Breast Cancer Research

RESEARCH Open Access

Autophagy is required for mammary tumor recurrence by promoting dormant tumor cell survival following therapy

Samantha Dwyer[1,2], Jason Ruth[1,2], Hans E. Seidel[1,2], Amelie A. Raz[1,2] and Lewis A. Chodosh[1,2,3,4*]

Abstract

Background Mortality from breast cancer is principally due to tumor recurrence. Recurrent breast cancers arise from the pool of residual tumor cells, termed minimal residual disease, that survive treatment and may exist in a dormant state for 20 years or more following treatment of the primary tumor. As recurrent breast cancer is typically incurable, understanding the mechanisms underlying dormant tumor cell survival is a critical priority in breast cancer research. The importance of this goal is further underscored by emerging evidence suggesting that targeting dormant residual tumor cells in early-stage breast cancer patients may be a means to prevent tumor recurrence and its associated mortality. In this regard, the role of autophagy in dormant tumor cell survival and recurrence remains unresolved, with conflicting reports of both pro-survival/recurrence-promoting and pro-death/recurrence-suppressing effects of autophagy inhibition in dormant tumor cells. Resolving this question has important clinical implications.

Methods We used genetically engineered mouse models that faithfully recapitulate key features of human breast cancer progression, including minimal residual disease, tumor dormancy, and recurrence. We used genetic and pharmacological approaches to inhibit autophagy, including treatment with chloroquine, genetic knockdown of ATG5 or ATG7, or deletion of *BECN* and determined their effects on dormant tumor cell survival and recurrence.

Results We demonstrate that the survival and recurrence of dormant mammary tumor cells following therapy is dependent upon autophagy. We find that autophagy is induced in vivo following HER2 downregulation and remains activated in dormant residual tumor cells. Using genetic and pharmacological approaches we show that inhibiting autophagy by chloroquine administration, ATG5 or ATG7 knockdown, or deletion of a single allele of the tumor suppressor *Beclin 1* is sufficient to inhibit mammary tumor recurrence, and that autophagy inhibition results in the death of dormant mammary tumor cells in vivo.

Conclusions Our findings demonstrate a pro-tumorigenic role for autophagy in tumor dormancy and recurrence following therapy, reveal that dormant tumor cells are uniquely reliant upon autophagy for their survival, and indicate that targeting dormant residual tumor cells by inhibiting autophagy impairs tumor recurrence. These studies identify a pharmacological target for a cellular state that is resistant to commonly used anti-neoplastic agents and suggest autophagy inhibition as an approach to reduce dormant minimal residual disease in order to prevent lethal tumor recurrence.

*Correspondence:
Lewis A. Chodosh
chodosh@pennmedicine.upenn.edu

Full list of author information is available at the end of the article

Keywords Autophagy, Breast cancer, Dormancy, Recurrence, HER2/neu, Mouse model

Introduction

Breast cancer is the leading cause of cancer death among women worldwide [1]. Mortality from breast cancer is principally due to tumor recurrence following a period of clinical remission after surgical removal of the primary tumor and adjuvant therapy. This latent period, which can extend 20 years or more, is thought to reflect the survival and persistence of residual cancer cells in a reversibly quiescent state, referred to as cellular dormancy [2–4]. Ultimately, these surviving residual tumor cells (RTCs), also termed minimal residual disease (MRD), can re-emerge from their dormant state and resume growth, leading to cancer recurrence. At present, the mechanisms that enable dormant RTCs to survive and recur are poorly understood. Since recurrent breast cancer is typically incurable, understanding the biology of dormant RTCs is of paramount clinical importance.

Breast cancer dormancy may occur either in the context of primary mammary tumor cells exposed to a foreign microenvironment (i.e., microenvironment-induced dormancy) or in residual mammary tumor cells that survive therapy (i.e., therapy-associated dormancy) [4]. Relatively little is known about the biology of dormant tumor cells, particularly in patients, in part due to the difficulty in identifying, isolating, and analyzing dormant tumor cells in vivo. Accordingly, mouse models have been developed for each type of dormancy, including inducible genetically engineered mouse (GEM) models that permit targeted oncogene inhibition [5–8], and models in which tumor cells interact with a foreign microenvironment that is non-permissive for growth, such as the D2.OR-D2A1 paired cell line model [9–11].

To study therapy-associated dormancy, we have developed and characterized GEM models for mammary tumorigenesis that faithfully recapitulate key features of the natural history of human breast cancer, including minimal residual disease, tumor dormancy, and recurrence [5–7, 12–15]. In these models, bitransgenic *MMTV-rtTA; TetO-HER2/neu* (*MTB/TAN*) or *MMTV-rtTA; TetO-Wnt1* (*MTB/TWnt1*) mice conditionally express the *HER2* or Wnt1 oncogenes in a mammary epithelial-specific and doxycycline-dependent manner. Following primary tumor development, oncogene downregulation triggered by doxycycline withdrawal results in the regression of primary mammary tumors to a non-palpable state as a consequence of their addiction to the oncogenic signaling pathways that led to their formation. However, analogous to the phenomena of tumor dormancy and recurrence in breast cancer patients, a small population of tumor cells survives oncogene downregulation and persists in the mammary gland in a state of cellular dormancy [6, 15]. After a variable latency period, these residual cells spontaneously give rise to actively growing recurrent tumors in a stochastic manner that ultimately results in the death of the animal [5–7, 13, 14].

Supporting the clinical relevance of these models, neoadjuvant therapy-refractory tumor cells that persist in patients following either chemotherapy or endocrine therapy exhibit changes in gene expression similar to those associated with cellular dormancy in GEM models [15]. Moreover, the same gene expression changes that occur in dormant mouse RTCs, referred to as the mouse core dormancy signature, are strongly associated with risk of early, as well as late, relapse in breast cancer patients [6, 15]. Further, this association is observed across breast cancer subtypes and sites of recurrence, suggesting that the biology of these models is neither specific for, nor restricted to, a particular subtype of human breast cancer – nor local versus distant relapse.

Additionally, functional interrogation of these GEM models has identified multiple pathways that contribute to tumor recurrence in mice, each of which is also associated with risk of metastatic recurrence in breast cancer patients, across breast cancer subtypes, and in a direction predicted by studies in mice [13–19]. Further, dormant RTCs in mice share a gene expression signature with bone marrow disseminated tumor cells (DTCs) isolated from prostate cancer patients [20], as well as DTCs from human xenograft models for head and neck, prostate, and breast cancers [21–23]. In aggregate, these findings support the clinical relevance of GEM models and suggest that they are informative for interrogating the biology of RTCs and the mechanisms that underlie distant, as well as local, relapse in patients.

The above GEM models enable in vivo mechanistic approaches to elucidating the molecular and cellular processes that contribute to the survival and recurrence of dormant RTCs. One such candidate process is macroautophagy. Macroautophagy (referred to hereafter as autophagy) is an evolutionarily conserved catabolic process involving the lysosomal degradation of organelles and proteins that serves to recycle damaged cellular components and generate energy [24]. Notably, the *Beclin 1* tumor suppressor is required for autophagy and is monoallelically deleted in 50% of sporadic human breast carcinomas, suggesting that autophagy may be tumor suppressive [25, 26]. Consistent with this, overexpression of BECLIN1 impairs the growth of breast cancer cell lines in vitro as well as their tumorigenicity in vivo, and monoallelic loss of *Becn1* in mice has been shown to promote primary mammary tumorigenesis in certain contexts [26–28].

In addition to these tumor suppressive functions, however, autophagy can also promote the survival of transformed mammary epithelial cells in vivo and in vitro, underscoring the complex role of autophagy in cancer whereby autophagy may paradoxically both promote and suppress breast cancer progression [29–32]. Based on these and other observations, autophagy has been proposed to constitute a survival mechanism for cancer cells subjected to a variety of cellular stresses by providing these cells with an alternate energy source through controlled cellular autodigestion and recycling of damaged cellular components [33–36].

In this context, emerging evidence has implicated autophagy as a potential survival mechanism in mammary tumor cells in which dormancy has been induced by exposure to a foreign microenvironment [37–39]. Notably, however, two studies using the D2.OR dormancy-prone cell line to investigate the role of autophagy in microenvironment-induced dormancy yielded opposing results in which autophagy inhibition in dormant tumor cells either suppressed or promoted metastatic outgrowth [37, 38]. Thus, the role of autophagy in breast cancer dormancy remains unclear, particularly with respect to the impact of autophagy inhibition on dormant tumor cell survival and dormancy exit – a question of critical importance for the clinical translation of therapeutic approaches aimed at preventing breast cancer recurrence by targeting tumor dormancy.

In this study, we use genetic and pharmacological approaches in genetically engineered mouse models for breast cancer to study the role of autophagy in cellular dormancy and tumor recurrence following therapy. These studies reveal that inhibiting autophagy following therapy results in the death of dormant mammary tumor cells in vivo and that autophagy inhibition by treatment with chloroquine, knockdown of ATG5 or ATG7, or deletion of a single allele of *BECN* are each sufficient to suppress mammary tumor recurrence. In aggregate, findings from this study support a tumor promoting role for autophagy in breast cancer dormancy and recurrence that is unique to the dormant state.

Results

Dormant mammary tumor cells undergo autophagy in vivo

The anti-HER2 targeted therapy trastuzumab has been reported to induce autophagy in breast cancer cell lines in vitro [40]. To confirm that genetic downregulation of HER2 recapitulates the effects of pharmacological HER2 inhibition, tumor cells from a primary mammary adenocarcinoma in an *MTB/TAN* mouse [13] were cultured in the presence of doxycycline to maintain HER2 levels. Doxycycline withdrawal induced acute *HER2* downregulation, which was accompanied by an acute 3.0-fold 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 (Fig. 1A and [41]). Consistent with the induction of autophagy, electron microscopy revealed an increased number of double-membraned autophagosomes in tumor cells following doxycycline withdrawal (Fig. 1, B and C).

To confirm these results, we generated *MTB/TAN* primary tumor cells stably expressing the autophagy marker EGFP-LC3. Induction of autophagy induces the incorporation of cleaved, lipidated LC3-II into autophagosomes, which alters LC3 subcellular localization from diffusely cytoplasmic to punctate [41]. 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 (Fig. 1, D and E). Together, these results indicate that acute HER2 downregulation triggers autophagy in vitro.

To determine whether cytoplasmic contents sequestered in autophagosomes reached the lysosome and were degraded in primary *MTB/TAN* tumor cells, acute doxycycline withdrawal was combined with chloroquine treatment. Chloroquine (CQ) raises lysosomal pH and inhibits protein degradation within the autolysosome such that cells with flux through the autophagic pathway show additional increases in LC3-II levels when treated with chloroquine [34, 42]. Indeed, combined chloroquine treatment and HER2 downregulation in primary *MTB/TAN* tumor cells further augmented LC3-II levels beyond those in cells subjected to HER2 downregulation alone (Figs. 1A, 4.2-fold for combined chloroquine treatment and HER2 downregulation vs. 3.0-fold for HER2 downregulation alone). These findings suggest that acute HER2 downregulation induces autophagy as well as flux through the autophagic pathway.

We next asked whether acute HER2 downregulation induces autophagy in vivo. Doxycycline was withdrawn from *MTB/TAN* mice bearing primary mammary tumors for 48 h to downregulate HER2 expression and immunoblotting was used to analyze levels of p62/SQSTM1, which recognizes ubiquitin-marked proteins and sequesters them for degradation through autophagy. As a consequence, p62 itself is degraded in cells undergoing autophagy [43, 44]. Consistent with our in vitro observations, acute HER2 downregulation resulted in a rapid 59.9% reduction in p62 protein levels (Fig. 2A). Furthermore, primary orthotopic tumors generated from *MTB/TAN* tumor cells expressing EGFP-LC3 exhibited an increase in the number of EGFP-positive punctae per tumor cell following acute doxycycline withdrawal (Fig. 2B). These results indicate that autophagy is induced in primary tumor cells following acute HER2 downregulation.

In *MTB/TAN* mice bearing primary mammary tumors, HER2 downregulation results in tumor regression to a

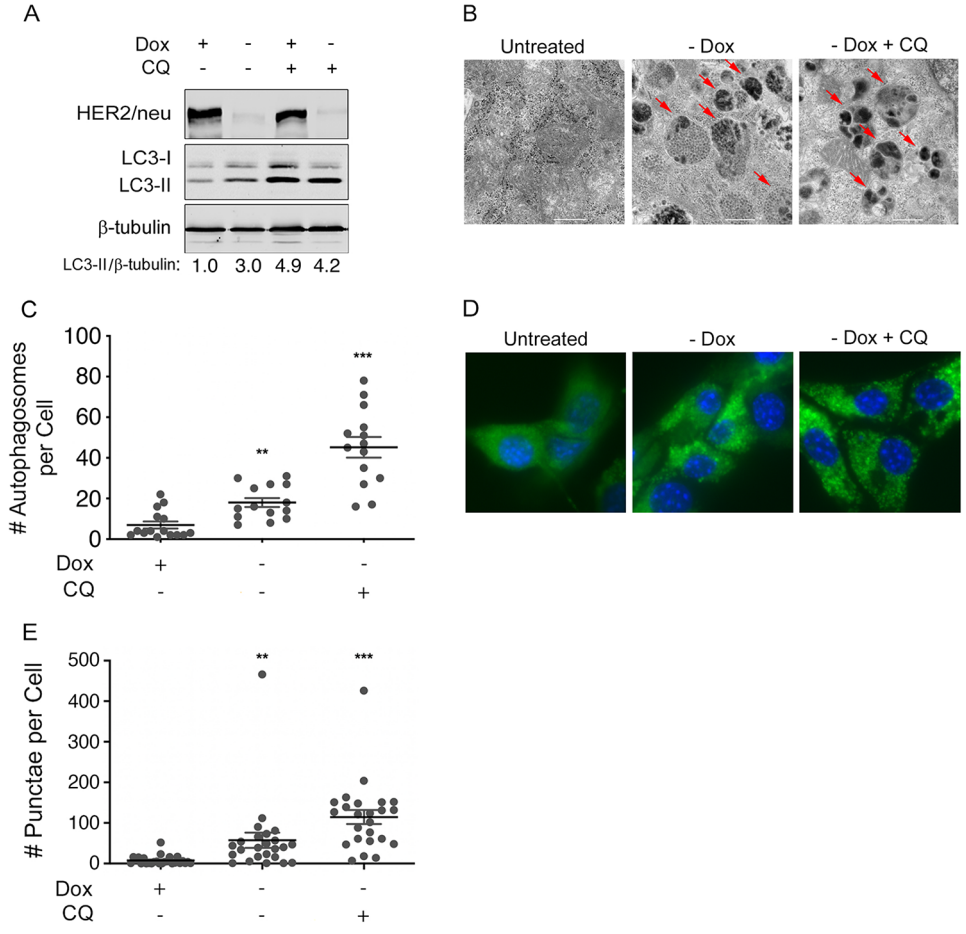

Fig. 1 HER2 down-regulation induces autophagy in primary tumor cells in vitro. (**A-E**) *MTB/TAN* primary tumor cells subjected to doxycycline withdrawal for 24 h. Treatment with 50 μM chloroquine (CQ) for 24 h was used as a positive control for the induction of autophagy. (**A**) HER2 levels and conversion of LC3-I to LC3-II determined by western blotting. β-tubulin is shown as a loading control. (**B**) Representative images of doublemembraned autophagosomes (arrows) visualized by electron microscopy. Bar = 500 nm. (**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 ± SEM $^{**}P < 0.001$, $^{***}P < 0.0001$

non-palpable state, but leaves behind a small number of residual tumor cells that survive HER2 downregulation and persist in a dormant state in residual lesions within the mammary gland (Supplemental Fig. 1) [6, 14]. These cells are quiescent, yet remain competent to resume growth, resulting in recurrent tumors [6, 13, 14, 16]. Fluorescence microscopy performed on dormant EGFP-LC3-labeled tumor cells within residual lesions in mice harboring fully regressed orthotopic tumors revealed an increase in the number of EGFP-positive punctae per tumor cell compared to actively growing orthotopic primary tumors (Fig. 2B). Together, these observations suggest that autophagy is triggered in tumor cells following acute HER2 downregulation in vivo and in vitro and that dormant residual tumor cells undergo autophagy in vivo.

Pharmacological or genetic inhibition of autophagy inhibits mammary tumor recurrence

Our observations that autophagy occurs in vivo, is triggered by acute HER2 downregulation in actively growing primary tumor cells in mice, and persists in dormant mammary tumor cells were equally consistent with models in which autophagy is tumor suppressive or tumor promoting. To distinguish between these possibilities, we first treated *MTB/TAN* tumor-bearing mice with chloroquine. As chloroquine has been used safely in millions of people worldwide for the prevention and treatment of malaria and has a favorable therapeutic index, this drug represents an attractive approach to inhibiting autophagy in vivo [45].

To determine the effect of chloroquine treatment on mammary tumor recurrence, female *nu/nu* mice maintained on doxycycline were injected orthotopically with primary *MTB/TAN* tumor cells. Following primary tumor formation, tumor regression was induced by

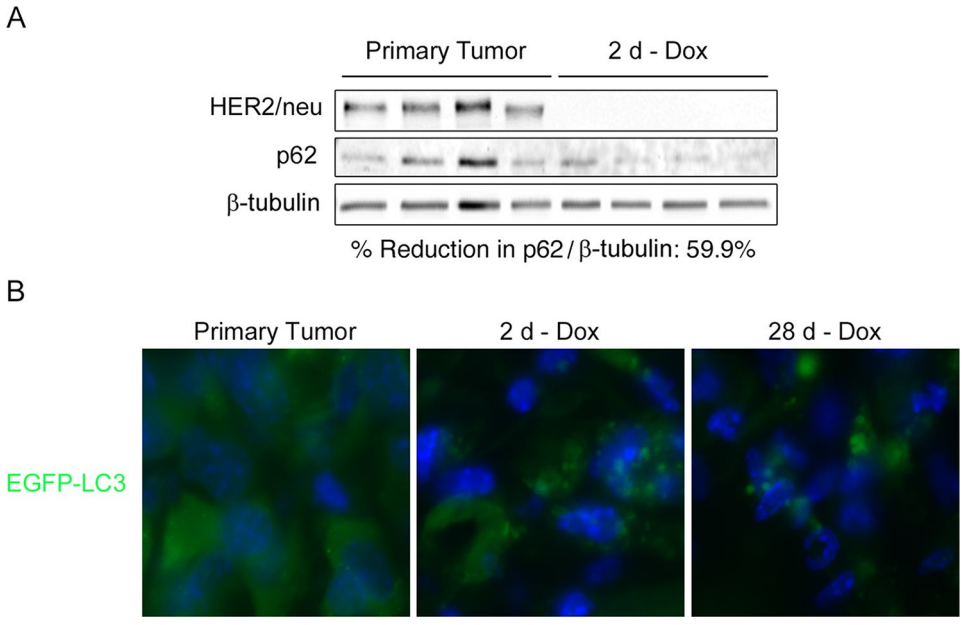

Fig. 2 HER2 down-regulation induces autophagy in primary and dormant tumor cells *in vivo*. (**A**) HER2 and p62 levels evaluated by western blotting in orthotopic *MTB/TAN* primary tumors in the presence of doxycycline or following doxycycline withdrawal for 2 d. β-tubulin is shown as a loading control. (**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

doxycycline withdrawal and HER2 downregulation [13]. Daily treatment with chloroquine was initiated to coincide with HER2 downregulation (Fig. 3A). Mice bearing fully regressed primary tumors were then monitored for recurrence. Daily chloroquine administration markedly delayed the onset of tumor recurrence in mice, with the median latency for tumor recurrence increasing from 84 to 140 days (H.R. = 3.12, 95% CI 1.45–6.72, P=0.004;

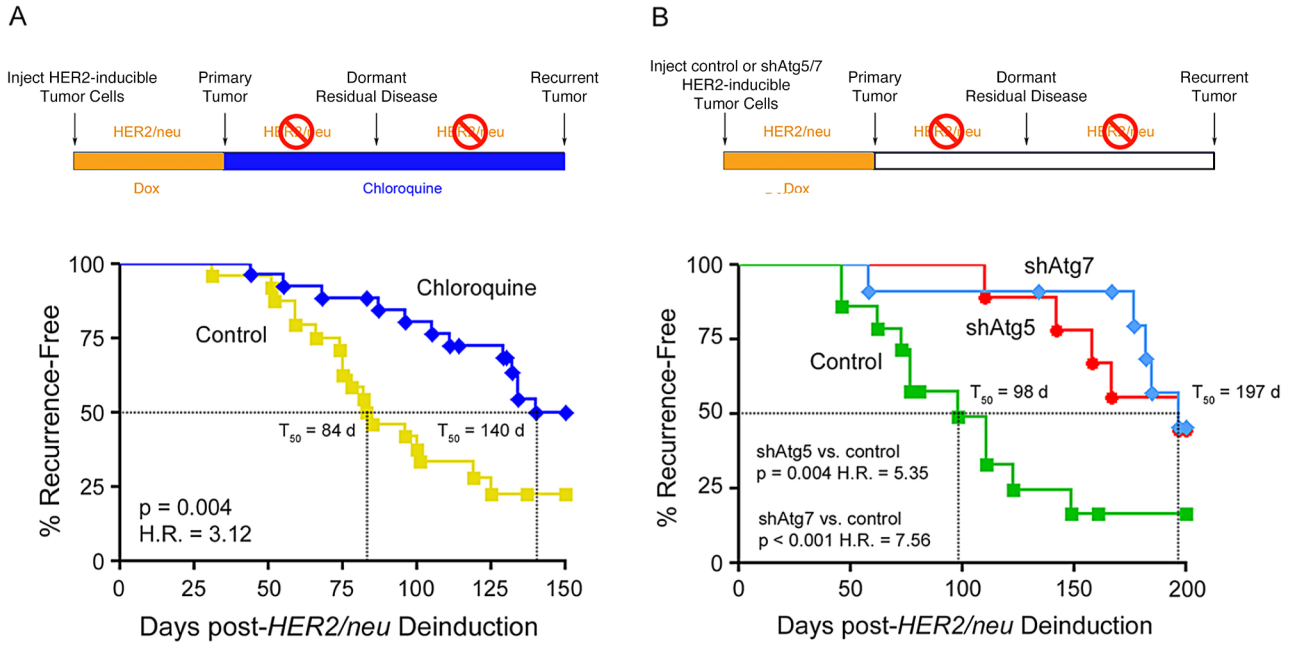

Fig. 3 Pharmacological or genetic inhibition of autophagy inhibits mammary tumor recurrence. (**A**) Schematic of orthotopic recurrence model and timing of chloroquine treatment. Recurrence-free survival of female *nu/nu* mice harboring *MTB/TAN* orthotopic primary tumors induced to regress by doxycycline withdrawal and treated with vehicle (n=24) or 60 mg/kg/d chloroquine (n=26), as described. (**B**) Schematic and recurrence-free survival of female *nu/nu* mice harboring fully regressed orthotopic tumors derived from vector control (n=14), shRNA targeting *Atg5* (shAtg5; n=9), or shRNA targeting *Atg7* (shAtg7; n=11)-expressing *MTB/TAN* tumor cells. Median recurrence latencies are indicated

Fig. 3A). This finding suggests that autophagy promotes, rather than inhibits, mammary tumor recurrence.

Since pharmacological agents may have off-target effects, to confirm and 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 [46]. ATG5 and ATG7 are components of a 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 required for formation of the autophagosome.

To determine if genetically inhibiting autophagy delays tumor recurrence, *MTB/TAN* primary tumor cells were generated that expressed shRNAs targeting either *Atg5* or *Atg7*. qPCR and immunoblotting confirmed knockdown of ATG5 and ATG7 (Supplemental Fig. 2). These cells were then injected orthotopically into the mammary glands of *nu/nu* mice on doxycycline, as were cells transduced with a vector control, to form primary tumors. As above, doxycycline was withdrawn from mice bearing orthotopic tumors of the same size to induce HER2 downregulation, which resulted in the regression of tumors to a non-palpable state. Mice were then monitored for recurrence.

This analysis revealed that genetic inhibition of autophagy by knocking down either ATG5 or ATG7 dramatically impaired tumor recurrence (H.R. = 5.35, 95% CI 1.72–16.62, $P=0.004$; H.R.= 7.56, 95% CI 2.33–24.58, $P<0.001$; Fig. 3B). These results are consistent with the effects of autophagy inhibition by chloroquine and further suggest that autophagy is required for the recurrence of HER2-induced tumors.

Beclin 1 is required for mammary tumor recurrence

The observations that ATG5 knockdown, ATG7 knockdown, and chloroquine treatment each result in a delay in mammary tumor recurrence is consistent with a model in which autophagy plays a pro-tumorigenic role in breast cancer recurrence. However, multiple lines of evidence suggest that autophagy is tumor suppressive, several of which are derived from studies in which *Becn1* was monoallelically deleted in tumor cells or in mice [26, 28, 30, 31, 47]. BECLIN1 is a haploinsufficient tumor suppressor whose heterozygous loss in mice results in increased susceptibility to lymphomas, liver cancer, lung cancer, and mammary epithelial hyperplasias [28, 47]. BECLIN1 is also essential for the initiation of autophagosome formation by means of its ability to form a complex with the Vps34 PI3 kinase and other proteins [20]. Since knockdown or loss of *BECLIN1* inhibits autophagy while increasing susceptibility to primary tumor formation, these data suggest a tumor suppressive role for BECLIN1 and autophagy.

In light of these conflicting data, we wished to determine whether – consistent with a tumor suppressive role for BECLIN1 and autophagy – loss of BECLIN1 would accelerate mammary tumor recurrence or whether – consistent with a tumor promoting role for autophagy as suggested by the effects of chloroquine treatment as well as ATG5 or ATG7 knockdown – loss of BECLIN1 would inhibit mammary tumor recurrence. To address this question, *Becn1*+/- mice were crossed to *MTB/TAN* inducible bitransgenic mice to generate cohorts of *MTB/TAN; Becn1*+/+ and *MTB/TAN; Becn1*+/- female mice. Primary mammary tumors were then induced by chronic activation of HER2 via doxycycline administration.

The incidence, latency, multiplicity and growth rate of primary HER2-induced tumors did not differ between *Becn1*+/+ and *Becn1*+/- mice (Fig. 4A and data not shown). Tumor-bearing mice were then deinduced and mice bearing fully regressed tumors were monitored for recurrence. The rate of primary tumor regression was unaffected by *Becn1* genotype (data not shown). In contrast, deletion of one allele of *Becn1* markedly delayed tumor recurrence with the median latency for tumor recurrence increasing from 77 days to 131 days (H.R. = 2.38, 95% CI 1.15–4.94, $P=0.019$; Fig. 4B). These results demonstrate that BECLIN1 is required for mammary tumor recurrence and further support our findings based on ATG5 or ATG7 knockdown and chloroquine treatment that autophagy is required for mammary recurrence.

Selection against dormant tumor cells with impaired autophagy

Having established a role for autophagy in the recurrence of HER2-induced mammary tumors, we wished to determine the cellular basis for this requirement. Based on evidence supporting a role for autophagy in responses to cellular stress, we considered the possibility that autophagy contributes to the survival of tumor cells subjected to HER2 pathway inhibition. To test this hypothesis, HER2 was downregulated for 24 h in vitro in *MTB/TAN* primary tumor cells expressing an shRNA targeting *Atg5* or a control vector and the percentage of viable cells was determined. The survival of tumor cells transduced with a control vector was not altered by HER2 downregulation in the presence of 0.5% serum (Fig. 5A). In contrast, a substantial increase in cell death was observed in tumor cells expressing HER2 when ATG5 was knocked down, and the combination of ATG5 knockdown with HER2 downregulation resulted in a dramatic impairment in cell survival due to increased apoptosis (Fig. 5A and data not shown). These findings suggest that autophagy is required for the survival of primary mammary tumor cells subjected to acute HER2 downregulation in vitro, which is consistent with a prior report [40].

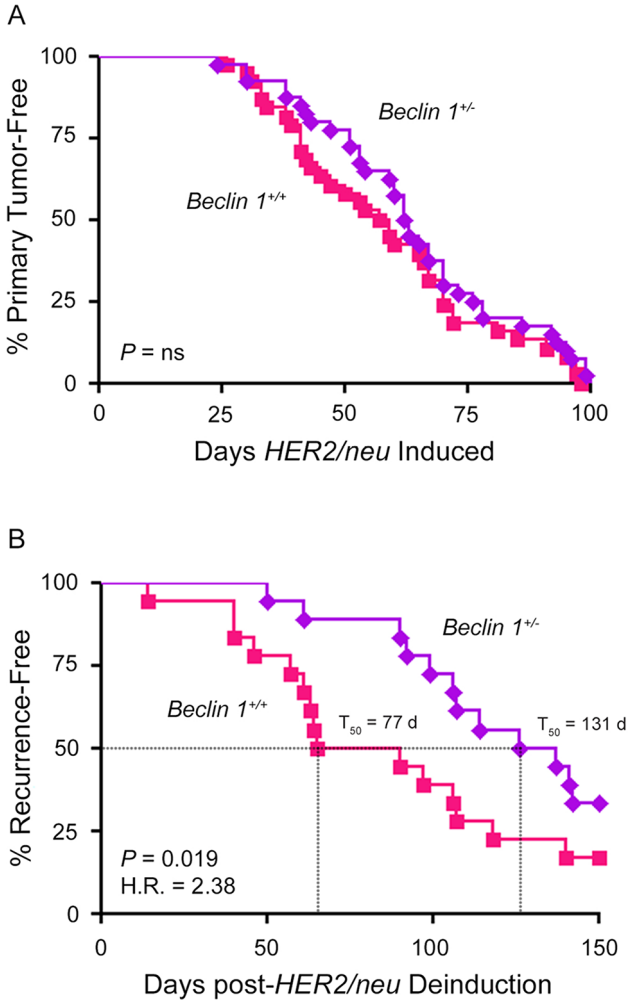

Fig. 4 Beclin 1 is required for mammary tumor recurrence in intact mice. (**A**) Primary tumor-free survival of female *MTB/TAN; Beclin 1*$^{+/+}$ (*n* = 38) and *MTB/TAN; Beclin 1*$^{+/-}$ mice (*n* = 40) administered doxycycline to induce HER2 beginning at 6 wk of age. (**B**) Recurrence-free survival of female *MTB/TAN; Beclin 1*$^{+/+}$ (*n* = 18) and *MTB/TAN; Beclin 1*$^{+/-}$ mice (*n* = 18) that harbored fully regressed primary tumors following doxycycline withdrawal. Median recurrence latencies are indicated

We next wished to test the role of autophagy in promoting tumor cell survival in vivo during the process of mammary tumor recurrence. *MTB/TAN* primary tumor cells expressing an shRNA targeting *Atg5* were labeled with an H2B-mCherry reporter, whereas *MTB/TAN* primary tumor cells transduced with an empty vector were labeled with an H2B-EGFP reporter. These two fluorescent populations of isogenic cells were admixed in equal parts (Supplemental Fig. 3), injected into the mammary glands of *nu/nu* mice maintained on doxycycline, and allowed to generate orthotopic primary tumors. Doxycycline was then withdrawn to initiate the process of tumor regression and fluorescence microscopy was used to determine the ratio of mCherry-labeled ATG5-knockdown cells to EGFP-labeled control cells in primary tumors, residual tumor lesions 14 days or 28 days following HER2 downregulation, and recurrent tumors (Fig. 5, B-D).

As predicted based on the unperturbed nature of HER2-induced primary tumorigenesis in *Becn1+/-* mice, we observed no selection for or against cells with ATG5 knockdown in primary tumors (Fig. 5, C and D). Furthermore, we observed no selection for or against cells expressing an *Atg5* shRNA in residual lesions 14 days post-HER2 downregulation, a time point that roughly corresponds to the completion of tumor regression and early stages of tumor dormancy (Fig. 5, C and D and Supplemental Fig. 1). In contrast, cells with ATG5 knockdown were present at a lower than expected ratio in residual lesions 28 days following HER2 downregulation, a time point at which residual tumor cells have been in a dormant state for ~3 wks (Fig. 5, C and D and Supplemental Fig. 1). This competitive disadvantage was even more pronounced in recurrent tumors, in which only 1% of fluorescent cells were mCherry-positive (Fig. 5, C and D).

To rule out differential effects of the H2B-mCherry and H2B-EGFP reporters, this experiment was repeated after exchanging fluorescent protein labels between *Atg5* shRNA and control cells. This confirmed the pronounced competitive disadvantage of *Atg5* shRNA tumor cells compared with control cells during late dormancy and recurrence time points (Supplemental Fig. 3). Together, these data indicate that primary tumor cells in which autophagy is impaired are at a strong, cell-intrinsic selective disadvantage following HER2 downregulation, but not until tumor cells have entered a dormant state.

Autophagy is required for dormant residual tumor cell survival in vivo

Our observations to this point suggested that autophagy is required for the survival of dormant tumor cells following oncogene downregulation. To test directly whether tumor dormancy represents a cellular state in which cells are particularly reliant upon autophagy for their survival, we determined the impact of chloroquine treatment on dormant tumor cells generated by HER2 downregulation. Primary *MTB/TAN* tumor cells were grown in 10% serum plus doxycycline, 1% serum plus doxycycline, or 1% serum in the absence of doxycycline. After 3 wk, cells grown in 1% serum in the absence of doxycycline were Ki67-negative, but could be induced to reenter the cell cycle solely by the re-addition of doxycycline, demonstrating that these cells are reversibly growth arrested (Supplemental Fig. 4).

After 3 wk in the above media, cells were treated with chloroquine for 1 week and cell viability was assessed. Dormant tumor cells maintained in 1% serum in the absence of HER2 expression were markedly more

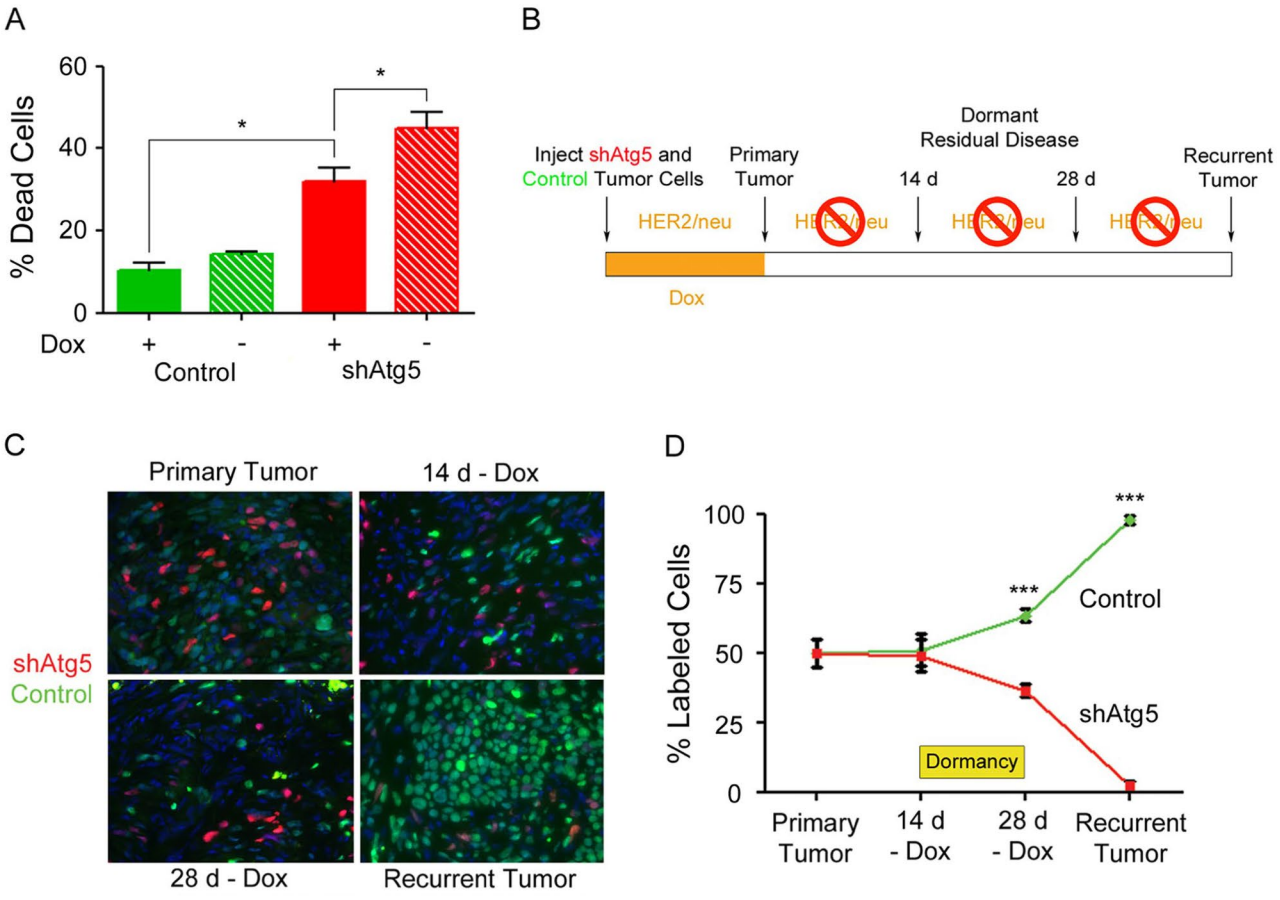

Fig. 5 Atg5 is required for dormant mammary tumor cell survival. (**A**) Viability of primary *MTB/TAN* tumor cells expressing an shRNA targeting *Atg5* (shAtg5) or empty vector grown in 0.5% serum with or without doxycycline for 24 h. Data represent mean ± SEM **P* < 0.05. (**B-D**) Mammary fat pads of female *nu/nu* mice on doxycycline were injected with an equal ratio of primary *MTB/TAN* tumor cells that were either transduced with shAtg5 and labeled with H2B-mCherry or were transduced with a vector control and labeled with H2B-EGFP. (**B**) Schematic of orthotopic competition assay and timing of tumor harvest. (**C**) Representative fluorescence microscopy images of primary tumors (*n* = 6), residual lesions 14 d post-deinduction (*n* = 5), residual lesions 28 d post-deinduction (*n* = 5), and recurrent tumors (*n* = 3) from (**B**). Magnification, x400. (**D**) Percentage of H2B-EGFP-positive and H2B-mCherry-positive tumor cells was determined at each time point in (**B**). Data represent mean ± SEM ****P* < 0.0001

sensitive to chloroquine treatment than proliferating cells expressing HER2 maintained in either 1% serum or 10% serum (Fig. 6A). These findings suggest that dormant tumor cells are more dependent upon autophagy for survival than actively proliferating cells.

We then tested if the degradation of metabolic substrates by autophagy was required to maintain oxidative phosphorylation and the viability of dormant mammary tumor cells treated with chloroquine. A cell-permeable form of pyruvate, methylpyruvate, was added to the culture media of dormant *MTB/TAN* tumor cells at the time of chloroquine treatment. Methylpyruvate, once taken up by a cell, can be oxidized in the tricarboxylic acid (TCA) cycle to fuel ATP production. The addition of methylpyruvate to dormant tumor cells rescued the cell death observed in response to chloroquine treatment (Fig. 6B). This suggests that the inhibition of autophagy decreased dormant tumor cell viability, at least in part due to the suppression of cellular bioenergetics.

We next wished to determine if dormant mammary tumors cells are dependent upon autophagy for survival in vivo. To address this, mice harboring orthotopic EGFP-labeled dormant residual tumor cells were treated with chloroquine for 2 wk beginning 21 d after HER2 downregulation. Mammary glands were then harvested, digested to form a single-cell suspension, and the number of EGFP-positive residual tumor cells was determined by flow cytometry. This analysis revealed that mice treated with chloroquine for 2 wk within the period of dormancy harbored 38% fewer dormant tumor cells ($P<0.05$) than mice treated with a vehicle control (Fig. 6C). Given the absence of tumor cell proliferation at these time points, these data strongly suggest that autophagy is required for the survival of dormant mammary tumor cells in vivo.

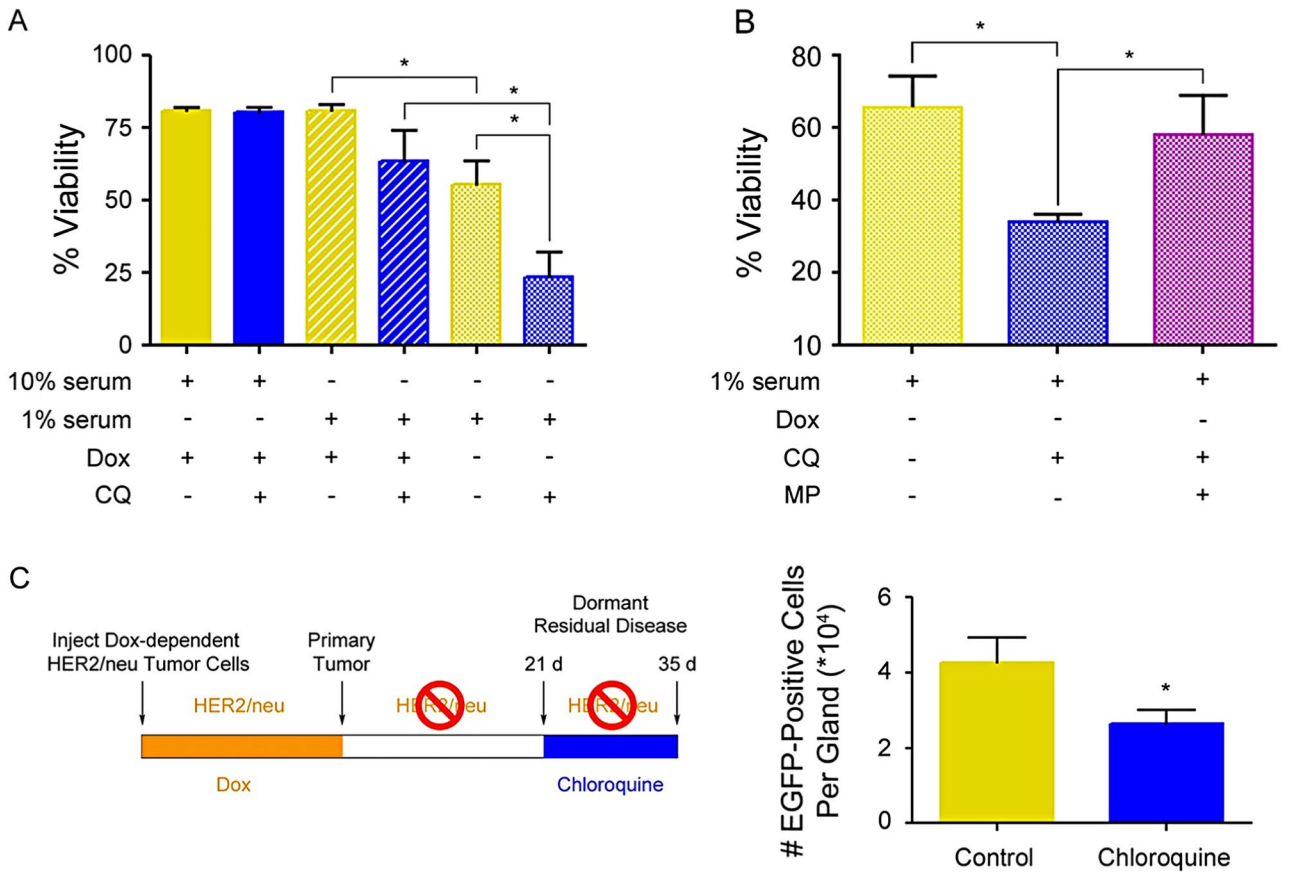

Fig. 6 Chloroquine delays recurrence by decreasing survival of dormant mammary tumor cells. (**A**) Viability of primary *MTB/TAN* tumor cells grown in 10% serum plus doxycycline, 1% serum plus doxycycline, or 1% serum without doxycycline for 3 wk, then treated with 50 μM chloroquine (CQ) or vehicle control for 1 wk. (**B**) Viability of *MTB/TAN* tumor cells grown in 1% serum without doxycycline for 3 wk, then treated with 50 μM chloroquine or 50 μM chloroquine plus 10 mM methylpyruvate (MP) for 1 wk. (**C**) Schematic of orthotopic dormancy model and timing of chloroquine treatment. Female *nu/nu* mice harboring primary orthotopic tumors generated from primary *MTB/TAN* cells were deinduced for 21 days and then treated daily with chloroquine 60 mg/kg ($n=21$) or vehicle control ($n=23$) for 2 wk. At 35 d post-deinduction, the number of EGFP-positive tumor cells per gland was determined using flow cytometry. Data represent mean ± SEM. $^{**}P<0.001$, $^{*}P<0.05$

Autophagy inhibition in mice bearing dormant minimal residual disease suppresses recurrence

Our findings to this point suggested that dormant residual tumor cells may be uniquely dependent upon autophagy for their survival. If correct, our data would predict that inhibitors of autophagy would be most effective not in actively growing tumors, but in dormant residual tumor cells. If so, chloroquine could be used on that basis to reduce the pool of viable dormant residual tumor cells and thereby prevent or delay tumor recurrence.

To test this hypothesis, we again employed chloroquine in an orthotopic recurrence assay, with the exception that chloroquine treatment was initiated 28 days following doxycycline withdrawal when tumors had completed their regression and residual tumor cells exist in a dormant state [6, 14], rather than at the time of HER2 downregulation when tumor cells are still proliferating and tumor regression has not yet begun (Fig. 7A). Mice were then monitored for tumor recurrence.

This analysis revealed that autophagy inhibition restricted specifically to the period of tumor dormancy markedly delayed recurrence (H.R. = 2.88, 95% CI 1.39–5.98, $p=0.005$; Fig. 7B). Notably, the magnitude of the effect of chloroquine administration beginning 28 days after HER2 downregulation was nearly identical to that observed for the effect of chloroquine administration beginning immediately at the time of HER2 downregulation (Fig. 3A). This finding was consistent with results from the fluorescent cell competition assay (Fig. 5, B-D and Supplemental Fig. 3), indicating that cells with impaired autophagy are not selected against during primary tumor formation or within the first 14 d following HER2 downregulation. Similarly, the magnitude of the effect of chloroquine administration beginning 28 days after HER2 downregulation was similar to that observed for the effect of deleting one allele of *Becn1*, which also failed to affect the rate of primary tumorigenesis in *MTB/TAN* mice (Fig. 4). Together, these observations

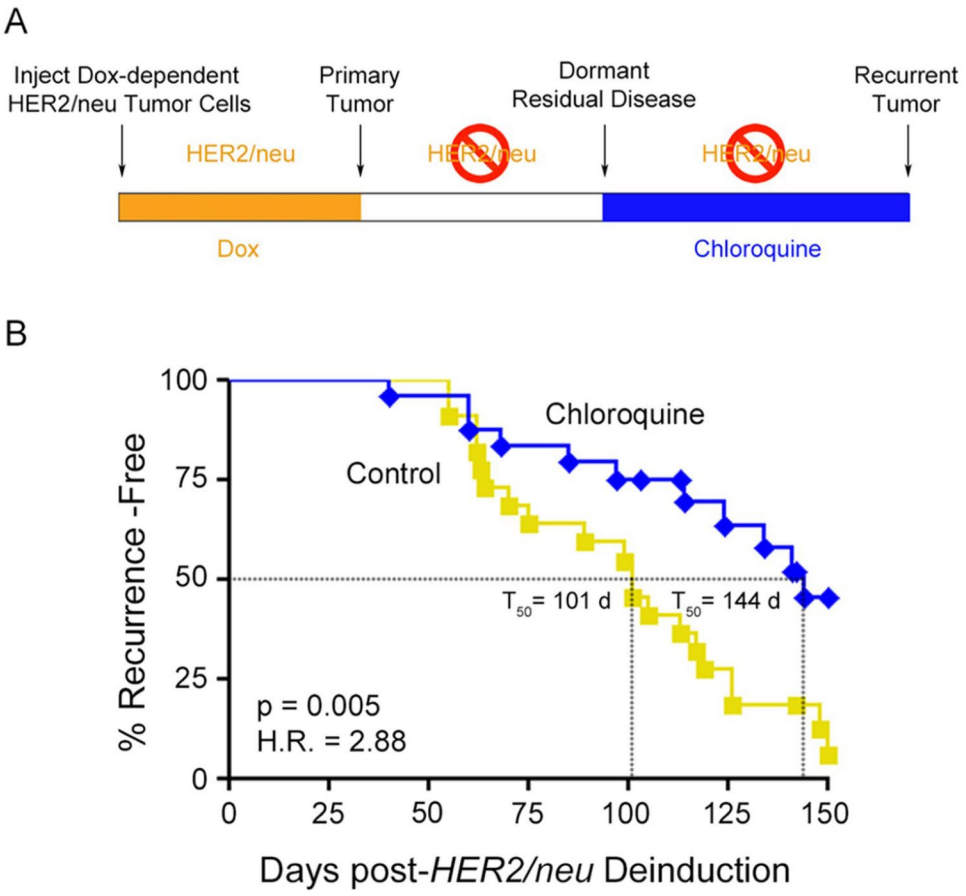

Fig. 7 Chloroquine treatment of mice bearing dormant minimal residual disease impairs recurrence. (**A**) Schematic of orthotopic recurrence model and timing of chloroquine treatment. (**B**) Recurrence-free survival of mice bearing fully regressed orthotopic tumors that were treated daily with vehicle control (*n* = 22) or 60 mg/kg chloroquine (*n* = 24) as described. Median latencies for tumor recurrence are indicated

provide further support for a model in which the effects of autophagy inhibition on tumor cell survival are largely confined to tumor dormancy.

Discussion

While abundant evidence exists supporting a role for autophagy in primary tumorigenesis and the acute response to cytotoxic and targeted therapies, the role of autophagy in tumor dormancy and recurrence is less well understood. The clinical relevance of this question is highlighted by the fact that minimal residual disease in the form of disseminated tumor cells (DTCs) is detectable in the bone marrow of more than 30% of early stage breast cancer patients and is associated with poorer recurrence-free survival as well as overall survival [48, 49]. While uncertainty exists, DTCs have been reported to exist in a non-proliferative state and, consistent with these cells existing in a state of cellular dormancy, at least some of these cells appear capable of resuming proliferation to give rise to recurrent cancers [4, 50–54]. Since breast cancer recurrence is responsible for the vast majority of deaths from this disease, eliminating dormant tumor cells by targeting their mechanisms of survival or preventing their reactivation is an attractive approach to improving patient outcomes by preventing recurrence.

In the present study, using genetic and pharmacological approaches to probe the role of autophagy in cellular dormancy following targeted therapy we have demonstrated that inhibiting autophagy by chloroquine administration, ATG5 or ATG7 knockdown, or deletion of a single allele of the tumor suppressor *Becn1* are each sufficient to inhibit mammary tumor recurrence. This requirement for autophagy in tumor recurrence was attributable to its ability to serve as a survival mechanism for the small number of primary tumor cells that survive HER2 pathway downregulation and persist in a dormant state as minimal residual disease. Consistent with this cellular mechanism of action, autophagy was induced in dormant residual tumor cells in vivo and in vitro following HER2 downregulation and pharmacological inhibition of autophagy in mice bearing dormant minimal residual disease significantly decreased the number of surviving dormant tumor cells and substantially delayed mammary tumor recurrence. Together, these findings support a

pro-tumorigenic role for autophagy and *Becn1* in breast cancer recurrence following targeted therapy by promoting the survival of dormant residual tumor cells.

Notably, two recent studies using the D2.OR/D2A1 paired mammary tumor cell line model for microenvironment-induced dormancy yielded opposing results, wherein inhibiting autophagy in dormant D2.OR cells was reported to result in either the suppression or promotion of metastatic outgrowth of dormant tumor cells in the lung following seeding by tail vein injection, or in 3D culture [37, 38]. Moreover, studies using a HER2/neu-overexpressing mammary tumor cell line reported that autophagy inhibition achieved by ATG5 knockdown resulted in early escape from dormancy and recurrence following Adriamycin treatment, whereas transient autophagy inhibition by chloroquine treatment during Adriamycin treatment had no effect, suggesting either neutral or anti-tumorigenic effects of autophagy inhibition during dormancy [55]. In addition, ectopic expression of the Ras-related gene, ARHI, in xenografts from a human ovarian cancer cell line has been reported to induce reversible tumor mass dormancy associated with increased autophagy and suppression of net tumor growth, again suggesting a tumor suppressive function for autophagy in the context of tumor mass dormancy [56].

In contrast, our findings that inhibiting autophagy by either pharmacological or genetic means significantly delayed tumor recurrence in mice bearing dormant minimal residual disease, and that inhibiting autophagy resulted in the death of dormant tumor cells in vivo and in vitro, indicate that autophagy is pro-tumorigenic in the context of tumor dormancy and recurrence as it occurs following targeted therapy. This finding contrasts with multiple lines of evidence suggesting that autophagy is a tumor suppressive mechanism during primary carcinogenesis, though we found no such suppression for HER2-induced primary tumorigenesis in *Becn1*-/+ mice [26, 28, 30, 31, 47]. These seemingly opposing, ostensibly context-specific functions of autophagy highlight the distinct biological properties of primary tumor cells and dormant residual tumor cells, and provides strong evidence for the existence of meaningful differences in therapeutic targets between these two states.

In this regard, we found that dormant mammary tumor cells are uniquely reliant on autophagy for their survival compared to actively growing tumor cells. Indeed, primary tumorigenesis induced by HER2 was unaffected in *Becn1+/-* mice and we observed no selection for or against cells with ATG5 knockdown during primary orthotopic tumor outgrowth, nor the rate of primary orthotopic tumor growth in ATG5- or ATG7-knockdown cells.

More surprisingly, and contrary to findings that autophagy supports cell survival in response to acute cellular stress, we observed no appreciable selection for or against cells with ATG5 knockdown within the first 14 days following HER2 downregulation, during which time the vast majority of tumor cells die secondary to oncogene addiction. In contrast, selection against tumor cells with ATG5 knockdown was readily apparent during the period of cellular dormancy that followed tumor regression, and chloroquine administration killed dormant residual tumor cells while having little, if any, effect on actively proliferating primary or recurrent tumor cells (Supplemental Fig. 5 and data not shown). Consistent with this, the magnitudes of recurrence inhibition in mice deleted for one allele of *Becn1* or chronically treated with chloroquine beginning at the time of HER2 downregulation were each nearly identical to that observed for chloroquine administration restricted to the period of tumor dormancy. This suggests a model in which tumor suppressive effects of autophagy inhibition result from effects on dormant tumor cells rather than actively proliferating tumor cells or tumor cells responding to the acute stress of HER2 pathway downregulation.

The above findings constitute important evidence supporting autophagy as a potential therapeutic target that is specific to dormant minimal residual disease. Indeed, in light of our demonstration that autophagy inhibition reduces the number of surviving dormant tumor cells both in vivo and in vitro, and our findings that autophagy inhibition in mice bearing dormant residual tumor cells improves recurrence-free survival, we surmise that the ability of autophagy inhibition to improve recurrence-free survival in mice is attributable, at least in part, to its ability to deplete the population of dormant residual tumor cells that otherwise contribute to disease recurrence. In support of this interpretation, we observed no effects of autophagy inhibition either on primary tumorigenesis or on growth rates of primary or recurrent tumors. Nor did we observe selection for or against cells with impaired autophagy during primary tumorigenesis or during tumor regression. Consistent with this, we found that autophagy inhibition by chloroquine restricted specifically to the period of tumor dormancy significantly delayed recurrence and that the magnitude of this effect was similar to that observed for initiation of chloroquine treatment immediately at the time of HER2 downregulation. Together, these observations provide further support for a model in which the effects of autophagy inhibition on tumor cell survival are largely confined to tumor dormancy.

Knockdown or loss of BECLIN1 inhibits autophagy while increasing susceptibility to primary tumor formation in certain contexts; conversely, overexpression of BECLIN1 inhibits tumor growth [26–28]. These

observations suggest a tumor suppressive role for both BECLIN1 and autophagy. Interestingly, Vera-Ramirez et al. reported that while knocking down ATG7 expression significantly reduced the metastatic capacity of D2.0R cells compared to controls, knocking down BECLIN1 in D2.0R cells did not affect metastatic burden in vivo, leading these authors to conclude that autophagy occurs in dormant D2.0R cells via a non-canonical pathway independent of BECLIN1 [37]. In contrast, we found highly concordant effects on tumor dormancy and recurrence of deleting one allele of *Becn1*, knocking down ATG5, knocking down ATG7, or treating dormant tumor cells with chloroquine, whereby each of these autophagy-inhibiting approaches suppressed tumor recurrence, ostensibly via their ability to induce death in dormant tumor cells. Whether the differences between these results are attributable to differences in microenvironment-induced versus therapy-associated dormancy, or other differences between these model systems will require further study.

Notably, we observed similar effects of autophagy inhibition on dormant tumor cell survival and rates of tumor recurrence for shRNA knockdown of ATG5 or ATG7 in tumor cells in an orthotopic model and for constitutive heterozygous deletion of *Becn1* in intact mice. Whereas the former approach is restricted to tumor cells, the latter occurs in all cells of the mouse. Accordingly, *Becn1* heterozygous deletion would theoretically encompass non-cell autonomous effects of autophagy inhibition on tumor dormancy and recurrence, as would chloroquine treatment. Indeed, the magnitude of effects of *Becn1* heterozygous deletion and chloroquine treatment on recurrence-free survival were extremely similar. As the effects of shRNA knockdown of either ATG5 or ATG7 on recurrence-free survival were somewhat greater than those of chloroquine treatment or heterozygous *Becn1* deletion, one possibility is that autophagy was more effectively inhibited by ATG5 or ATG7 knockdown than Beclin1 heterozygous deletion or chloroquine treatment, although we cannot rule out the possibility that autophagy inhibition may have opposing tumor cell autonomous and non-autonomous effects.

Interesting recent evidence suggests that possibility that tumor cells that exist in a state of cellular dormancy may exhibit properties of senescent cells, and that senescence may constitute one pathway by which tumor cells subjected to therapeutic stress are induced to enter, or maintain, a reversibly quiescent state [57–59] This possibility is particularly intriguing in light of the demonstration that dormant tumor cells in our model are dependent upon autophagy for their survival, since autophagy and senescence commonly occur together and since there is some evidence to suggest that autophagy may be required for the maintenance of senescence [60, 61]. Whether dormant residual tumor cells in our model exhibit senescent features and, if so, whether this accounts for their dependence on autophagy will require further study.

Most anti-neoplastic therapeutic modalities preferentially target actively proliferating cancer cells. In light of the identification of cellular dormancy as a stage of tumor progression that is particularly reliant upon autophagy for survival, our findings suggest that dormant residual tumor cells may be uniquely susceptible to agents, such as chloroquine, that inhibit this process. To date however, most pre-clinical and clinical trials have principally employed autophagy inhibitors to target actively proliferating stages of disease that our findings predict may be intrinsically resistant to autophagy inhibition [56, 62–64]. Accordingly, our results suggest that autophagy inhibitors may be more effective in clinical contexts characterized by cellular tumor dormancy, and support autophagy inhibition as a tractable therapeutic strategy for preventing breast cancer recurrence by depleting the population of dormant residual tumor cells that otherwise give rise to incurable recurrent tumors.

Methods

Cell culture

The doxycycline inducible *MTB/TAN* primary tumor cell line was generated and cultured as described [7]. Chloroquine disphosphate salt and methylpyruvate were purchased from Sigma-Aldrich. Viability was calculated using Vi-CELL (Beckman Coulter).

Immunoblotting

Protein lysates were prepared by homogenizing tumors or cell lines in lysis buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1% Triton X-100) supplemented with Halt™ Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific). For Western blot analysis, membranes were probed with Alexa-Fluor-conjugated secondary antibodies (Molecular Probes). Bound antibodies were detected using the Odyssey detection system (LI-COR Biosciences). The following primary antibodies were used for Western blotting: anti-ErbB2 (Cell Signaling), anti–LC3 (Cell Signaling), anti–β-tubulin (Biogenex), anti–p62 (Progen Biotechnik), anti-ATG12 (Cell Signaling), and anti-ATG7 (Cell Signaling). Images were quantified using ImageJ.

Microscopy

For transmission electron microscopy, cells for electron microscopic examination were fixed with 2.5% glutaraldehyde, 2.0% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, overnight at 4°C. After subsequent buffer washes, the samples were post-fixed in 2.0% osmium tetroxide for 1 h at RT, and rinsed in DH_2O

prior to *en bloc* staining with 2% uranyl acetate. After dehydration through a graded ethanol series, the tissue was infiltrated and embedded in EMbed-812 (Electron Microscopy Sciences). Thin sections were stained with uranyl acetate and lead citrate and examined with a JEOL 1010 electron microscope fitted with a Hamamatsu digital camera and AMT Advantage image capture software. At least 25 cells were analyzed for each condition.

For fluorescence microscopy, cells were seeded onto 2-well chamber glass slides. Tissue was snap-frozen in O.C.T. (TissueTek) and cut into 8 μM frozen sections. Samples were fixed in 4% paraformaldehyde for 10 min then stained with Hoechst 33,258 (Sigma-Aldrich) to visualize nuclei. For EGFP-LC3 expressing cells, at least 25 cells for each condition were analyzed by quantifying EGFP-LC3 punctae using Cell Profiler [32]. Briefly, nuclei were segmented, and the number of punctae per nucleus was determined. Manual validation of nuclear segmentation and punctae quantification were performed on a training set prior to the automated analysis of samples. For EGFP-LC3-labeled tissues, four tumors were analyzed per time point and at least three non-consecutive slides per tumor were imaged. For the orthotopic fluorescent cell competition assay, the admixture of cells was plated onto 2-well chamber glass slides and six fields were quantified prior to injection into *nu/nu* mice for tumor generation. For each tumor or residual tissue sample, four non-serial sections per tumor and six images per section were obtained. The number of fluorescently labeled cells per image was determined using Image-Pro Plus 7 (Media Cybernetics).

For immunofluorescence analysis, cells were seeded on 2-well chamber glass slides, fixed in 4% paraformaldehyde for 10 min, and permeabilized with 0.5% Triton/PBS for 20 min. For tissue, 8 μM paraffin tissue sections were prepared using a standard xylene-based de-waxing procedure. Sections were subjected to antigen retrieval in the 2100 Retriever (Electron Microscopy Sciences). Cell and tissue samples then were blocked in 10% goat serum/4% BSA/PBS for 1 h and incubated overnight at 4 °C with primary antibody diluted in 4% BSA/PBS. Samples were stained with Alexa-Fluor-conjugated secondary antibodies (Molecular Probes) diluted in 4% BSA/PBS for 1 h at RT in addition to Hoechst 33,258 (Sigma-Aldrich) to visualize nuclei. Anti-Ki67 (DAKO) and anti-EGFP (Novus Biologicals) primary antibodies were used for immunofluorescence. Fluorescence and immunofluorescence microscopy were performed on a DM 5000B Automated Upright Microscope (Leica) and images were captured using a DFC350 FX monochrome digital camera (Leica).

Retrovirus production and infection

H2B-EGFP and EGFP-LC3 (Tamotsu Yoshimori, Ph.D., National Institute of Genetics, Shizouka-ken, Japan) were subcloned into pK1 retroviral vector as previously described [7]. Oligonucleotides for shRNAs targeting *Atg5* and *Atg7* were obtained from Open Biosystems. The following sequences were used: *Atg5* shRNA: TGCTGTTGACAGTGAGCGCAGAACCTGCCTTCTCACAAACTAGTGAAGCCACAGATGTAGTTTGTGAGAAGGCAGGTTCTTTGCCTACTGCCTCGGA; *Atg7* shRNA: TGCTGTTGACAGTGAGCGCTGCTACTAGATGAAGACAATATAGTGAAGCCACAGATGTATATTGTCTTCATCTAGTAGCAATGCCTACTGCCTCGGA. Oligonucleotides were cloned into the LMP vector (Open Biosystems) as described [33]. LMP vectors were generated that expressed H2B-EGFP or H2B-mCherry in place of EGFP by subcloning. The oligonucleotide targeting *Atg5* was also cloned into these vectors for use in the orthotopic competition assay.

Retroviruses were generated by transfection of Plat-E cells [34]. In brief, Plat-E cells were plated at 5×10^6 cells per 10-cm dish 1 d before transfection. Lipofectamine® 2000 transfection reagent (Life Technologies) plus 24 μg of the retroviral plasmid of interest was added to cells. Media was changed 6 h later and replaced with 5 ml fresh media. Viral supernatants were harvested two days later, centrifuged at 2,000 r.p.m., split into 1 ml aliquots, and snap frozen at -80 °C.

MTB/TAN primary tumor cells were plated at 1×10^5 cells per well in 6-well plates. The following day, 1 ml of viral supernatant, 3 ml of media, and 8 μg/ml polybrene were added to each well. Cells were then centrifuged at 2,000 r.p.m. for 2 h at RT and then incubated overnight at 37 °C. Media was changed the following day. Selection with 1 μg/ml puromycin was initiated 24 h later to isolate stable transfectants.

Mice and recurrence assays

Animal care and all animal experiments were performed with the approval of, and in accordance with, guidelines of the University of Pennsylvania IACUC. Orthotopic recurrence assays were performed as described [6]. 1×10^6 cells were injected into the inguinal mammary fat pads of athymic *nu/nu* mice (Taconic) maintained on 2 mg/ml doxycycline (Research Products International) and 5% sucrose in drinking water. For fluorescent cell competition assays, H2B-EGFP and H2B-mCherry-labeled cells were admixed in a 1:1 ratio and 1×10^6 cells of the resulting mixture were injected into the inguinal mammary fat pads of *nu/nu* mice. Animals were monitored twice per week for primary or recurrent tumor formation. For chloroquine treatment, animals were matched based on time of deinduction and then randomly assigned to a treatment cohort. Tumor volume

was calculated using the formula $(W^{2*}L)/2$ where $L \geq W$. Area under the curve (AUC) was calculated using the formula $[(vol_1+vol_2)/2]^*(day_2-day_1)$. Mean recurrent tumor growth rate was calculated using this formula $[(sumAUC)-(vol_1^*(day_n-day_1))]/(day_n-day_1)^2$.

Becn1$^{+/-}$ C57BL/6 mice (Zhenyu Yue, Ph.D., Mount Sinai School of Medicine, New York, New York, USA) were backcrossed 6 generations onto the FVB background. These animals were then crossed to *MTB/TAN* mice. 6-wk-old female offspring of the desired genotypes from each litter were induced with 2 mg/ml doxycycline (Research Products International) and 5% sucrose in the drinking water. Animals were monitored for mammary tumor development and recurrence once per week.

RNA isolation and qRT-PCR

RNA was isolated from cells using RNeasy® RNA Mini Kit (Qiagen) and reverse transcription was performed using the SuperScript® First-Strand Synthesis System for RT-PCR (Life Technologies) according to manufacturer's instructions. qRT-PCR was performed on the 7900 HT Fast Real-Time PCR system using 6-carboxyfluorescein–labeled Taqman probes (Applied Biosystems) specific for *Atg5*, *Atg7*, and *Tbp* as a reference. Relative expression levels were calculated using the comparative Ct method [35].

Flow cytometry

Mammary glands were harvested, manually minced, and incubated at 37 °C for 1 h in MEGM (Lonza), 1X B-27 (Gibco), 20 ng/ml bFGF (Sigma-Aldrich), 4 µg/ml heparin (Stem Cell Technologies), 5% Super Calf Serum (Gemini Bio-Products), and 1X Collagenase/Hyaluronidase (Stem Cell Technologies). Tissue was then suspended in red blood cell lysis buffer for 5 min, washed with 1X DPBS (cellgro), digested in 0.25% trypsin-EDTA (Gibco) for 2 min, washed, and digested in 1X DPBS (cellgro), 2% Super Calf Serum (Gemini Bio-Products), 1 mM EDTA, 1 mg/ml dispase II (Stem Cell Technologies), and 100 mg/ml DNAse I (Qiagen) for 5 min. After washing, cells were resuspended in 1X DPBS (cellgro), 2% Super Calf Serum (Gemini Bio-Products), and 1 mM EDTA and analyzed for EGFP-fluorescence using a FACSCalibur™ flow cytometer (BD Biosciences). Total fluorescently-labeled cell number was calculated using CountBright™ Absolute Counting Beads for flow cytometry (Molecular Probes).

Statistical analyses

Student's unpaired t-test was used for statistical analysis or Mann-Whitney *U* test when data was not normally distributed. Log-rank test was used when analyzing survival curves. P value < 0.05 was considered statistically significant. Hazard ratio with 95% confidence interval was calculated for survival curves.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13058-024-01878-7.

Supplementary Material 1

Acknowledgements

We thank Tamotsu Yoshimori for EGFP-LC3 construct, Zhenyu Yue for Beclin 1+/- mice, James Alvarez and Elizabeth Yeh for helpful discussions, Jianping Wang for histology assistance, and Dewight Williams and the Electron Microscopy Resource Laboratory of the University of Pennsylvania.

Author contributions

SD and LAC conceived of and designed the study, analyzed and interpreted the data, and wrote the manuscript. SED conducted experiments and acquired data, including experiments involving mice. JR assisted in data acquisition. HES and AAR contributed to experiments and data acquisition for experiments in involving mice.

Funding

This work was supported by grants from the NCI, the Department of Defense Breast Cancer Research Program, and the Breast Cancer Research Foundation.

Data availability

The datasets generated and/or analyzed in the current study are available in the manuscript and supporting files.

Declarations

Ethical approval

Not applicable.

Competing interests

The authors declare no competing interests.

Author details

[1]Department of Cancer Biology, Perelman School of Medicine at the University of Pennsylvania, Room 614 BRB II/III, 421 Curie Boulevard, Philadelphia, PA 19104-6160, USA
[2]Abramson Family Cancer Research Institute, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA 19104, USA
[3]Abramson Cancer Center, University of Pennsylvania, Philadelphia, PA 19104, USA
[4]Department of Medicine, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA 19104, USA

Received: 6 March 2024 / Accepted: 2 August 2024
Published online: 18 October 2024

References

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global Cancer statistics 2020: GLOBOCAN estimates of incidence and Mortality Worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2021;71(3):209–49.
2. Hadfield G. The dormant cancer cell. Br Med J. 1954;2(4888):607–10.
3. Karrison TG, Ferguson DJ, Meier P. Dormancy of mammary carcinoma after mastectomy. J Natl Cancer Inst. 1999;91(1):80–5.
4. Dalla E, Sreekumar A, Aguirre-Ghiso JA, Chodosh LA. Dormancy in breast Cancer. Cold Spring Harb Perspect Med. 2023.
5. Moody SE, Sarkisian CJ, Hahn KT, Gunther EJ, Pickup S, Dugan KD, et al. Conditional activation of Neu in the mammary epithelium of transgenic mice results in reversible pulmonary metastasis. Cancer Cell. 2002;2(6):451–61.

6. Ruth JR, Pant DK, Pan TC, Seidel HE, Baksh SC, Keister BA, et al. Cellular dormancy in minimal residual disease following targeted therapy. Breast Cancer Res. 2021;23(1):63.
7. Gunther EJ, Moody SE, Belka GK, Hahn KT, Innocent N, Dugan KD, et al. Impact of p53 loss on reversal and recurrence of conditional wnt- induced tumorigenesis. Genes Dev. 2003;17(4):488–501.
8. Janghorban M, Yang Y, Zhao N, Hamor C, Nguyen TM, Zhang XH, et al. Single-cell analysis unveils the role of the Tumor Immune Microenvironment and Notch Signaling in Dormant minimal residual disease. Cancer Res. 2022;82(5):885–99.
9. Morris VL, Tuck AB, Wilson SM, Percy D, Chambers AF. Tumor progression and metastasis in murine D2 hyperplastic alveolar nodule mammary tumor cell lines. Clin Exp Metastasis. 1993;11(1):103–12.
10. Morris VL, Koop S, MacDonald IC, Schmidt EE, Grattan M, Percy D, et al. Mammary carcinoma cell lines of high and low metastatic potential differ not in extravasation but in subsequent migration and growth. Clin Exp Metastasis. 1994;12(6):357–67.
11. Naumov GN, MacDonald IC, Weinmeister PM, Kerkvliet N, Nadkarni KV, Wilson SM, et al. Persistence of solitary mammary carcinoma cells in a secondary site: a possible contributor to dormancy. Cancer Res. 2002;62(7):2162–8.
12. Gunther EJ, Belka GK, Wertheim GB, Wang J, Hartman JL, Boxer RB, et al. A novel doxycycline-inducible system for the transgenic analysis of mammary gland biology. FASEB J. 2002;16(3):283–92.
13. Moody SE, Perez D, Pan TC, Sarkisian CJ, Portocarrero CP, Sterner CJ, et al. The transcriptional repressor snail promotes mammary tumor recurrence. Cancer Cell. 2005;8(3):197–209.
14. Abravanel DL, Belka GK, Pan TC, Pant DK, Collins MA, Sterner CJ, et al. Notch promotes recurrence of dormant tumor cells following HER2/neu-targeted therapy. J Clin Invest. 2015;125(6):2484–96.
15. Sreekumar A, Lu M, Choudhury B, Pan TC, Pant DK, Lawrence-Paul MR, et al. B3GALT6 promotes dormant breast cancer cell survival and recurrence by enabling heparan sulfate-mediated FGF signaling. Cancer Cell. 2024;42(1):52–69. e7.
16. Feng Y, Pan TC, Pant DK, Chakrabarti KR, Alvarez JV, Ruth JR, et al. SPSB1 promotes breast cancer recurrence by potentiating c-MET signaling. Cancer Discov. 2014;4(7):790–803.
17. Alvarez JV, Pan TC, Ruth J, Feng Y, Zhou A, Pant D, et al. Par-4 downregulation promotes breast cancer recurrence by preventing multinucleation following targeted therapy. Cancer Cell. 2013;24(1):30–44.
18. Chen S, Paul MR, Sterner CJ, Belka GK, Wang D, Xu P, et al. PAQR8 promotes breast cancer recurrence and confers resistance to multiple therapies. Breast Cancer Res. 2023;25(1):1.
19. Ecker BL, Lee JY, Sterner CJ, Solomon AC, Pant DK, Shen F, et al. Impact of obesity on breast cancer recurrence and minimal residual disease. Breast Cancer Res. 2019;21(1):41.
20. Chery L, Lam HM, Coleman I, Lakely B, Coleman R, Larson S, et al. Characterization of single disseminated prostate cancer cells reveals tumor cell heterogeneity and identifies dormancy associated pathways. Oncotarget. 2014;5(20):9939–51.
21. Marshall JC, Collins JW, Nakayama J, Horak CE, Liewehr DJ, Steinberg SM, et al. Effect of inhibition of the lysophosphatidic acid receptor 1 on metastasis and metastatic dormancy in breast cancer. J Natl Cancer Inst. 2012;104(17):1306–19.
22. Kobayashi A, Okuda H, Xing F, Pandey PR, Watabe M, Hirota S, et al. Bone morphogenetic protein 7 in dormancy and metastasis of prostate cancer stem-like cells in bone. J Exp Med. 2011;208(13):2641–55.
23. Bragado P, Estrada Y, Parikh F, Krause S, Capobianco C, Farina HG, et al. TGF-beta2 dictates disseminated tumour cell fate in target organs through TGF-beta-RIII and p38alpha/beta signalling. Nat Cell Biol. 2013;15(11):1351–61.
24. Mizushima N. Autophagy: process and function. Genes Dev. 2007;21(22):2861–73.
25. Aita VM, Liang XH, Murty VV, Pincus DL, Yu W, Cayanis E, et al. Cloning and genomic organization of beclin 1, a candidate tumor suppressor gene on chromosome 17q21. Genomics. 1999;59(1):59–65.
26. Liang XH, Jackson S, Seaman M, Brown K, Kempkes B, Hibshoosh H, et al. Induction of autophagy and inhibition of tumorigenesis by beclin 1. Nature. 1999;402(6762):672–6.
27. Cicchini M, Chakrabarti R, Kongara S, Price S, Nahar R, Lozy F, et al. Autophagy regulator BECN1 suppresses mammary tumorigenesis driven by WNT1 activation and following parity. Autophagy. 2014;10(11):2036–52.
28. Qu X, Yu J, Bhagat G, Furuya N, Hibshoosh H, Troxel A, et al. Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene. J Clin Investig. 2003;112(12):1809–20.
29. Flynn AB, Schiemann WP. Autophagy in breast cancer metastatic dormancy: Tumor suppressing or tumor promoting functions? J Cancer Metastasis Treat 2019;5.
30. Karantza-Wadsworth V, Patel S, Kravchuk O, Chen G, Mathew R, Jin S, et al. Autophagy mitigates metabolic stress and genome damage in mammary tumorigenesis. Genes Dev. 2007;21(13):1621–35.
31. Degenhardt K, Mathew R, Beaudoin B, Bray K, Anderson D, Chen G, et al. Autophagy promotes tumor cell survival and restricts necrosis, inflammation, and tumorigenesis. Cancer Cell. 2006;10(1):51–64.
32. Liu J, Debnath J. The evolving, multifaceted roles of autophagy in cancer. Adv Cancer Res. 2016;130:1–53.
33. Smith AG, Macleod KF. Autophagy, cancer stem cells and drug resistance. J Pathol. 2019;247(5):708–18.
34. Lum JJ, Bauer DE, Kong M, Harris MH, Li C, Lindsten T, et al. Growth factor regulation of autophagy and cell survival in the absence of apoptosis. Cell. 2005;120(2):237–48.
35. Han W, Pan H, Chen Y, Sun J, Wang Y, Li J, et al. EGFR tyrosine kinase inhibitors activate autophagy as a cytoprotective response in human lung cancer cells. PLoS ONE. 2011;6(6):e18691.
36. Vera-Ramirez L. Cell-intrinsic survival signals. The role of autophagy in metastatic dissemination and tumor cell dormancy. Semin Cancer Biol. 2020;60:28–40.
37. Vera-Ramirez L, Vodnala SK, Nini R, Hunter KW, Green JE. Autophagy promotes the survival of dormant breast cancer cells and metastatic tumour recurrence. Nat Commun. 2018;9(1):1944.
38. La Belle Flynn A, Calhoun BC, Sharma A, Chang JC, Almasan A, Schiemann WP. Autophagy inhibition elicits emergence from metastatic dormancy by inducing and stabilizing Pfkfb3 expression. Nat Commun. 2019;10(1):3668.
39. Sosa MS, Bragado P, Aguirre-Ghiso JA. Mechanisms of disseminated cancer cell dormancy: an awakening field. Nat Rev Cancer. 2014;14(9):611–22.
40. Vazquez-Martin A, Oliveras-Ferraros C, Menendez JA. Autophagy facilitates the development of breast cancer resistance to the anti-HER2 monoclonal antibody trastuzumab. PLoS ONE. 2009;4(7):e6251.
41. Kabeya Y, Mizushima N, Ueno T, Yamamoto A, Kirisako T, Noda T, et al. LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. Embo J. 2000;19(21):5720–8.
42. Poole B, Ohkuma S. Effect of weak bases on the intralysosomal pH in mouse peritoneal macrophages. J Cell Biol. 1981;90(3):665–9.
43. Pankiv S, Clausen TH, Lamark T, Brech A, Bruun JA, Outzen H, et al. p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. J Biol Chem. 2007;282(33):24131–45.
44. Mathew R, Karp CM, Beaudoin B, Vuong N, Chen G, Chen HY, et al. Autophagy suppresses tumorigenesis through elimination of p62. Cell. 2009;137(6):1062–75.
45. Pullman TN, Craige B, Alving AS, Whorton CM, Jones R. Comparison of Chloroquine, Quinacrine (Atabrine), and quinine in the treatment of Acute attacks of Sporozoite-Induced Viv Ax Malaria (Chesson Strain). J Clin Invest. 1948;27(3 Pt 2):46–50.
46. Mizushima N, Yoshimori T, Ohsumi Y. The role of Atg proteins in autophagosome formation. Annu Rev Cell Dev Biol. 2011;27:107–32.
47. Yue Z, Jin S, Yang C, Levine AJ, Heintz N. Beclin 1, an autophagy gene essential for early embryonic development, is a haploinsufficient tumor suppressor. Proc Natl Acad Sci USA. 2003;100(25):15077–82.
48. Braun S, Pantel K, Muller P, Janni W, Hepp F, Kentenich CR, et al. Cytokeratin-positive cells in the bone marrow and survival of patients with stage I, II, or III breast cancer. N Engl J Med. 2000;342(8):525–33.
49. Hartkopf AD, Brucker SY, Taran FA, Harbeck N, von Au A, Naume B, et al. Disseminated tumour cells from the bone marrow of early breast cancer patients: results from an international pooled analysis. Eur J Cancer. 2021;154:128–37.
50. Pantel K, Schlimok G, Braun S, Kutter D, Lindemann F, Schaller G, et al. Differential expression of proliferation-associated molecules in individual micrometastatic carcinoma cells. J Natl Cancer Inst. 1993;85(17):1419–24.
51. Solakoglu O, Maierhofer C, Lahr G, Breit E, Scheunemann P, Heumos I, et al. Heterogeneous proliferative potential of occult metastatic cells in bone marrow of patients with solid epithelial tumors. Proc Natl Acad Sci U S A. 2002;99(4):2246–51.
52. Muller V, Stahmann N, Riethdorf S, Rau T, Zabel T, Goetz A, et al. Circulating tumor cells in breast cancer: correlation to bone marrow micrometastases,

heterogeneous response to systemic therapy and low proliferative activity. Clin Cancer Res. 2005;11(10):3678–85.
53. Klein CA. Framework models of tumor dormancy from patient-derived observations. Curr Opin Genet Dev. 2011;21(1):42–9.
54. Agudo J, Aguirre-Ghiso JA, Bhatia M, Chodosh LA, Correia AL, Klein CA. Targeting cancer cell dormancy. Nat Rev Cancer. 2023.
55. Aqbi HF, Tyutyunyk-Massey L, Keim RC, Butler SE, Thekkudan T, Joshi S, et al. Autophagy-deficient breast cancer shows early tumor recurrence and escape from dormancy. Oncotarget. 2018;9(31):22113–22.
56. Lu Z, Luo RZ, Lu Y, Zhang X, Yu Q, Khare S, et al. The tumor suppressor gene ARHI regulates autophagy and tumor dormancy in human ovarian cancer cells. J Clin Invest. 2008;118(12):3917–29.
57. Saleh T, Gewirtz DA. Considering therapy-induced senescence as a mechanism of tumour dormancy contributing to disease recurrence. Br J Cancer. 2022;126(10):1363–5.
58. Saleh T, Bloukh S, Carpenter VJ, Alwohoush E, Bakeer J, Darwish S et al. Therapy-Induced Senescence: An Old Friend becomes the enemy. Cancers (Basel). 2020;12(4).
59. Duy C, Li M, Teater M, Meydan C, Garrett-Bakelman FE, Lee TC, et al. Chemotherapy induces Senescence-Like resilient cells capable of initiating AML recurrence. Cancer Discov. 2021;11(6):1542–61.
60. McGrath MK, Abolhassani A, Guy L, Elshazly AM, Barrett JT, Mivechi NF, et al. Autophagy and senescence facilitate the development of antiestrogen resistance in ER positive breast cancer. Front Endocrinol (Lausanne). 2024;15:1298423.
61. Triana-Martinez F, Loza MI. and Dominguez E. Beyond Tumor suppression: Senescence in Cancer Stemness and Tumor Dormancy. Cells 2020;9(2).
62. Amaravadi RK. Autophagy-induced tumor dormancy in ovarian cancer. J Clin Invest 2008.
63. Amaravadi RK, Lippincott-Schwartz J, Yin XM, Weiss WA, Takebe N, Timmer W, et al. Principles and current strategies for targeting autophagy for cancer treatment. Clin Cancer Res. 2011;17(4):654–66.
64. Amaravadi RK, Kimmelman AC, Debnath J. Targeting Autophagy in Cancer: recent advances and future directions. Cancer Discov. 2019;9(9):1167–81.

Publisher's Note