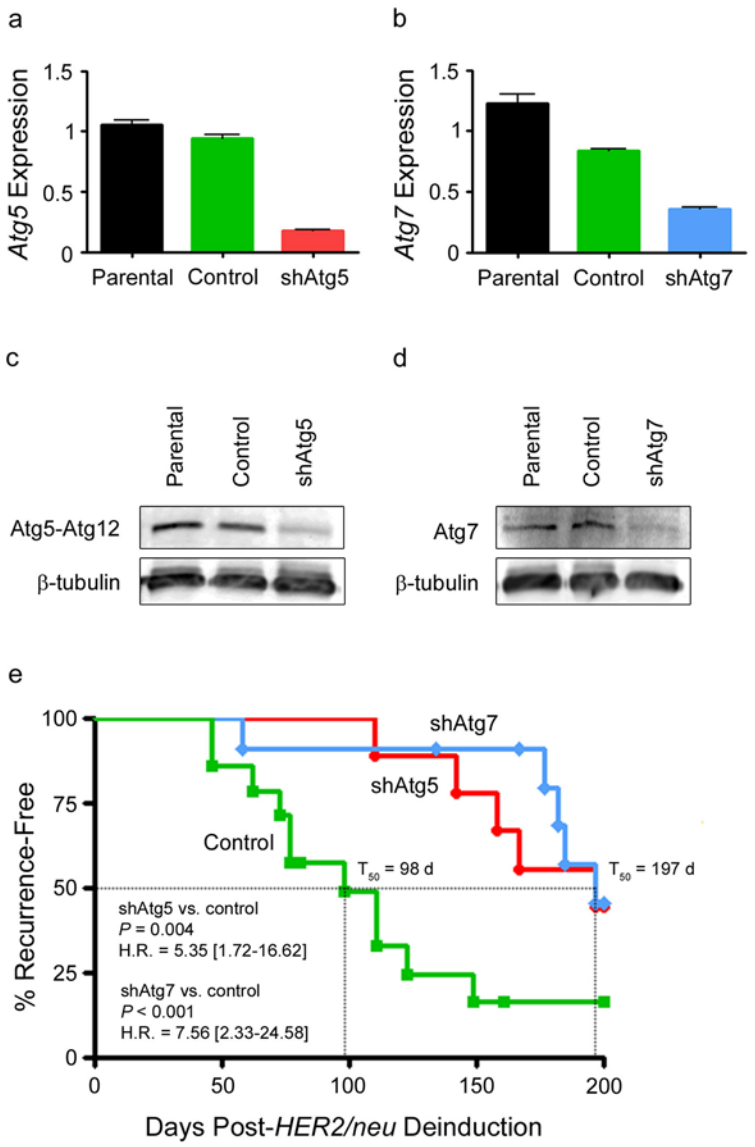

Figure 14 Atg5 and Atg7 are required for mammary tumor recurrence. (a,b) qRT-PCR analysis of (a) *Atg5* or (b) *Atg7* expression levels in parental primary *MTB/TAN* tumor cells, or *MTB/TAN* tumor cells expressing a vector control, or shRNAs targeting *Atg5* or *Atg7* (shAtg5 or shAtg7). Expression is normalized to *Tbp*. Data represent mean ± s.e.m. (c,d) Western blot analysis of (c) Atg5-Atg12 and (d) Atg7 levels in parental primary *MTB/TAN* tumor cells, or vector control, shAtg5, or shAtg7-expressing *MTB/TAN* tumor cells. β-tubulin is shown as a loading control. (e) Recurrence-free survival of female *nu/nu* mice harboring fully regressed orthotopic tumors derived from vector control (n = 14), shAtg5 (n = 9), or shAtg7-expressing *MTB/TAN* tumor cells (n = 11). Mean recurrence latencies indicated.

18. Extent of Clinical Activities Initiated and Completed. Items 18(A) and 18(B) should be completed for all research projects. If the project was restricted to secondary analysis of clinical data or data analysis of clinical research, then responses to 18(A) and 18(B) should be "No."

18(A) Did you initiate a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
__X___No

18(B) Did you complete a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
__X___No

If "Yes" to either 18(A) or 18(B), items 18(C) – (F) must also be completed. (Do NOT complete 18(C-F) if 18(A) and 18(B) are both "No.")

18(C) How many hospital and health care professionals were involved in the research project?

_____Number of hospital and health care professionals involved in the research project

18(D) How many subjects were included in the study compared to targeted goals?

_____Number of subjects originally targeted to be included in the study
_____Number of subjects enrolled in the study

Note: Studies that fall dramatically short on recruitment are encouraged to provide the details of their recruitment efforts in Item 17, Progress in Achieving Research Goals, Objectives and Aims. For example, the number of eligible subjects approached, the number that refused to participate and the reasons for refusal. Without this information it is difficult to discern whether eligibility criteria were too restrictive or the study simply did not appeal to subjects.

18(E) How many subjects were enrolled in the study by gender, ethnicity and race?

Gender:
_____Males
_____Females
_____Unknown

Ethnicity:
_____Latinos or Hispanics
_____Not Latinos or Hispanics
_____Unknown

Race:
_____American Indian or Alaska Native
_____Asian
_____Blacks or African American
_____Native Hawaiian or Other Pacific Islander
_____White
_____Other, specify:_____
_____Unknown

18(F) Where was the research study conducted? (List the county where the research study was conducted. If the treatment, prevention and diagnostic tests were offered in more than one county, list all of the counties where the research study was conducted.)

19. Human Embryonic Stem Cell Research. Item 19(A) should be completed for all research projects. If the research project involved human embryonic stem cells, items 19(B) and 19(C) must also be completed.

19(A) Did this project involve, in any capacity, human embryonic stem cells?
_____Yes
X____ No

19(B) Were these stem cell lines NIH-approved lines that were derived outside of Pennsylvania?
_____Yes
_____ No

19(C) Please describe how this project involved human embryonic stem cells:

20. Articles Submitted to Peer-Reviewed Publications.

20(A) Identify all publications that resulted from the research performed during the funding period and that have been submitted to peer-reviewed publications. Do not list journal abstracts or presentations at professional meetings; abstract and meeting presentations should be listed at the end of item 17. **Include only those publications that acknowledge the Pennsylvania Department of Health as a funding source** (as required in the grant agreement). List the title of the journal article, the authors, the name of the peer-reviewed publication, the month and year when it was submitted, and the status of publication (submitted for publication, accepted for publication or published.). Submit an electronic copy of each publication or paper submitted for publication, listed in the table, in a PDF version 5.0.5 (or greater) format, 1,200 dpi. Filenames for each publication should include the number of the research project, the last name of the PI, the number of the publication and an abbreviated research project title. For example, if you submit two publications for PI Smith for the “Cognition and MRI in Older Adults” research project (Project 1), and two

publications for PI Zhang for the "Lung Cancer" research project (Project 3), the filenames should be:

Project 1 – Smith – Publication 1 – Cognition and MRI
Project 1 – Smith – Publication 2 – Cognition and MRI
Project 3 – Zhang – Publication 1 – Lung Cancer
Project 3 – Zhang – Publication 2 – Lung Cancer

If the publication is not available electronically, provide 5 paper copies of the publication.

Note: The grant agreement requires that recipients acknowledge the Pennsylvania Department of Health funding in all publications. Please ensure that all publications listed acknowledge the Department of Health funding. If a publication does not acknowledge the funding from the Commonwealth, do not list the publication.

Title of Journal Article:	Authors:	Name of Peer-reviewed Publication:	Month and Year Submitted:	Publication Status (check appropriate box below):
1. None				☐Submitted ☐Accepted ☐Published

20(B) Based on this project, are you planning to submit articles to peer-reviewed publications in the future?

Yes___X_____ No_____

If yes, please describe your plans:

As follow-up studies are performed under the auspices of future projects, the research findings described herein will be submitted for publication and will acknowledge Pennsylvania Department of Health funding, as appropriate.

21. Changes in Outcome, Impact and Effectiveness Attributable to the Research Project. Describe the outcome, impact, and effectiveness of the research project by summarizing its impact on the incidence of disease, death from disease, stage of disease at time of diagnosis, or other relevant measures of outcome, impact or effectiveness of the research project. If there were no changes, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

None, at present.

22. Major Discoveries, New Drugs, and New Approaches for Prevention Diagnosis and Treatment. Describe major discoveries, new drugs, and new approaches for prevention,

diagnosis and treatment that are attributable to the completed research project. If there were no major discoveries, drugs or approaches, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

No major discoveries.

23. Inventions, Patents and Commercial Development Opportunities.

23(A) Were any inventions, which may be patentable or otherwise protectable under Title 35 of the United States Code, conceived or first actually reduced to practice in the performance of work under this health research grant? Yes_____ No___X_____

If "Yes" to 23(A), complete items a – g below for each invention. (Do NOT complete items a - g if 23(A) is "No.")

a. Title of Invention:

b. Name of Inventor(s):

c. Technical Description of Invention (describe nature, purpose, operation and physical, chemical, biological or electrical characteristics of the invention):

d. Was a patent filed for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No____

If yes, indicate date patent was filed:

e. Was a patent issued for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No____
If yes, indicate number of patent, title and date issued:
Patent number:
Title of patent:
Date issued:

f. Were any licenses granted for the patent obtained as a result of work performed under this health research grant? Yes_____ No____

If yes, how many licenses were granted?_____

g. Were any commercial development activities taken to develop the invention into a commercial product or service for manufacture or sale? Yes___ No____

If yes, describe the commercial development activities:

23(B) Based on the results of this project, are you planning to file for any licenses or patents, or undertake any commercial development opportunities in the future?

Yes_____ No_____X_____

If yes, please describe your plans:

24. Key Investigator Qualifications. Briefly describe the education, research interests and experience and professional commitments of the Principal Investigator and all other key investigators. In place of narrative you may insert the NIH biosketch form here; however, please limit each biosketch to 1-2 pages. *For Nonformula grants only – include information for only those key investigators whose biosketches were not included in the original grant application.*

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. DO NOT EXCEED FOUR PAGES.

NAME	POSITION TITLE
Lewis A. Chodosh	Professor and Chairman of Cancer Biology
eRA COMMONS USER NAME (credential, e.g., agency login) chodoshl	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	MM/YY	FIELD OF STUDY
Yale University	B.S	1981	Biochemistry
Harvard Medical School	M.D.	1989	Medicine
Massachusetts Institute of Technology	Ph.D.	1989	Biology, Dr. Phillip Sharp
Massachusetts General Hospital	Medicine	1991	Medicine Residency
Massachusetts General Hospital	Endocrinology	1994	Endocrine Fellowship
Harvard Medical School	Postdoc	1994	Breast Cancer, Dr. Philip Leder

B. Positions and Honors

Positions and Employment

1989-1991 Intern and Resident in Internal Medicine, Massachusetts General Hospital
1991-1994 Clinical Fellow in Endocrinology, Massachusetts General Hospital, Harvard Medical School
1992-1994 Postdoctoral Fellow, Dept. of Genetics, Harvard Medical School, (Laboratory of Dr. Philip Leder)
1994-2002 Assistant Professor/Associate Professor, Dept of Molecular and Cellular Engineering, UPENN
1994-2006 Assistant Professor/Associate Professor, Departments of Cell & Developmental Biology and Medicine, Division of Endocrinology, Diabetes and Metabolism, University of Pennsylvania
1994- Attending Staff Physician, Hospital of the University of Pennsylvania School of Medicine
2002-2006 Associate Professor, Department of Cancer Biology, Univ. of Pennsylvania School of Medicine
2002-2007 Vice Chair, Department of Cancer Biology, University of Pennsylvania School of Medicine
2005-2010 Director, UPENN/DOD Breast Cancer Center of Excellence
2005- Director, Cancer Genetics, Abramson Family Cancer Research Institute
2005- Program Leader, Breast Cancer Program, Abramson Cancer Center
2006- Professor, Departments of Cancer Biology, Cell & Developmental Biology, and Medicine Division of Endocrinology, Diabetes & Metabolism, Univ. of Pennsylvania School of Medicine
2006- Associate Director for Basic Science, Abramson Cancer Center
2008- Chairman, Department of Cancer Biology, University of Pennsylvania School of Medicine

Other Experience and Professional Memberships

1992 Diplomate in Internal Medicine, American Board of Internal Medicine
1997 Diplomate in Endocrinology and Metabolism, American Board of Internal Medicine
1998- External Advisory Committee, Nurses' Health Study I and II
2005- Senior Editor, *Breast Cancer Research*
2008- Editor-in-Chief, *Breast Cancer Research*
2002- American Society for Clinical Investigation
2008- Association of American Physicians

Honors

1977 National Merit Scholar
1977 Harvard Book Award
1977 Cornell Ingenuity in Mathematics and Science Award
1981 *Summa cum laude*, Yale University
1981 Phi Beta Kappa, Yale University
1981 Distinction in Molecular Biophysics and Biochemistry, Yale University
1981 Emerson Tuttle Cup for Distinguished Academic Achievement, Yale University

1983-1989	Medical Scientist Training Program, Harvard Medical School
1989	Leon Reznick Memorial Prize for excellence in research, Harvard Medical School
1989	Soma Weiss Research Assembly Award, Harvard Medical School
1989	Magna cum laude, Harvard Medical School
1992-1994	Merck Research Laboratories M.D. Ph.D. Fellowship
1996-1999	Charles E. Culpeper Medical Scholar
1998-2002	U.S. Army Breast Cancer Research Program Career Development Award
2002-	American Society for Clinical Investigation
2002	AACR-Sidney Kimmel Symposium for Cancer Research Scholar Award
2003-2009	Co-Chair, AACR Special Conference on Advances in Breast Cancer Research
2008-	J. Samuel Staub Professor of Cancer Biology
2008-	Association of American Physicians

C. Selected Peer-reviewed Publications (selected from 111 peer-reviewed publications)

1. Liu Z, Wang M, Alvarez JV, Bonney ME, Chen CC, D'Cruz C, Pan TC, Tadesse MG and Chodosh LA. Singular value decomposition-based regression identifies activation of endogenous signaling pathways in vivo. *Genome Biology*, 9:R180, 2008. PMC2646284.
2. Wertheim GBW, Yang TW, Pan TC, Ramne A, Liu Z, Gardner HP, Dugan KD, Kristel P, Kreike B, van de Vijver MJ, Cardiff RD, Reynolds C and Chodosh LA. The Snf1-related kinase, Hunk, is essential for mammary tumor metastasis. *PNAS*, 106:15855-15860, 2009. PMC2734880.
3. Chen CC, Boxer RB, Stairs DB, Portocarrero CP, Horton RH, Alvarez JV, Birnbaum MJ and Chodosh LA. Akt is required for Stat5 activation and mammary differentiation. *Breast Cancer Research*, 17;12:R72, 2010. PMC3096959.
4. Yeh ES, Yang TW, Jung JJ, Gardner HP, Cardiff RD and Chodosh LA. Hunk is required for HER2/neu-induced mammary tumorigenesis. *JCI*, 121:886-879, 2011. PMC3049391.
5. Whiteaker JR, Lin C, Kennedy J, Hou L, Trute M, Sokal I, Yan P, Schoenherr RM, Zhao L, Voytovich UJ, Kelly-Spratt KS, Krasnoselsky A, Gafken PR, Hogan JM, Jones LA, Wang P, Amon L, Chodosh LA, Nelson PS, McIntosh MW, Kemp CJ, Paulovich AG. A targeted proteomics-based pipeline for verification of biomarkers in plasma. *Nature Biotechnology*, 29:625-34, 2011. PMC3232032.
6. Zhang XY, Pfeiffer HK, Mellert HS, Stanek TJ, Sussman RT, Kumari A, Yu D, Rigoutsos I, Thomas-Tikhonenko A, Seidel HE, Chodosh LA, Packham G, Baserga R, McMahon SB. Inhibition of the single downstream target BAG1 activates the latent apoptotic potential of MYC. Molecular and Cellular Biology, 31:5037-45, 2011. PMC3233020.
7. Markosyan N, Chen EP, Ndong VN, Yao Y, Sterner CJ, Chodosh LA, Lawson JA, Fitzgerald GA, Smyth EM. Deletion of cyclooxygenase 2 in mouse mammary epithelial cells delays breast cancer onset through augmentation of type 1 immune responses in tumors. *Carcinogenesis*, 32:1441-9, 2011.
8. Chodosh LA. Breast cancer: current state and future promise. *Breast Cancer Research*, 13:113, 2011. PMC3326550.
9. Lieberman BP, Ploessl K, Wang L, Qu W, Zha Z, Wise DR, Chodosh LA, Belka G, Thompson CB and Kung HF. PET imaging of glutaminolysis in tumors by 18F-(2S,4R)4-fluoroglutamine. *Journal of Nuclear Medicine*, 52:1947-1955, 2011.
10. Qu W, Oya S, Lieberman BP, Ploessl K, Wang L, Wise DR, Divgi CR, Chodosh LA, Thompson CB, Kung HF. Preparation and characterization of L-[5-11C]-glutamine for metabolic imaging of tumors. *Journal of Nuclear Medicine*. 53:98-105, 2012.
11. Magnitsky S, Belka GK, Sterner C, Pickup S, Chodosh LA and Glickson JD. Lactate detection in inducible and orthotopic Her2/neu mammary gland tumours in mouse models. *NMR in Biomedicine*, Jul 5, 2012.
12. Chen CC, Stairs DB, Boxer RB, Belka GK, Horseman ND, Alvarez JV and Chodosh LA. Autocrine prolactin induced by the Pten-Akt pathway is required for lactation initiation and provides a direct link between the Akt and Stat5 pathways. *Genes & Development*, 26:2154-68, 2012. PMC3465737.
13. Chitnis NS, Pytel D, Bobrovnikova-Marjon E, Pant D, Zheng H, Maas NL, Frederick B, Kushner JA, Chodosh LA, Koumenis C, Fuchs SY, Diehl JA. miR-211 Is a Prosurvival MicroRNA that Regulates chop Expression in a PERK-Dependent Manner. Molecular Cell, 48:353-64, 2012. PMC3496065.