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14. ABSTRACT Late recurrent disease, which arises from tumor cells that lie dormant for extended periods before reawakening into lethal metastases, is a principal cause of mortality in breast cancer patients. We are dissecting how autophagy, a tightly controlled lysosomal digestion process, impacts breast cancer dormancy and metastatic colonization. Previously, we discovered that autophagy in breast cancer cells prevents metastatic colonization in vivo. Over the last year, we genetically ablated autophagy in a transgenic mammary cancer model that exhibits delayed kinetics with respect to the development of metastasis. Our results in this slow progression model also indicate that tumor cell autophagy impedes metastatic colonization. In addition, we identified the selective autophagy receptor, NBR1, as a key mediator of metastatic colonization in vivo as well as focal adhesion turnover in breast cancer cells in vitro. These results support our model that tumor cell autophagy restricts late recurrent disease by preventing the ability of dormant, disseminated tumor cells to exit from quiescent states by impeding focal adhesion remodeling and function. Lastly, we have found that systemic inhibition of autophagy, using the anti-malarial chloroquine, leads to reduced pulmonary metastasis. This contrasts with our results obtained with genetic autophagy inhibition specific to tumor cells and motivates the hypothesis that autophagy inhibition in host stromal constituents, rather than tumor cells, may direct the potentially beneficial effects of anti-malarial treatment in preventing breast cancer metastasis.

15. SUBJECT TERMS
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Table of Contents

I. INTRODUCTION:

Late recurrence continues to be a major barrier to eradicating breast cancer. Current evidence supports that such late recurrences in breast cancer likely arise from disseminated tumor cells that lie clinically dormant for extended periods of time [1]. These wayward breast cells either persist as solitary dormant cells, or form avascular micrometastases, which also remain dormant and clinically undetectable, presumably until they recruit appropriate micro-environmental factors to expand [1,2]. Nevertheless, to date, it remains largely unclear what biological processes govern the critical steps in late recurrent disease. These steps include: 1) the entry of breast cancer cells into dormancy, 2) the maintenance and survival of these cells during lengthy periods of quiescence, and ultimately, 3) their exit from dormancy to produce overt metastatic disease [2,3]. The overall goal of our research is to evaluate how changes in autophagy, a tightly-regulated lysosomal digestion process, impact one or more of these critical steps in the development of late recurrent breast cancer via the regulation of quiescent versus proliferative behavior exhibited by cancer cells. To address this issue, we have been taking multiple approaches using both three-dimensional culture models and in vivo mouse transgenic models to assay tumor dormancy and metastatic outgrowth in breast cancer. During years 1 through 3 of this award, we have obtained support for the intriguing hypothesis that autophagy inhibition promotes metastasis in multiple immunocompetent mammary cancer models. Moreover, our in vitro studies using 3D culture models uncovered that autophagy inhibition promoted the active proliferation of quiescent cells [4]. In addition, during years 1-3, we created new in vivo strategies to model late recurrent breast cancer, utilizing spontaneous orthotopic transplantation and experiment metastasis assays of tumors derived from the Polyoma middle T (PyMT)-based transgenic model generated in a pure C57B/6 background, which exhibits slower kinetics of tumor progression and metastasis.

Based on these results, we focused on the following scientific goals during year 4 of this EOHS award:

(1) Our primary focus during year 4 was the continuation of our mechanistic studies to dissect the potential mechanisms by which autophagy suppresses metastatic colonization and outgrowth. We followed up both of major lines of investigation from years 1-3, which had illuminated two potential mechanisms by which autophagy restricts metastasis. First, autophagy exerted cell autonomous control of focal adhesion turnover and signaling during early colonization and proliferative outgrowth. Second, based on our studies in 3D models demonstrating a role for autophagy in the control of secretion [5], the altered secretion of factors in autophagy-deficient cells modulated metastatic colonization. As detailed in sub-task 2c, these studies have yielded several interesting results. Most importantly, we identified the autophagy cargo receptor, NBR1, as a key mediator of early metastatic seeding and colonization in breast cancer [6]. Furthermore, we have obtained evidence that autophagy inhibition promotes the early steps of metastatic seeding via the control of focal adhesion turnover and we have uncovered a novel role for NBR1 in supporting focal adhesion turnover. The initial results of these studies were submitted for publication and they are currently in revision for *Journal of Cell Biology*.

(2) We initiated studies to assess whether autophagy suppresses macro-metastatic outgrowth and promotes dormant behavior using the slow progression PyMT-based models that we had generated for this project. These studies are ongoing, but the early results using experimental metastasis assays are consistent with the hypothesis that decreased autophagy promotes the exit of dormant tumor cells to produce overt and active metastatic disease.

(3) We began to determine the effects of antimalarial treatment on the onset of metastasis in mice transplanted with autophagy-competent neoplastic breast cells. In contrast to genetic autophagy inhibition specific to tumor cells, treatment with the anti-malarial chloroquine (CQ) leads to reduced pulmonary metastasis. We find this result to be very exciting because it indicates that CQ, which is being repurposed for the treatment of diverse cancers due to its long history of use in humans[7], may have utility as a strategy to reduce late recurrent metastatic outgrowth in breast cancer patients. They also raise the hypothesis that anti-malarials exert their

anti-metastatic effects via autophagy inhibition in host stromal constituents, rather than through its effects on breast cancer cell autophagy. We will test this important prediction over the upcoming year.

II. RESEARCH ACCOMPLISHMENTS BODY:

Task 1. Establish three-dimensional mammary epithelial cell culture and in vivo mouse model systems for late breast cancer recurrence.

a. Establish three-dimensional mammary epithelial organotypic cell culture model system cultured on basement membrane. (Months 1-12).

This sub-task was **completed** during year 1 and the findings were reported in the 2012 Annual Progress Report. Our studies of oncogenic-PI3K transformed breast cancer cells were published in *Oncogene* [4]*;* the full manuscript was included in the Appendix of the 2012 Annual Progress Report. Importantly, these data suggested that the inhibition of autophagy paradoxically promotes the active growth of quiescent cells, thus arguing against our originally proposed hypothesis.

b. **Quantify rates of proliferation and apoptosis in human breast cancer cell lines grown in three-dimensional epithelial organotypic cell culture** (Months 1-24).

These studies were completed during years 1-3 and reported in the 2012, 2013 and 2014 Annual Progress Reports. The major results were published in the prestigious journal *Cancer Discovery* [5]; the full manuscript was included in the 2014 Annual Progress Report as an Appendix.

Overall, these studies revealed two opposing, context-dependent functions for autophagy that potentially influence late recurrent metastatic progression. On the one hand, in breast tumors driven by the PI3K pathway, autophagy restricts proliferation and maintains a quiescent state. On the other, in tumors with hyperactivation of the Ras/MAPK pathway, autophagy has minimal effects on proliferation; rather, it promotes invasive behavior and alters epithelial differentiation and secretion. Our in vivo studies have been focused on precisely defining and elaborating these two opposing biological functions in vivo during analysis of the autophagy pathway in the transgenic breast models we have generated.

c. Optimize protocols for the stable ex vivo transduction of fluorescent (e.g., GFP), luminescent (e.g. luciferase), and drug resistant (e.g., puromycin) marker proteins into human cancer cell lines. (Months 1-12).

This sub-task was **completed** during year 1. The optimized protocols were detailed in the 2012 Annual Progress Report.

d. **For human cells in subtask 1b that exhibit a low proliferation index in 3D culture, introduce cells (containing fluorescent and/or drug resistance marker proteins) into the systemic circulation of immunodeficient mice and determine the latency period to the onset of metastasis. (Months 13-36; Revised timeline: Months 37-60).**

Based on our studies in subtask 1b during years 1 and 2, we identified the luminal breast cancer lines MCF7 and T47D as the most useful cells to potential utilize for 1d and 1e. Because of the unexpected results regarding autophagy that we obtained in our 3D culture studies, as well as in vivo results from PyMT and 4T1 cells described in subtask 2b, we deferred the initiation of these studies until we obtained more robust data from the PyMT model in sub-tasks 2b-c. Hence, we were able to prioritize the studies

from PyMT tumors regarding late recurrent disease because it is an immune competent, syngeneic model. The initial results are described below and support that autophagy impedes, rather than promotes, late recurrent growth. We have continued these studies using the immune competent models we have developed.

Based on those studies, our plan has been to address salient issues using the immunodeficient models in this task; we anticipate that the MCF7 cell line, which harbors an oncogenic PI3K mutation, will be the most appropriate human cell line to confirm and extend if autophagy impedes late recurrent growth in the PyMT model. Until recently, one of the primary limitations in using these cell lines has been the ability to genetically inhibit autophagy using stable RNA interference approaches. In our initial studies, detailed in the 2012 Annual Progress Report, we had difficulty achieving stable depletion of autophagy modulators (ATGs) for extended periods of time in MCF7 cells. As a result, we utilized anti-malarials, such as chloroquine, for in vitro studies. These studies are shown in Figure 1 and they demonstrated an intriguing result--that chloroquine (CQ) inhibited MCF7 cell proliferation at levels comparable to anti-estrogen treatment with 4-hydroxytamoxifen (4-OHT). 4OHT produced a 4.8-44.0% reduction in structure size in a dose dependent manner (Figure 1A). Upon CQ treatment, MCF7 structure size was reduced up to 33% in a dose-dependent manner (Figure 1B). 5µM CQ also elicited a 2-fold reduction in Ki-67 activity, which was comparable in efficacy to that obtained upon treatment with 5µM 4-OHT (Figure 1C and D).

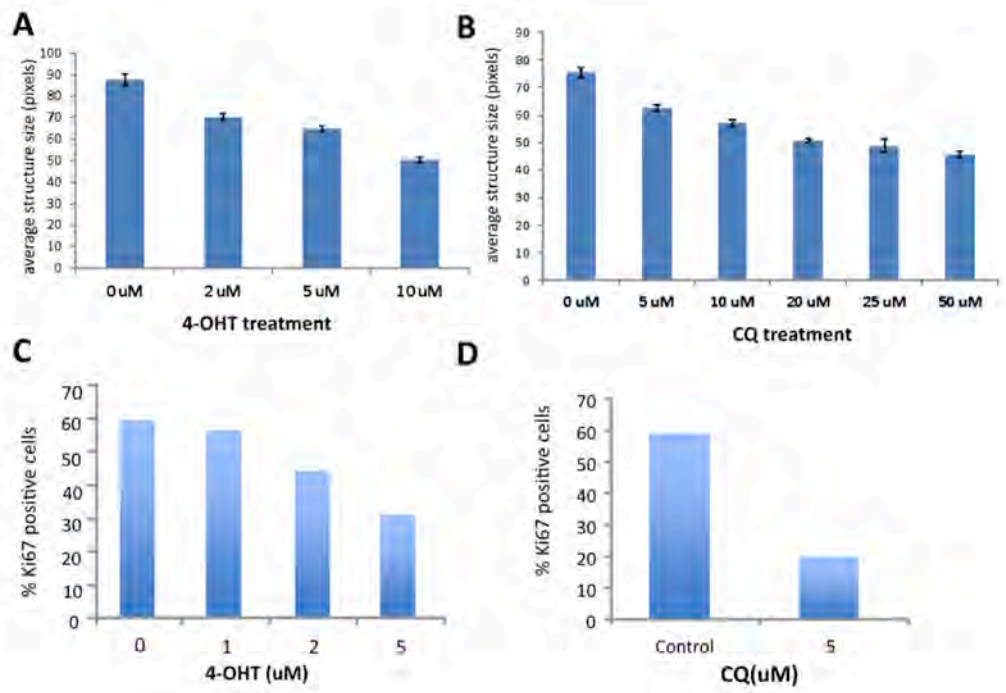

Figure 1: CQ inhibits MCF7 structure size and proliferation at compared levels to anti-estrogen treatment with 4-OHT. MCF7 3D cultures were treated with 4-OHT and CQ at the indicated concentrations (A, B): Structure size on day 11 of 3D culture. A total of 300 acini from three independent experiments were measured for each condition. Results represent mean ± SEM. (C, D): Ki-67 proliferation index following 11 days of treatment with 4-OHT and CQ.

Although these studies were consistent with a role for autophagy in supporting proliferation, they contrasted with our published results using PI3K-H1047R transformed MCF10A organotypic cultures upon CQ treatment in subtask 1a [4]. This raised an important question of whether the pharmacological effects of anti-malarials were specifically due to autophagy inhibition, or if other cellular processes contributed?

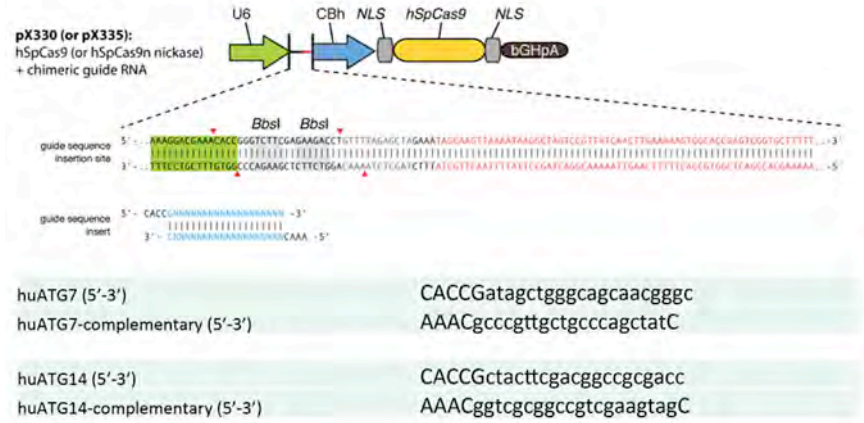

Fig 2. CRISP/Cas9-Mediated Genomic Deletion of cATGs. Top: Construct used for genomic editing was developed at Broad Institute, MIT (http://crispr.genome-engineering.org). Bottom: sgRNA targeting sequences against ATG7 and ATG14 used for genomic editing of human cell lines, as shown in Fig. 4.

In order to answer this question, as well as to achieve the goals in subtask 1d, a critical barrier necessary to overcome was to develop of a robust genetic approach to inhibit autophagy for extended periods in human cells. Over the past year, we have been able to overcome the limitations we previously encountered with stable RNA interference using clustered regularly interspaced short palindromic repeats (CRISPR)/ Cas9-mediated genomic editing in human cells [8]. In contrast to RNA interference, CRISPR results in stable genetic changes in cell lines. We have generated the small guide RNA reagents (sgRNAs) targeting key autophagy genes in human cell lines in a tactical fashion (Figure 2). As shown in figure 3, we have generated multiple clones in mammary cells that are lacking either ATG7 or ATG14, two core autophagy genes. These cells exhibit robust autophagy inhibition as evidenced by the strong accumulation of p62/SQSTM1, a substrate degraded via autophagy. We are now in the process of generating autophagy-knockout MCF7 clones into which we will introduce fluorescence (e.g. CFP, zsGreen) for tracking purposes in vivo. Over the upcoming year, we will utilize these new cell lines for the in vivo experiments proposed in sub-task 1d.

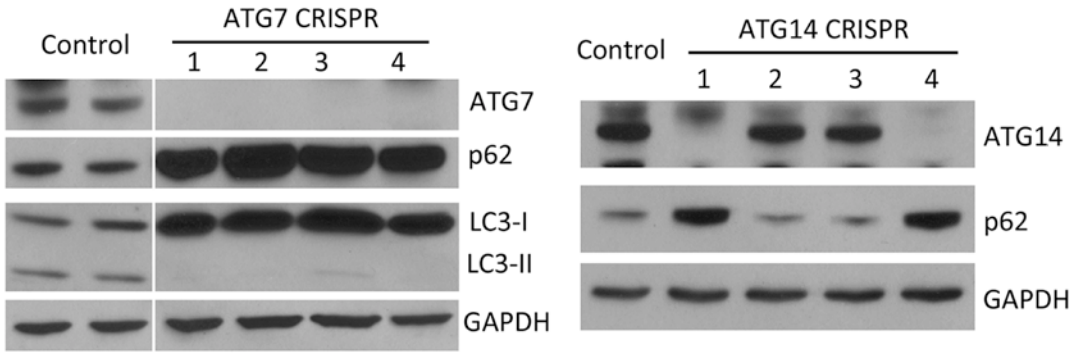

Fig. 3. CRISPR/Cas9-mediated gene deletion. CRISPR mediated genetic deletion of ATG7 (clones 1 through 4) and ATG14 (clones 1 and 4) in MCF10A mammary cells. ATG loss impairs autophagy, causing p62 accumulation in both cell types. ATG7 regulates early steps in autophagosome initiation; as a result, it also results in the loss of the LC3-II marker in cells. Control=scrambled sgRNA.

e. Isolate late onset macro-metastatic tumors and dormant tumor cells from subtask 1d, and obtain gene expression profiles. To obtain dormant cells from each cohort in subtask 1d, five to seven (5-7) tumor cell bearing mice will be euthanized at an intermediate time point (anticipated to occur at six to nine (6-9) months post initial injection but prior to overt metastatic disease), cancer cells isolated from host tissues, and if required, expanded ex vivo (Months 48-60).

These studies are planned for the upcoming year. Since subtask 1d was delayed to pursue studies in the

PyMT model, we have revised the timeline for subtask 1e accordingly.

f. Generate transgenic mice expressing MMTV-PyMT (Polyoma Middle T) and ROSA26-LSL-RFP in a pure genetic background (C57B/6). (Months 1-12).

We **completed** the generation of these compound transgenic mice in a pure C57B/6 genetic background, which was described in the 2012 and 2013 Annual Progress Reports.

g. Establish routine isolation and short-term culture conditions for normal and neoplastic mouse mammary epithelium. (Months 1-12).

This sub-task was **completed** during year 1. The optimized protocols have been detailed in the 2012 Annual Progress Report.

h. Optimize protocols for the stable ex vivo transduction of Cre recombinase and fluorescent, luminescent, and/or drug selection marker proteins into normal and neoplastic mouse mammary epithelium. (Months 1-12).

This sub-task was **completed** during year 1. The optimized protocols were detailed in the 2012 Annual Progress Report.

i. Isolate neoplastic epithelium from female transgenic mice generated in subtask 1f and transplant into syngeneic C57B/6 host recipient animals. (Months 13-24).

This sub-task was **completed** during year 2 and reported in the 2013 Progress Report.

j. Determine the latency period to the onset of metastasis for recipient mice generated in subtask 1i. Fifteen mice (15) will be functionally evaluated for altered characteristics of histopathologic progression from primary to metastatic disease (mammary morphogenesis, epithelial cell proliferation/cell death, overt tumor formation, tumor burden, tumor type, and development/latency to metastases) (Months 25-36; Revised timeline: Months 25-48).

In the previous year, when we transplanted mice with PyMT cells for 6 months, we were unable to detect overt tumors in intact lung tissue. However, as reported in the 2014 Annual Progress Report, we generated single cell suspensions from these lungs; via immunofluorescence microscopic analysis, we detected isolated CFP-positive tumor cells residing within the lung tissue of recipient mice; moreover, upon selection with G418, we observed the outgrowth of CFP-positive PyMT tumor colonies ex vivo, thereby confirming the presence of disseminated tumor cells. This tumor cell seeding suggested that metastatic disease would potentially develop over a longer time period. Upon carrying out the experiment over a longer period of 8 months, we have begun to observe macro-metastatic lesions derived from primary PyMT tumors in the lungs of C57B/6 hosts; the histology of the lungs from five representative animals, are shown in Figure 4, corroborate small, but overt, metastatic lesions. These experiments validate that the transplantation of PyMT cells can give rise to overt metastasis over time. Thus, we have obtained the first evidence that this C57B/6-based PyMT transplantation model exhibits features of a dormancy period and late recurrence.

We will further analyze the histopathological characteristics of these metastases and continue to employ this model to generate the data for subtask 1k. Nevertheless, both our published studies in subtask 1a-b and our ongoing studies in Task 2 have led to the working hypothesis that decreased autophagy promotes the exit of quiescent tumor cells to produce overt metastatic disease. Importantly, we have been able to reproduce the salient phenotypes supporting this model using experimental metastasis assays, which are shorter in duration (2-3 months versus 8-10 months). Hence, to more expeditiously pursue these studies,

we will complement our ongoing studies for subtasks 1j-k with experimental metastasis assays.

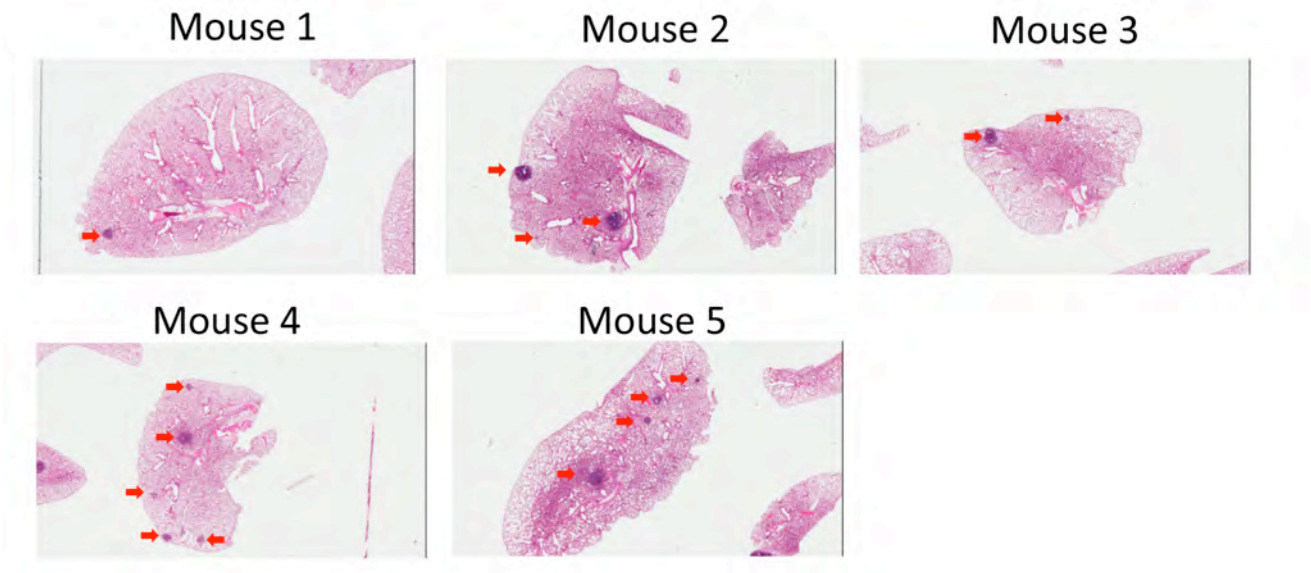

Fig. 4: Development of pulmonary macrometastasis from PyMT-derived tumor cells. Mice were orthotopically transplanted with primary tumor cells harvested from PyMT tumors (generated in subtask 2a) and lungs were analyzed at 240 d post-transplant. H&E staining of lung tissue reveals metastatic lesions (red arrows) Lung tissues from five individual mice are shown; quantification of metastatic burden is still in progress.

k. Isolate late onset macro-metastatic tumors and dormant tumor cells from mice in subtask 1i, and obtain gene expression profiles. To obtain dormant cells from subtask 1i, ten (10) tumor cell bearing mice from 1i will be euthanized at an intermediate time point (anticipated to occur at six to nine (6-9) months post fat pad transplantation but prior to overt metastatic disease), cancer cells isolated from host tissues, and if required, expanded ex vivo (Months 37-48, Revised timeline: 48-60).

These studies are being conducted over the upcoming year. As discussed above, in addition to analyzing metastasis from spontaneous assays, we will evaluate metastasis derived using experimental metastasis approaches.

Task 2. Determine the requirement for autophagy in the survival of dormant breast cancer cells.

a. Generate transgenic mice expressing MMTV-PyMT (Polyoma Middle T), ROSA26-LSL-RFP, and conditional null alleles (floxed) of autophagy regulators (e.g., atg12 and atg5) in a pure genetic background (C57B/6). (Months 1-24).

We already completed the interbreeding of the mice to generate the compound transgenic mice necessary for these studies in year 2, which we described in the 2013 Annual Progress Report. In year 3, we also generated compound transgenic mice contain MMTV-PyMT, *atg12* floxed alleles, tamoxifen-inducible Cre recombinase (CAG-Cre^{ERT}) [9], as well as a fluorescent reporter (R26-LSL-RFP) to monitor Cre excision [10], which we described in the 2014 Annual Progress Report. Moreover, as shown in studies from both sub-tasks 1j and 2b, we are able to isolate cells from tumors derived from these donor animals and store them for extended periods in cryostorage. Subsequently, these tumor cells can be expanded and reintroduced into syngeneic recipient animals, both spontaneous transplantation into mammary fat pads

and direct inoculation into the systemic circulation. The cell-based reagents we have generated were detailed in the 2013 and 2014 Annual Progress Reports. Overall, subtask 2a has been successfully completed and we now possess a frozen bank of PyMT tumor tissue in which we can genetically ablate autophagy in a tumor cell specific manner for our proposed subtasks. Moreover, in the case CAG-Cre-ER animals, we will conduct ATG deletion studies in a temporal-specific manner as the need arises.

b. Using techniques optimized in subtasks 1g-i, isolate neoplastic epithelium from female transgenic mice generated in subtask 2a, transduce with Cre recombinase, and transplant into cleared mammary fad pads of syngeneic C57B/6 host recipient animals. For this subtask, we anticipate that at least ten (10) mice from each transgenic donor will be utilized for epithelial isolation and at least twenty five (25) host recipient animals will be utilized for fat pad transplantation (Months 19-36; revised timeline: 19-60).

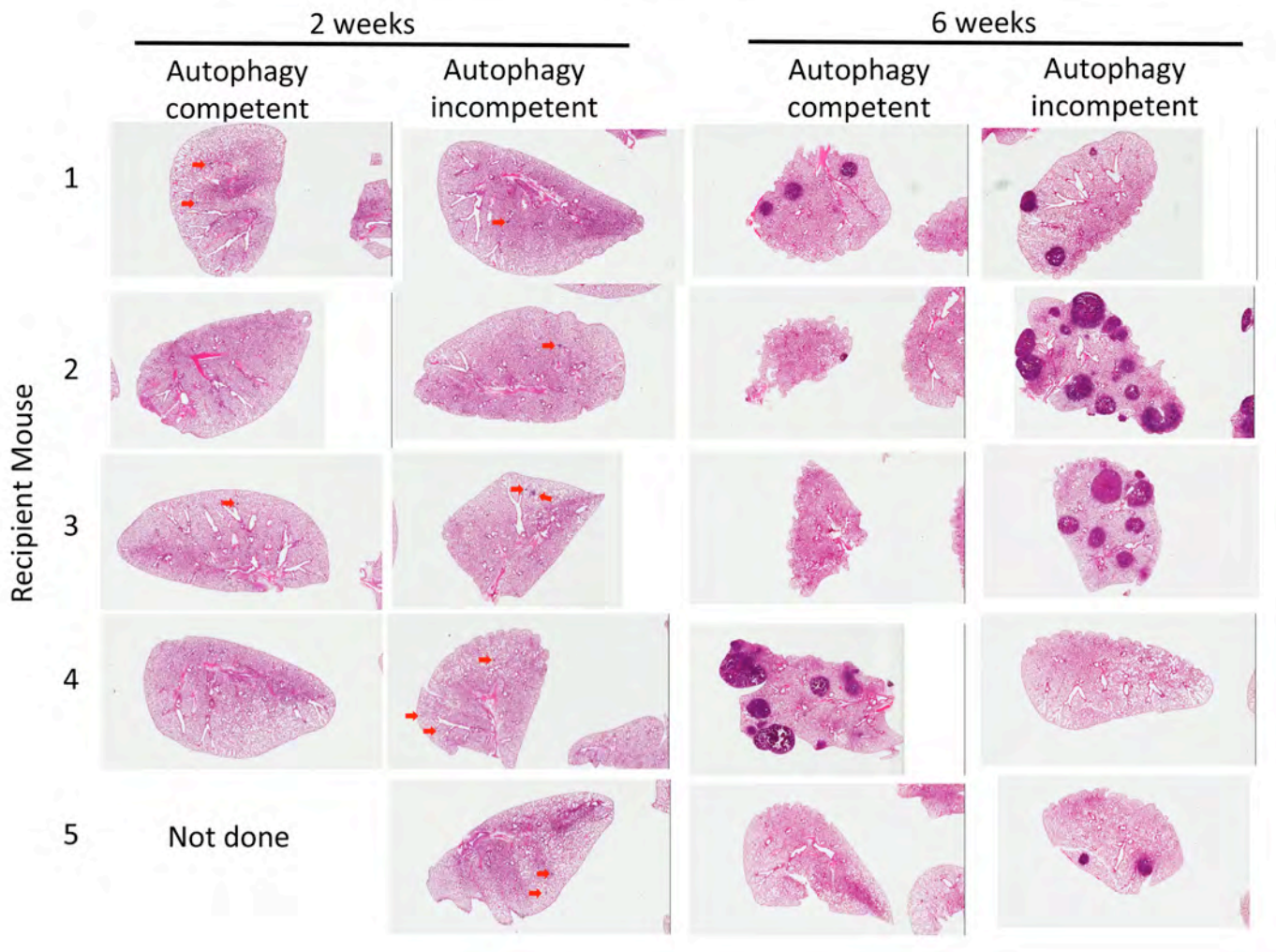

Fig 5: Effects of genetic deletion of ATG12 in PyMT mouse mammary cancer cells on metastasis to lung. Tail veil injection of PyMT cells that were autophagy-competent (ATG12 wild type, infected with empty vector) and autophagy-deficient (Ad-Cre infected to delete ATG12 ex vivo). Lungs were harvested at the indicated time points following inoculation. H & E staining of lung tissue reveals increased numbers of metastasis and increased size of metastatic lesions in autophagy-deficient cells. 5 recipient mice are shown for each time point and condition; for the 2 week time point, only four recipient animals were obtained for the autophagy-competent controls due to the lack of available donor cells for injection following ex vivo transduction. Additional biological replicates and the quantification of metastatic burden is currently in progress.

As discussed in the 2014 Annual Progress report, we began complementing the spontaneous metastasis assays with experimental metastasis assays to ascertain the specific roles of autophagy-deficiency during early lung colonization. This change was motivated by our emerging data over the past two years that the genetic inhibition of autophagy accelerates, rather than attenuates, metastatic burden in the lung using other more rapid progression models, such as the PyMT-R221 (FVB/n genetic background), and 4T1 (BALB/c genetic background) tumor cell models. Further mechanistic studies using those models are discussed in subtask 2c.

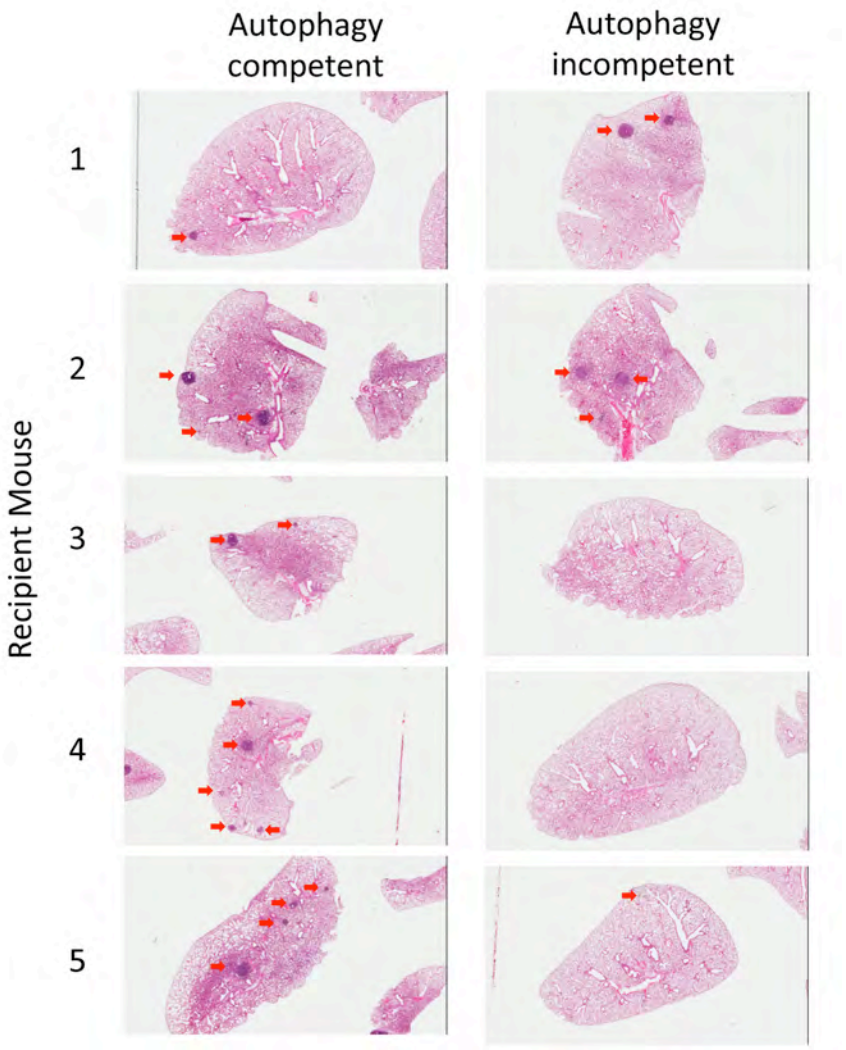

Fig. 6: Development of pulmonary macrometastasis from autophagy incompetent primary PyMT-derived tumor cells. Autophagy-competent cells are ATG12 wild type (infected with empty vector) and autophagy-deficient cells are ATG12 deficient. H&E staining of lung tissue reveals metastatic lesions (red arrows) after 240 d. Lung tissues from five individual mice of each cohort are shown. The lungs of the autophagy-competent controls are the same as those shown in Fig. 4 as part of the studies in subtask 1j.

Over the last year, we prioritized the experimental metastasis assays using the neoplastic epithelium generated from subtask 2a to assess whether the effects of autophagy on late metastatic colonization are reproduced in the model of late recurrence we have been developing. Our ongoing studies using this model support our hypothesis that autophagy ablation promotes the transition from early colonization and dormant behavior towards more aggressive outgrowth. Primary tumor cells from MMTV-PyMT, *atg12f/f*

donors were subject to adenoviral Cre or control adenovirus (empty vector) treatment using the protocols from subtask 1h; subsequently, 5 x 10^5 cells were introduced into the systemic circulation of C57B/6 mice (n=10 total host animals for each cohort). Five animals per cohort were euthanized at two time points--2 weeks and 6 weeks following initial inoculation. The lung histology from these animals, shown in Figure 5, suggests that metastatic colonization is increased upon ATG12 deletion in PyMT tumor cells. Because these results are promising, we have initiated additional biological repeats of this experiment, which are ongoing. In order to a obtain statistically robust result, our plan is to analyze 10-15 animals per time point from each cohort; similar to subtask 2c, we will conduct the precise quantification of lung metastatic burden, including the numbers of metastasis, the size of individual metastatic lesions, and if needed, the rates of proliferation and apoptosis in these lesions.

We view these experiments to be very exciting because they corroborate the importance of tumor cell autophagy in restricting metastatic outgrowth in a primary, slow-progressing model. We have also conducted spontaneous metastasis assays using PyMT-ATG12 floxed cells in parallel with the studies described for subtask 1j. As discussed in that subtask, our goal has been to determine the optimal time point to evaluate late recurrent metastasis in this model. Similar to controls, ATG12 deficient, autophagy-incompetent tumors develop metastasis over long-term periods. However, in contrast to the experimental metastasis assays, we have not observed an obvious increase in macrometastasis in the spontaneous assays, as illustrated in Figure 6. Because these studies involve a small sample size, it is difficult to ascertain their significance at this juncture; accordingly, we are repeating the experiments to more rigorously corroborate this result. Nevertheless, if we do find that this result is valid, these discrepancies between the spontaneous and experimental metastasis models point to a specific effect of autophagy on colonization at distant tissue sites, which has been a key question we have sought to address through our mechanistic studies In subtask 2c.

c. Determine whether the deletion of autophagy regulators from transplanted neoplastic breast cells affects the onset of metastasis in recipient mice from subtask 2b. Fifteen (15) mice from each cohort will be functionally evaluated for altered characteristics of histopathologic progression from primary to metastatic disease (mammary morphogenesis, epithelial cell proliferation/cell death, overt tumor formation, tumor burden, tumor type, and development/latency to metastases) (Months 25-48).

Over the last two years, we unexpectedly found that genetic inhibition of autophagy in tumor cells led to increased metastasis in the lung; these findings were first reported in our 2013 Annual Progress Report. These studies were initially conducted using the PyMT-R221-based model in which tumor cells were transplanted into syngeneic hosts for analysis of metastasis phenotypes. To inhibit autophagy, we expressed shRNA against ATG7 or ATG12 for stable RNAi-mediated silencing of this essential autophagy regulator. Using both experimental and spontaneous metastasis models, we discovered that mice transplanted with autophagy-deficient PyMT cells exhibited a significantly increase in overall number of

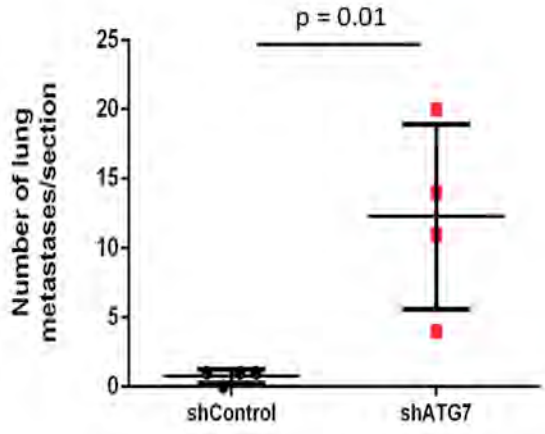

Fig. 7: ATG knockdown in 4T1 mouse mammary cancer cells enhances spontaneous metastasis to lung. Mice were orthotopically transplanted into the mammary fat pad with 4T1 cells that were autophagy-competent (shCTL) and autophagy-deficient (shATG7). Lungs were harvested at 21 days post-transplant and the number of lung metastasis per section was quantified (10 randomly sampled lung sections per quantified from each animal, n=4 animals per cohort).

macrometastases.

Based on this initial analysis, an important priority became to corroborate these findings in an independent model. We chose to do so using second well-established immune competent model of mouse mammary carcinoma progression and metastasis-the 4T1 model. Similar to PyMT, the 4T1 mammary cancer model exhibits high rates of metastasis to distant sites, including the lung, with established kinetics [11]. We reported initial results in the 2014 Annual Progress Report. To summarize those previous results, we achieved stable, robust autophagy inhibition in these cells via lentiviral mediated stable shRNA knockdown of ATGs, which was evidenced by the loss of PE-lipidated LC3 (LC3-II) and the accumulation of p62/SQSTM1. Upon orthotopic transplantation into syngeneic BALB/c hosts, primary 4T1 tumors rapidly grow over a 3-4 week period; no differences in primary tumor growth were observed upon autophagy inhibition. Nevertheless, the initial analysis of the lungs revealed increased metastasis in animals transplanted with ATG-deficient tumors compared to controls. We have quantified these effects of autophagy inhibition on metastasis, which is shown in Figure 7. Overall, these results establish that autophagy impedes rather than promotes metastasis in two established immune-competent models of breast cancer metastasis in vivo—4T1 and PyMT. As we have discussed above, these findings in both of these mammary cancer models contrast with some of our published results from studies in sub-task 1b, which were conducted using HRasV12-transformed cells and reported in the 2012 Annual Progress Report [5]. Our previous results may be context specific, because Ras-transformed cells require autophagy for growth and metabolism [3]. Consistent with this idea, we obtained contrasting results from subtask 1a demonstrating that autophagy restricts proliferation and maintains a quiescent state in vitro in breast cancer cells with activating mutations in the PI3K pathway [4].

Nevertheless, our in vivo results support a model in which autophagy suppresses metastasis at a specific stage, most notably early colonization at the foreign tissue site, which is viewed by the cancer field as a key and likely rate-limiting step in metastasis [12]. This has clinical implications because it suggests that enhanced metastasis may be a long-term risk of autophagy inhibitors like hydroxychloroquine (HCQ). Thus, a key unresolved issue is resolving the mechanisms by which reduced autophagy suppresses early metastatic colonization in breast cancer. ***In order to address this important question, the mechanistic experiments in subtask 2c have been our major scientific focus during year 4; the results from these experiments are described in detail below.***

Pro-metastatic functions of the autophagy cargo receptor NBR1: Autophagy cargo receptors (ACRs) mediate selective autophagic degradation of intracellular proteins, and in the process, they are degraded via autophagy [13]. In cancer, the accumulation of autophagy cargo adapters, specifically p62/SQSTM1, has been shown to drive primary tumor growth in autophagy-deficient cells [14-17]. Notably, during the first year of this award, we demonstrated in sub-task 1a that p62 accumulation was a key mediator of oncogenic PI3K-driven proliferation in our 3D culture model; these results were published and reported in our 2012 Progress Report [4]. Despite these profound effects on primary tumor growth and oncogenic proliferation, the effects of p62/SQSTM1 on metastasis are unknown. Moreover, it remains unclear whether autophagy cargo adapters other than p62 impact metastasis. During year 3, we evaluated whether the pulmonary metastasis from the autophagy-deficient models exhibited increased levels of autophagy cargo receptors and confirmed an increase in p62/SQSTM1 as well as in a second autophagy cargo receptor, NBR1 (Neighbor of BRCA1) in metastatic cells culture from the lungs of mice transplanted with PyMT-shATG7 tumors; these results were previously detailed in the 2014 Annual Progress Report.

These results broached the intriguing hypothesis that the accumulation of specific autophagy cargo receptors promotes metastatic outgrowth in autophagy deficient cells. Accordingly, we predicted that the loss of one of these ACRs, namely NBR1 and/or p62/SQSTM1, would inhibit metastatic seeding or colonization. To test this prediction, we began to develop loss-of-function strategies to stably deplete autophagy cargo receptors in breast cancer cells. Over the past year, we successfully developed the

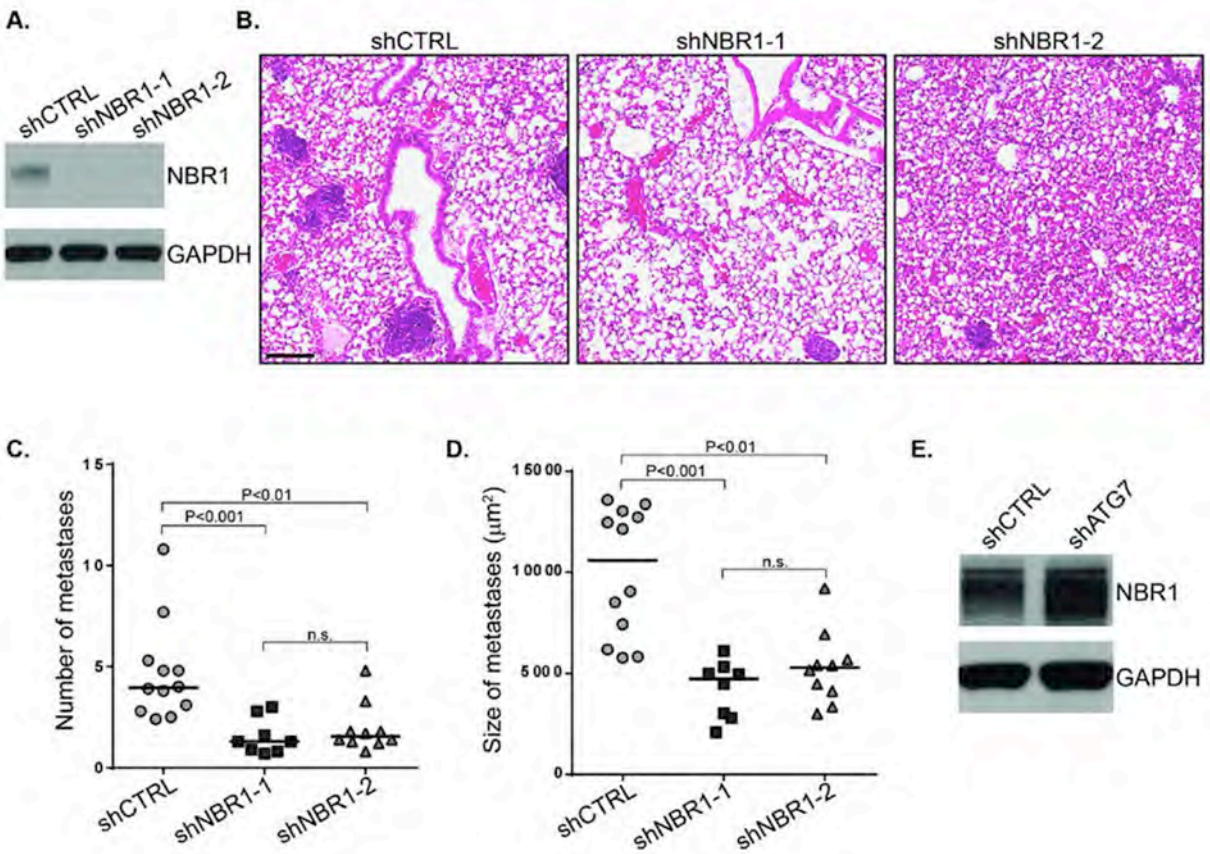

Fig. 8: NBR1 depletion impedes pulmonary metastasis in PyMT-R221 cells. (A) Immunoblot verifying knockdown of NBR1 in R221A cells using two independent shRNAs. GAPDH is the loading control. (B) Representative lung H&E from experimental metastasis studies in mice injected with cells expressing control shRNA or shRNAs against NBR1. (C) Quantification of the number of lung metastases per field shown in B. Four total fields from three sections per mouse were counted. Data is presented as median (line) and each data point represents a single mouse. n=12 mice for shCTRL, n=8 mice for shNBR1-1, and n=10 mice for shNBR1-2 pooled from two independent experiments. P value determined using a non-parametric Kruskall-Wallis test followed by Dunn's post-test. n.s.=not significant. (D) Quantification of the size of lung metastases from fields of view shown in B. Four total fields from three sections per mouse were analyzed. Data is presented as median (line) and each data point represents a single mouse. n=12 mice for shCTRL, n=8 mice for shNBR1-1, and n=10 mice for shNBR1-2 pooled from two independent experiments. P value determined using a non-parametric Kruskall-Wallis test followed by Dunn's post-test. n.s.=not significant. (E) Immunoblot showing accumulation of NBR1 in cells inhibited for autophagy via knockdown of ATG7. GAPDH is the loading control.

reagents to stably knockdown NBR1 and evaluated its role in the metastasis the PyMT-R221 model using experimental metastasis assays. NBR1 was depleted from R221A cells via stable expression of two independent shRNAs (Fig. 8A); control and NBR1 deficient cells were inoculated into the lateral tail vein. Upon quantification of lung histology at two weeks post-injection, NBR1 loss-of-function, significantly inhibited metastasis (Fig. 8B). This was characterized by a significant decrease in both the size and number of metastases (Fig. 8C-D). These data are consistent with the concept that NBR1 functions to support metastasis at foreign tissue sites Moreover, because we have observed that NBR1 levels are elevated in autophagy-deficient R221A cells (Fig. 8E), we propose that its accumulation in autophagy deficient cells may support metastatic states in vivo. Intriguingly, with regard to late recurrent disease, NBR1 can inhibit p38 MAPK signaling, which is a known promoter of metastatic dormancy [2,18], thereby, broaching a model in which NBR1 accumulation upon autophagy inhibition may be sufficient to enhance metastasis, potentially through down-regulation of p38 signaling. To establish whether NBR1 is unique in

this regard, we seek to evaluate whether p62/SQSTM1 similarly contributes as a pro-metastatic factor; we still need to generate the shRNA reagents required for these experiments, which we plan to conduct over the upcoming year. In ongoing work, we are further testing this role for NBR1 as a novel pro-metastatic factor to define the molecular basis of metastasis suppression by autophagy; as detailed below, we have uncovered a novel role for NBR1 in focal adhesion turnover that may be an important mediator of its pro-metastatic functions.

Effects of autophagy inhibition and NBR1 on focal adhesion (FA) remodeling: In parallel to our in vivo work with NBR1, we also continued our mechanistic studies of autophagy in focal adhesion remodeling that were initiated during year 3. As discussed below, our studies in year 4 have uncovered a novel role for NBR1 in enabling autophagy-mediated focal adhesion turnover in breast cancer cells, which intriguingly correlate with our findings that NBR1 impacts metastatic colonization (Figure 8). As a result, these studies became a major focus during the past year.

These mechanistic studies were originally motivated by published work by other labs demonstrating a key role for focal adhesions and focal adhesion-based signaling in the outgrowth of metastatic colonies [19-22]. In our 2014 Annual Progress Report, we demonstrated that autophagy inhibition led to Increased focal adhesion size in both PyMT-R221 and $HRAS^{V12}$ MCF10A mammary epithelial cells. Because FAs undergo cycles of turnover and remodeling in which they both assemble and disassemble in order for cells to migrate, these increases in FA size suggest defective turnover in autophagy-deficient cells [23,24]. We also conducted real-time dynamic imaging studies, which revealed that ATG knockdown significantly reduced FA disassembly and increased FA lifetime. These results were originally reported in the 2014 Annual Report and broached the intriguing hypothesis that efficient focal adhesion turnover during metastasis may be regulated by the autophagy pathway.

To initially follow up the novel results from year 3, we further dissected the role of the autophagy cargo receptors in focal adhesion turnover. To initially establish the individual autophagy cargo receptors regulate migration, we transiently depleted for several of the major known receptors, including p62/SQSTM1, NBR1, optineurin (OPTN), and nuclear dot protein 52 (NDP52) (Fig 9A) in HRASV12-transformed mammary epithelial cells and performed scratch-wound closure assays in Figure 10. Importantly, we identified NBR1 as the only cargo receptor whose knockdown significantly attenuated wound closure (Figs. 10A-B), consistent with its role in metastasis in vivo. To verify this result, we generated stable pools of cells with shRNA against NBR1 (Fig. 9B). In agreement with our findings using transient, siRNA-mediated depletion, stable NBR1 knockdown also significantly inhibited migration (Fig. 10C-D). Importantly, in contrast to ATG knockdown, NBR1 depletion did not affect basal autophagy levels (Fig. 9C).

To further dissect the role of NBR1 in motility, we measured FA dynamics in paxillin-mCherry-expressing cells upon stable NBR1 knockdown. Similar to our previously reported results observed with ATG depletion last year, NBR1 loss-of-function decreased the rates of FA assembly and disassembly by 32% and 41%, respectively, leading to an overall 81% increase in FA lifetime compared to controls (Fig. 11A-B). Consistent with this role for NBR1 in facilitating FA turnover, endogenous NBR1 co-localized with anti-paxillin-labeled FAs, and GFP tagged NBR1 associated with dynamic leading edge FAs in live migrating cells (Fig. 11C-D).

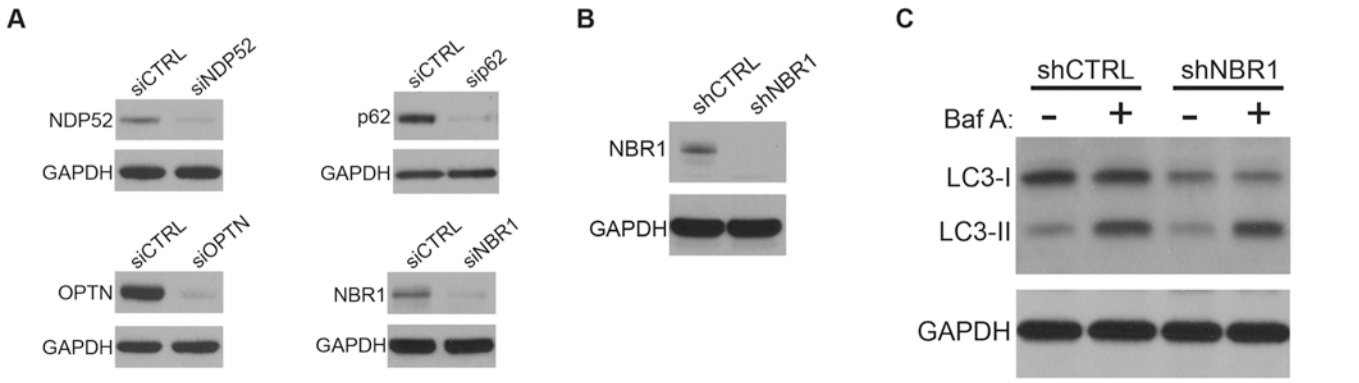

Fig. 9: RNAi-mediated depletion of autophagy cargo receptors. (A) siRNA-mediated depletion of the indicated autophagy cargo receptors used for scratch-wound closure assays. GAPDH is loading control. (B) shRNA knockdown of NBR1. (C) LC3-II turnover in the absence or presence of bafilomycin A (Baf A, 20 nM for 30 min). GAPDH is loading control.

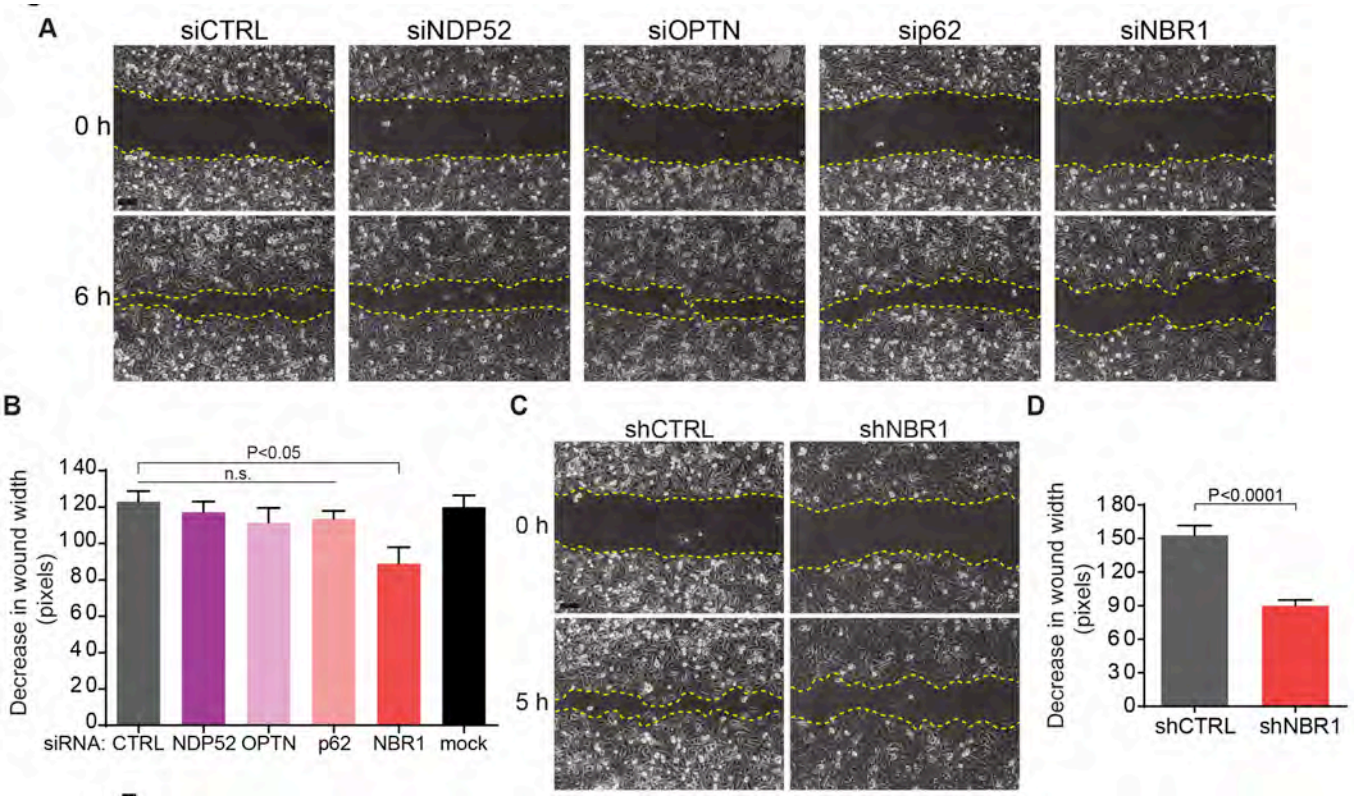

Fig. 10: NBR1 facilitates cell migration and FA turnover. (A) Representative phase contrast microscopy images at time of wounding (0 h) and 6 h post-wounding for cells expressing control siRNA (CTRL) or siRNA against NDP52, OPTN, p62, or NBR1. Dashed yellow lines highlight wound boundaries. Bar, 100 µm. (B) Quantification of wound closure over 6 h by cells expressing indicated siRNAs (mean+s.e.m.). The decrease in wound width was determined by subtracting the final width at 6 h from the initial width at 0 h. n=8 wounds for siCTRL, n=8 wounds for siNDP52, n=8 wounds for siOPTN, n=6 wounds for sip62, n=6 wounds for siNBR1, and n=5 wounds for mock (no siRNA), pooled from four independent experiments. P values calculated using one-way ANOVA followed by Tukey's post-test. n.s.=not significant. (C) Representative phase contrast microscopy images of cells expressing shCTRL or shNBR1 at time of wounding (0 h) and at 5 h post-wounding. Dashed yellow lines highlight wound boundaries. Bar, 100 µm. (D) Quantification of wound closure over 5 h by shCTRL and shNBR1 cells (mean+s.e.m.). n=12 wounds for shCTRL and n=15 wounds for shNBR1, pooled from three independent experiments. P value determined using Student's unpaired t-test.

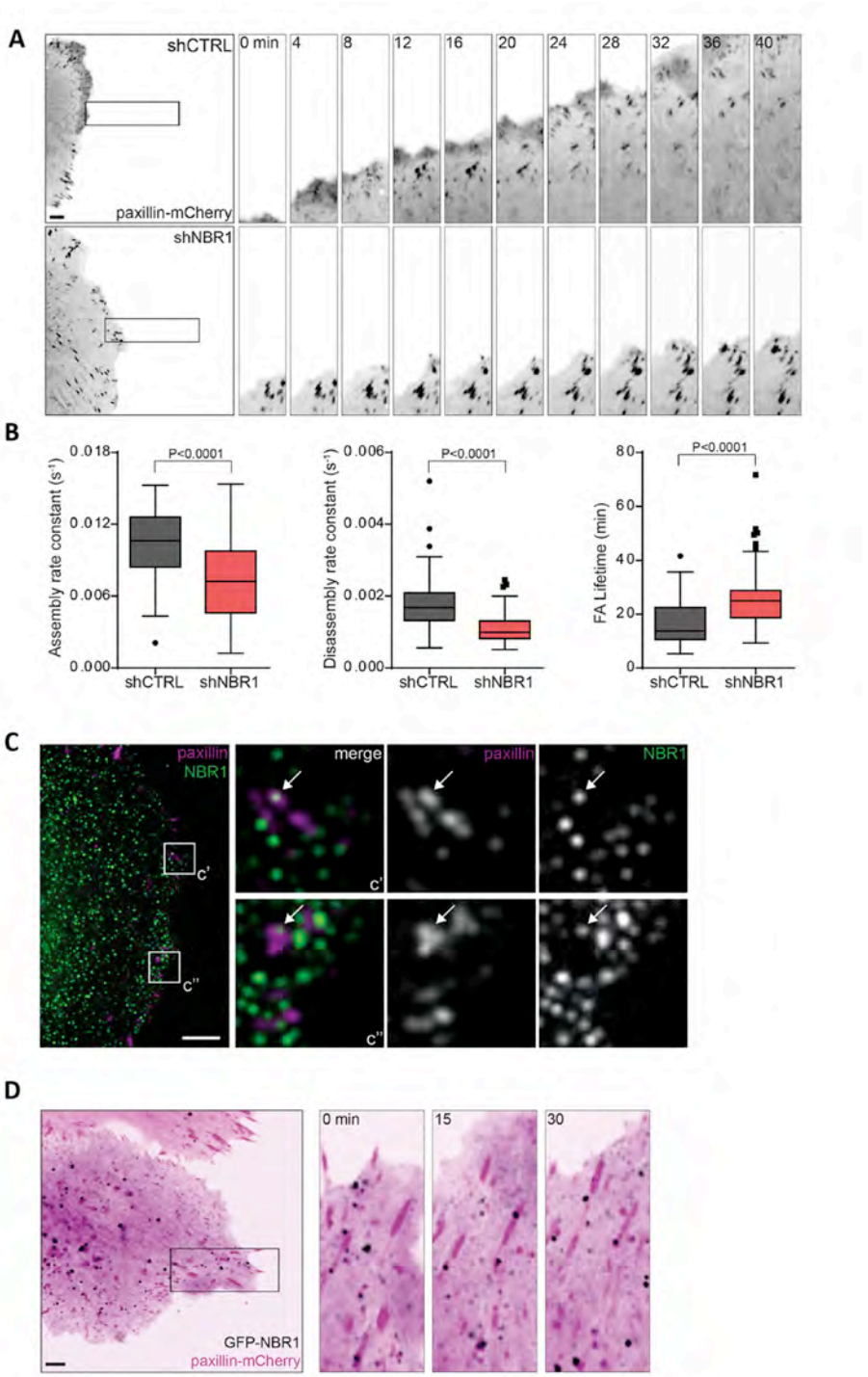

Fig. 11: NBR1 facilitates FA turnover. A) Spinning disk confocal microscopy time-lapse sequences of cells expressing paxillin-mCherry (black) to monitor FA dynamics. Left panels show representative cells expressing shCTRL (top) or shNBR1 (bottom). Image sequences of boxed regions on the right have been rotated such that the cell edge with dynamic FAs is moving upwards vertically. Elapsed time (min) in top left of images. Bar, 5 mm. (D) Quantification of FA assembly rate constants (left), disassembly rate constants (middle), and lifetime (right) for FAs in shCTRL or shNBR1 cells. Data presented as median (line), first and third quartile (box), and whiskers extend to +/- 1.5 times the interquartile range. P value calculated using a non-parametric Mann-Whitney test. n=53 FAs for shCTRL and n=58 FAs for shNBR1, pooled from three independent experiments. (C) Representative immunofluorescence images of wound edge cells stained for endogenous paxillin (magenta) to mark FAs and endogenous NBR1 (green). Whole cell merged image shown at left and enlarged boxed insets of merged and single-channel paxillin and NBR1 images shown at right. Arrow points to co-location in insets. Bar, 5 µm. (E) Spinning disk confocal microscopy of a migrating cell expressing GFP-NBR1 (black) and paxillin-mCherry (magenta). Boxed region is shown as enlarged insets to the right rotated such that the cell edge is moving upwards vertically.

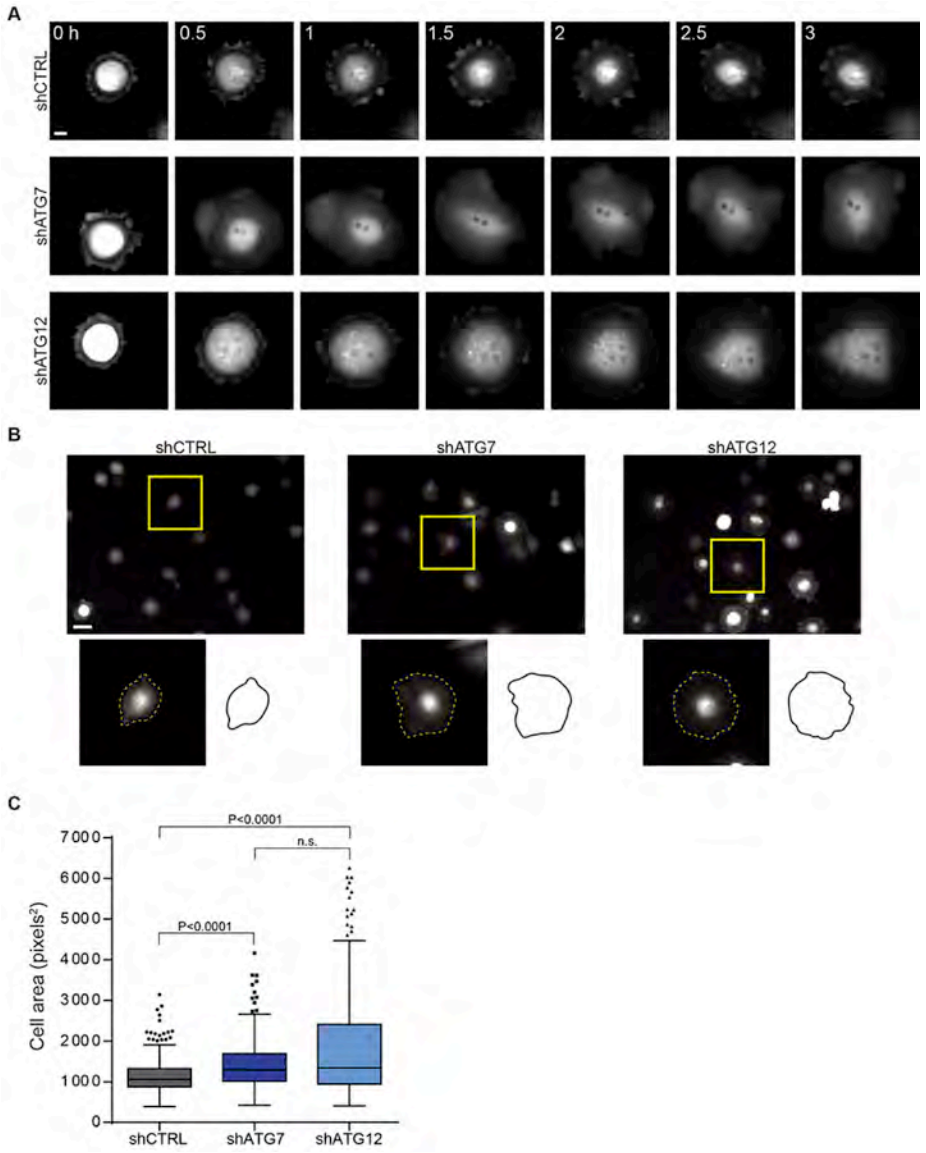

Fig 12: Autophagy inhibition results in enhanced cell spreading. (A) Spinning disk confocal microscopy time-lapse sequences of cells expressing ZsGreen during spreading after replating. Representative cells shown over a 3h time course. Elapsed time (h) indicated in top left of images. Bar, 10µm. (B) Representative images of ZsGreen expressing cells fixed 1h post-replating used for quantification of cell area in (C). Whole field images shown with enlarged boxed insets of individual cells at bottom left. Tracing of individual cell in inset shown at bottom right. Bar, 50 µm. (C) Quantification of area of cells fixed 1h post-replating. Area determined by manually outlining individual ZsGreen-expressing cell borders. Data presented as median (line), first and third quartile (box), and whiskers extend to +/- 1.5 times the interquartile range. n=315 cells for shCTRL, n=351 cells for shATG7, and n=306 cells for shATG12, pooled from 3 independent experiments. P values calculated using a non-parametric Kruskall-Wallis test followed by Dunn's post-test.

In addition to affecting motility, increased stabilization of FAs is also associated with enhanced cell spreading, which has been shown to impact both metastatic seeding and colonization via diverse mechanism [25,26]. Accordingly, we performed cell-spreading assays to further assess the functional impact of FA stabilization upon autophagy inhibition. We generated control and ATG knockdown cells expressing ZsGreen for tracking purposes, and using live-cell imaging, we monitored the spreading of cells upon replating over 3 h. These assays revealed that autophagy-deficient cells underwent prolonged spreading, resulting in an increased cell area compared to controls (Fig. 12A). Quantification of the area of ZsGreen-expressing cells fixed at 1h post-replating further confirmed that ATG-depleted cells exhibited a

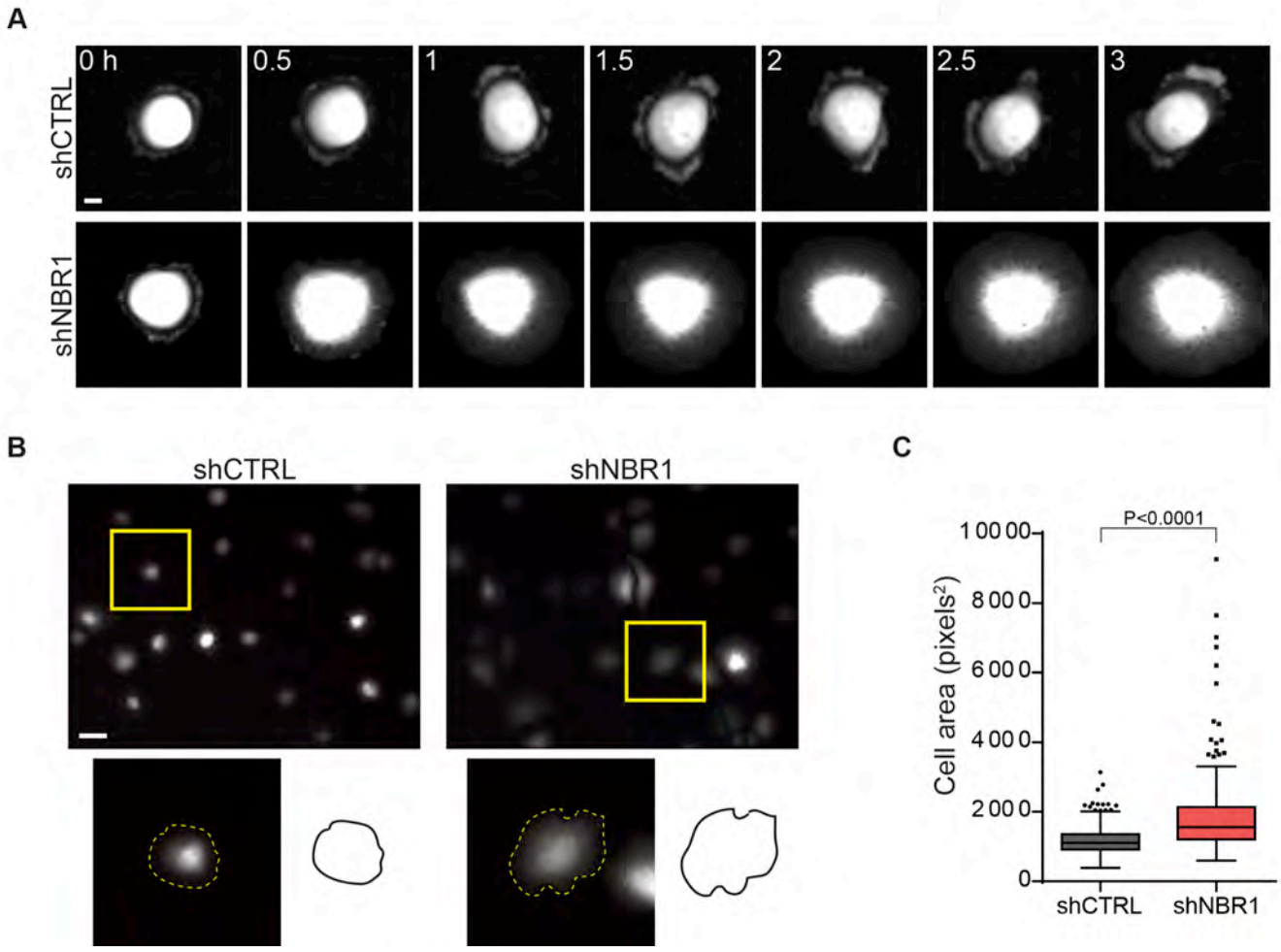

Fig. 13: NBR1 knockdown leads to enhanced cell spreading. (A) Spinning disk confocal microscopy time-lapse sequences of cells expressing ZsGreen during spreading after replating. Representative images of shCTRL (top) and shNBR1 (bottom) cells are shown over 3 h. Elapsed time (h) indicated at top left. Bar, 10 µm. (B) Representative images of ZsGreen-labeled cells fixed at 1 h post-replating. Whole field images shown with boxed insets of individual cells enlarged at bottom left. Tracing of individual cell in inset at bottom right. Bar, 50 µm. (C) Quantification of area of shCTRL- and shNBR1-expressing cells fixed 1 h post-replating. Area determined by manually outlining individual ZsGreen-expressing cell borders. Data presented as median (line), first and third quartile (box), and whiskers extend to +/- 1.5 times the interquartile range. n=211 cells for shCTRL and n=195 cells for shNBR1, pooled from two independent experiments. Note that these experiments were run in conjunction with two out of the three experimental repeats in Fig. 3, B and C; thus, quantitative data for shCTRL from those experiments are also included as part of Fig. S3 C. P values were calculated using a non-parametric Kruskall-Wallis test followed by Dunn's post-test.

significant increase in cell area compared to autophagy-competent cells (Fig. 12B and C). Together with our quantitative analysis of FAs in migrating cells, these findings point to a broader role for autophagy in modulating adhesion-dependent phenotypes that are particularly relevant for metastatic seeding and colonization at distant tissue sites. Furthermore, we extended these findings to analyze NBR1-depleted ZsGreen-expressing cells and found that they similarly underwent prolonged spreading compared to control cells and exhibited increased cell area at 1h post-replating (Fig. 13 A-C). Collectively, these results demonstrate that NBR1 loss-of-function phenocopies the effects of autophagy inhibition on both FA turnover and adhesion-dependent processes, indicating NBR1 and autophagy may coordinately facilitate FA remodeling through a common pathway of NBR1-mediated selective autophagy. Notably, these findings are unique to NBR1 among the known ACRs we have tested and potentially concordant with the in vivo phenotypes we have observed during NBR1 loss-of-function in metastasis.

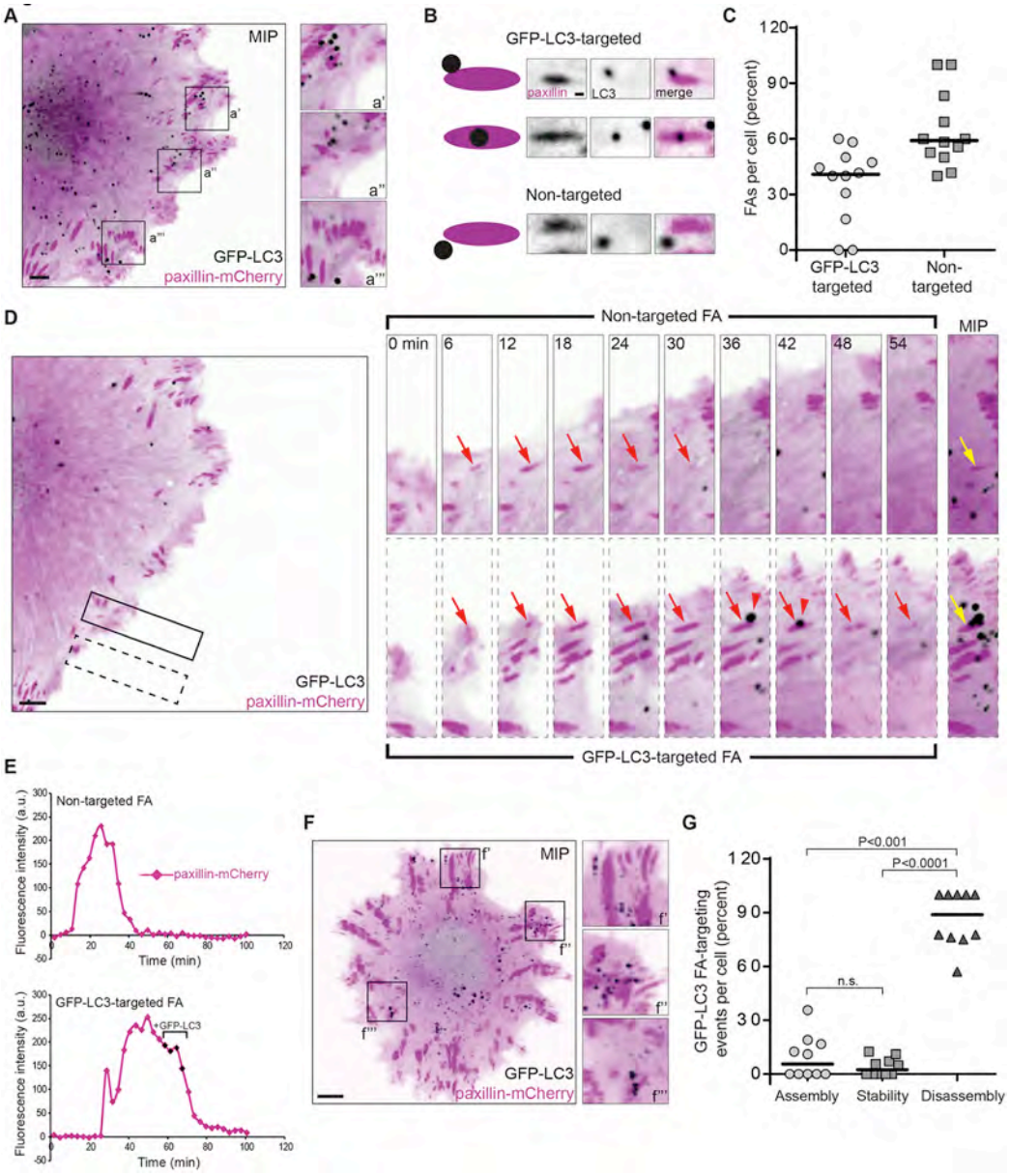

Fig. 14: Autophagosomes associate with dynamic FAs. (A) Spinning disk confocal microscopy of a migrating cell expressing GFP-LC3 (black) to label autophagosomes and paxillin-mCherry (magenta) to label FAs. Left panel shows maximum intensity projection (MIP) of a cell over 21 min illustrating multiple associations between autophagosomes and FAs. Boxed inset areas are enlarged in right panel. Bar, 5 µm. (B) Criteria for distinguishing GFP-LC3-targeted FAs versus non-targeted FAs used for the analysis in (C). Left illustration depicts a representation of targeted-FAs (top and middle) and non-targeted FAs (bottom). Bar, 0.5 µm. (C) Quantification of dynamic leading edge FAs per cell targeted by autophagosomes. FAs were randomly chosen independent of the GFP-channel and then manually tracked from their appearance to disappearance for evidence of direct contact by GFP-LC3 vesicles. Scatter plots show individual single cells (n=12 total cells) and median (line), representing n=129 total FAs analyzed from 2 independent experiments. (D) Spinning disk confocal microscopy time-lapse sequences of representative targeted (box with dotted border, bottom) and non-targeted (box with solid border, top) FAs. Arrows track single FAs over time, with autophagosome targeting indicated by arrowheads. Elapsed time (min) shown in top left of images. Right-most panels show MIP for each FA (arrow) shown in the corresponding time-lapse sequence. Bar, 5 µm. (E) Representative paxillin-mCherry fluorescence intensity (y-axis) plots over time (x-axis) for the FAs shown in (D). Frames in which GFP-LC3 was in direct contact with FAs are indicated by black data points and bracketing (bottom plot). (F) Spinning disk confocal microscopy of a spreading cell expressing GFP-LC3 (black) and paxillin-mCherry (magenta). Left panel shows MIP of a cell over 40 min to show autophagosomes near dynamic FAs throughout the cell periphery during spreading. Boxed inset areas are shown enlarged at right. Bar, 5 µm. (G) Temporal analysis of GFP-LC3 targeting to FAs. Plots show individual cells (n=12 total cells) and median (line), representing n=114 total targeting events analyzed from two independent experiments. P values were calculated using a non-parametric Kruskall-Wallis test followed by Dunn's post-test. n.s.=not significant.

Overall, these results provided evidence that autophagy serves as a mechanism for FA turnover and implicated the selective autophagy cargo receptor, NBR1, as a key mediator of this process. Typically, autophagy functions through the local and direct sequestration of cellular material into the forming autophagosome, which eventually fuses with lysosomes for cargo degradation [27,28]. Hence, we reasoned that autophagy-dependent FA turnover might entail the close local apposition of autophagosomes with dynamic FAs during migration. To test this prediction, we generated cells co-expressing paxillin-mCherry and GFP-LC3, which marks autophagosomes, and observed that autophagosomes localized throughout the leading edge of migrating cells (Fig. 14A). We enumerated adhesions targeted by GFP-LC3, defined as GFP-LC3 puncta in direct contact with paxillin-mCherry-labeled FAs, and found that 40% of dynamic FAs were directly targeted by autophagosomes (Fig. 14B-E). Of note, we believe that these experiments may underestimate the actual number of targeted FAs due to the rapid intracellular dynamics of GFP-LC3-labeled vesicles. Additionally, during cell spreading, we similarly observed GFP-LC3 puncta to be associated with dynamic FAs throughout the periphery of the cell, further confirming that autophagosomes are locally targeted to FAs (Fig. 14F).

We next determined if autophagosome targeting to FAs proceeds in a temporally specific manner. Interestingly, a limited number of targeting events occurred during FA assembly or when FAs were relatively stable; rather, the vast majority of GFP-LC3 targeting events occurred during FA disassembly (Fig. 14D, E, and G). Together with our data indicating that autophagy is functionally required for FA turnover, these results support that autophagy impacts leading edge FAs by proximally facilitating disassembly. This autophagy-dependent FA remodeling may involve the local sequestration of FA components into the autophagosome to promote FA destabilization and disassembly. Remarkably, although we have found that autophagosomes are found preferentially associated with disassembling FAs, it is still important to note that ATG knockdown functionally impairs both FA assembly and disassembly. Thus, while disassembly may be proximally modulated by autophagy, we cannot rule out that autophagy regulates FA assembly by more indirect mechanisms that do not necessarily involve the localization of autophagosomes to FAs.

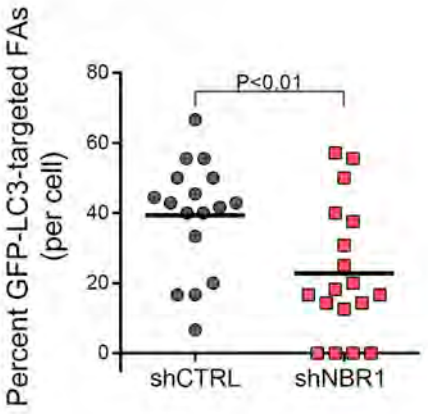

Fig 15: NBR1 is required for efficient targeting of autophagosomes to leading edge FAs. Quantification of dynamic leading edge FAs per cell targeted by autophagosomes in shCTRL and shNBR1 cells expressing GFP-LC3 to mark autophagosomes and mCherry-paxillin to mark FAs during live cell imaging. FAs were randomly chosen independent of the GFP-channel and then manually tracked from their appearance to disappearance for evidence of direct contact by GFP-LC3 vesicles. Scatter plots show individual single cells (n=19 total cells) and median (line) analyzed from 2 independent experiments.

Nonetheless, our results expand on the growing importance of selective autophagy regulators, namely NBR1, in controlling essential cellular functions. To further assess whether NBR1 was required for efficient targeting of autophagosomes to FAs, we evaluated the ability of GFP-LC3 to target to mCherry-labeled FAs in shNBR1 cells. These results confirm that NBR1 loss-of-function attenuates the ability of autophagosomes to target leading edge FAs (Fig. 15). Our findings that loss of NBR1 function inhibits migration and leading edge FA turnover, that only autophagy-competent NBR1 is sufficient to specifically drive FA disassembly, and that NBR1 localizes near dynamic FAs all support that cargo receptor-mediated autophagy plays a role in fine-tuning migratory capacity by optimizing adhesion-site turnover. Like other autophagy cargo receptors, NBR1 enables the selective capture of cellular substrates into autophagosomes. This recruitment may ultimately trigger autophagic sequestration or consumption of FA components, either of which would be expected to promote FA disassembly. Indeed, selective autophagy is an

attractive candidate for mediating turnover of large macromolecular complexes, such as FAs, as it is the major homeostatic pathway through which bulky cellular cargo, such as organelles and protein aggregates, are sequestered and degraded [28]. In conclusion, these results establish NBR1-mediated selective autophagy as a key mechanism of autophagy-dependent FA turnover, which we hypothesize serves as an important mechanism for NBR1 to promote the outgrowth of quiescent cells into overt and active metastatic cells. We will test this exciting hypothesis using our in vivo models over the upcoming year. ***We recently submitted the salient results from these mechanistic cell biological studies for publication at <u>The Journal of Cell Biology</u> and the reviewer comments were positive; we are currently revising this manuscript over the upcoming year.***

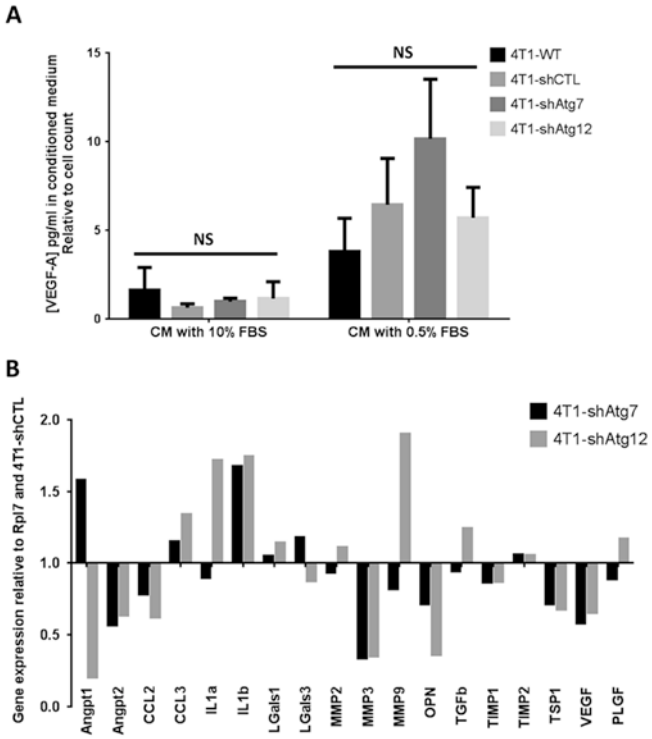

Fig 16: Autophagy deficiency does not impact angiogenic factor expression. (A) ELISA for VEGF-A levels from conditioned media produced from 4T1 cells. (n=3). (B) qPCR for angiogenic factor mRNA expression in 4T1 cells. Data from shATG7 and 12 cells were normalized to expression in shCTRL cells.

<u>Vascular and microenvironmental changes in autophagy-deficient tumors:</u> During year 3, we found that primary 4T1 tumors lacking ATGs exhibited alterations in the tumor vasculature. FITC-conjugated dextran injection studies and endogenous fibrinogen staining (a clotting factor deposited as a provisional matrix and thus a surrogate marker for vascular leakage) both revealed decreased extravascular fluorescence in 4T1-shAtg7 tumors, indicative of reduced vessel leakage. Because our studies in sub-task 1b demonstrated an important role for autophagy in the elaboration of secreted factors [5], we focused on testing the hypothesis that microenvironmental changes secondary to the impaired secretion of specific factors in autophagy-deficient 4T1 tumors were responsible for the reduced vessel leakage. We first assayed whether autophagy deficient cells displayed altered levels of the key angiogenic factors but did not detect significant differences in the secretion of VEGF-A or in the gene expression of multiple angiogenic factors (Figure 16A and B). In parallel to these studies, we repeated the dextran injection studies in mice lacking T lymphocytes (Rag KO mice). In contrast to immunocompetent BALB/c mice, where only autophagy-

deficient mice exhibited the reduced vascular leakage phenotype (Figure 17, left), both autophagy-competent and autophagy-deficient tumors 4T1 tumors exhibited reduced vessel leakage in the RagKO background (Figure 17, right). These results raised the intriguing possibility that autophagy deficiency was directing these vascular changes via non cell-autonomous role of tumor cell autophagy exerted on the immune microenvironment.

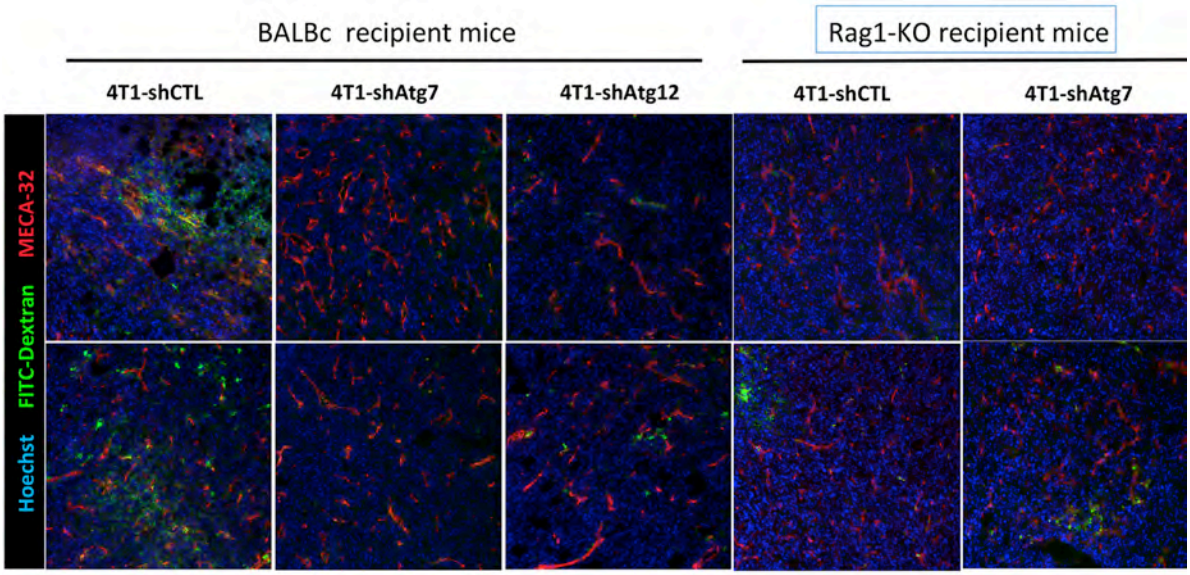

Fig. 17: Effects of 4T1 tumor cell autophagy deficiency on vascular permeability in immunocompetent versus immune- deficient (Rag1-KO) mice. FITC-Dextran (green) was injected into the tail veins of tumor-bearing mice and visualized by microscopy. Vessels were stained with antibodies against the endothelial marker MECA32 (red). In BALB/c host animals, a decrease in extravascular FITC-dextran, suggestive of decreased vascular permeability, is observed in Atg7/12 knockdown tumors compared to controls. In RAG1-KO host animals, both control and autophagy-deficient tumors exhibit decreased permeability, intimating the requirement for an intact immune system in tumor vascular leakage.

As a result, we further analyzed the effects of autophagy deficiency on the immune system in the 4T1 model. Cancer cells were injected orthotopically into the mammary fat pad of syngeneic host mice and allowed to form tumors over 2-3 weeks. The resected tumors were digested and T cell infiltration was measured by flow cytometry (Figure 18A). In addition, tumor-bearing animals were injected with Brefeldin A to trap secreted proteins 6 hours prior to resection; thereafter, tumors were digested and T cell activation was measured via key flow cytometry for surface and intracellular markers in both CD4+ and CD8+ subsets (Figure 18B). These results demonstrate that autophagy-deficiency does not impact either T cell infiltration or T cell activation in the 4T1 model. In addition, they argue against our hypothesis that autophagy-dependent priming of the immune cell microenvironment contributes to changes in vascular function and metastasis. Although disappointing, from a clinical standpoint, these results are reassuring because they indicate that tumor cell autophagy inhibition will not adversely impact tumor-specific immune responses. We have begun to evaluate whether similar results are observed with anti-malarial treatment in our studies in subtask 2e.

In summary, the results from our mechanistic areas of investigation during year 4 suggest that autophagy inhibition promotes the early steps of metastatic seeding via the control of focal adhesion turnover; moreover, the effects of the autophagy cargo receptor, NBR1, on metastasis correlate with its ability to promote FA disassembly. Over the upcoming year, we will scrutinize the cause-effect relationships between the NBR1-mediated selective autophagy on adhesion in vitro and metastatic progression and escape from dormancy in vivo. In contrast, we have not identified a clear non cell-autonomous signal in

autophagy-deficient tumor cells that may be responsible for increased metastatic colonization and escape from dormancy at the foreign tissue site. We anticipate that further delineating these pro-metastatic activities will mechanistically inform how autophagy impacts tumor cell exit from dormancy and toward overt metastatic disease. Thus, during the upcoming years, one of the important goals for both sub-tasks 2c and 2d will be to follow-up salient results gleaned from the PyMT and 4T1 models in the ongoing late recurrence studies using the transplanted mice from sub-task 2b.

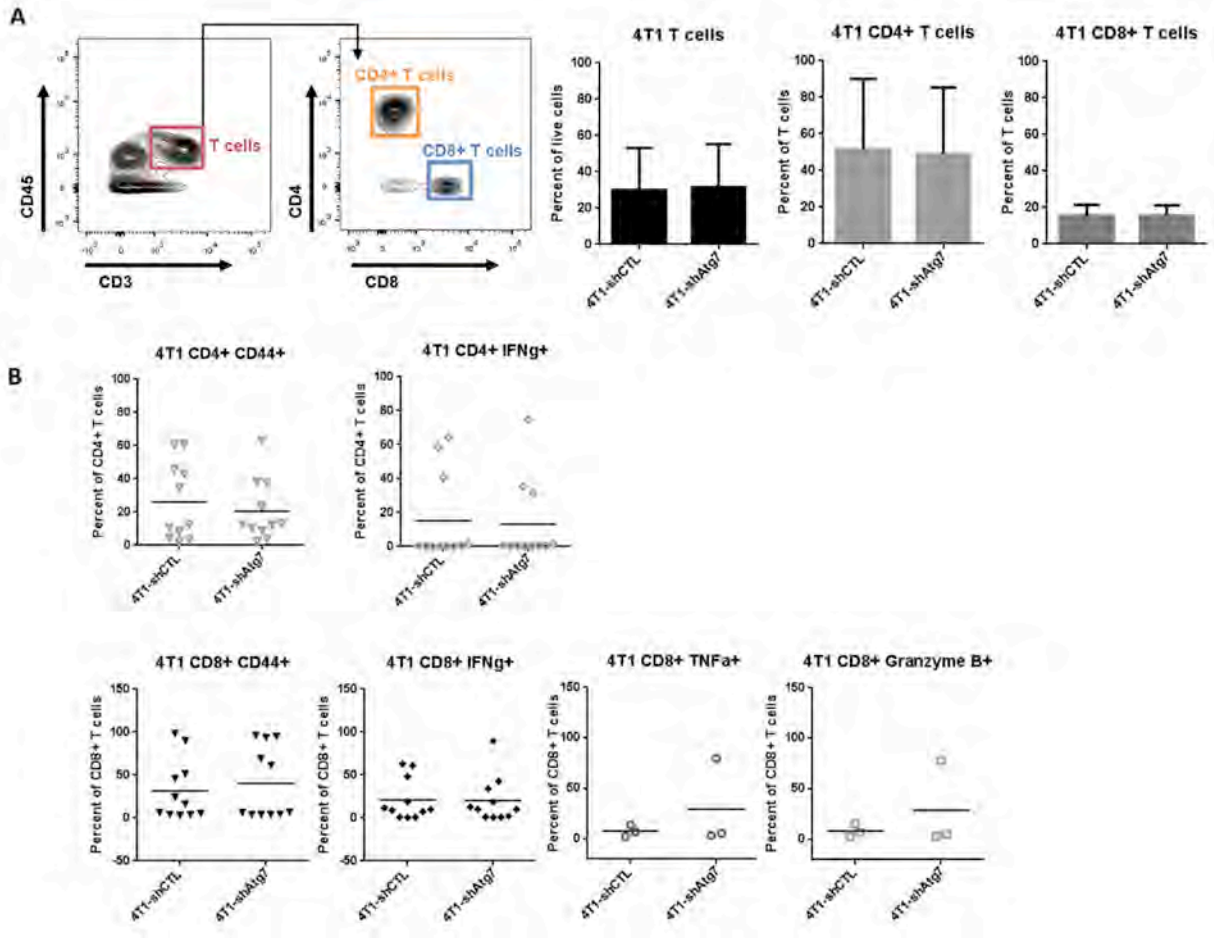

Fig. 18. T cell infiltration and activation is unchanged in autophagy-deficient mouse mammary tumors. 4T1 cells were injected into the mammary fat pad. Resected tumors were digested and T cell infiltration was measured by flow cytometry. (A) Flow cytometry gating strategy to define T cell populations in 4T1 tumors. T cell infiltration in primary mouse tumors was unchanged. Two-way ANOVA was not significant. Error bars represent standard deviation. (B) Mice bearing 4T1 tumors were injected with Brefeldin A (IV) to trap secreted proteins and tumors were resected after 6h. Tumors were digested and T cell activation was measured by flow cytometry for surface and intracellular markers. Activation was measured from CD4+ and CD8+ T cell populations defined in Fig. 18A. T cell activation in autophagy-deficient 4T1 tumors was unchanged. Each data point represents an individual mouse tumor; mice from three independent experiments were analyzed. Two-way ANOVA was not significant.

d. If late onset metastasis does occur in autophagy deficient tumor cell recipients from subtasks 2b-c, isolate late onset macro-metastatic tumors and dormant tumor cells and obtain gene expression profiles. To obtain dormant cells from each cohort in subtask 2b, ten (10) tumor cell bearing mice from 2b will be euthanized at an intermediate time point (anticipated to occur at 6-9 months post fat

pad transplantation but prior to overt metastatic disease), cancer cells isolated from host tissues, and if required, expanded ex vivo (Months 37-48; Revised timeline: Months 49-60).

These studies are planned for the upcoming year. Since the studies using the primary PyMT model in subtask 2b were delayed to pursue further experimental work in the PyMT-R221 and 4T1 models as well as conduct the mechanistic studies detailed in subtask 2c, we have revised the timeline for subtask 2d accordingly.

e. If metastasis is significantly reduced or eliminated in autophagy deficient tumor cell recipients from subtasks 2b-c, determine the effects of antimalarial treatment on the onset of metastasis in mice transplanted with autophagy competent neoplastic breast cells, using appropriate transplantation models determined from subtask 1d and/or subtask 1i-j (Months 37-60).

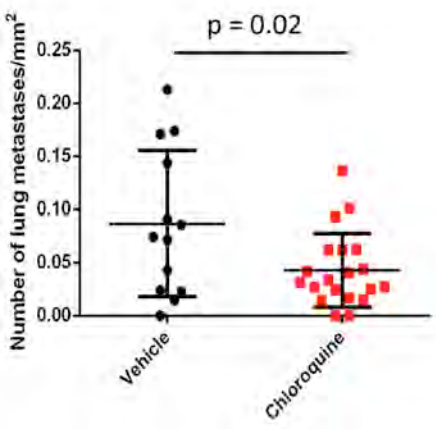

Fig. 19: Chloroquine (CQ) treatment of 4T1 mouse mammary cancers reduces spontaneous metastasis to lung. 4T1 cells orthotopically transplanted into the mammary fat pad and mice were treated with vehicle (water) or CQ starting at day 7. Lungs were harvested at 21 days post-transplant and lung metastasis were quantified.

Although our studies to date have indicated that metastasis is enhanced, rather than reduced, upon genetic inhibition of tumor cell autophagy, we continued to pursue this sub-task because our unexpected findings raised the possibility that anti-malarials such as chloroquine (CQ), which are currently being aggressively repurposed as pharmacological autophagy inhibitors in diverse cancers, including breast cancer, may harbor long-term risks by enhancing metastasis in patients [7]. For these studies, we initially focused on the 4T1 spontaneous metastases model. Mice were transplanted with 4T1 tumor cells; starting at day 7 following transplant, mice treated daily with either vehicle (water) or CQ at 60 mg/kg via intraperitoneal (IP) injection. As shown in Figure 19, these studies reveal that treatment with the anti-malarial CQ leads to a significant reduction in pulmonary metastasis. These studies are both exciting and reassuring because they intimate that CQ can be utilized to reduce metastatic outgrowth in breast cancers; moreover, there are consistent with our in vitro results in human cell lines in subtasks 1d. In the upcoming year, we will extend these interesting results to MCF7 cells as a complement to our genetic studies of autophagy inhibition using CRISPR/Cas deleted cells.

In addition to understanding the effects of autophagy on metastasis, the use of the 4T1 model for these studies also afforded us the capability to interrogate the effects of antimalarial treatment on immune cell function in a well-established immune competent transplant model. This allowed us to illuminate an extremely important clinical issue because of potential concerns about the untoward effects of autophagy inhibition on tumor immunity. As shown in Figure 20, pharmacological inhibition of autophagy in 4T1 tumors did not significant impact T cell infiltration or function. Thus, although previous studies in other cancer types have alluded to a critical role for autophagy in eliciting a robust anti-cancer immune response, our results using both genetic and pharmacological autophagy inhibitors argue against this possibility [29]. Furthermore, they indicate that anti-malarials do not adversely impact T cell function and these agents will not antagonize immune checkpoint blockade strategies in the treatment in breast and other cancers.

Finally, it is important to recognize that these data using CQ do contrast with our results in 4T1 cells using genetic autophagy inhibition (ATG knockdown) from subtasks 2b and 2c. We speculate that two reasons may explain this discrepancy. First, as we have discussed above in subtask 1d, cellular processes other than autophagy may contribute to the pharmacological effects of anti-malarials. In fact, we have recently

published a study, funded by other grants from the NIH, demonstrating that the pentose phosphate pathway is an important determinant of anti-malarial-induced cell death in lung cancer cells [30]. Second, anti-malarials may exert their anti-cancer effects via autophagy inhibition in host stromal constituents, rather than in tumor cells. We find this possibility to be very intriguing; accordingly, for our studies in task 3, we will utilize a systemic autophagy-deletion model (B-actin Cre-ER, *atg12f/f* which we already created in years 2-3 in order to generate the PyMT model with inducible autophagy deletion for sub-task 2a) to address this important and novel concept.

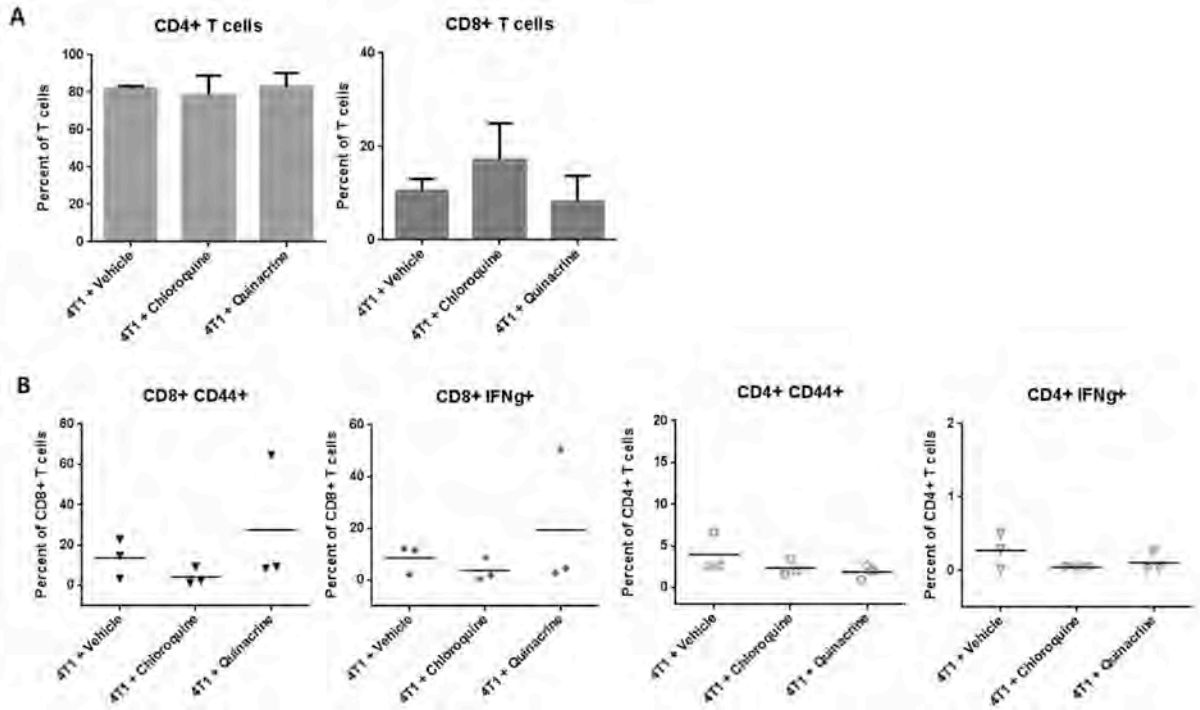

Fig 20: Antimalarial treatment does not impact T cell infiltration or activation in 4T1 tumors. 4T1 cancer cells were transplanted into the mammary fat pad of BALB/c mice were injected. Tumor-bearing mice were treated with the antimalarials chloroquine or quinacrine, or with vehicle control starting at day 7 following transplantation. Tumors were resected at day 14. Brefeldin A (BFA) was used in all *ex vivo* reagents during flow cytometric analysis. Tumors were analyzed for CD4 and CD8 T cell infiltration (B) and activation (B, CD44 and IFNgamma). Two-way ANOVA was not significant.

Task 3: Determine if dietary restriction or mammalian target of rapamycin (mTOR) inhibition can prevent the expansion of dormant cells into overt metastases.

a. Isolate neoplastic epithelium from female transgenic mice generated in subtask 1f, transduce with Cre recombinase, and transplant into cleared mammary fad pads of syngeneic C57B/6 host recipient animals. For this subtask, ten (10) donor mice will be utilized for epithelial isolation. Twenty-five (25) host recipient animals per cohort necessary for subtasks 3b and 3d will be utilized for fat pad transplantation (Months 37-60).

We have recently initiated these studies. These studies use the primary PyMT model and were slightly delayed to confirm the development of macro-metastatic outgrowth, as discussed in sub-task 2, as well pursue further experimental work in the PyMT-R221 and 4T1 models and to focus the mechanistic studies detailed in subtask 2c. Moreover, the results obtained with anti-malarial treatment in subtask 2e broach that the experimental strategy for Task 3 may benefit from the analysis of systemic deletion of ATGs in the host,

not just in the tumor epithelium. Results will be forthcoming during year 5 of the project.

b. Determine the effects of dietary restriction (DR), defined as a 40% reduction in caloric intake, on the onset of overt and latent metastasis for recipient mice generated in subtask 3a. We anticipate that twenty-five (25) mice each from two (2) cohorts (cohort 1: subject to DR; cohort 2: controls not subject to DR) will be functionally evaluated for altered characteristics of histopathologic progression from primary to metastatic disease (mammary morphogenesis, epithelial cell proliferation/cell death, overt tumor formation, tumor burden, tumor type, and development/latency to metastases) (Months 49-60).

These studies have not been initiated.

c. From mice in subtask 3b, isolate late onset macro-metastases (if they occur) and dormant tumor cells and obtain gene expression, biosynthetic, and metabolic profiles. To obtain dormant cells from each cohort in subtask 3b, ten (10) tumor cell bearing mice from 3b will be euthanized at an intermediate time point (anticipated to occur at 6-9 month post fat pad transplantation but prior to overt metastatic disease), cancer cells isolated from host tissues, and if required, expanded ex vivo (Months 49-60).

These studies have not been initiated.

d. Determine the effects of mTOR inhibition on the onset of overt and latent metastasis for recipient mice generated in subtask 3a. Twenty-five (25) mice per cohort will be functionally evaluated for altered characteristics of histopathologic progression from primary to metastatic disease (mammary morphogenesis, epithelial cell proliferation/cell death, overt tumor formation, tumor burden, tumor type, and development/latency to metastases) (Months 49-60).

These studies have not been initiated.

e. From mice in subtask 3d, isolate late onset macro-metastases (if they occur) and dormant tumor cells and obtain gene expression, biosynthetic, and metabolic profiles. To obtain dormant cells from each cohort in subtask 3b, ten (10) tumor cell bearing mice from 3d will be euthanized at an intermediate time point (anticipated to occur at 6-9 month post fat pad transplantation but prior to overt metastatic disease), cancer cells isolated from host tissues, and if required, expanded ex vivo (Months 49-60).

These studies have not been initiated.

III. KEY RESEARCH ACCOMPLISHMENTS:

Year 1 (Months 1-12):

1. We optimized an overlay (“on-top”) three-dimensional organotypic culture system on laminin-rich reconstituted basement membrane suitable for the culture of oncogene-expressing mammary epithelial cells and human breast cancer cells.
2. We uncovered that the anti-malarial chloroquine (CQ) inhibits quiescent behavior in response to mTOR pathway inhibition in mammary epithelial cells expressing a tumor-derived, activating mutation in phosphatidylinositol 3-kinase (PI3K), H1047R. These data support a model that autophagy inhibition may paradoxically promote the active growth of quiescent cells via the MAPK pathway secondary to the accumulation of the autophagy substrate, p62/SQSTM1. **Published in Chen et al. *Oncogene*, 2012.**

3. Based on the 3D proliferation rates of 6 human breast cancer cell lines tested during the first year, we identified two ER+ cell lines MCF7 and T47D as lead candidates for further evaluation as potential models for in vivo dormant behavior.
4. We discovered that the antimalarials chloroquine (CQ) and quinacrine (Qu) are able to inhibit the 3D growth and proliferation of MCF7 cells at levels comparable to tamoxifen (OHT).
5. We optimized protocols for the lentiviral-mediated delivery of reporter proteins and the drug resistance markers to breast cancer cells.
6. We optimized protocols for the successful isolation of mammary organoids from normal and PyMT tumor-bearing mice.
7. We developed protocols to deliver adenoviral Cre in order to excise the ATG12 conditional allele (*atg12f/f*) ex vivo from organoids derived from normal mammary gland and PyMT tumors. Upon reintroduction of cells into the mammary fad pads of recipient mice, we confirmed both ATG12 deletion and defective autophagy in the resultant tumors arising from ATG12 deleted tumor cells.
8. We have generated Polyoma Middle T (PyMT), ROSA26-LSL-RFP, and the ATG conditional mouse strains (*atg12f* and *atg5f*) in a pure C57B/6 genetic background.

Year 2 (Months 12-24):

9. We have extended our three-dimensional culture studies of PI3K-H1047R and evaluated the effects of ATG depletion on the morphogenesis of breast epithelial cells transformed with oncogenic Ras. Autophagy inhibition does not impact proliferation in this model, indicating that the effects of autophagy on quiescent behavior in 3D culture are oncogene-dependent.
10. In Ras-transformed breast cancer cells, we have found that the depletion of autophagy-related genes suppresses invasion in three-dimensional culture and reduces pulmonary metastases *in vivo*.
11. Based on conditioned media experiments in 3D culture, we have found that autophagy-deficient Ras cells, fail to secrete pro-invasive factors. We have also discovered that reduced autophagy diminishes the secretion of the pro-migratory cytokine, interleukin-6, which is necessary and sufficient to restore invasion of autophagy-deficient cells.
12. We have completed the interbreeding to generate compound transgenic mice containing Polyoma middle T (PyMT), ATG conditional alleles, and ROSA26-LSL-RFP in a C57B/6 genetic background. We have harvested primary cells from tumor bearing mice, validated the ability to genetically delete ATGs, as well as reintroduce these cells into a syngeneic C57B/6 host.
13. We have confirmed micro-metastatic lesions upon tail vein injection of PyMT cells and the presence of solitary tumor cells within lung tissue of recipient mice at an early time point of a spontaneous metastasis assay, suggesting that this approach will be useful to study recurrence over extended periods of time.
14. We have created a bank of frozen primary PyMT-derived tumor cells in which we can ablate autophagy (via Cre deletion of *atg12* or *atg5*) as well as carry out transplantation studies in the upcoming years.
15. We have created an immortalized cell line from a PyMT *atg12f/f* tumor isolated in our laboratory, which will be valuable for dissecting the mechanisms responsible for the phenotypes from experiments using primary tumor-derived tissue in years 3-5.
16. Using stable RNAi-mediated depletion of ATG7 in a PyMT cell line, we have found that autophagy inhibition accelerates the outgrowth of overt metastasis in the lungs of syngeneic recipient mice. Overall, this result provides in vivo corroboration for our 3D studies of PI3K-H1047R during year 1 and suggests that increasing autophagy in the host animal may be useful in preventing late recurrent disease.

Year 3 (Months 25-36):

17. We uncovered that autophagy promotes the transcription and production of the secreted factors, WNT5A and MMP2, in breast epithelial cells transformed with oncogenic Ras. Overall, these results support that autophagy in promoting cancer cell invasion via the coordinate production of multiple

secreted factors in addition to IL6, which may impact late recurrent disease and metastatic outgrowth in vivo. **Published in Lock et al., *Cancer Discovery*, 2014.**

18. We expanded our bank of frozen primary PyMT-derived tumor cells in which we can ablate autophagy (via Cre deletion of *atg12* or *atg5*) as well as continue to carry out transplantation studies in the upcoming years.
19. We initiated transplantation experiments using the primary PyMT tumors we had created in previous years. These mice will be analyzed for the onset of pulmonary macro-metastasis during the upcoming year. Analysis of an intermediate time point confirmed the persistence of solitary tumor cells within lung tissue of recipient mice from a spontaneous metastasis assay and broached the possibility of late metastatic recurrence over extended periods of time.
20. We generated compound transgenic mice containing a tamoxifen-inducible Cre recombinase (CAG-Cre^{ERT}) along with Polyoma middle T (PyMT), *atg12f/f*, and ROSA26-LSL-RFP, all in a C57B/6 genetic background. Upon harvesting tumors from these mice, we have confirmed efficient ATG12 deletion ex vivo upon treatment with 4-hydroxytamoxifen (4OHT) as well as confirmed the complete ablation of autophagy. This new model will allow for the temporal specific deletion of ATGs in vivo.
21. Using stable RNAi-mediated depletion of ATG7 and ATG12 in the 4T1 cell line, we corroborated that autophagy inhibition accelerates the outgrowth of overt metastasis in the lungs of syngeneic recipient mice. Overall, this result validates our year 2 results from the PyMT-R221 model using an immune competent transplantation model and suggests that increasing autophagy in the host animal may be useful in preventing late recurrent disease. Over the upcoming years, a major goal will be to evaluate this new concept using the primary slow progression PyMT models.
22. We have uncovered potential mechanisms for the pro-metastatic phenotypes observed in autophagy deficient cells. These include: 1) activation of pro-metastatic pathways due to the accumulation of the autophagy cargo receptors p62/SQSTM1 and NBR1; 2) changes in angiogenesis due to modulation of secreted angiogenic factors; and 3) autophagy-dependent changes in focal adhesion remodeling that may impact metastatic seeding or colonization.
23. Using the 3D overlay model developed in year 1, sub-task 1a, we find that autophagy-deficient PyMT-R221 cells exhibit enhanced spreading during low density re-seeding, providing additional evidence that autophagy may restrict metastasis by impeding early seeding and colonization events in vivo.

Year 4 (Months 37-48):

24. We have successfully employed clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 genomic targeting key autophagy genes in human cell lines in a tactical fashion, hence overcoming a long-standing technical barrier for achieving sustained, long-term autophagy inhibition in human breast cancer cells. We will utilize this strategy for the in vivo experiments in sub-task 1d.
25. We have observed macro-metastatic lesions derived from primary PyMT tumors in the lungs of C57B/6 hosts, thus validating that the primary PyMT cells can give rise to overt metastasis over time. Thus, we have obtained the first evidence that the C57B/6-based PyMT transplantation model that we developed over the initial year exhibits features of a dormancy period and late recurrence.
26. To expedite our understanding of autophagy in metastatic colonization gleaned from mouse breast cancer cell lines, we utilize experimental metastasis assays using the PyMT neoplastic epithelium generated in previous years. Our ongoing studies using this model provide initial support for our hypothesis that autophagy ablation promotes the transition from early colonization and dormant behavior towards more aggressive outgrowth.
27. In mechanistic studies to define the molecular basis of metastasis suppression by autophagy, we identified the autophagy cargo receptor, NBR1, as a key mediator of early metastatic seeding and colonization in breast cancer. In parallel, we have uncovered a novel role for NBR1 in supporting focal adhesion turnover and are delineating whether its accumulation in autophagy-deficient cells contribute to its pro-metastatic functions via effects on focal adhesion remodeling during metastatic colonization.

28. In addition to identifying NBR1 as a mediator of both focal adhesion turnover and metastasis, we conducted real time dynamic imaging studies to rigorously interrogate the role of autophagy in breast cancer cell focal adhesion turnover. We have uncovered that autophagic targeting to FAs is temporally specific with the vast majority of targeting events occurred during FA disassembly Along with our data indicating that autophagy is functionally required for FA turnover, these results support that autophagy impacts leading edge FAs by proximally facilitating disassembly. Finally, we have demonstrated that NBR1-mediated selective autophagy acts of a key mechanism of autophagy-dependent FA disassembly in breast cancer cells. **The salient results from these studies are currently in revision for *The Journal of Cell Biology*.** In this manuscript, we propose that NBR1 enables autophagy-dependent FA turnover, which serves as a crucial mechanism by which autophagy in tumor cells restricts the outgrowth of quiescent cells into actively growing metastatic cells.
29. We have also tested the role of autophagy-dependent secretion in the control of the tumor angiogenic and immune microenvironment. Autophagy-deficiency does not impact angiogenic factor production, T cell infiltration or T cell activation in the 4T1 model, thus argue against our hypothesis that autophagy-dependent priming of the immune cell microenvironment contributes to changes in vascular function and metastasis. In further support, systemic pharmacological inhibition of autophagy using anti-malarials does not significant impact T cell infiltration or function in 4T1 tumors. From a clinical standpoint, these results are interesting because they suggest that the use of autophagy inhibition to prevent late recurrence disease will not adversely impact tumor-specific immune responses.
30. In contrast to genetic autophagy inhibition, treatment with the anti-malarial CQ leads to reduced pulmonary metastasis. These studies intimate that CQ can be utilized to reduce late stage metastatic outgrowth in breast cancers. In the upcoming, year, we will assess whether anti-malarials exert their anti-metastatic effects via autophagy inhibition in host stromal constituents, rather than in breast cancer cells.

IV. REPORTABLE OUTCOMES:

Publications

Months 1-12 (provided as Appendix material in 2012 Progress Report):

1. **J. Debnath** (2011). The Multifaceted Roles of Autophagy in Breast Cancer. *J. Mamm. Gland Biol. Neopl.* 16 (3): 173-87. PMID: 21779879 PMCID: PMC3170851.

2. N. Chen, Eritja N., Lock R., **Debnath J.** (2012). Autophagy Restricts Proliferation Driven By Oncogenic Phosphatidylinositol 3-Kinase in Three-Dimensional Culture. *Oncogene.* doi: 10.1038/onc.2012.277. 2012 Jul 9. Epub ahead of print. PMID: 22777351 PMCID: PMC3470740.

Months 12-24 (provided as Appendix material in 2013 Progress Report):

1. Murrow, L, **Debnath, J.** (2013) Autophagy as a Stress Response and Quality Control Mechanism: Implications For Cell Injury and Human Disease. *Annu Rev Pathol.,* 2013, 8:105-137. PMID: 23072311. PMCID: PMC3971121.

2. M.S. Sosa, P. Bragado, **J. Debnath*,** J. A. Aguirre-Ghiso*. Regulation of Tumor Cell Dormancy By Tissue Microenvironments and Autophagy (2013). Adv. Exp. Med. Biol, 734: 73-89. PMID: 23143976. PMCID: PMC3651695. ***Co-senior author.**

3. N. Chen, **Debnath J.** (2013). IkB Kinase (IKK) Triggers Detachment-Induced Autophagy In Mammary Epithelial Cells Independently of the PI3K/AKT/MTORC1 Pathway. *Autophagy.* 9(8): 1214-27. PMCID: PMC3748193

Months 25-36 (provided as Appendix material in 2013 Progress Report):

1. R. Lock, Kenific, C.M., Leidal, A.M., Salas, E., and **Debnath, J.** (2014) Autophagy dependent production of secreted factors facilitates RAS-driven invasion. *Cancer Discovery*. 4(4): 466-79. PMCID: PMC3980002.

2. Goldsmith, J, Levine, B., and **Debnath, J.** (2014). Autophagy and cancer metabolism. *Methods Enzymol.* 542: 25-57. PMID: 24862259.

3. Leidal, A.M. and **Debnath, J.** (2014). "Doubling down" on the autophagy pathway to suppress tumor growth. *Genes and Development.* 2014; 28(11): 1137-9. PMID: 24888584.

Months 37-48 (provided as Appendix B):

1. Kenific, C.M. and **Debnath J** (2015). Cellular and metabolic functions for autophagy in cancer cells. *Trends Cell Biol.* 25(1): 37-45. PMCID: PMC4275311.

2. Kaur, J. and **Debnath, J.** (2015). Autophagy at the crossroads of catabolism and anabolism. *Nat Rev Mol Cell Biol.* 16 (8): 461-72. PMID: 26177004. PMCID In Progress.

Presentations (Months 25-36):

International and National Conferences:

2015 Invited Speaker, Forum on Autophagy and Cancer, American Association of Cancer Research Annual Meeting, Philadelphia, PA
2015 Invited Speaker, Gordon Conference on Mammary Gland Biology, Mount Snow, VT
2015 Speaker, Samuel Waxman Cancer Research Foundation Annual Meeting, New York, NY
2015 Invited Speaker and Co-organizer, Keystone Symposium on Autophagy, Breckenridge, CO
2015 Invited Speaker, European Molecular Biology Organization (EMBO) International Conference on Autophagy Signaling in Health and Disease, Sardinia, Italy
2015 Invited Plenary Speaker, PISA 2015, Recent Advances in Cell Injury, Inflammation, and Neoplasia, American Society for Investigative Pathology, Baltimore, MD

Invited Lectures and Seminars:
2014 Invited Seminar, University of Chicago, Ben May Cancer Biology Program, Chicago, IL
2014 Invited Seminar, Distinguished Speaker Series, Department of Translational Molecular Pathology, University of Texas M.D. Anderson Cancer Center, Houston, TX
2015 Invited Speaker, University of Cincinnati, Department of Cancer Biology, Cincinnati, OH
2015 Invited Speaker, University of Pittsburgh Cancer Institute, Pittsburgh, PA
2015 Invited Speaker, Sanford-Burnham Institute, La Jolla, CA

Patents and Licenses (Months 1-48):

None.

Degrees Obtained (Months (1-48):

2015 Candia Kenific, Ph.D. in Biomedical Sciences, University of California, San Francisco.
Thesis title: Regulation of metastatic phenotypes by autophagy.

Reagent Development (Months 1-48):

Generation of MMTV-PyMT mice on the C57B/6 strain background.
Generation of *atg12f*, LSL-RFP mice on the C57B/6 strain background.
Generation of *atg5f* mice on the C57B/6 strain background.
Generation of *atg5f*, LSL-RFP mice on the C57B/6 strain background.
Generation of MMTV-PyMT, *atg12f*, LSL-RFP mice on the C57B/6 strain background.
Generation of mammary carcinoma cell line derived from an MMTV-PyMT, *atg12f*, β-actin CFP, ROSA26-LSL-RFP host tumor suitable for transplantation into C57B/6 syngeneic host animals.
Generation of MMTV-PyMT, atg12f, LSL-RFP, CAG-Cre^{ERT} mouse on a C57B/6 strain background.

Funding Applied For Based On Work Supported By Era of Hope (Months 37-48):

1. Debnath, Jayanta (PI):	NIH R01 CA126792 Status: AWARDED (2/1/2015-1/31/2020)
2. Andrew Leidal (Post-doc):	Banting Post-doctoral Fellowship, Canadian Institutes of Health Research Status: AWARDED (7/1/2015-6/30/2017)

Employment and Research Opportunities (Months 37-48):

1) Nan Chen (Post-doc 2011-12)	Staff Research Scientist, Memorial Sloan Kettering Cancer Center, New York, NY

After Dr. Chen worked on this project during year 1 (2011-12), she took a leave of absence from 2012-2015 to take care of her first child. She recently returned as a research scientist at MSKCCC in summer 2015.

2) Ritu Malhotra (Post-doc 2012-14)	Research Scientist, Siemens Healthcare, Mountain View, CA
3) Candia Kenific (Graduate Student 2011-15)	Post-doctoral Fellowship, Lab of Professor David Lyden, Weil Cornell Medical Center

V. CONCLUSION:

The biological processes that govern the critical steps in late recurrent disease in breast cancer remain largely unclear [3]. This project focuses on how the fundamental stress pathway autophagy impacts quiescent versus proliferative behavior exhibited by breast cancer cells during metastatic colonization. Over the past year, we continued to delineate the role of autophagy during metastatic progression using PyMT and 4T1 mouse tumor cell lines transplanted into syngeneic immune competent hosts. Our previous work on this project unexpectedly found that autophagy inhibition promotes metastasis in vivo in both of these immune competent mammary cancer models, thereby corroborating our in vitro findings from organotypic 3D models from the initial years of this EOHS award. Over the last year, our major focus was to better define the mechanisms contributing to this pro-metastatic phenotype. Importantly, we identified the autophagy cargo receptor, NBR1, as a key mediator of early metastatic seeding and colonization in breast cancer. Furthermore, we obtained evidence that autophagy inhibition promotes the early steps of metastatic seeding via the control of focal adhesion turnover and we uncovered a novel role for NBR1 in supporting focal adhesion turnover in vitro. To elaborate on these exciting

results, we conducted real time dynamic imaging studies to rigorously interrogate the role of autophagy in breast cancer cell focal adhesion turnover. We uncovered that autophagy primarily targets focal adhesions during the disassembly phase. Taken together with our data that autophagy is functionally required for FA turnover, these results support that autophagy impacts leading edge FAs by proximally facilitating disassembly. Finally, we have demonstrated that NBR1 is a critical mediator of this selective autophagic process that promotes FA disassembly in breast cancer cells. During the upcoming year, we will assess whether the accumulation of NBR1 activates cell-autonomous signals, including proliferative signals emanating from the focal adhesion, which drives metastatic colonization at the foreign tissue site.

Our findings also have broached the possibility that pharmacological autophagy inhibitors, such as hydroxychloroquine (HCQ) harbor long-term risks by enhancing metastasis. As a result, over the past year, we began to test the effects of CQ on metastatic colonization in vivo. In contrast to genetic autophagy inhibition, treatment with the anti-malarial CQ reduced pulmonary metastasis. These studies are reassuring because they support that CQ may still have utility in reducing late stage metastatic outgrowth in breast cancers. We speculate that two reasons may explain the discrepancy between our results obtained from genetic versus pharmacological autophagy inhibition in these mammary cancer models. First, cellular processes other than autophagy contribute to the pharmacological effects of anti-malarials. Alternatively, anti-malarials may predominantly exert their anti-cancer effects via autophagy inhibition in host stromal constituents. We find this second possibility to be very intriguing. In the upcoming year, we will assess whether the genetic autophagy inhibition in the host leads to anti-metastatic effects, in contrast to the pro-metastatic phenotypes we have observed upon inhibiting autophagy in breast cancer cells.

Furthermore, we continued to investigate whether the inhibition of tumor cell autophagy impacts the tumor microenvironment via the control of secretory pathways. These studies were motivated by our previous work in 3D culture models demonstrating the importance of autophagy-dependent secretion during invasion in vitro [5]. Because we found that autophagy-deficient breast tumors exhibited alterations in the tumor vasculature, we tested whether the impaired secretion of specific factors in autophagy-deficient tumors was responsible for the reduced vessel leakage. However, over the last year, we were unable to detect any significant changes in the angiogenic secretome. In complementary studies of the immune microenvironment, we also discovered that autophagy deficiency does not impact T cell infiltration or T cell activation, thereby arguing against our hypothesis that autophagy-dependent priming of the immune cell microenvironment contributes to changes in vascular function and metastasis. In further support, systemic pharmacological inhibition of autophagy using anti-malarials did not significant impact T cell infiltration or function. Although these studies were negative, from a clinical viewpoint, these results are important because they suggest that the use of autophagy inhibition to prevent late recurrence disease will not adversely impact tumor-specific immune responses.

Overall, our findings to date suggest that autophagy may promote late recurrent disease by promoting the exit of tumor cells from quiescent states to produce overt metastatic disease. To address this question, we have generated a MMTV-PyMT mammary cancer progression model in a pure C57B/6 strain to more effectively model late recurrence and assess the effects of autophagy on dormant tumor cell behavior [31]. During the past year, we confirmed the development of metastasis following transplantation of tumor cells into the mammary fat pad of syngeneic recipient mice. In ongoing studies, we are using this model to study the effects of autophagy inhibition on late recurrent metastatic disease.

Impact: Late recurrent breast cancer remains a principal cause of lethality in breast cancer patients. Our studies using two established immune-competent models of breast cancer metastasis demonstrate that tumor cell autophagy impedes the active outgrowth of metastatic cells during early colonization at the foreign tissue site. In contrast, our initial studies over the last year suggest that systemic treatment with anti-malarials as pharmacological autophagy inhibitors leads to a reduction in metastatic outgrowth. Based on these results, we propose that anti-malarials exert their anti-cancer effects via systemically inhibiting autophagy in the entire host, including stromal cells necessary for effective metastatic growth in the host. Further testing this hypothesis has

important therapeutic implications because it will validate whether these agents can be effectively repurposed to reduce late stage metastatic outgrowth in breast cancers. In addition, we have generated a PyMT-based mouse model for late recurrent disease. If we are able to corroborate similar functional requirements for autophagy inhibition in tumor versus host cells in this model of late recurrent disease, this will provide the conceptual foundation for targeting autophagy in the prevention of late recurrent metastasis in breast cancer patients.

VI: BIBLIOGRAPHY:

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22. Shibue T, Weinberg RA: **Integrin beta1-focal adhesion kinase signaling directs the proliferation of metastatic cancer cells disseminated in the lungs**. *Proc Natl Acad Sci U S A* 2009, **106**:10290-10295.
23. Webb DJ, Donais K, Whitmore LA, Thomas SM, Turner CE, Parsons JT, Horwitz AF: **FAK-Src signalling through paxillin, ERK and MLCK regulates adhesion disassembly**. *Nat Cell Biol* 2004, **6**:154-161.
24. Webb DJ, Parsons JT, Horwitz AF: **Adhesion assembly, disassembly and turnover in migrating cells -- over and over and over again**. *Nat Cell Biol* 2002, **4**:E97-100.
25. Cavalcanti-Adam EA, Volberg T, Micoulet A, Kessler H, Geiger B, Spatz JP: **Cell spreading and focal adhesion dynamics are regulated by spacing of integrin ligands**. *Biophys J* 2007, **92**:2964-2974.
26. Friedl P, Wolf K: **Plasticity of cell migration: a multiscale tuning model**. *J Cell Biol* 2010, **188**:11-19.
27. Feng Y, He D, Yao Z, Klionsky DJ: **The machinery of macroautophagy**. *Cell Res* 2014, **24**:24-41.
28. Murrow L, Debnath J: **Autophagy as a stress-response and quality-control mechanism: implications for cell injury and human disease**. *Annu Rev Pathol* 2013, **8**:105-137.
29. Michaud M, Martins I, Sukkurwala AQ, Adjemian S, Ma Y, Pellegatti P, Shen S, Kepp O, Scoazec M, Mignot G, et al.: **Autophagy-dependent anticancer immune responses induced by chemotherapeutic agents in mice**. *Science* 2011, **334**:1573-1577.
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VII: APPENDICES:

A. Curriculum Vitae of Jayanta Debnath.
B. Collected publications from Months 37-48.

Prepared: September 23, 2015

University of California, San Francisco

CURRICULUM VITAE

Name: Jayanta Debnath, MD

Position: Associate Professor, Step 3
Pathology
School of Medicine

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Core Campus, HSW, 450B
University of California, San Francisco
San Francisco, CA 94143

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Fax: 415-514-0878
email: jayanta.debnath@ucsf.edu
www: pathology.ucsf.edu/debnath

EDUCATION

1988 - 1992	Georgia Institute of Technology	B.S.	Highest Honors, Chemistry
1992 - 1998	Harvard Medical School	M.D.	Magna cum laude
1995 - 1997	National Cancer Institute, NIH	HHMI Research Scholar	Harold Varmus Lab
1998 - 1999	Brigham and Women's Hospital	Intern	Pathology
1999 - 2000	Brigham and Women's Hospital	Resident	Pathology
2000 - 2003	Brigham and Women's Hospital	Fellow	Pathology
2000 - 2005	Harvard Medical School	Postdoctoral Fellow	Cell Biology (Joan Brugge Lab)

LICENSES, CERTIFICATION

2003	Massachusetts Medical License Board of Registration in Medicine (expired 2006)
2003	American Board of Pathology, Board Certification in Anatomic Pathology
2005	California Medical Board, Physician and Surgeon

PRINCIPAL POSITIONS HELD

2003 - 2005	Harvard Medical School	Instructor	Cell Biology

2005 - 2011	University of California, San Francisco	Assistant Professor	Pathology
2011 - present	University of California, San Francisco	Associate Professor	Pathology

OTHER POSITIONS HELD CONCURRENTLY

2005 - present	UCSF Medical Center	Staff Pathologist

HONORS AND AWARDS

1988	President's Scholar (Full academic scholarship)	Georgia Institute of Technology
1989	Outstanding freshman, sophomore, junior, and senior awards, School of Chemistry	Georgia Institute of Technology
1991	Undergraduate Research Fellowship in Chemistry	National Science Foundation
1992	Phi Kappa Phi Scholarship Cup (Valedictorian)	Georgia Institute of Technology
1995	NIH-HHMI Research Scholar	Howard Hughes Medical Institute
1997	Research Award for Continued Medical Studies	Howard Hughes Medical Institute
1998	Honors in a Special Field, magna cum laude	Harvard Medical School
1998	Soma Weiss Medical Student Research Day Speaker Award	Harvard Medical School
2000	HHMI Postdoctoral Fellowship for Physicians	Howard Hughes Medical Institute
2003	Mentored Clinical Scientist Development Award (KO8)	National Cancer Institute
2003	Pfizer Scholar-In-Training Award	American Association of Cancer Research
2006	Culpeper Scholar Award in the Medical Sciences	Partnership for Cures
2006	AACR Genentech BioOncology Career Award	AACR
2006	HHMI Physician-Scientist Early Career Award	Howard Hughes Medical Institute
2007	Stewart Family Trust Award	UCSF Cancer Center
2009	Aspen Cancer Conference Fellow	Aspen Cancer Conference
2011	Era of Hope Scholar Award	DOD Breast Cancer Research Program
2013	Elected to Membership in ASCI	American Society for Clinical Investigation

2013	Waxman Award	Samuel Waxman Cancer Research Foundation

KEYWORDS/AREAS OF INTEREST

autophagy, apoptosis, oncogenes, breast cancer, three-dimensional culture, extracellular matrix, integrins, ubiquitin-like molecules

PROFESSIONAL ACTIVITIES

CLINICAL

2005-present, Attending Pathologist, Department of Pathology Autopsy Service, UCSF Medical Center, San Francisco, CA

SUMMARY OF CLINICAL ACTIVITIES

I serve as an attending in the autopsy service at Moffitt Hospital at UCSF (6 weeks per year).

PROFESSIONAL ORGANIZATIONS

Memberships

2000 - present	American Society for Cell Biology
2004 - 2005	American Society of Investigative Pathology
2012 - present	American Society of Investigative Pathology
2006 - present	American Association of Cancer Research
2013 - present	American Society of Clinical Investigation (Elected)

Service to Professional Organizations

2004 -	American Society for Cell Biology	Participant, Capitol Hill Day (Advocacy for NIH budget)
2006 -	WICB Career Luncheon, American Society for Cell Biology	Discussion Leader
2009 -	Georgia Institute of Technology College of Science Advisory Board	Advisor
2009 -	2010 AACR Annual Meeting Program Committee (Cell Growth Signaling Pathways Subsection), American Association of Cancer Research	Subcommittee Member
2011 -	6th Era of Hope Conference, Orlando, FL, DOD Breast Cancer Research Program	Technical Program Committee (Abstract Placement For Meeting)
2011 -	2012 AACR Annual Meeting Program Committee (Cell Death Subsection) American Association of Cancer Research	Subcommittee Member

2013 -	2014 AACR Annual Meeting Program Committee (Cell Death Subsection) American Association of Cancer Research	Subcommittee Member
2015 -	2015 American Society of Clinical Oncology Annual Meeting Educational Book	Panel of Expert Scientific Reviewers
-	Meetings and Sessions Organized:	
2010 -	UCSF Diller Cancer Center Bay Area Workshop, "Cancer Cell Growth and Metabolism", UCSF Diller Family Comprehensive Cancer Center	Co-organizer (Conference Chair) with Davide Ruggero, UCSF
2010 -	National Cancer Institute (NIH) Workshop on Autophagy and Cancer	Co-chair with Eileen White, CINJ
2011 -	2011 ASCB Annual Meeting Special Session on Extracellular Matrix Regulation of Programmed Cell Death, Denver, CO	Co-organizer with Mike Overholtzer, MSKCC
2014 -	Experimental Biology 2014, ASIP Symposium on Cancer Pathobiology, San Diego, CA	Symposium Organizer and Chair
2015 -	2015 Keystone Symposium on Autophagy	Co-organizer (conference chair) with Eric Baehrecke, U. Mass.

SERVICE TO PROFESSIONAL PUBLICATIONS

2011 - 2015 Ad hoc referee for approximately 60 manuscripts per year. Since 2011, journals include:

- Autophagy, 31 papers
- Cancer Cell, 3 papers
- Cancer Discovery, 8 papers
- Cancer Research, 18 papers
- Clinical Cancer Research, 2 papers
- Cell, 5 papers
- Cell Death and Differentiation, 5 papers
- Cell Metabolism, 1 papers
- Current Biology, 2 papers
- Developmental Cell, 2 papers
- Disease Models and Mechanisms, 2 papers
- eLife, 1 paper
- EMBO Journal, 3 papers
- EMBO Reports, 2 papers
- Experimental Cell Research, 1 paper
- Genes and Development, 5 papers

- Journal of Cell Science, 5 papers
- Journal of Cell Biology, 2 papers
- Journal of Clinical Investigation, 6 papers
- Mitochondrion, 1 paper
- Molecular Biology of the Cell, 7 papers
- Molecular Cancer Therapeutics, 2 papers
- Molecular and Cellular Biology, 10 papers
- Molecular Cell, 23 papers
- Molecular Oncology, 1 paper
- Molecular Therapy, 1 paper
- Nature, 7 papers
- Nature Cell Biology, 7 papers
- Nature Chemical Biology, 1 paper
- Nature Communications, 2 papers
- Nature Reviews Cancer, 1 papers
- Nature Structural and Molecular Biology, 5 papers
- Oncogene, 27 papers
- PNAS, 3 papers
- PLOS One, 1 papers
- Radiation Research, 2 papers
- Science, 2 papers
- Science Signaling, 1 papers
- Science Translational Medicine, 3 papers
- Traffic, 1 paper
- Trends in Cell Biology, 1 paper

2010 - 2011 Editorial Board, Autophagy

2011 - present Associate Editor, Autophagy (92 papers)

2014 - Associate Editor, Molecular and Cellular Oncology

2015 - Advisory Editor, Oncotarget

INVITED PRESENTATIONS

INTERNATIONAL

1997 Invited Lecture, International Meeting on Cytoplasmic Tyrosine Kinases, Stockholm, Sweden

2001 Invited Speaker, American Society for Cell Biology National Meeting, Minisymposium of Programmed Cell

Death, Washington, D.C.

2005 Session Chair, Autophagy and Cell Death, Gordon Research Conference on Autophagy, Il Ciocco, Italy

2006 Invited Speaker, American Society for Cell Biology National Meeting, Minisymposium on Programmed Cell Death, San Diego, CA

2007 Invited Lecture and Session Chair, ASCB/ECI Engineering Cell Biology Meeting, Cambridge, MA

2008 Invited Lecture, Autophagy in Cell Death and Aging Session, Gordon Research Conference on Autophagy, Ventura, CA

2009 Invited Speaker, American Society of Cell Biology National Meeting, Minisymposium on Autophagy and Lysosomes, San Diego, CA

2009 Session Co-chair, Minisymposium on Autophagy and Cancer, American Association of Cancer Research Annual Meeting, Denver, CO

2010 Invited Lecture, Stanley Korsmeyer Symposium on Autophagy and Apoptosis, American Association of Cancer Research Annual Meeting, Washington, D.C.

2010 Invited Lecture and Session Chairperson, Workshop on 3D Cultures, American Association of Cancer Research Annual Meeting, Washington, D.C.

2010 Invited Speaker, Selective Autophagy Session, Gordon Research Conference on Autophagy, Il Ciocco, Italy.

2011 Co-chair and speaker, American Society of Cell Biology National Meeting Special Session on "ECM Regulation of Programmed Cell Death," Denver, CO

2011 Invited Speaker, American Society for Cell Biology National Meeting, Minisymposium on Ubiquitin Related Proteins, Denver, CO

2011 Invited Speaker and Session Chair, Zing Conference on Autophagy, Mayan Riviera, Mexico.

2012 Invited Panelist, Session on “Autophagy and Disease” Gordon Research Conference on Autophagy, Ventura, CA.

2012 Invited Speaker, Experimental Biology 2012, ASIP Session on Autophagy, San Diego, CA

2012 Invited Speaker, 6th International Symposium on Autophagy, Okinawa, Japan

2012 Invited Speaker, American Society for Cell Biology National Meeting, Minisymposium on Cancer Cell Biology, San Francisco, CA

2014 Invited Speaker, Gordon Research Conference on Autophagy, Il Ciocco, Italy

2014 Invited Speaker, Educational Session on Autophagy and Cancer, American Association of Cancer Research Annual Meeting, San Diego, CA

2014 Invited Speaker and Symposium Chair, Experimental Biology 2014, ASIP Symposium on Cancer Pathobiology, San Diego, CA

2014 Invited Speaker, Educational Session on Signaling Pathways and Therapeutics, American Society of Clinical Oncology Annual Meeting, Chicago, IL

2015 Invited Speaker, Forum on Autophagy and Cancer, American Association of Cancer Research Annual Meeting, Philadelphia, PA

2015 Invited Speaker, Gordon Conference on Mammary Gland Biology, Mount Snow, VT

2015 Speaker, European Molecular Biology Organization (EMBO) International Conference on Autophagy Signaling in Health and Disease, Sardinia, Italy

2016 Invited Speaker, International Association of Breast Cancer Research 2016 Meeting, Portland, OR

NATIONAL

2001 Invited Lecture, National Cancer Institute Workshop on Estrogen Negative Breast Cancer, Bethesda, MD

2003 Invited Speaker, Keystone Symposium, Molecular Targets in Cancer Therapy, Banff, Alberta, Canada

2004 Platform Presentation, Twentieth Oncogene Meeting, Frederick, MD

2006 Invited Lecture, Timberline Symposium, Timberline, OR

2008 Faculty Speaker, HHMI Medical Fellows Meeting, Chevy Chase, MD

2009 Speaker, Stand Up To Cancer (SU2C) Breast Cancer Dream Team Meeting, AACR, Philadelphia, PA

2010 Invited Speaker, Keystone Symposium on Cell Death. Vancouver, BC, Canada

2010 Co-chair and Invited Speaker, National Cancer Institute Workshop on Autophagy and Cancer, Bethesda, MD

2010 Keynote Speaker, 8th Annual Clinical Investigator Trainee (CIST) Meeting, HHMI, Chevy Chase, MD

2011 Invited Speaker, NCI/CCSB Workshop on Systems Biology of Tumor Dormancy, Boston, MA

2013 Invited Plenary Speaker, Applied Pharmaceutical Toxicology Meeting, Genentech Inc., South San Francisco, CA

2014 Invited Speaker, National Breast Cancer Coalition Artemis Meeting on Tumor Dormancy, Calistoga, CA

2014 Invited Speaker, National Breast Cancer Coalition Leadership Summit, Alexandria, VA

2014 Speaker, Samuel Waxman Cancer Research Foundation Annual Meeting, New York, NY

2014 Invited Speaker, Keystone Symposium on Autophagy and Disease, Austin, TX

2015 Speaker, Samuel Waxman Cancer Research Foundation Annual Meeting, New York, NY

2015 Invited Speaker and Co-organizer, Keystone Symposium on Autophagy, Breckenridge, CO

2015 Invited Plenary Speaker, PISA 2015, Recent Advances in Cell Injury, Inflammation, and Neoplasia, American Society for Investigative Pathology, Baltimore, MD

2015 Invited Speaker, Basic Science Symposium on Autophagy, American Association for the Studies of Liver Diseases Annual Meeting, San Francisco, CA

2016 Invited Speaker, Banbury Meeting on Autophagy and Cancer, Cold Spring Harbor Laboratory, NY

REGIONAL AND OTHER INVITED PRESENTATIONS

2003 Invited Lecture, University of Vermont Cancer Center Symposium, Burlington, VT

2003 Invited Lecture, Society for Developmental Biology, Woods Hole, MA

2004 Invited Lecture, Department of Molecular Biomedical Research, University of Ghent, Belgium

2004 Invited Lecture, Department of Biochemistry and Molecular Biology, UMDNJ-New Jersey Medical School, Newark, NJ

2004 Invited Lecture, Cancer Biology and Genetics Program, Sloan-Kettering Institute, New York, NY

2005 Seminar, UCSF Rock Hall Research In Progress Series

2005 Speaker, UCSF BMS Graduate Program Retreat, Granlibaken, CA

2006 Seminar, UCSF Breast Oncology Program, San Francisco, CA

2006 Seminar, UCSF Molecular Medicine Program Seminar

Series, San Francisco, CA

2006 Invited Speaker, UCSF Cancer Center Seminar Series, San Francisco, CA

2007 Invited Lecture, Wayne State University Department of Pharmacology (Mar 2007), Detroit, MI

2007 Invited Lecture, Northwestern University, Department of Endocrinology and Molecular Medicine (Jan 2007), Chicago, IL

2007 Invited Lecture, Stanford University, Geriatric Research, Education, and Clinical Center, Palo Alto, CA

2008 Invited Lecture, Glaxo Smith Kline Pharmaceuticals, Collegeville, PA

2008 Invited Keynote Speaker, Symposium on Cell Death, University of Colorado Heath Sciences Center, Denver, CO

2008 Invited Keynote Speaker, University of California Davis Medical Center Breast Cancer Symposium, Sacramento, CA

2008 Invited Lecture, UNMC Eppley Comprehensive Cancer Center, Omaha, NE

2009 Invited Lecture, Han-Mo Koo Memorial Seminar, Van Andel Institute, Grand Rapids,MI

2009 Invited Lecture, San Francisco Veteran's Administration Medical Center, San Francisco, CA

2009 Invited Lecture, Buck Institute for Age Research, Novato, CA

2009 Invited Lecture, Diller Family Cancer Center Building Inaugural Scientific Symposium, San Francisco, CA

2009 Invited Lecture, Georgia Institute of Technology School of Biology, Atlanta, GA

2009 Invited Lecture, Winship Cancer Institute, Emory University School of Medicine, Atlanta, GA

2010 Invited Lecture, Lerner Research Institute and Taussig Cancer Center, Cleveland Clinic, Cleveland, OH

2010 Invited Lecture, Ontario Cancer Institute, University of Toronto, Toronto, ON, Canada

2010 Invited Lecture, Clontech Laboratories, Mountain View, CA

2010 Invited Lecture, University of Massachusetts Medical Center Department of Cancer Biology, Worchester, MA

2010 Invited Lecture, University of Colorado Denver Cancer

Center, Denver, CO

2010 Invited Lecture, San Francisco State University Department of Biology Seminar Series, San Francisco, CA

2010 Invited Lecture, UCSF Heme/Onc Research Seminar Series, San Francisco, CA

2010 Invited Seminar, Division of Cancer Treatment and Diagnosis, National Cancer Institute, Bethesda, MD

2010 Speaker and Co-organizer, Helen Diller Family Comprehensive Cancer Center Workshop on Metabolism and Cancer, UCSF, San Francisco, CA

2011 Invited Speaker, Novartis Institute of Biomedical Research, Cambridge, MA

2011 Speaker, UCSF Biomedical Sciences Graduate Program Retreat, Granlibakken, CA

2012 Invited Speaker, University of Southern California Keck School of Medicine, Cellular Homeostasis Lecture Series, Los Angeles, CA

2012 Invited Seminar, Department of Oncological Sciences, Mount Sinai School of Medicine, New York, NY

2012 Invited Speaker, UCSF Cancer Center Seminar Series, San Francisco, CA

2012 Invited Speaker, University of Minnesota Department of Biochemistry, Molecular Biology, and Biophysics Seminar Series, Minneapolis, MN

2012 Invited Speaker, Molecular Pharmacology & Chemistry Research Seminar Series Memorial Sloan-Kettering Cancer Center, New York, NY

2012 Invited Seminar, Department of Biological Sciences and Center for the Study for Gene Structure and Function, Hunter College, City University of New York, New York, NY

2012 Invited Seminar, Cleave Biosciences, Burlingame, CA

2012 Invited Speaker, 1st Annual Helen Diller Family Cancer Center Annual Retreat, Santa Cruz, CA

2012 Invited Seminar, Department of Physiology, University of Texas Health Science Center, San Antonio, TX

2013 Invited Keynote Speaker, 2013 Vancouver Autophagy Symposium, Vancouver, BC, Canada

2013 Invited Seminar, Department of Cell and Developmental Biology, Oregon Health & Sciences University, Portland, OR.

2013 Invited Speaker, UCSF Breast Oncology Program, San Francisco, CA

2013 Invited Seminar, Amgen Department of Oncology, San Francisco, CA

2014 Invited Seminar, Institut Pasteur, Paris, France

2014 Invited Seminar, Program in Cancer Biology, University of Hawaii Cancer Center, Honolulu, HI

2014 Invited Seminar, University of Chicago, Ben May Cancer Biology Program, Chicago, IL

2014 Invited Seminar, Distinguished Speaker Series, Department of Translational Molecular Pathology, University of Texas M.D. Anderson Cancer Center, Houston, TX

2015 Invited Speaker, University of Cincinnati, Department of Cancer Biology, Cincinnati, OH

2015 Invited Speaker, University of Pittsburgh Cancer Institute, Pittsburgh, PA

2015 Invited Speaker, Sanford-Burnham Institute, La Jolla, CA

2016 Invited Speaker, Frontiers in Oncology Grand Rounds, University of Maryland, Baltimore, MD

CONTINUING EDUCATION COURSES ATTENDED

2003 Osler Pathology Review Course, Tampa, FL

2004 San Antonio Breast Cancer Symposium, San Antonio, TX

2011 AACR Annual Meeting, Orlando, FL

2013 ASCI/AAP Annual Meeting, Chicago, IL

2015 ASCI/AAP Annual Meeting, Chicago, IL

GOVERNMENT AND OTHER PROFESSIONAL SERVICE

2008 -	Hong Kong Research Grant Council	External grant reviewer
2008 -	National Medical Research Council, Singapore	External grant reviewer
2008 -	Austrian Science Fund	External grant reviewer
2008 -	Department of Defense Breast Cancer Research Program	Ad hoc programmatic reviewer, Integration Panel
2008 -	United Kingdom Breast Cancer Campaign	External grant reviewer
2009 -	National Medical Research Council, Singapore	External grant reviewer
2009 -	Hong Kong Research Grant Council	External grant reviewer
2009 -	Health Research Board of Ireland	External grant reviewer
2009 -	Department of Defense Breast Cancer Research	Ad hoc programmatic

	Program	reviewer, Integration Panel
2009 -	NIH Challenge Grants (RC1)	Ad hoc Reviewer (Stage 1)
2010 -	Department of Defense Breast Cancer Research Program	Ad hoc programmatic reviewer, Integration Panel
2010 -	French National Research Agency	External grant reviewer
2010 -	New Jersey Cancer Commission	Program project reviewer
2010 -	Health Research Board of Ireland	External grant reviewer
2010 -	Medical Research Council, United Kingdom	External grant reviewer
2010 -	NIH Director's Challenge (RC4)	Ad hoc Reviewer (Stage 1)
2011 -	Cancer Research UK (CRUK)	External grant reviewer
2011 -	Department of Defense Breast Cancer Research Program	Ad hoc programmatic reviewer, Integration Panel
2011 -	Italian Association of Cancer Research (AIRC)	External grant reviewer
2011 -	Wellcome Trust, United Kingdom	External grant reviewer
2011 -	Danish Cancer Society	External grant reviewer
2011 -	NIH MBPP (Membrane Biology and Protein Processing) Study Section	Ad hoc Reviewer
2011 -	Hong Kong Research Grant Council	External grant reviewer
2012 -	Cancer Research UK (CRUK)	External grant reviewer
2012 -	National Medical Research Council, Singapore	External grant reviewer
2012 -	Italian Association of Cancer Research (AIRC)	External grant reviewer
2012 -	Hong Kong Research Grant Council	External grant reviewer
2012 -	Howard Hughes Medical Institute (HHMI) Medical Student Fellows Program	Fellowship program reviewer
2012 -	Department of Defense Breast Cancer Research Program	Ad hoc programmatic reviewer, Integration Panel
2012 -	Luxemborg National Research Fund (FNR)	External grant reviewer
2012 -	NIH TCB (Tumor Cell Biology) Study Section	Ad hoc reviewer
2012 -	Biotechnology and Biomedical Sciences Research Council (BBSRC), United Kingdom	External grant reviewer
2013 -	Swiss National Science Foundation, Switzerland	External grant reviewer
2013 -	Italian Association of Cancer Research (AIRC)	External grant reviewer
2013 -	National Medical Research Council, Singapore	External grant reviewer
2013 -	Hong Kong Research Grant Council	External grant reviewer
2013 -	NIH SBIR Grants Study Section	Ad hoc reviewer
2013 -	NIH TCB (Tumor Cell Biology) Study Section	Ad hoc reviewer

2013 -	NIH Cancer Biology Special Emphasis Panel	Ad hoc reviewer
2013 -	Department of Defense Breast Cancer Research Program	Ad hoc programmatic reviewer, Integration Panel
2013 - 2019	NIH TCB (Tumor Cell Biology) Study Section	Permanent member
2014 -	Department of Defense Breast Cancer Research Program	Ad hoc programmatic reviewer, Integration Panel
2014 -	Health Research Board of Ireland	External grant reviewer
2014 -	Italian Association of Cancer Research (AIRC)	External grant reviewer
2014 -	Deutsche Krebshilfe (German Cancer Aid)	External grant reviewer
2015 -	Austria Science Fund-Wittgenstein Award	External grant reviewer
2015 -	Hong Kong Research Grant Council	External grant reviewer
2015 -	The Terry Fox Cancer Research Foundation, Canada	Program project reviewer
2015 -	French National Cancer Institute	Program project reviewer
2015 -	French National Research Agency	External grant reviewer
2015 -	Department of Defense Breast Cancer Research Program	Member, Integration Panel

UNIVERSITY AND PUBLIC SERVICE

UNIVERSITY SERVICE

UCSF CAMPUS-WIDE

2009 - present	UCSF Graduate Division Community Building Workshop (formerly called Diversity Workshop)	Small group leader
2011 - present	UCSF Diversity Committee	Member
2014 - present	Faculty Advisory Committee, Initiative for Maximizing Student Development, NIGMS-IMSD grant, UCSF Graduate Division	
2010 - 2014	Placement of Summer Research Training Program (SRTP) Student Into UCSF Labs (formalized as SRTP Admissions and Placement Committee in 2015)	
2015 - present	Summer Research Training Program Admissions and Placement Committee, UCSF Graduate Division	Member

SCHOOL OF MEDICINE

2005 - 2010	Interviewer, Molecular Medicine Fellowship Program	

2008 - 2009	Stewart Trust Grant Review Committee Member	HDFCCC
2009 -	Laboratory Medicine (Breast Oncology Program Director) Search Committee	Member (Recruited Laura Van't Veer)
2012 - present	ACS Individual Research Award Grant Review Committee Member	HDFCCC
2013 - present	Research Allocation Program Training (RAPtr) Committee	Member
-		
-	Service for Biomedical Sciences (BMS) Graduate Program:	
2005 - present	Interviewer, BMS Graduate Program	
2006 - present	Biomedical Sciences (BMS) Graduate Program Admissions Committee	Member
2007 - present	BMS Seminar Committee	Member
2007 - present	BMS Student Advisory Committee	Member
2008 - present	Under-represented Minority Subcommittee, BMS Admissions Committee	Member
2009 - present	BMS Graduate Program Internal Selection Committee for Fellowships and Awards	Member
2012 - present	Guidelines Committee for Preliminary Qualifying Exam, Biomedical Sciences Program (with Allan Balmain and Tony Defranco)	Member
2014 - 2015	BMS Admissions Committee	Chairperson
2015 - present	BMS Admissions Committee	Co-chair with Robert Blelloch, UCSF
2015 - present	BMS Executive Committee	Member

DEPARTMENTAL SERVICE

2005 - 2007	Departments of Pathology and Lab Medicine Website	Member
2005 - present	Interviewer, Anatomic Pathology Residency Program	Interviewer
2008 - 2011	Pathology/Diller Cancer Center Faculty Search Committee	Member (Recruited Bradley Stohr and Scott Seeley)
2009 -	Dept of Anatomy (Werb) T32 Cancer Research Training Grant (Postdoctoral Fellows) Selection	Ad-Hoc Reviewer
2011 - present	Pathology Bridge Funding Committee	Member
2012 -	Pathology and Laboratory Medicine Physician Scientist Pathway Research Day	Faculty Speaker
2012 -	Dept of Anatomy (Werb) T32 Cancer Research Training Grant (Postdoctoral Fellows) Selection	Ad-Hoc Reviewer

2013 -	Departments of Anatomy and Pathology Faculty Search Committee	Member (Recruited Eric Snyder)
2013 -	Pathology and Laboratory Medicine Physician Scientist Pathway Research Day	Faculty Speaker
2013 -	Dept of Anatomy (Werb) T32 Cancer Research Training Grant for Post-doctoral Fellows	Associate Director
2013 -	Department of Pathology Faculty Search Committee	Member
2014 -	Pathology and Laboratory Medicine Physician Scientist Pathway Research Day	Faculty Speaker
2015 -	Pathology and Laboratory Medicine Physician Scientist Pathway Research Day	Faculty Speaker
2015 -	Department of Pathology Faculty Search Committee for Experimental and Liver/GI Pathologist	Member (no faculty successfully recruited)
2015 -	Departments of Anatomy and Pathology Faculty Search Committee	Member
2015 -	Department of Pathology Faculty Search Committee for Physician Scientist	Chair
-		
-	Georgia Tech & Harvard Medical School	
1998 - 2001	Regional Selection Committee, Georgia Tech President's Scholarship Program	
2003 - 2003	Organizing committee, Harvard Medical School on "Open Access" Publishing	

PUBLIC SERVICE

2007 -	Annual Biomedical Research Conference for Minority Students Poster session judge and BMS minority recruitment, Austin, TX
2007 -	ReachMD "Clinician's Roundtable" Radio Interview
2007 -	Georgia Institute of Technology President's Scholarship Program Keynote Speaker, PSP Annual Luncheon, Atlanta, GA
2009 -	Interviewed for Science Careers (from the journal Science) for article on "Redefining Tenure at Medical Schools" by Chesea Wald.
2009 -	Interviewed for "Living History" Video, Georgia Institute of Technology Alumni Association
2010 -	Panelist, Career Panel Discussion 8th Annual Clinical Investigator Trainee Meeting, NIH, Bethesda, MD.

2013 - Dinner Speaker, Regional Meeting of HHMI Medical Student Research Fellows, San Francisco, CA.

SUMMARY OF SERVICE ACTIVITIES

My university service is primarily devoted to the Biomedical Sciences (BMS) Graduate Program and toward the recruitment and retention of underrepresented minority students into graduate programs at UCSF.

BMS Graduate Program Service: I have long-term service on numerous committees in the BMS Graduate Program, including the Seminar Planning committee (since 2007), Internal Selection Committee for Awards and Fellowships (since 2009), Preliminary Qualifying Exam Guidelines Committee (since 2012) and the admissions committee (since 2007).

For 2014-15, I served as the Chair of the Admissions Committee for the BMS Graduate Program and will continue to serve in this capacity in 2015 with Dr. Robert Blelloch. In fall 2015, I will become a member of the BMS Executive Committee.

Diversity Efforts for the BMS Program and UCSF Graduate Division: I have served on the URM Admissions Subcommittee for the BMS Graduate Program since its inception in 2009. This committee is tasked to identify and recruit the top diversity candidates from the large and highly competitive overall applicant pool. Since 2012, I have served on the Faculty Diversity Committee (chaired by Professor Carol Gross), which serves to insure diversity and community building among the basic science graduate programs at UCSF. This committee organizes the Annual Community Building Workshop for entering graduate students as well as assists in the curriculum for the Summer Research Training Program (SRTP), a program that annually brings URM undergraduates to UCSF to conduct summer research in basic science faculty labs. In 2014, I became a member of the Faculty Advisory Committee for the Initiative for Maximizing Student Development Grant, an NIGMS-funded program in the UCSF Graduate Division to recruit and retain URM graduate students. Finally, since 2010, I have been one of the principal faculty responsible for placing SRTP undergraduate students into BMS faculty member labs each summer; in 2015, this became a formally organized committee in the UCSF Graduate Division called the SRTP Admissions and Placement Committee.

RAPtr Committee: Since 2012, I have served on the RAPtr Committee for the School of Medicine, which is responsible for the review and selection of proposals from medical students to receive funding to conduct summer and year-long research projects.

TEACHING AND MENTORING

TEACHING

FORMAL SCHEDULED CLASSES FOR UCSF STUDENTS

Qtr	Academic Yr	Course Number and Title	Teaching Contribution	Units	Class Size
Fall	2000 - 2004	Harvard Medical School Medical Student Pathology	Lab Instructor	0	8
FA	2005 -	BMS260, BMS Cell Biology	Discussion Leader	4	8
FA	2006 -	BMS260, BMS Cell Biology	Discussion Leader	4	8
FA	2007 -	BMS260, BMS Cell Biology	Discussion Leader	4	8
FA	2008 -	BMS260, BMS Cell Biology	Discussion Leader	4	8
FA	2010 -	BMS260, BMS Cell Biology	Discussion Leader	4	8
FA	2006 -	Infection, Immunity, and Inflammation	Lab Instructor	0	25
FA	2007 -	Infection, Immunity, and Inflammation	Lab Instructor	0	25
FA	2008 -	Infection, Immunity, and Inflammation	Lab Instructor	0	25
FA	2009 -	Infection, Immunity, and Inflammation	Lab Instructor	0	25
FA	2010 -	Infection, Immunity, and Inflammation	Lab Instructor	0	25
FA	2012 -	Infection, Immunity and Inflammation	Lab Instructor	0	25
FA	2013 -	Infection, Immunity and Inflammation	Lab Instructor		25
FA	2014 -	Infection, Immunity, and Inflammation	Lab Instructor		25
FA	2006 -	Cancer	Discussion Leader	0	15
FA	2007 -	Cancer	Discussion Leader	0	15
FA	2008 -	Cancer	Discussion Leader	0	15
FA	2009 -	Cancer	Discussion Leader	0	15
FA	2010 -	Cancer	Discussion Leader	0	15
FA	2011 -	Cancer	Discussion Leader		15
FA	2013 -	Cancer	Discussion Leader		15
FA	2014 -	Cancer	Discussion Leader		15
WI	2007 -	Life Cycle/Epilogue	Lab Instructor	0	24
WI	2008 -	Life Cycle/Epilogue	Lab Instructor	0	24
WI	2007 -	Biochem 297, Molecular Pathology/Biology of Neoplasia	Lab Instructor	0	15
WI	2008 -	Biochem 297, Molecular Pathology/Biology of Neoplasia	Lab Instructor	0	15
FA	2011 -	Physio 181, Demystifying Medicine	Lecturer (1	2	25

Qtr	Academic Yr	Course Number and Title	Teaching Contribution	Units	Class Size
			lecture)		
WI	2014 -	Physio 181, Demystifying Medicine	Lecturer (1 lecture)	2	25
WI	2009 -	BMS230, Molecular and Cellular Biology of Cancer	Lecturer (1-2 lectures)	4	25
FA	2010 -	BMS230, Molecular and Cellular Biology of Cancer	Lecturer (1-2 lectures)	4	25
FA	2012 -	BMS230, Advanced Topics In Cancer Research	Co-Course Director and Lecturer (1 lecture and 1 discussion section)	4	8
FA	2013 -	BMS 230, Advanced Topics In Cancer Research	Co-Course Director and Lecturer (2 lectures and 1 discussion section)	4	25
FA	2014 -	BMS 230, Advanced Topics In Cancer Research	Co-Course Director and Lecturer (2 lectures and 1 discussion section)	4	25
FA	2015 -	BMS 230, Advanced Topics in Cancer Research	Course Director and Lecturer (2 lectures and 1 discussion section)	4	25

INFORMAL TEACHING

2005 - 2006 BMS Journal Club Faculty Coach (Guided paper discussions and oral presentations for 3 graduate students (3 hours per student)), University of California, San Francisco

2005 - present Autopsy service attending pathologist (Supervised 2-4 residents/medical students six weeks per year), UCSF Medical Center

2006 - 2007 Qualifying exam committee member (Served on qualifying exam committee for 4 BMS graduate students (7-10 hrs per student)), University of California, San Francisco

2006 - 2007 BMS Journal Club Faculty Coach (Guided oral presentations for 4 graduate students (2-3 hours per student)), University of California, San Francisco

2006 - 2006 Discussion Leader, "Mentoring Mania" Symposium, UCSF (Led one time (1 hour) discussion with 5 post-docs), UCSF Medical Center

2007 - 2008 Qualifying exam committee member (Served on qualifying exam committee for 1 BMS and 1 CCB graduate student (7-10 hrs per student)), University of California, San Francisco

2007 - 2008 Chair, qualifying exam committee (Chair of qualifying exam committee for Seth Bechis (BMS/MSTP student), 10 hrs total), University of California, San Francisco

2007 - 2008 BMS Journal Club Faculty Coach (Guided oral presentations for one graduate student (2-3 hours per student)), University of California, San Francisco

2007 - 2009 Thesis Advisory Committee, Cathy Collins (Ph.D. candidate, MSTP/BMS) (Meet 1-2 times yearly, 2 hrs per meeting), University of California, San Francisco

2007 - 2010 Thesis Advisory Committee, (Kate Nestor, Ph.D. candidate, BMS) (1 meeting/year, 2h per mtg), University of California, San Francisco

2007 - 2011 Thesis Advisory Committee, Daniel Garcia (Ph.D. candidate, BMS) (1 meeting/year, 2h per mtg), University of California, San Francisco School of Medicine

2007 - 2011 Thesis Advisory Committee, Brandon Tavshajian (Ph.D. candidate, CCB) (1 meeting/year, 2h per mtg), University of California, San Francisco School of Medicine

2008 - 2009 Chair, Qualifying exam committee (Chair of qualifying exam committee for Sarah Gierke (BMS), 10h total), University of California San Francisco

2008 - 2009 Faculty Mentor for NSF and BMS260 proposals, BMS Graduate Program (Advised two first-year BMS sutdents in writing NSF and BMS260 proposals (10 hours per student)), University of California San Francisco

2008 - 2009 BMS Journal Club Faculty Coach (Guided oral presentations for five graduate students (2-3 hours per student)), University of California, San Francisco

2008 - 2009 Faculty Mentor for BMS225A proposal, BMS Graduate Program (Advised one first-year BMS sutdents in writing BMS225A proposal (10 hours total)), University of California San Francisco

2008 - 2009 Chair, Qualifying exam committee (Chair of qualifying exam committee for Jonathan Chou (MSTP/BMS), 10 hrs total), University of California, San Francisco

2009 - 2010 Qualifying exam committee member (Served on qualifying exam committee for 2 BMS graduate students (7-10 hrs per student)), University of California, San Francisco

2009 - 2010 BMS Journal Club Faculty Coach (Guided oral presentations for three graduate students (2-3 hours per student)), University of California, San Francisco

2009 - 2012 Thesis Advisory Committee, Jonathan Chou (Ph.D. candidate, MSTP/BMS) (1 meeting/year, 2h per mtg), University of California, San Francisco School of Medicine

2010 - 2013 Chair, Thesis Advisory Committee, Lionel Lim (Ph.D. candidate, BMS) (1 meeting/year, 2h per mtg), University of California, San Francisco School of Medicine

2009 - 2010 Chair, Qualifying exam committee (Chair of qualifying exam committee for Megan Salt (BMS), 10 hrs total), University of California, San Francisco

2010 - 2011 Qualifying Exam committee member (Serve on qualifying exam committee for 1 BMS, 1 PIBS, 1 Biophysics, and 1 Neuroscience graduate student, 7-10 hrs per student), University of California, San Francisco.

2010 - 2011 BMS Journal Club Faculty Coach (Guided oral presentations for two graduate

students (2-3 hours per student)), University of California, San Francisco

2011 - 2012 Qualifying Exam committee member (Serve on qualifying exam committee for 1 DSCB Student and 2 BMS Students, 7-10 hrs per student), University of California, San Francisco.

2011 - 2012 BMS and CCB Journal Club Faculty Coach (Guided oral presentations for three graduate students (2-3 hours per student)), University of California, San Francisco

2012 - present Thesis Advisory Committee, Si-Han Chen (Ph.D. candidate, Biophysics) (1 meeting/year, 2h per mtg), UCSF

2012 - present Thesis Advisory Committee, Julia Marguiles (Ph.D. candidate, Neuroscience) (1 meeting/year, 2h per mtg), UCSF

2012 - 2015 Chair, Thesis Advisory Committee, Mike Ando (Ph.D. candidate, BMS) (1 meeting/year, 2h per mtg), UCSF

2012 - 2015 Thesis Advisory Committee, Brittany Anderton (Ph.D. candidate, BMS) (1 meeting/year, 2h per mtg), UCSF

2012 - 2013 BMS Journal Club Faculty Coach (Guided oral presentations for one graduate students (2-3 hours per student)), University of California, San Francisco

2012 - 2013 Qualifying Exam committee member (Serve on qualifying exam committee for 1 UCB/UCSF Bioengineering Student), University of California, San Francisco.

2012 - 2013 Faculty Mentor for BMS225A proposal, BMS Graduate Program (Advised one first-year BMS student in writing BMS225A proposal (10 hours total)), University of California San Francisco

2012 - 2013 Chair, Qualifying exam committee (Chair of qualifying exam committee for Gianne Souza (BMS), 10h total), University of California San Francisco

2013 - 2015 Chair, Thesis Advisory Committee, Florie Charles Mar (Ph.D. candidate, BMS) (1 meeting /year, 2h per mtg), UCSF

2013 - 2015 Thesis Advisory Committee, Darien Reed (Ph.D. candidate, MSTP/Tetrad) (1 meeting/year, 2h per mtg), UCSF

2013 - 2014 Thesis Advisory Committee, Alec Cerchiari (Ph.D. candidate, Bioengineering Student) (1 meeting/year, 2h per mtg), UCB/UCSF Bioengineering Program.

2013 - 2015 Chair, Thesis Advisory Committee, Renee Rivas (Ph.D. candidate, MSTP/BMS) (1 meeting/year, 2h per mtg), University of California San Francisco

2013 - Invited Speaker, UCSF Medical Scientist Training Program (MSTP) Grand Rounds, University of California, San Francisco

2012 - 2013 Chair, Qualifying exam committee (Chair of qualifying exam committee for Christine Sheridan (BMS), 10h total), University of California San Francisco

2013 - 2014 Chair, Qualifying exam committee (Chair of qualifying exam committee for Amanda Paulson (BMS), 10h total), University of California San Francisco

2013 - 2014 BMS Journal Club Coach (Guided oral presentations for three BMS graduate students, 2-3 hours per student), University of California San Francisco

2013 - 2014 Department of Pathology MOD Conference Mentor (Guided oral presentation by Manana Kvezerelli, Anatomic Pathology Resident, 1-2 hrs), University of

California San Francisco

2013 - 2014 Qualifying Exam committee member (Served on qualifying exam committee for 1 BMS, 1 Biophysics and 1 CCB student), University of California, San Francisco.

2013 - 2014 Chair, Qualifying exam committee (Chair of qualifying exam committee for David Pardo (BMS), 10 h total), University of California, San Francisco.

2014 - 2015 BMS Journal Club Coach (Guided oral presentations for one BMS graduate student, 2-3 hours per student), University of California San Francisco

2015 - present Thesis Advisory Committee, Joe Udoechu (Ph.D. candidate, BMS) (1 meeting/year, 2h per mtg), UCSF

2015 - present Thesis Advisory Committee, T.J. Hu (Ph.D. candidate, BMS) (1 meeting/year, 2h per mtg), UCSF

2015 - present Thesis Advisory Committee, Svetlana Keylin (Ph. D. candidate, DSCB) (1 meeting/year, 2h per mtg), UCSF

2015 - present Thesis Advisory Committee, Ted Ho (Ph. D. candidate, Biophysics) (1 meeting/year, 2h per mtg), UCSF

2015 - present Thesis Advisory Committee, David Pardo (Ph. D. candidate, BMS) (1 meeting/year, 2h per mtg), UCSF

2014 - 2015 Qualifying Exam Comittee Member (Served on qualifying exam committee for 3 BMS students) University of California San Francisco

2014 - 2015 Chair, Qualifying Exam Committee (Chair of qualifying exam committee for Vassily Kutyavin (BMS), 10 h total), University of California, San Francisco.

2015 - present Thesis Advisory Committee, Alex Samocha (Ph. D. candidate, BMS) (1 meeting/year, 2h per mtg), UCSF

TEACHING NARRATIVE

My formal teaching expertise encompasses the areas of cell biology, cancer biology, and pathology.

Graduate school: I primarily teach graduate level courses for the Biomedical Sciences Graduate Program. From 2005-10, I served as a discussion leader to a group of 8-10 first year students in the BMS 260 Cell Biology. Since 2010, I have given lectures and led discussion groups for the BMS 230 Cancer Biology course, both prior to and while serving as the course director.

I also advise graduate students in the BMS program in my laboratory, both rotation students and students working toward a thesis in my laboratory. I have served as a chair or as a member of multiple Qualifying Exam Committees for 2nd year UCSF graduate students (in the BMS, DSCB, PIBS, CCB, Neuroscience, Bioengineering and Biophysics programs) and currently serve as a chair or member of twelve Thesis Advisory Committees for advanced graduate students in the BMS, DSCB, Neuroscience and Biophysics Programs. In addition, I have served serve as a faculty coach to students for their oral presentations at the BMS Journal Club, and as a mentor for student's written proposals in various BMS courses.

Course DIrector for BMS230, Advanced Topics In Cancer Research: ***This course is the primary graduate level elective cancer biology course taught at UCSF; it is offered every fall to first and second year as well as more advance graduate students at UCSF.***

In Fall 2012, I became the co-course director of the BMS 230 Cancer Biology course with Professor Martin McMahon at the HDFCCC; we reorganized the curriculum, previously a survey course, into a series of focused, in-depth advanced topics in cancer research. These topics will change on a yearly basis in order to cover timely issues in cancer biology. In Fall 2015, I will become the sole course director for BMS230 due to the departure of Dr. McMahon from UCSF.

Medical School: In the medical school curriculum at UCSF, I teach as a lab instructor in the Immunology Block (I3) and small group discussion leader for the pathology section in the Cancer Block (M3). As part of my clinical duties, I also supervise and teach anatomic pathology residents and medical students as an attending in the autopsy service at Moffitt Hospital at UCSF (6 weeks per year).

MENTORING

PREDOCTORAL STUDENTS SUPERVISED OR MENTORED

Dates	Name	Program or School	Role	Current Position
2001 - 2005	Carolyn Wrobel	Harvard Medical School	Advisor for rotation and during graduate thesis work, Brugge Lab	Assistant Professor, Depaul University
2001 - 2001	Diedra Wrighting	Harvard Medical School	Rotation Advisor, Brugge Lab	Research Scientist, Broad Institute, MIT
2001 - 2001	Sean Beausoliel	Harvard Medical School	Rotation Advisor, Brugge Lab	Research Scientist, Cell Signaling Technologies
2005 - 2007	Chris Fung	University of California, San Francisco	Supervised post undergraduate research	Resident, Emergency Medicine, University of Michigan
2006 - 2011	Lilly Radoshevich	University of California, San Francisco	Thesis Advisor, BMS Graduate Student	EMBO Post-doctoral Fellowship (Pascal Cossart, HHMI and Pasteur Institut, Paris, France)
2006 - 2011	Rebecca Lock	University of California, San Francisco	Rotation Advisor/Thesis Advisor	CTF Young Investigator Post-doctoral Fellowship (Karen Cichowski, Harvard Medical School)
2007 - 2013	Eduardo Salas	University of California, San Francisco	Supervised post undergraduate research	Research Scientist, Gilead Corporation, Foster City, CA
2007 - 2009	Cynthia Jimenez	University of California, San Francisco School of Medicine	M.D. With Thesis Committee Member	Surgical pathologist (private practice), Sacramento, CA
2008 - 2008	Ada Li	University of California, San Francisco	Summer Research Mentor (SEP High School Program)	Undergraduate, UCLA

Dates	Name	Program or School	Role	Current Position
2008 - 2015	Candia Kenific	University of California, San Francisco	Rotation advisor/Thesis Advisor	Post-doctoral Fellow, (David Lyden, Weill Cornell Medical Center starting Fall 2015)
2009 - 2014	Lyndsay Murrow	University of California, San Francisco	Rotation advisor/Thesis Advisor	Damon Runyon Post-doctoral Fellow (Zev Gartner, UCSF)
2009 - 2013	Laura Westrake	Van Andel Research Institute	Thesis committee member (external advisor)	Post-doc, Gia Voltz Lab, University of Colorado Boulder
2009 - 2009	Estefania Fernandez	University of California, San Francisco	Summer Research Mentor (SRTP Undergraduate Program)	MD/PhD (MSTP) Program, Washington Univ-St. Louis
2010 - 2010	Nuria Eritja	University of California, San Francisco	Short term stay advisor	Post-doc, Spain
2011 - 2011	Shivali Gupta	University of California, San Francisco	Summer Research Mentor (SRTP Undergraduate Program)	MPH, University of California, Berkeley
2011 - 2012	Sanaaz Sadegh	University of California, San Francisco	Advisor, International Undergraduate Internship	Graduate Student, University of Southern California
2012 - present	Hanna Kuznetsov	University of California, San Francisco	Rotation advisor/Thesis advisor	BMS Graduate Student
2012 - 2013	Kimberley Woo	University of California, Berkeley	Undergraduate Research Advisor	Undergraduate, UC Berkeley
2012 - 2013	Jordan Wu	University of California, Berkeley	Undergraduate Research Advisor	Junior Specialist, Debnath Lab, UCSF
2013 - present	Jordan Wu	University of California, San Francisco	Supervised post-undergraduate research	Junior Specialist, Debnath Lab, UCSF
2013 - present	Juliet Goldsmith	University of California, San Francisco	Rotation advisor/Thesis advisor	BMS Graduate Student
2013 -	Jeff Chukwuneke	University of California, San Francisco	Summer Research Mentor (SRTP Undergraduate Program-Amgen Scholars)	Medical Student, Columbia University College of Physicians and Surgeons
2014 -	Caroline Park	Albert Einstein College of Medicine, Bronx, NY	External examiner, PHD thesis	MSTP Student, Cuervo Lab, Albert Einstein
2014 - present	Timothy Marsh	University of California, San Francisco	Rotation advisor/Thesis advisor	BMS Graduate Student
2014 -	Rocio Saavedra	University of Puerto Rico, PR	Summer Research Mentor (SRTP Undergraduate	Graduate Student, Yale University, New Haven, CT

Dates	Name	Program or School	Role	Current Position
			Program-NSF)	
2014 - 2015	Florie Charles Mar	University of California, San Francisco	Thesis Advisor (Co-mentor with Dr. Brad Stohr)	Portola Biosciences, South San Francisco, CA
2015 - present	Jennifer Liu	University of California, San Francisco	Rotation Advisor/Thesis Advisor	MD/PhD (MSTP) Student, UCSF
2015 -	Mackinzie Stanley	Whitman College, WA	Summer Research Internship Mentor	Undergraduate, Whitman College

POSTDOCTORAL FELLOWS AND RESIDENTS DIRECTLY SUPERVISED OR MENTORED

Dates	Name	Fellow	Faculty Role	Current Position
2007 - 2011	Nan Chen	University of California, San Francisco	Postdoctoral Advisor	Research scientist, Minnesota.
2007 - 2010	Kimberley Evason	University of California, San Francisco School of Medicine	Advisor, UCSF Molecular Medicine Program	Instructor, Department of Pathology
2007 - 2010	W. Patrick Devine	University of California, San Francisco School of Medicine	Advisor, UCSF Molecular Medicine Program	Post-doc, Gladstone Institute (Benoit Bruneau)
2007 - present	Ritu Malhotra	University of California, San Francisco	Postdoctoral Advisor	Research Scientist, Siemens Healthcare, Mountain View, CA
2008 - present	Srirupa Roy	University of California, San Francisco School of Medicine	Postdoctoral Advisor	Post-doctoral fellow
2011 - present	Jasvinder Kaur	University of California, San Francisco School of Medicine	Postdoctoral Advisor	Post-doctoral fellow
2011 - present	Jennifer Rudnick	University of California, San Francisco School of Medicine	Postdoctoral Advisor	Post-doctoral fellow
2013 - present	Andrew Leidel	University of California, San Francisco School of Medicine	Postdoctoral Advisor	Post-doctoral fellow
2013 - present	David Solomon	University of California, San Francisco School of Medicine	Sponsor, UCSF Physician Scientist Scholar Program Application	Neuropathology Fellow, UCSF
2014 - present	Craig Forrester	University of California, San Francisco School of Medicine	Member, Scholarship Oversight Committee, UCSF	Pediatric Heme/Onc Fellow and Post-doc, Ruggero Lab

Dates	Name	Fellow	Faculty Role	Current Position
			Pediatrics	

FACULTY MENTORING

Dates	Name	Position While Mentored	Mentoring Role	Current Position
2011 - 2013	Andrew Hseih	Instructor, UCSF Heme/Onc	Career development and NIH K08 mentoring committee	Assistant Professor, Fred Hutch Cancer Center
2014 - 2015	Eric Snyder	Assistant Professor	Department of Pathology Faculty Mentor	Assistant Professor, University of Utah, Dept of Pathology
2014 - present	Anatoly Urisman	Clinical Instructor	Department of Pathology Faculty Mentor	Clinical Instructor, UCSF Pathology

MENTORING NARRATIVE

My laboratory is dedicated to the education of post-doctoral fellows, graduate students, and undergraduate students. My principal mentoring role is as a PhD advisor to graduate students in the BMS program. Currently, four BMS graduate students are pursuing a thesis in my laboratory, among which three are UCSF Discovery Fellows. In addition, I currently have four post-doctoral fellows in my lab, and I regularly host rotation students, summer undergraduate students, visiting graduate students and fellows for short-term stays in my lab. My record of mentorship is best evidenced by the track record of my trainees in obtaining first-author publications in high impact journals (e.g., Cell, Cancer Discovery, Nature Cell Biology) as well as receiving prestigious pre-doctoral (e.g., NIH F32, NSF) and post-doctoral (e.g., ACS, DOD, CIHR, Banting) fellowships. Notably, to date, all of the PhD graduates from my lab are pursuing academic post-doctoral fellowships.

In the Department of Pathology, I serve as a career advisor to residents and fellows interested in becoming academic experimental pathologists. In 2013, I began service as Associate Director (Basic Science) for the "Molecular and Cellular Mechanisms of Cancer" T32 Training Grant (T32 CA108462, PI: Zena Werb), a multi-departmental training grant for post-doctoral fellows pursuing cancer research at UCSF and the HDFCCC.

OTHER

COMPETITIVE FELLOWSHIPS AND AWARDS OBTAINED BY TRAINEES WHILE THEY WERE IN DEBNATH LAB:

Lilly Radoshevich (PhD Student), Sandler/Genentech Predoctoral Fellowship, 2007-08.

Rebecca Lock (PhD Student), DOD Breast Cancer Predoctoral Fellowship, 2008-11.

Rebecca Lock (PhD Student), CRCC Predoctoral Fellowship, 2008-09 (declined).

Lilly Radoshevich (PhD Student), Phi Beta Kappa Scholarship, 2010.

Lilly Radoshevich (PhD Student), Keystone Scholarship (Travel award), 2010.

Lyndsay Murrow (PhD Student), NSF Graduate Fellowship, 2010-2013.

Candia Kenific (PhD Student), Genentech Predoctoral Fellowship, 2010-11.

Rebecca Lock (PhD Student), HDFCCC Student Invitee to Cancer Molecular Therapeutics Research Association Conference, 2011.

Candia Kenific (PhD Student), University of California Cancer Research Coordinating Committee Fellowship, 2011-12.

Rebecca Lock (PhD Student), DOD Era of Hope Meeting Outstanding Poster Award, 2011.

Jennifer Rudnick (Post-doc), NIH T32 Training Grant Recipient, 2011-13.

Candia Kenific (PhD Student), NRSA Graduate Student Fellowship (F31CA167905), National Cancer Institute, 2012-2015

Jennifer Rudnick (Post-doc), ACS Postdoctoral Fellowship Recipient, 2013-16 (terminated early to start DOD BCRP Fellowship).

Jennifer Rudnick (Post-doc), DOD Breast Cancer Postdoctoral Fellowship Recipient, 2013-16.

Hanna Kuznetsov (PhD Student), NSF Graduate Fellowship, 2013-2016.

Jasvinder Kaur (Post-doc), Keystone Scholarship (Travel award), 2014.

Hanna Kuznetsov (PhD Student), Discovery Fellow, UCSF Graduate Division, 2014.

Juliet Goldsmith (PhD Student), NSF Graduate Fellowship, 2014-2017.

Hanna Kuznetsov (PhD Student), HHMI-CTSI GEMS Fellowship, 2014 (declined).

Juliet Goldsmith (PhD Student), Discovery Fellow, UCSF Graduate Division, 2014.

Florie Mar (PhD Student, co-mentored with Brad Stohr), Discovery Fellow, UCSF Graduate Division, 2014.

Andrew Leidal (Post-doc), Banting Post-doctoral Fellowship, Canadian Institute of Health Research, 2015-17.

Andrew Leidal (Post-doc), Canadian Institute of Health Research Post-doctoral Fellowship, 2015 (declined).

Timothy Marsh (PhD Student), Discovery Fellow, UCSF Graduate Division, 2015.

SUMMARY OF TEACHING AND MENTORING HOURS

2014 - 2015	290 total hours of teaching (including preparation)
	Formal class or course teaching hours: 40 hours
	Informal class or course teaching hours: 250 hours
	Mentoring hours: 150 hours
	Other Hours:
2015 - 2016	290 total hours of teaching (including preparation)
	Formal class or course teaching hours: 40 hours
	Informal class or course teaching hours: 250 hours
	Mentoring hours: 150 hours
	Other Hours:
2016 - 2017	Total anticipated hours of teaching: 400 hours

RESEARCH AND CREATIVE ACTIVITIES

RESEARCH AWARDS

CURRENT

R01 CA126793-06 (Principal Investigator)	02/2015 - 01/2020
NIH/NCI	$200, 000 direct/yr1
Autophagy in adhesion and metastasis	$1,585,000 total
R01 CA188404 (Principal Investigator, (MPI: Debnath and Bergers))	09/2014 - 06/2019
NIH/NCI	$269,000 direct/yr1
Autophagy as a microenvironmental regulator of tumorigenesis and resistance.	$2,100,000 total
W81XWH-11-1-0130 (Principal Investigator)	09/2011 - 08/2016
DOD BCRP Era of Hope Scholar Award	$505,000 direct/yr1
Eliminating Late Recurrence to Eradicate Breast Cancer.	$3,775,682 total
SWCRF (Prinicipal Investigator)	07/2013 - 06/2016
Samuel Waxman Cancer Research Foundation	$20,000 direct/yr1
Effects of Autophagy on Carcinoma Differentiation and Aggression	$195,000 total
8-Ball (Principal Investigator)	9/2013 - 8/2016
8-Ball Foundation	$100,000 direct/yr1

Metabolic Adaptation In Gastrointestinal Stromal Tumor (GIST)	$250,000 total

PENDING

R21 CA (Principal Investigator)	02/2016 - 01/2018
NIH/NCI	$125,000 direct/yr1
Dechiphering Autophagy-Dependent Secretion Using Proximity-Based Biotinylation	$275,000 total

PAST

1K08CA098419-01 (Principal Investigator)	2003 - 2009
NIH/NCI	$127,150 direct/yr1
Oncogenes and Luminal Apoptosis Within Mammary Acini	$653,265 total
Culpeper Medical Scholar (Principal Investigator)	2006 - 2009
Foundation	$108,000 direct/yr1
The Role and Regulation of Autophagy in Epithelial Cell Death	$324,000 total
AACR \Genentech BioOncology Career Award (Principal Investigator)	2006 - 2009)
Foundation	$50,000 direct/yr1
The Role and Regulation of Autophagy Downstream of HER Family Pathways	$100,000 total
Stewart Trust	2007 - 2008
Foundation (Principal Investigator)	
Discovering New Autophagy Modulators For Cancer Chemotherapy	$50,000 direct/yr1
	$50,000 total
UC Cancer Research Coordinating Committee (Principal Investigator)	2007 - 2008
CRCC	$50,000 direct/yr1
Autophagy During HMEC Agonescence	$50,000 total
PBBR (Sandler) Integrative Research Award (Principal Investigator, Debnath and Ronen)	2009 - 2010
Foundation	$80,000 direct/yr1
Defining the Metabolic Consequences of Autophagy Using Magnetic Resonance Spectroscopy	$80,000 total
TRDRP 18XT-0106 (Principal Investigator)	2009 - 2011

UCOP	$125,000 direct/yr1
Autophagy and K-Ras Mutant Lung Cancer Cells	$250,000 total
R01 CA126792-S1 (Principal Investigator)	2009 - 2011
NIH (ARRA Supplement)	$66,000 direct/yr1
Autophagy and Epithelial Cell Fate During Anoikis and 3D Morphogenesis. Recovery Act Supplement	$201,000 total
HHMI Early Career Award For Physician Scientists (Principal Investigator)	2006 - 2012
Regulation of ATG12 During Autophagy.	$75,000 direct/yr1
	$375,000 total
AACR/SU2C - Stand Up To Cancer Breast Cancer Dream Team (Co-Investigator)	2009 - 2012
An Integrated Approach to Targeting Breast Cancer Molecular Subtypes and Their Resistance Phenotypes.	$Salary support only (5% effort) direct/yr1
PBBR/Sanofi (Principal Investigator (Debnath and Xu))	2011 - 2013
PBBR New Frontiers Research Award (Sanofi UCSF LIFTT Award)	$76,000 direct/yr1
Autophagy in Hypothalamus-Mediate Energy Balance and Obesity	$149,000 total
UCSF Breast Oncology Program (Principal Investigator)	2013 - 2014
UCSF Breast Oncology Program/Breast Cancer SPORE Developmental Research Project	$40,000 direct/yr1
Autophagy-Dependent Secretion and Breast Cancer Progression	$40,000 total
W81XWH-12-1-0505 (Principal Investigator)	2012 - 2014
DOD BCRP Innovator and Scholar Concept Award	$150,000 direct/yr1
Targeting Autophagy in the Tumor Stroma To Eradicate Breast Cancer	$300,000 total
R01 CA126792-01 (Principal Investigator)	2009 - 2015
NIH	$174,000 direct/yr1
Autophagy and Epithelial Cell Fate During Anoikis and 3D Morphogenesis.	$1,414,500 total

PEER REVIEWED PUBLICATIONS

1. **Debnath J**, Husain PA, May SW. Activation of an adrenergic pro-drug through sequential stereoselective action of tandem target enzymes. Biochem Biophys Res Commun. 1992 Nov 30; 189(1):33-9. PMID: 1449487

2. Husain PA, **Debnath J**, May SW. HPLC-based method for determination of absolute configuration of alpha-chiral amines. Anal Chem. 1993 May 15; 65(10):1456-61. PMID: 8517551

3. Van Etten RA, **Debnath J**, Zhou H, Casasnovas JM. Introduction of a loss-of-function point mutation from the SH3 region of the Caenorhabditis elegans sem-5 gene activates the transforming ability of c-abl in vivo and abolishes binding of proline-rich ligands in vitro. Oncogene. 1995 May 18; 10(10):1977-88. PMID: 7539119

4. **Debnath J,** Chamorro M, Czar MJ, Schaeffer EM, Lenardo MJ, Varmus HE, Schwartzberg PL. rlk/TXK encodes two forms of a novel cysteine string tyrosine kinase activated by Src family kinases. Mol Cell Biol. 1999 Feb; 19(2):1498-507. PMID: 9891083.

5. Schaeffer EM*, **Debnath J***, Yap G, McVicar D, Liao XC, Littman DR, Sher A, Varmus HE, Lenardo MJ, Schwartzberg PL. Requirement for Tec kinases Rlk and Itk in T cell receptor signaling and immunity. Science. 1999 Apr 23; 284(5414):638-41. PMID: 10213685 ***co-first author.**

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2. M.S. Sosa, P. Bragado, **J. Debnath**,* J. A. Aguirre-Ghiso*. Regulation of Tumor Cell Dormancy By Tissue Microenvironments and Autophagy. 2013, Adv. Exp. Med. Biol, 734: 73-89. ***co-senior author.**

RESEARCH PROGRAM

My laboratory is internationally recognized for its studies of autophagy, a tightly regulated cellular self-digestion pathway, and how this process regulates epithelial cell fate, oncogenic transformation and carcinoma progression. In eukaryotic cells, autophagy primarily functions as a critical survival response and metabolic adaptation pathway during nutrient deprivation or stress; as a result, interest in manipulating autophagy to treat human diseases, such as cancer, has rapidly intensified.

Autophagy and cell-matrix adhesion:* Cancer cells are resistant to anoikis, a form of apoptosis observed in epithelial cells deprived of extracellular matrix (ECM) contact. Several years ago, we discovered that autophagy serves as a key mechanism of anoikis resistance. In follow-up work, we discovered that autophagy also facilitates glycolytic metabolism during oncogenic transformation. Based on these findings, we are dissecting how autophagy contributes to the metabolic fitness of oncogene-transformed cells, allowing them to survive and expand in response to ECM deprivation and other microenvironmental stresses. More recently, we have uncovered a new role for autophagy in selectively promoting the disassembly and turnover of integrin-associated focal adhesions during cell migration and adhesion, which may have important implications in invasion and metastasis.

***Autophagy in cancer progression and metastases:* My laboratory is also delineating how autophagy impacts breast cancer progression in vivo using mouse cancer models. We have created mice containing conditional null mutant alleles that allow us to delete autophagy in a tissue specific manner and crossed them with established mouse models**

of metastatic breast cancer to define the role of autophagy in cancer progression in vivo. Our studies focus on dissecting the functional requirements for autophagy in both tumor cells as well as key stromal constituents of the larger tumor microenvironment during primary tumor growth and metastasis. We are particularly interested in the role of autophagy in breast cancer cells that lie dormant for extended periods, and ultimately, metastasize at foreign tissue sites.

Novel biochemical and biological functions of ATGs: ***Despite widespread interest in exploiting autophagy for therapeutic purposes, we have much to learn about how this process works in mammalian cells and tissues. Autophagy is a tightly regulated by highly conserved gene products called ATGs. However, our recent results implicate these ATGs in diverse cellular functions, many of which are distinct from their long-established roles in catabolism. Using cell biological, biochemical and yeast genetic approaches, we are: 1) dissecting new roles for ATGs in the control of unconventional secretion; 2) probing genetic interactions between autophagy and mitochondrial protein quality control pathways; 3) elucidating the cellular functions of ATG12-ATG3 (a novel complex between two autophagy regulators that my laboratory discovered in 2010) in the control of endocytosis and exosome biogenesis; and 4) determining how autophagy impacts protein translation and anabolic capacity during starvation.***

SIGNIFICANT PUBLICATIONS

1. Murrow L, Malhotra R, and **Debnath J**. ATG12-ATG3 Interacts with Alix to Promote Basal Autophagic Flux and Late Endosome Function. Nature Cell Biol. 2015 Mar 2; 17(3):300-10.

Significance: This manuscript follows up our discovery of the ATG12-ATG3 conjugate and identifies a new biochemical interaction between ATG12-ATG3 and the ESCRT associated protein, Alix.. This paper expands our understanding of the interconnections between the core autophagy and ESCRT machineries in the control of basal autophagy, endosome-to-lysosome trafficking, and identifies a new role for autophagy pathway components in exosome biogenesis and secretion.

Role: Senior author. Lyndsay Murrow, a PhD graduate student from my laboratory, and I designed the overall project and all of the individual experiments in the paper. Lynsday wrote the first draft of the paper, which I edited to create the final version.

2. Lock R, Kenific CM, Leidal AM, Salas E, **Debnath J**. Autophagy-Dependent Production of Secreted Factors Facilitates Oncogenic RAS-Driven Invasion. Cancer Discov. 2014 Apr; 4(4):466-79.

Significance: This paper delineates a previously unrecognized function for autophagy in facilitating oncogenic RAS-driven invasion; an intact autophagy pathway is required for the elaboration of multiple secreted factors favoring invasion, including the proinflammatory cytokine IL6.

Role: Senior author. Becky Lock, a PhD graduate from my laboratory, and I designed the overall project. We received major input on experiments from Candia Kenific, a BMS graduate student, and Andrew Leidal, a post-doc in my laboratory. All of the authors designed and carried out the major experiments in the paper. Becky wrote the first draft of the paper, which I edited, with input from the other co-authors, to create the final version.

3. Warr MR, Binnewies M, Flach J, Reynaud D, Garg T, Malhotra R, **Debnath J**, Passegué E. FOXO3A directs a protective autophagy program in haematopoietic stem cells. Nature. 2013 Feb 21; 494(7437):323-7.

Significance: The paper demonstrates for the first time that the autophagy pathway serves as an important survival mechanism in both young and old hematopoietic stem cells (HSCs) in response to nutrient starvation and metabolic stress.

Role: Co-author. This was close collaboration with the Passague lab over three years. I conceived and designed the overall project with Matt Warr, a post-doctoral fellow, and Emmannuelle Passague. Matt and I developed and optimized the assays to monitor autophagy in mouse hematopoietic stem cells. My lab also generated the Atg12 conditional knockout critical for these studies. Matt was the primary individual that carried out the experiments. Matt, Emmanuelle, and myself analyzed the results and wrote the paper.

4. Lock R, Roy S, Kenific CM, Su JS, Salas E, Ronen SM, **Debnath J**. Autophagy facilitates glycolysis during Ras-mediated oncogenic transformation. Mol Biol Cell. 2011 Jan 15; 22(2):165-78.

Significance: This paper demonstrates an unexpected connection between autophagy and glycolysis that facilitates adhesion-independent transformation driven by a strong oncogenic insult-mutant Ras. Inhibiting autophagy due to the genetic deletion or RNAi-mediated depletion of multiple autophagy regulators (ATGs) attenuates Ras-mediated adhesion-independent transformation and proliferation, as well as reduces glycolytic capacity. Overall, these results point to a unique mechanism by which autophagy may promote Ras-driven tumor growth in specific metabolic contexts.

Role: Senior author. Becky Lock, PhD graduate from my laboratory, and I designed the overall project, with major input on the glycolysis studies from Srirupa Roy, a post-doctoral fellow in my laboratory, and Sabrina Ronen, UCSF Professor of Radiology. Becky and Srirupa designed and carried out the major experiments in the paper, with assistance from the other co-authors. Becky wrote the first draft of the paper, which I edited, with input from the other co-authors, to create the final version.

5. Radoshevich L, Murrow L, Chen N, Fernandez E, Roy S, Fung C, **Debnath J**. ATG12 conjugation to ATG3 regulates mitochondrial homeostasis and cell death. Cell. 2010 Aug 20; 142(4):590-600.

Significance: This paper challenges a long-held view in the autophagy field that ATG12, an ubiquitin-like modifier required for autophagy, possesses a single substrate, called ATG5. This paper uncovers that ATG12 is conjugated to ATG3, another enzyme required for autophagy. As individual proteins, both ATG12 and ATG3 are essential for early autophagosome formation. In contrast, the ATG12-ATG3 protein complex dramatically alters the mitochondrial network and the response to mitochondrial cell death. Overall, these results unveil a previously unrecognized role for ATG12-ATG3 in mitochondrial homeostasis, and implicate the ATG12 conjugation system in cellular functions distinct from the early steps of autophagosome formation.

Role: Senior author. Lilly Radoshevich, a PhD graduate student from my laboratory, and I designed the overall project and all of the individual experiments in the paper. Lilly and the other co-authors carried out the experiments. Lilly wrote the first draft of the paper, which I edited to create the final version.

Review

CellPress

Cellular and metabolic functions for autophagy in cancer cells

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Autophagy is a lysosomal degradation pathway that acts as a dynamic regulator of tumorigenesis. Specifically, autophagy has been shown to impede early cancer development while facilitating advanced tumor progression. Recent studies have uncovered several tumor-promoting functions for autophagy; these include the maintenance of multiple metabolic pathways critical for aggressive tumor growth and the promotion of tumor cell survival downstream of the unfolded protein response. Furthermore, autophagy supports anoikis resistance and cancer cell invasion. At the same time, because autophagy cargo receptors, which are essential for selective autophagy, lie upstream of diverse cancer-promoting signaling pathways, they may profoundly influence how alterations in autophagy affect tumor development. This review focuses on how these tumor cell autonomous functions of autophagy broadly impact tumorigenesis.

Overview of autophagy and tumorigenesis

Macroautophagy (hereafter called autophagy) is an evolutionarily conserved pathway of lysosomal-mediated cellular self-digestion. It involves the formation of a double-membrane vesicle, the autophagosome, which engulfs cytoplasmic components and delivers them to the lysosome for degradation (Box 1). Landmark studies in yeast have identified over 30 autophagy-related genes (*atg*s); in response to stress and starvation, numerous signaling pathways impinge on these ATGs to induce autophagy. The resulting lysosomal digestion and recycling of cellular contents is proposed to refuel cells with metabolic building blocks that are critical for survival during stress [1–3]. Additionally, during normal cellular homeostasis, autophagy functions as a primary route of degradation for damaged organelles and protein aggregates [4]. Because of these conserved functions in eukaryotic cells, autophagy has been proposed to act as a crucial cellular adaptation pathway that promotes tumorigenesis by facilitating the survival of cancer cells under duress [5–7].

Nonetheless, over the past decade, studies of how autophagy impacts cancer development have yielded conflicting results. Initial studies demonstrating that loss of the essential autophagy regulator *beclin1* (*atg6*) results in increased tumorigenesis in mice provided genetic evidence that autophagy serves tumor suppressive functions [8,9]. Additionally, *BECN1* was proposed to be a haploinsufficient tumor suppressor in humans, but a recent analysis of human cancer sequencing data questions these original findings [10–12]. Further support for a role for autophagy in limiting tumorigenesis came from elegant studies in which the deletion of *atg5* or *atg7* led to spontaneous development of premalignant liver tumors due to accumulation of oxidative stress and activation of genome damage responses [13,14]. However, because tumor progression requires cancer cells to thrive in untoward environments, tumor-supporting functions for autophagy have also been uncovered [7]. Now, we appreciate that autophagy serves dual roles during tumorigenesis; its homeostatic function limits genome-damaging events that would otherwise favor tumor initiation, while its ability to help cells mitigate stress facilitates advanced tumor progression [5,6]. Importantly, studies in genetically engineered mouse models (GEMMs) of cancer have provided additional support for these opposing functions of autophagy; during oncogene activation, genetic deletion of ATGs enhances early tumor development but impairs advanced tumorigenesis [15–18] (Table 1). In addition, novel insight into how autophagy controls tumor cell fate and regulates cell phenotypes other than growth and survival has emerged. Here, we focus on how GEMMs have improved our understanding of how autophagy controls tumor cell metabolism and cell survival, as well as highlighting new cell biological functions for autophagy in tumor cells during cancer progression.

Control of tumor cell metabolism by autophagy

Rapidly proliferating tumor cells have increased anabolic demands, which are met by metabolic changes induced upon activation of oncogenes and loss of tumor suppressors [19]. At its most fundamental level, autophagy couples catabolic breakdown of cellular content with anabolic pathways of macromolecule synthesis by supplying the cell with intracellular metabolites generated via lysosomal-mediated degradation. Despite this salient feature of autophagy, its importance in tumor cell metabolism was not appreciated until recently.

Studies of oncogenic Ras transformation were the first to demonstrate a role for autophagy in supporting tumor cell proliferation and in maintaining metabolic function in the context of oncogene activation. In mouse embryonic

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Box 1. Autophagosome formation and maturation in mammals

Autophagosome formation and maturation is a highly regulated process that occurs through a series of distinct steps controlled by ATGs (Figure IA). Initiation of autophagosome formation is regulated by the ULK and class III PI3K complexes (Figure IB). The ULK complex consists of ULK1/2, which are the mammalian orthologs of ATG1, as well as mATG13, FIP200 (ATG17), and ATG101 [82]. Under nutrient-rich conditions, the ability of the ULK complex to initiate autophagy is inhibited by mTORC1, which phosphorylates and inactivates ULK1/2 [83]. Upon starvation, mTORC1 activity is suppressed, leading to disassociation from and activation of the ULK complex. The class III PI3K complex, consisting of the lipid kinase Vps34, Beclin1 (ATG6), ATG14L, and p150, is then activated by the ULK complex [84]. The PI3K complex functions to generate phosphatidylinositol 3-phosphate (PI3P) at the site of early autophagosome formation for recruitment of additional ATGs that will subsequently mediate elongation and closure of the autophagosome membrane.

Elongation and closure is controlled by two ubiquitin-like conjugation pathways that conjugate ATG12 to ATG5 and LC3 to the lipid phosphatidylethanolamine (PE) [85] (Figure IC). Conjugation of ATG12 to ATG5 is regulated by the E1- and E2-like activities of ATG7 and ATG10, respectively. The ATG12–ATG5 complex then associates with ATG16 to form a multimeric complex that localizes to the outer surface of the autophagosomal membrane. LC3 is conjugated to PE by ATG7 and the E2-like enzyme ATG3, and the ATG12–ATG5–ATG16 complex functions in an E3-like fashion to promote LC3 lipidation by PE. PE is inserted into the autophagosome membrane, and LC3–PE is localized to both the inner and outer membranes. Importantly, these core ATGs that directly control elongation of the autophagosome membrane are commonly targeted for experimental purposes, either by genetic deletion or RNAi-mediated depletion, to conduct functional studies of autophagy during tumorigenesis. Additionally, LC3-PE (also termed LC3-II) is commonly used as a marker of autophagosomes to monitor the induction or inhibition of autophagy [86].

Ultimately, the autophagosome fuses with endocytic and lysosomal compartments, leading to formation of the autolysosome (Figure IA). Autophagic cargo is then degraded through the activity of lysosomal proteases. The mechanisms underlying these late-stage maturation steps are only beginning to emerge, but studies aimed at identifying essential regulators of autolysosome formation have unveiled roles for common mediators of cellular membrane fusion, including Rab and soluble *N*-ethylmaleimide-sensitive factor (NSF) attachment protein receptor (SNARE) proteins [87,88]. Further elucidation of genes involved in these late stages will facilitate more comprehensive functional analyses of the autophagy pathway in cancer.

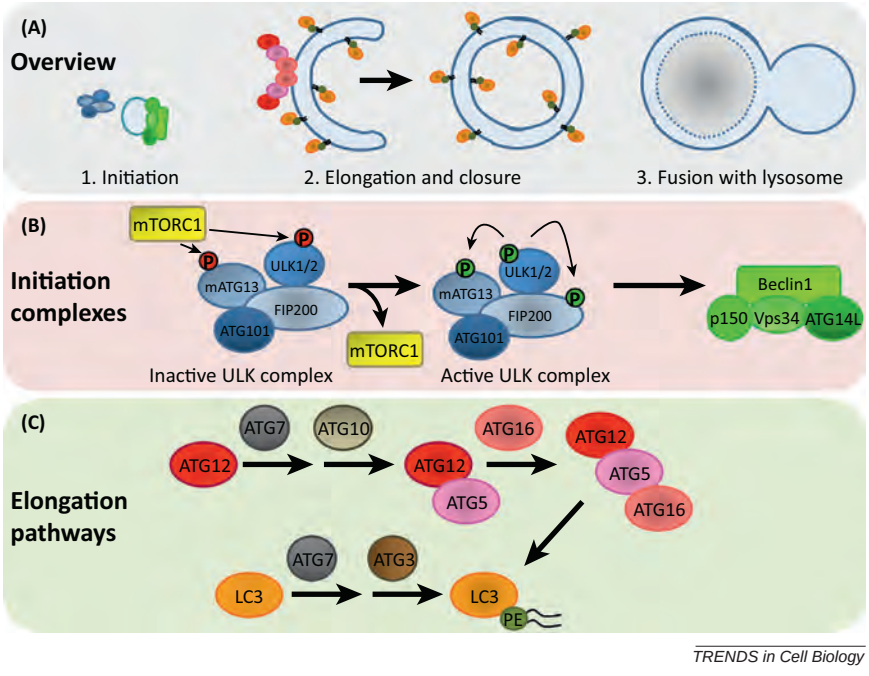

Figure I. Autophagosome formation and maturation in mammals. **(A)** Overview. **(B)** The ULK and PI3K complexes initiate autophagosome formation. **(C)** Ubiquitin-like conjugation pathways promote elongation of the autophagosome membrane.

fibroblasts (MEFs) transformed with oncogenic HRas and MDA-MB-231 human breast carcinoma cells, which harbor oncogenic KRas, genetic autophagy inhibition reduced anchorage-independent transformation, slowed proliferation, and decreased glycolysis [20]. Similar results were obtained in a transgenic model of breast tumorigenesis driven by the polyoma middle T (PyMT) oncogene; deletion of *FIP200*, which is essential for autophagy initiation, impaired glycolysis in these tumor cells *in vitro* and reduced mammary tumorigenesis *in vivo* [21]. An additional requirement for autophagy in cancer cell metabolism was subsequently shown using HRas-transformed immortalized baby mouse kidney (iBMK) cells and pancreatic ductal adenocarcinoma (PDAC) cell lines with activated Ras [22,23]. Remarkably, these studies described an increase in autophagy with oncogenic activation of Ras, suggesting that sustained autophagy allows Ras-transformed tumor cells to meet their high metabolic demands. Accordingly, inhibiting autophagy in these models led to multiple defects in mitochondrial metabolism, including decreased production of tricarboxylic acid (TCA) cycle intermediates, reduced mitochondrial respiration, and diminished ATP production. Although these various studies of Ras transformation uncovered different requirements for autophagy during glycolysis versus mitochondrial metabolism, collectively they demonstrated that autophagy is important for

Table 1. Effects of *atg* deletion on cancer progression and metabolism in GEMMs of cancer

GEMM		*Atg* deletion[a]	Phenotype upon autophagy inhibition		Refs
Cancer type	Genotype		Tumor progression[b]	Tumor cell metabolism[c]	
Mammary carcinoma	*MMTV-PyMT*	*FIP200* (MMTV-Cre)	Decreased initiation and progression	Impaired glycolysis	[21]
Non-small cell lung cancer	*lox-stop-lox-Kras^{G12D}; Tp53$^{flox/flox}$*	*Atg7* (intranasal adenoviral Cre)	Decreased progression, oncocytoma formation	Impaired mitochondrial metabolism and fatty acid oxidation, lipid accumulation	[24]
Non-small cell lung cancer	*lox-stop-lox-Kras^{G12D}*	*Atg5* (intranasal adenoviral Cre)	Increased initiation, decreased progression, oncocytoma formation	Impaired mitochondrial metabolism	[15]
Non-small cell lung cancer	*frt-stop-frt-Kras^{G12D}; Tp53$^{frt/frt}$*	*Atg7* (Ubc-CreERT2)	Decreased progression, oncocytoma formation	Lipid accumulation	[25]
Non-small cell lung cancer	*Braf^{V600E}; Tp53$^{flox/flox}$*	*Atg7* (intranasal adenoviral Cre)	Increased initiation, decreased progression, oncocytoma formation	Impaired mitochondrial glutamine metabolism	[18]
Pancreatic cancer	*lox-stop-lox-Kras^{G12D}; Pdx-cre*	*Atg5* or *Atg7* (Pdx-Cre)	Increased initiation, decreased progression	ND	[16]
Pancreatic cancer	*lox-stop-lox-Kras^{G12D}; Tp53$^{flox/flox}$; Pdx-cre*	*Atg5* or *Atg7* (Pdx-Cre)	Increased progression	Increased glycolysis	[16]
Pancreatic cancer	*lox-stop-lox-Kras^{G12D}; Tp53$^{flox/+}$; Pdx-cre*	*Atg5* (Pdx-Cre)	Increased initiation, decreased progression	ND	[17]

[a]The method of cre recombinase-mediated deletion is indicated in parentheses. MMTV-Cre expression is mammary epithelial cell specific, Ubc-CreERT2 expression is ubiquitous and tamoxifen-inducible, and Pdx-Cre is exocrine and endocrine pancreatic specific.

[b]Effects on tumor initiation are related to tumor onset and development of early-stage tumors. Effects on progression are related to advanced tumorigenesis.

[c]ND indicates that the metabolic phenotype was not determined in the context of *atg* deletion.

supporting the diverse metabolic demands of different tumor types.

The role of autophagy in sustaining Ras-regulated metabolism has also been explored in lung and pancreatic cancer GEMMs driven by oncogenic KRas or the Ras effector Braf (Table 1). Deletion of *atg5* or *atg7* in an oncogenic KRas-induced lung cancer model led to diminished overall lung tumor burden; notably, autophagy-deficient tumors exhibited oncocytic differentiation, marked by the accumulation of abnormal mitochondria within tumor cells [15,24,25]. In the absence of the tumor suppressor p53, this loss of mitochondrial homeostasis resulted in defective fatty acid oxidation and, consequently, impaired lipid metabolism when *atg7* was deleted [24]. Based on these results, the authors concluded that reduced lipid catabolism compromises the ability of autophagy-deficient tumor cells to cope with nutrient deprivation. In a Braf-driven lung cancer model, advanced tumor progression was similarly reduced by *atg7* deletion [18]. Autophagy-deficient cell lines derived from these tumors harbored aberrant mitochondria and addition of the metabolite glutamine rescued defects in mitochondrial metabolism, suggesting that autophagy-inhibited tumor cells exhibit slowed growth due to increased metabolic stress associated with a lack of intermediates that drive mitochondrial metabolic pathways. Defects in lipolysis were not observed here as in the KRas lung model, but these studies nonetheless corroborated the importance of autophagy in regulating metabolic homeostasis by broadly controlling proper mitochondrial function. Furthermore, autophagy inhibition also reduced growth and survival of central nervous system tumor cells with activated Braf. While the impact of autophagy on metabolism was not investigated here, the results obtained in the Braf lung model suggest an underlying mechanism by which autophagy may impact Braf-driven growth across multiple tumor types [26].

Studies of pancreatic cancer GEMMs driven by mutant KRas have revealed a seemingly complex and varying role for autophagy in controlling tumor cell metabolism. In the context of embryonic *p53* deletion in the pancreas, genetic and pharmacological inhibition of autophagy actually accelerates pancreatic tumor progression [16]. Cells isolated from these tumors lacking both ATG7 and p53 exhibited increased rates of glycolysis and increased levels of metabolites in the pentose phosphate pathway, a key side branch of glucose metabolism that facilitates tumor growth. By contrast, when *p53* inactivation occurred by somatic loss of heterozygosity (LOH), autophagy inhibition resulted in impaired PDAC progression, and pharmacological targeting of autophagy using the lysosomal inhibitor chloroquine led to defects in mitochondrial respiration across a panel of human PDAC cell lines, regardless of p53 status [17].

Much remains to be learned with regard to the precise mechanisms through which autophagy controls metabolism. While specific enzymes involved in glycolysis have been shown to regulate autophagy, no such regulation by autophagy on particular steps of the glycolytic pathway has been uncovered [27,28]. Additionally, although accumulation of abnormal mitochondria due to decreased mitophagy may explain the defects associated with mitochondrial metabolism upon autophagy inhibition, impaired mitophagy was not observed in autophagy-deficient PDAC cell lines that exhibited diminished oxidative phosphorylation [23]. This discrepancy suggests that there are mitophagy-independent pathways through which autophagy controls mitochondrial metabolism and that engagement of these various regulatory mechanisms may be context-dependent.

Moreover, in most studies, these metabolic defects have been characterized using tumor cell lines in culture. While this method certainly facilitates a detailed analysis of metabolic parameters, it may not accurately recapitulate the metabolic state of tumors *in vivo*. *In vivo* application of established NMR-based technologies to assay glycolysis during tumor formation or use of methods to measure metabolism in freshly isolated mitochondria from tumors will provide further insight into the role of autophagy in cancer metabolism [29,30].

Autophagy and the unfolded protein response in cancer

In addition to being critical for metabolic adaptation, autophagy has other functions in helping tumors cope with oncogene, environmental, and therapy-induced stresses, particularly during induction of the unfolded protein response (UPR) (Figure 1). The UPR is a cytoprotective pathway that alleviates stress associated with accumulation of misfolded proteins in the endoplasmic reticulum (ER) [31]. It is regulated by three sensors, including protein kinase RNA-like ER kinase (PERK), which phosphorylates the translation regulatory protein eukaryotic initiation factor 2α (eIF2α), ultimately leading to a block in translation to prevent further accumulation of unfolded proteins. Recently, oncogenic activation of c-Myc, which promotes increased translation, was shown to activate the UPR to accommodate this increase in protein synthesis [32]. Knockout of PERK led to cell death in the context of activated c-Myc, and this cytoprotective function was due to PERK-mediated activation of autophagy. Similar results were obtained in a *Drosophila* model of Myc overexpression in which induction of the UPR and PERK led to an autophagy-dependent increase in cell growth [33].

While the mechanism of autophagy induction by the UPR during Myc transformation remains unclear, hypoxia can also induce UPR-dependent upregulation of autophagy downstream of PERK via transcriptional mechanisms [34,35]. During hypoxia-induced UPR, increased expression of the transcription factors activating transcription factor 4 (ATF4) and CCAAT-enhancer-binding protein homologous protein (CHOP) leads to enhanced expression of unc-51-like kinase 1 (*ULK1*), which is required for initiation of autophagy, and microtubule-associated proteins 1A/1B light chain 3B (*MAP1LC3B*) and *ATG5*, which are both essential for autophagosome formation. Increased expression of essential ATGs downstream of the UPR is also seen during other stresses, such as extracellular matrix (ECM) detachment, suggesting transcriptional upregulation of ATGs may be a general route of autophagy upregulation by the UPR [36]. Moreover, because hypoxia occurs in multiple tumor types, this mechanism of cytoprotective autophagy induction may be common across many cancers. Similarly, inhibition of Braf by targeted therapy in melanoma activates autophagy downstream of PERK, and this induction mediates resistance of tumor cells to Braf inhibitors [37]. Thus, like hypoxia, activation of autophagy by therapy-induced UPR and PERK may be another route of cytoprotective autophagy induction in various cancers.

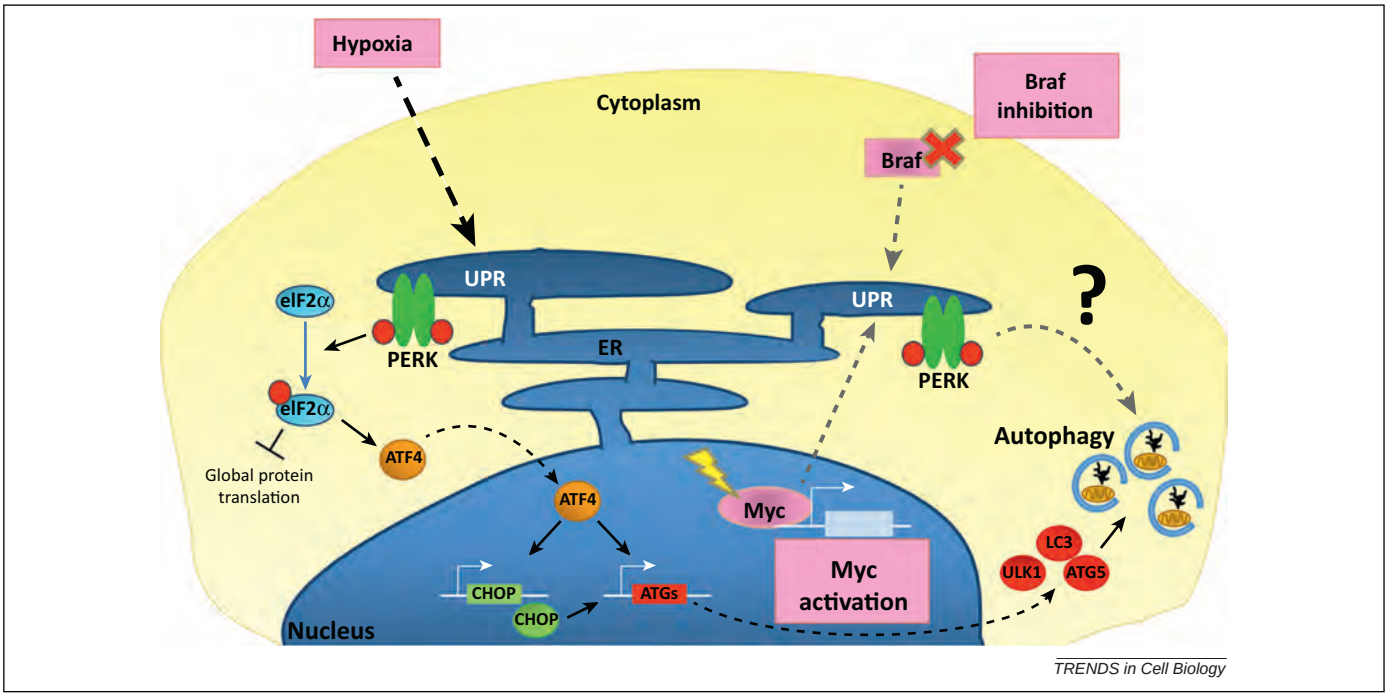

Figure 1. Unfolded protein response (UPR)-mediated induction of cytoprotective autophagy supports tumor cell survival and growth. Multiple stresses promote the UPR in tumor cells, such as hypoxia, activation of the oncogenic transcription factor Myc, and inhibition of the growth-promoting kinase Braf. In response to these stimuli, UPR-dependent activation of the protein kinase RNA-like endoplasmic reticulum (ER) kinase (PERK) has been most strongly implicated in the induction of autophagy as a cytoprotective pathway. Hypoxia activates PERK, which phosphorylates eukaryotic initiation factor 2α (eIF2α) to suppress global translation and to selectively induce activating transcription factor 4 (ATF4). ATF4 is a transcription factor that promotes expression of the transcription factor CCAAT-enhancer-binding protein homologous protein (CHOP). Together, ATF4 and CHOP drive expression of multiple core autophagy machinery genes, including unc-51-like kinase 1 (ULK1), microtubule-associated proteins 1A/1B light chain 3B (MAP1LC3B; LC3), and autophagy-related gene 5 (ATG5), which collectively promote autophagosome formation (Box 1). Although the mechanisms by which PERK induces autophagy during Myc activation and Braf inhibition have not been determined, activation of ATF4 and CHOP downstream of PERK may similarly induce the transcription of core autophagy regulators.

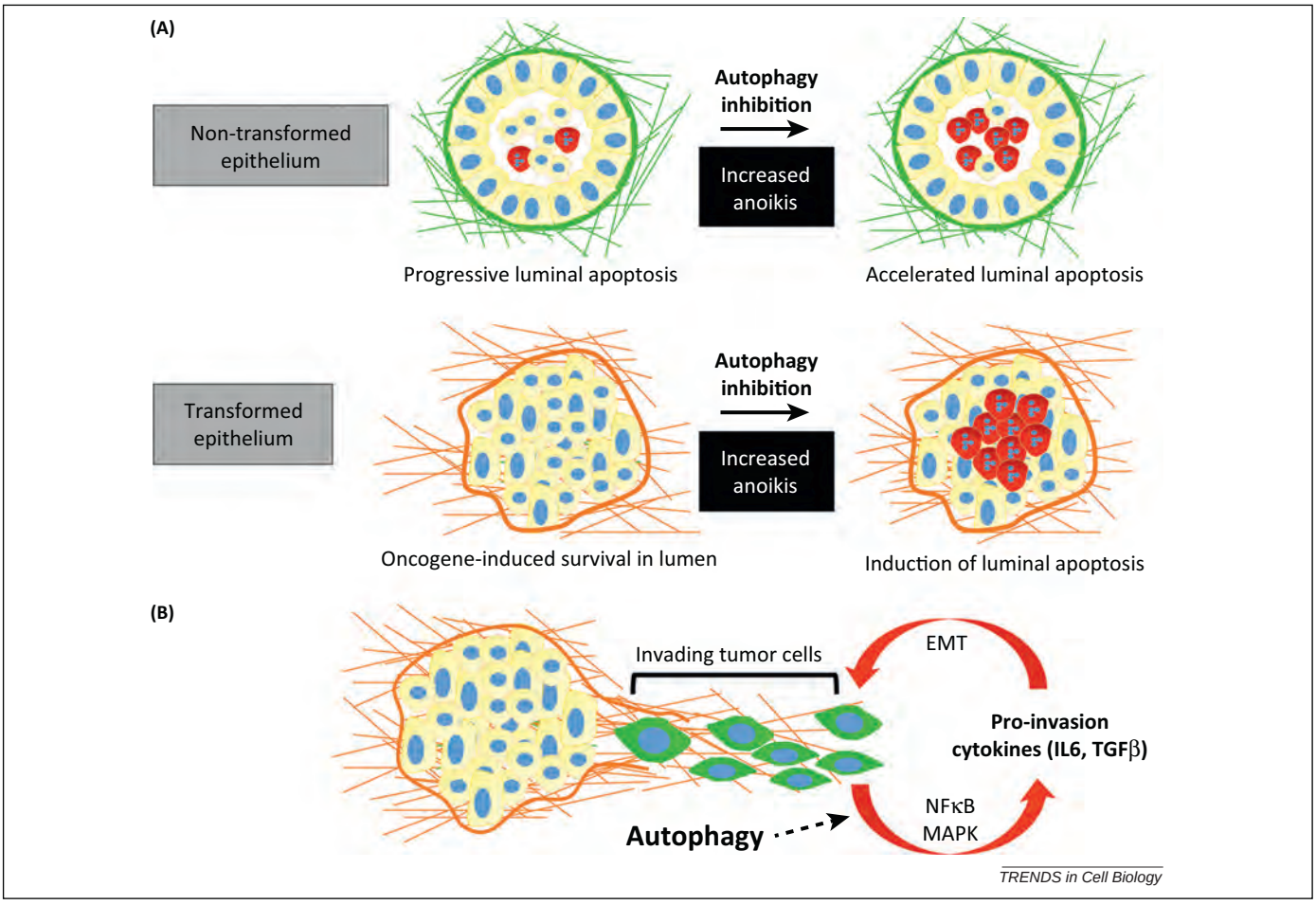

Figure 2. Autophagy promotes anoikis resistance and tumor cell invasion. **(A)** 3D culture of MCF10A cells leads to the formation of acini with hollow lumens. Luminal clearance occurs through anoikis of central cells (depicted in red) lacking extracellular matrix (ECM) contact. Oncogene activation protects luminal cells from anoikis, leading to the formation of structures with filled lumens. Autophagy promotes the survival of both normal and transformed epithelial cells deprived of ECM contact; therefore, inhibiting autophagy leads to increased anoikis. **(B)** Autophagy promotes tumor cell invasion by facilitating the secretion of multiple pro-invasive cytokines. Activation of the nuclear factor of kappa light polypeptide gene enhancer in B cells (NFκB) and mitogen-activated protein kinase (MAPK) pathways by autophagy has been shown to contribute to the increased production of these secreted factors. In turn, these cytokines may augment a pro-invasive gene signature program through the induction of epithelial-to-mesenchymal transition (EMT).

While autophagy may support tumor cells during hypoxia by diverse mechanisms, one can speculate that the ability of autophagy to facilitate glycolysis and supply metabolites, as has been observed during Ras transformation, may also be critical for metabolic adaption to oxygen deprivation. A similar requirement for autophagy during Myc-induced metabolic changes may also exist, since Myc transformation has been associated with enhanced glycolysis and glutamine metabolism [38]. Overall, an important outcome of UPR-mediated activation of autophagy may be to sustain tumor cell metabolism. Future studies interrogating connections between the various requirements for autophagy during diverse stresses and in the context of different oncogenes may uncover conserved mechanisms for control of tumorigenesis by autophagy.

Regulation of cellular invasion and metastasis by autophagy

Metastasis, the process by which tumor cells spread to foreign sites throughout the body, involves phenotypic changes that allow tumor cells to gain entry into and out of the vasculature and to survive stresses associated with traversing the circulation and growing in a foreign microenvironment [39,40]. In many cancer patients, metastasis is the primary cause of mortality, primarily because limited treatments for metastatic disease exist. Interestingly, autophagy impacts cell biological phenotypes that regulate metastasis, such as resistance to anoikis and invasion (Figure 2). Resistance to anoikis allows cells to survive stress associated with ECM detachment, which may occur while tumor cells are in the circulation or at the metastatic site where they cannot fully engage the foreign ECM [41]. Invasion allows tumor cells to access the vasculature for dissemination and to exit the circulation at metastatic sites [42,43].

Autophagy was first shown to promote the survival of non-transformed mammary epithelial cells during ECM detachment; subsequent studies revealed that detachment-induced autophagy is critical for adhesion-independent transformation [20,44]. Multiple Ras-transformed human cancer cell lines upregulate autophagy upon detachment, and autophagy inhibition compromises adhesion-independent growth and survival of cells harboring activated Ras. Similarly, when oncogenic phosphatidylinositide 3-kinase (PI3K)-transformed MCF10A cells were grown in 3D culture, autophagy inhibition led to increased

apoptosis of luminal cells deprived of ECM contact [45]. Recently, this requirement for autophagy during anoikis resistance was shown to be necessary for metastasis of hepatocellular carcinoma (HCC) cells [46]. Autophagy inhibition attenuated pulmonary metastasis of HCC cells following orthotopic transplantation into nude mice, and this defect correlated with increased anoikis of autophagy-deficient HCC cells.

Autophagy has also emerged as a regulator of cellular invasion and migration. In an organotypic model of invasion through a collagen matrix, knockdown of the essential autophagy regulator, ATG12, decreased invasive capacity of glioma cells [47]. Although this study did not delineate the mechanism of autophagy-mediated invasion, other studies have demonstrated multiple routes by which autophagy controls invasion. For example, in glioblastoma (GBM) stem cells, autophagy inhibition or knockdown of the autophagy regulator p62 decreased invasion and migration *in vitro* and led to metabolic defects [48]. Based on previous evidence indicating that glycolysis is important for GBM invasion, the authors proposed a model in which p62-dependent autophagy impacts metabolism to control invasion [49].

Further roles for autophagy in regulating invasion have been uncovered in other models. Invasion of HCC cells during starvation was shown to be autophagy-dependent, due to the ability of autophagy to stimulate transforming growth factor beta (TGFβ) and promote epithelial-to-mesenchymal transition (EMT), a well-established transcriptional program that supports metastasis [50]. A similar requirement for autophagy in controlling invasion was observed in Ras-transformed epithelial cells in 3D culture [51]. Autophagy inhibition attenuated invasion and caused a partial reversion of EMT. Additionally, impaired self-eating led to decreased secretion of multiple pro-invasive cytokines, including interleukin-6 (IL6). Notably, decreased invasion upon autophagy inhibition was partly restored with IL6 re-addition, demonstrating a specific need for the autophagy pathway in controlling secretion of this cytokine. Furthermore, autophagy-deficient Ras-transformed cells exhibited reduced pulmonary metastases. Overall, these findings uncovered a new role for autophagy during cancer cell invasion by promoting secretion and suggested that autophagy-dependent secretion may be important for metastasis *in vivo*. An additional report showed that induction of autophagy by toll-like receptors (TLRs) promotes secretion of pro-invasive factors, including IL6, in lung cancer cells, further corroborating a role for autophagy as a determinant of pro-invasive secretion [52].

The mechanism by which autophagy controls secretion during invasion remains poorly defined. Although these phenotypes may be secondary to autophagic turnover of secretory regulators, autophagy has been directly implicated in promoting both conventional and unconventional secretion in other contexts [53]. During TLR-mediated invasion, autophagy upregulates signaling pathways that promote secretion, such as nuclear factor of kappa light polypeptide gene enhancer in B cells (NFκB) and mitogen-activated protein kinase (MAPK), but how autophagy controls these pathways in this model is unclear [52]. Furthermore, IL6 can promote EMT and stimulate TGFβ signaling, which argues that autophagy-dependent secretion may also be important for HCC cell invasion [54].

Lastly, in contrast to the studies above, autophagy has been described as a suppressor of metastasis by preventing p62-dependent stabilization of the EMT-promoting transcription factor Twist1 [55]. Because EMT is regulated by multiple signaling pathways and transcription factors, these varying roles of autophagy in regulating EMT and invasion point to a complex relationship between self-eating and metastasis [56]. Hence, additional work is needed to establish the cellular functions for autophagy during *in vivo* cancer invasion and metastasis.

Regulation of tumorigenesis by autophagy cargo receptors

Autophagy cargo receptors mediate selective degradation of autophagy substrates by binding ubiquitinated targets and recruiting autophagosomes to this cargo. This recruitment generally occurs through interaction of receptors with LC3 via an LC3 interacting region (LIR), but the recent identification of the ferritin receptor nuclear receptor coactivator 4 (NCOA4), which lacks a canonical LIR, points to additional mechanisms by which cargo receptors interact with autophagosomes [57,58]. Importantly, because these receptors are themselves degraded during selective autophagy, inhibition of autophagy promotes their accumulation and results in aberrant regulation of their downstream pathways. This may have crucial implications for tumorigenesis, as illustrated by studies demonstrating tumor-promoting functions for the archetypal autophagy cargo receptor p62/sequestosome 1 (SQSTM1) [59,60].

p62/SQSTM1 is a versatile, multidomain adapter that regulates several signaling pathways to promote tumorigenesis [59,60] (Figure 3). Among these, NFκB-mediated control of proinflammatory signaling and regulation of the antioxidant response by nuclear factor erythroid 2-related factor 2 (Nrf2) have been most strongly linked to tumorigenesis in the context of autophagy inhibition. Through interaction with tumor necrosis factor (TNF) receptor associated factor (TRAF6), p62 promotes NFκB signaling, and this p62-mediated activation of NFκB is required for Ras-induced lung and pancreatic tumorigenesis [60–62]. Indeed, *atg5* knockout or overexpression of p62 in tumorigenic iBMK cells enhances tumor growth by modulating NFκB signaling [63]. Additionally, p62 regulates tumorigenesis of transformed MEFs during autophagy inhibition resulting from knockout of *FIP200* [64]. When p62 is depleted in *FIP200* null tumor cells, tumor growth is inhibited. Conversely, when p62 is re-expressed in *p62* knockout cells also deleted for *FIP200*, tumor growth is enhanced and NFκB signaling is upregulated. Similarly, regulation of the transcription factor Nrf2 by p62 is also important for tumorigenesis. p62 inhibits degradation of Nrf2, a key regulator of the oxidative stress response, by binding to Kelch-like erythroid-cell-derived protein with CNC homology (ECH)-associated protein 1 (Keap1), an adaptor for the E3 ubiquitin ligase that promotes Nrf2 degradation [65]. The ability of p62 to support tumor growth by activating Nrf2 is crucial for the spontaneous development of liver tumors due to *atg5* or *atg7* knockout [13,14,66].

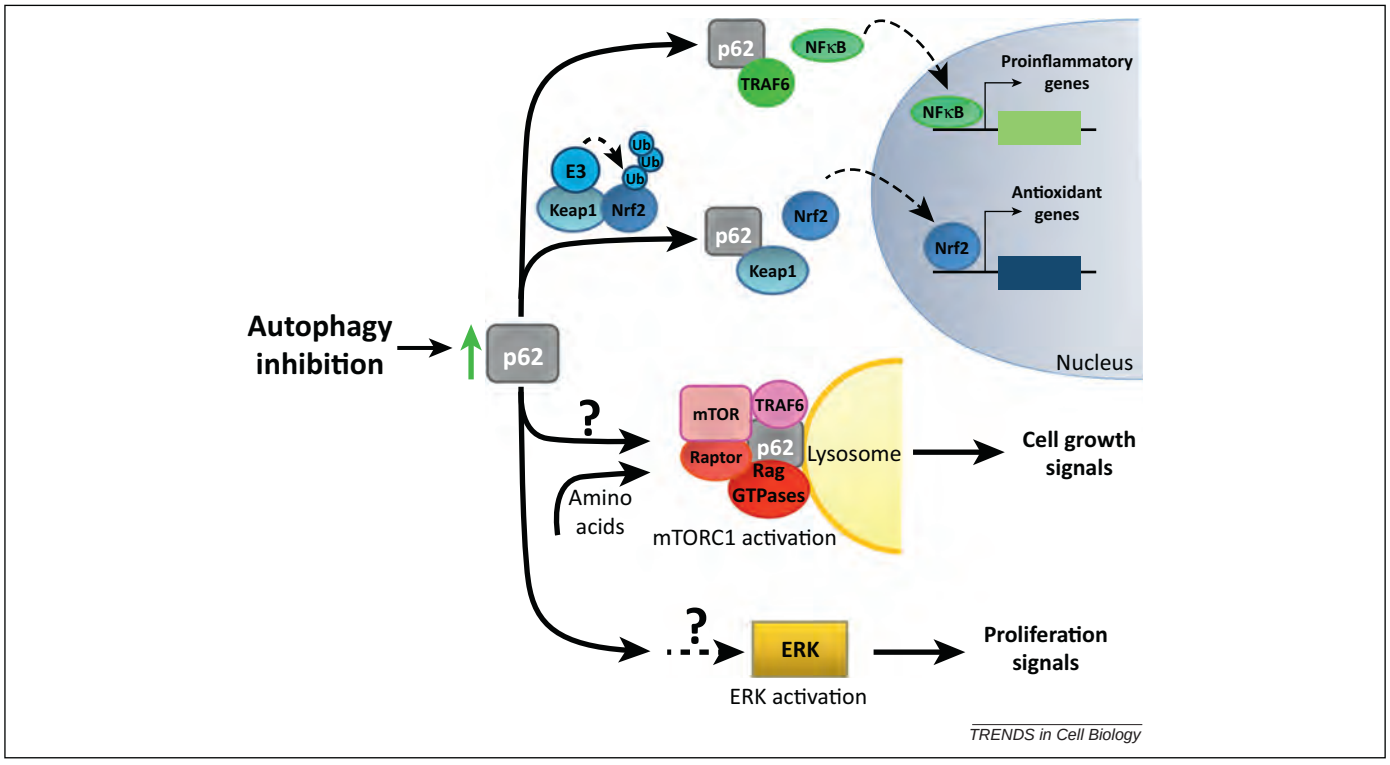

Figure 3. p62/sequestosome 1 (SQSTM1) activates multiple signaling pathways that support tumorigenesis. Because p62 accumulates with autophagy inhibition, impairment of autophagy has been correlated with increased activation of multiple pathways and with enhanced tumor cell growth. First, autophagy-related gene (*Atg*) deletion and p62 have been shown to promote nuclear factor of kappa light polypeptide gene enhancer in B cells (NFκB) activity. p62 promotes NFκB function by interaction with tumor necrosis factor (TNF) receptor associated factor (TRAF6). The increased expression of proinflammatory genes by NFκB supports tumorigenesis. Second, *Atg* deletion promotes nuclear factor erythroid 2-related factor 2 (Nrf2) stability and activity by increasing levels of p62, which interacts with and sequesters Kelch-like erythroid-cell-derived protein with CNC homology (ECH)-associated protein 1 (Keap1). Keap1 is an adaptor for the E3-ubiquitin ligase that promotes Nrf2 ubiquitination and proteosomal degradation. Nrf2-mediated expression of antioxidants promotes tumor growth. Furthermore, in response to nutrient signaling, p62 complexes with regulators of mammalian target of rapamycin (mTOR) to activate mTOR complex 1 (mTORC1) at the lysosome and promote cell growth. The impact of p62 on mTORC1 pathway activation during autophagy inhibition has not been established. Finally, RNAi-mediated depletion of ATGs or p62 overexpression enhances extracellular signal-regulated kinase (ERK) signaling to enable the proliferation of transformed cells. The mechanism underlying this increase in ERK activity is not understood.

p62 also controls additional pro-tumorigenic pathways, including those regulated by mammalian target of rapamycin complex 1 (mTORC1) and extracellular signal-regulated kinase (ERK). mTORC1 critically regulates tumor cell growth, and p62 activates mTORC1 by potentiating its ability to complex with Rag GTPases and TRAF6 and by facilitating its recruitment to lysosomes; this regulation of mTORC1 by p62 supports tumor growth *in vivo* and cell proliferation *in vitro* [67,68]. Autophagy inhibition or p62 overexpression can also enhance the growth of PI3K-transformed MCF10A cells in 3D culture [45]. In this model, p62-induced proliferation correlates with activation of mitogenic ERK signaling. These studies collectively point to a pro-tumorigenic function for p62 and highlight the varied regulatory roles of p62 during tumorigenesis.

In addition to p62, other cargo receptors, such as nuclear dot protein 52 kDa (NDP52), optineurin (OPTN), and neighbor of BRCA1 gene 1 (NBR1), mediate selective autophagy [57]. Similar to p62, NDP52 has been implicated in regulation of NFκB signaling in lung cancer cells [69]. Although this regulation was proposed to occur through NDP52-mediated selective autophagy, the precise mechanism is unknown. In lung cancer cells, the ubiquitylation of OPTN increases autophagy, promotes the degradation of p62 and damaged proteins, and suppresses lung tumorigenesis in nude mice [70]. Moreover, OPTN can inhibit NFκB signaling, but how this regulation affects tumor development has not been investigated [71]. While formal evidence for NBR1 in mediating tumor progression is lacking, it is noteworthy that NBR1 can support Nrf2 antioxidant signaling to potentially impact cancer [72]. Additionally, NBR1 regulates selective autophagic clearance of midbodies that form during cell division, and midbody accumulation contributes to increased growth of tumor cells *in vitro* [73]. Overall, further establishing the contribution of these selective autophagy regulators to cancer progression remains an important and exciting topic for future study.

Concluding remarks

Given the diversity of tumor types and the numerous oncogenic drivers involved in cancer, the role of autophagy during cancer progression will likely continue to remain complex and intensely debated. Thus, going forward it will be imperative to use appropriate models of disease to accurately determine the clinical contexts in which autophagy inhibition or activation should be considered therapeutically. Moreover, the controversial and context-dependent role of autophagy during initiation and advanced progression of tumors emphasizes the need for a comprehensive understanding of the cell biological processes regulating autophagy during tumorigenesis.

Certainly, the highly dynamic nature of autophagy and its regulation by many evolutionarily conserved genes and diverse signaling pathways suggests there are countless avenues by which cancer cells can modulate the pathway to their benefit.

Although research to date has identified many stress pathways in tumor cells that induce autophagy, better elucidating the relationship between autophagy and these pathways in cancer GEMMs remains an important goal. For example, understanding if there is a defined repertoire and coordination of stress-induced pathways that drive autophagy in particular tumor types may reveal unexpected routes for therapeutic modulation of autophagy in a tumor-specific manner. Such knowledge may also lead to development of therapeutic alternatives to hydroxychloroquine (HCQ)-mediated lysosomal inhibition, which is currently the major clinical option for inhibiting autophagy in patients [74–81]. Furthermore, autophagy has traditionally been viewed as a pathway that promotes tumor cell survival during stress; nevertheless, the aforementioned studies of invasion, secretion, and EMT have begun to illuminate new functions for autophagy in tumor cells. As these novel roles for autophagy during tumorigenesis continue to emerge, understanding the mechanisms by which self-eating controls these processes may expose opportunities for specific targeting of autophagy-dependent phenotypes in tumor cells.

Finally, this review specifically focuses on the functions of autophagy in tumor cells. However, because the effects of pharmacological inhibitors like HCQ are not confined to cancer cells, their long-term use may be limited by adverse effects associated with autophagy and lysosomal inhibition in normal cells. Indeed, a recent study of inducible systemic *atg7* deletion in adult mice demonstrates that acute autophagy ablation elicits the rapid regression of KRas lung tumors; however, extended periods of autophagy deficiency causes the deterioration of multiple tissues and lethal neurodegeneration [25]. To identify new strategies that selectively target autophagy in cancer cells without harming normal tissue, we must continue to define the cellular and metabolic functions of autophagy in both normal and tumor cells.

Acknowledgments

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CELL DEATH AND AUTOPHAGY

Autophagy at the crossroads of catabolism and anabolism

Jasvinder Kaur and Jayanta Debnath

Abstract | Autophagy is a conserved catabolic process that degrades cytoplasmic constituents and organelles in the lysosome. Starvation-induced protein degradation is a salient feature of autophagy but recent progress has illuminated how autophagy, during both starvation and nutrient-replete conditions, can mobilize diverse cellular energy and nutrient stores such as lipids, carbohydrates and iron. Processes such as lipophagy, glycophagy and ferritinophagy enable cells to salvage key metabolites to sustain and facilitate core anabolic functions. Here, we discuss the established and emerging roles of autophagy in fuelling biosynthetic capacity and in promoting metabolic and nutrient homeostasis.

Ubiquitin–proteasome system
(UPS). The cellular quality control pathway that tags and degrades unwanted or superfluous proteins.

Basal autophagy
A constitutive autophagic degradation process that proceeds in the absence of any overt stress or stimulus and serves important housekeeping roles.

Autophagosomes
Double membrane-bound vesicles that sequester cytoplasmic materials and target them for lysosomal degradation during macroautophagy.

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Autophagy, the process of cellular self-eating, has long been recognized as an important protein degradation pathway, particularly during starvation or stress. In contrast to the ubiquitin–proteasome system (UPS), which targets individual short-lived proteins, autophagy functions as a bulk process with the capacity to degrade long-lived proteins and organelles such as the endoplasmic reticulum, mitochondria, peroxisomes, the nucleus and ribosomes[1,2]. Evidence indicates that autophagic degradation promotes the recycling and salvage of cellular nutrients, thereby enabling cell survival during starvation. Autophagy, along with the UPS, is also a key mechanism for protein homeostasis and quality control. Indeed, basal autophagy within cells is important for the degradation of damaged and dysfunctional proteins and organelles; autophagy-deficient mice exhibit a build-up of misfolded, damaged proteins[3–5] (BOX 1).

Although protein degradation is a salient feature of autophagy, studies over the past decade have revealed that autophagy plays a key part in mobilizing diverse cellular energy and nutrient stores, including carbohydrates, lipids and minerals. Hence, a growing appreciation of the role of autophagy in controlling cellular metabolism in both normal and diseased cells has fuelled immense interest in elucidating how dysfunctional autophagy influences metabolic disorders and metabolic adaptation in diseases such as cancer. Here, we provide a brief overview of the autophagic process before reviewing the established and emerging roles of the catabolism of proteins, lipids (lipophagy), carbohydrates (glycophagy) and iron (ferritinophagy) in fuelling energy and nutrient stores. We also delineate how autophagy facilitates the adaptive metabolic response and supports anabolic pathways within cells.

Overview of the autophagic pathway

Autophagy refers to a collection of tightly regulated catabolic processes, all of which deliver cytoplasmic components to the lysosome for degradation, and that are broadly classified into three types: macroautophagy, microautophagy and chaperone-mediated autophagy (FIG. 1a). Macroautophagy involves the formation of double membrane-bound vesicles called autophagosomes that engulf cytoplasmic proteins and organelles; these autophagosomes are trafficked to lysosomes, at which point the sequestered cargo is degraded[6]. Microautophagy refers to the invagination of the lysosomal or endosomal membrane, resulting in the direct engulfment of substrates that are subsequently degraded by lysosomal proteases[7]. Chaperone-mediated autophagy is distinct from macroautophagy and microautophagy because cargo is not sequestered within a membrane delimited vesicle. Instead, proteins targeted by chaperone-mediated autophagy contain a KFERQ-like pentapetide motif that is recognized by the cytosolic chaperone heat shock cognate 70 kDa protein (HSC70); HSC70 promotes the translocation of these targets across lysosomal membranes into the lysosomal lumen via the lysosomal-associated membrane protein 2A (LAMP2A) receptor[8]. This Review focuses on macroautophagy, which we hereafter call autophagy. Although the molecular regulation of autophagy remains an active area of research, immense progress over the past decade has been made in two areas: our understanding of the biogenesis of the autophagosomal membrane and of the molecular control of selectivity. This section provides a brief overview of these two topics; more detailed reviews on the molecular regulation of autophagy are found elsewhere[2,9,10].

Box 1 | **Autophagy and protein quality control**

In addition to its importance in protein catabolism during starvation, basal autophagy is now recognized as a critical housekeeping pathway even in nutrient-rich conditions. This quality control mechanism is particularly crucial in postmitotic tissues, such as nerve and muscle, where autophagy is important for the removal of aggregated proteins and, therefore, for protecting the cells from the toxic effects of dysfunctional proteins that cannot be diluted via cell division[113]. Loss of autophagy in neurons or cardiac muscles can result in the accumulation of ubiquitylated proteins and inclusion bodies, leading to neurodegeneration and cardiac hypertrophy, respectively[114,115]. Constitutive autophagy in non-stressed conditions is therefore critical for the turnover of intracellular proteins and for maintaining cell homeostasis.

In serving these functions, autophagy cooperates with another key protein degradation pathway, the ubiquitin–proteasome system. Robust activation of autophagy has been observed upon the pharmacological inhibition of the proteasome or the genetic ablation of proteasome components[116,117]. In these situations, autophagy is proposed to remove oligomeric protein aggregates that are potentially deleterious to cells. Autophagy is also important for the replenishment of intracellular amino acid pools in response to proteasome inhibition[118], which is consistent with findings showing that proteasomes are critical for protein synthesis as they maintain amino acid levels[119]. Alternatively, proteasome activation can suppress autophagy by increasing amino acid levels and constitutively activating mammalian TOR complex 1 (mTORC1)[120].

Finally, autophagy has a pro-survival role during endoplasmic reticulum (ER) stress as it acts as an alternative mechanism for the clearance of misfolded or damaged proteins that cannot be cleared by the unfolded protein response. Autophagy selectively degrades aggregated proteins that accumulate in the ER lumen, such as the mutant secretory protein α1-antitrypsin[121]. Additionally, studies of yeast and mammalian cells demonstrate that autophagy mediates ER homeostasis by selectively segregating portions of this organelle network; this process has been termed ER-phagy or reticulophagy[122–125].

Autophagosome biogenesis. The earliest step of autophagy is characterized ultrastructurally by the sequestration of portions of cytoplasm into a double membrane-bound vesicle called the autophagosome. The autophagosome subsequently fuses with a lysosome, leading to the degradation of the sequestered cytosolic proteins and organelles[9]. Studies in yeast have identified more than 30 autophagy-related proteins (ATGs), many of which have identified mammalian orthologues[11,12]. As depicted in FIG. 1a, autophagosome formation comprises three main steps: initiation, nucleation and expansion of the isolation membrane. The intricate process of autophagosome formation begins at the phagophore assembly site (also known as the isolation membrane) where proteins of the UNC51-like kinase (ULK) complex (which is composed of ULK1 or ULK2 and ATG13, FAK family kinase interacting protein of 200 kDa (FIP200) and ATG101) assemble to initiate autophagosome formation[9]. Next, in the nucleation stage, the activated ULK complex targets a class III PI3K complex — consisting of beclin 1 (Atg6 in yeast), vacuolar protein sorting 15 (VPS15), VPS34 and ATG14 — to promote the local production of a pool of phosphatidylinositol 3-phosphate that is specific to autophagosomes. Interestingly, a recent study revealed that ATG14 also promotes the fusion of autophagosomes with the endolysosomal compartment[13]. Finally, in the expansion stage, the ATG12–ATG5–ATG16 complex is recruited to the autophagosome membrane where it facilitates the lipidation of microtubule-associated protein 1 light chain 3 (MAP1LC3; also known as LC3) with phosphatidylethanolamine; LC3 is the chief mammalian homologue of yeast Atg8, which is required for the expansion of the isolation membrane. Recent research indicates that the deacetylation and cytosolic translocation of a nuclear pool of LC3 is required for its lipidation with phosphatidylethanolamine during starvation-induced autophagy[14].

In contrast to yeast, in which the initial phagophore assembly site is likely to be assembled *de novo*[15,16], the origin of membranes contributing to autophagosome formation in mammalian cells has been intensely debated and scrutinized. Although evidence supports the idea that nucleation of the isolation membrane occurs at a distinct site emanating from the endoplasmic reticulum (ER), termed the omegasome[17], other sources of membrane contribute to autophagosome formation, including ER–Golgi intermediate compartments, ER–mitochondria junctions, mitochondria, endosomes and the plasma membrane[17–22]. Taken together, these studies highlight the complexities of autophagy initiation in mammals. Given the diversity of stimuli and stresses that can induce autophagy, an important area of future research is determining whether and how these varied membrane sources are utilized for autophagy in response to specific stimuli.

Selective autophagy. Nutrient starvation-induced autophagy was originally believed to non-selectively sequester and degrade cytoplasmic material. However, it is increasingly being appreciated that autophagy is a selective process, resulting in the targeted engulfment of specific cargoes such as mitochondria, peroxisomes and ribosomes, and protein aggregates. Selective autophagy is mediated by autophagy cargo receptors that bind cargo earmarked with degradation signals, most commonly ubiquitin in mammals, through their ubiquitin-binding domain (UBD). These receptors also commonly possess a motif called the LC3 interacting region (LIR), which mediates their binding to Atg8 (LC3 in mammals) isoforms present on newly developing autophagosomes (FIG. 1b). As a result, autophagy cargo receptors act as molecular bridges that capture ubiquitylated proteins targeted for degradation by the autophagy pathway and complement the UPS. For example, the autophagy cargo receptors p62 (also known as SQSTM1), NBR1 (next to *BRCA1* gene 1 protein) and histone deacetylase 6 (HDAC6) all promote the autophagic clearance of protein aggregates in a process known as aggrephagy, which is dependent on both the UBD and LIR[2,10]. In yeast, although orthologues of UBD-containing adaptor proteins such as p62 are missing, a recent mass spectrometric study identified Cue5 as a potential autophagy cargo receptor[23]. Cue5 possesses a coupling of ubiquitin-conjugation to ER degradation (CUE) domain, which is structurally related to mammalian UBDs and is capable of binding to ubiquitin as well as interacting with Atg8. Hence, Cue5 is a ubiquitin–Atg8 adaptor protein that functions analogously to p62 in mammals to mediate the selective autophagic degradation of ubiquitylated targets. Both yeast Cue5 and its mammalian orthologue, Toll interacting protein (TOLLIP), target aggregation-prone proteins that cannot be cleared by the UPS, such as huntingtin, for autophagic degradation[23].

Unfolded protein response
The activation of a stress response in the endoplasmic reticulum due to an increase in misfolded or aggregated proteins.

Autophagy-related proteins
(ATGs). Autophagy regulators.

Autophagy cargo receptors
Adaptor proteins that mediate the targeting of autophagosomes to cargo (for example, mitochondria and protein aggregates), often via ubiquitin and LC3-binding domains.

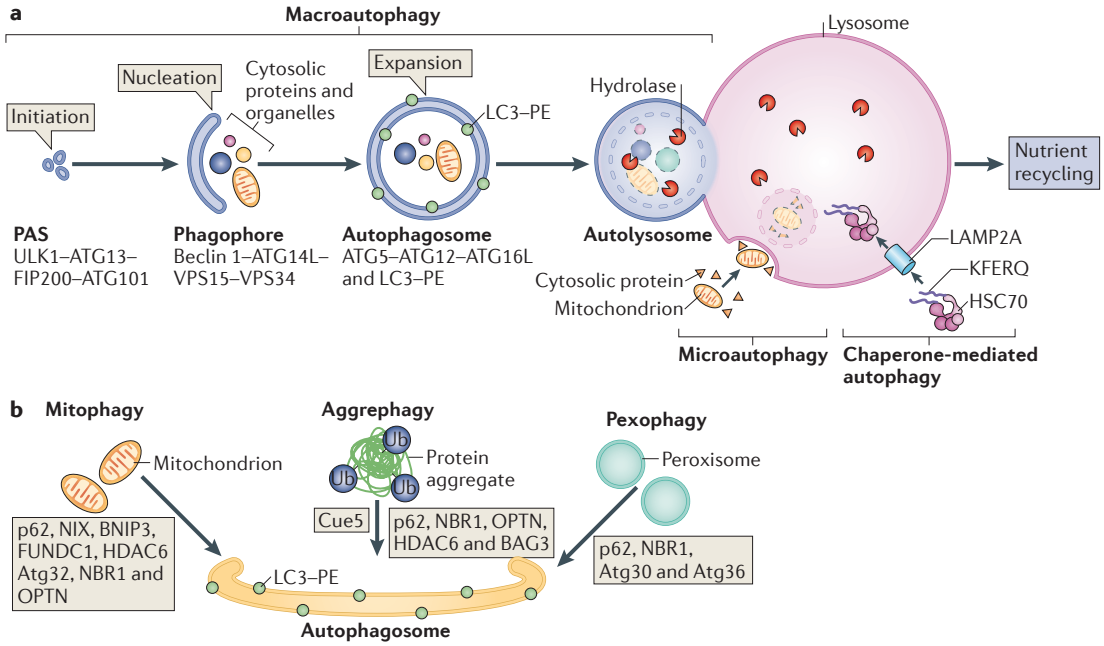

Figure 1 | **Overview of mammalian autophagy pathways. a** | In macroautophagy, initiation begins with the formation of the phagophore assembly site (PAS). This is mediated by the UNC51-like kinase (ULK) complex, which consists of ULK1 (or ULK2), autophagy-related protein 13 (ATG13), FAK family kinase interacting protein of 200 kDa (FIP200) and ATG101. Further nucleation requires the class III PI3K complex, which is composed of the vacuolar protein sorting 34 (VPS34) PI3K, along with its regulatory subunits ATG14L, VPS15 and beclin 1 (Atg6 in yeast). Phagophore membrane elongation and autophagosome completion requires two ubiquitin-like conjugation pathways. The first produces the ATG5–ATG12 conjugate, which forms a multimeric complex with ATG16L, whereas the second results in the conjugation of phosphatidylethanolamine (PE) to LC3 (the microtubule-associated protein 1 light chain 3, a principal mammalian homologue of yeast Atg8). PE-conjugated LC3 (LC3–PE) is required for the expansion of autophagic membranes, their ability to recognize autophagic cargoes and the fusion of autophagosomes with lysosomes. The resulting autophagosome fuses with endocytic and lysosomal compartments, ultimately leading to formation of the autolysosome. In microautophagy, substrates are directly engulfed at the boundary of the lysosomal membrane. In chaperone-mediated autophagy, substrates with the pentapeptide motif KFERQ are selectively recognized by the heat shock cognate 70 kDa protein (HSC70) chaperone and translocated to lysosomes in a LAMP2A-dependent manner. In all three processes, the autophagic cargo is degraded via lysosomal hydrolases. **b** | The selective autophagy of proteins and organelles is mediated by autophagy cargo receptors (listed within rectangles in the figure), which interact with both the autophagic substrate and the developing autophagosome via an LC3-interacting region (LIR). BAG3, BAG family molecular chaperone regulator 3; BNIP3, BCL2/adenovirus E1B 19 kDa protein-interacting protein 3; FUNDC1, FUN14 domain-containing protein 1; HDAC6, histone deactylase 6; NBR1, next to *BRCA1* gene 1 protein; NIX, NIP3-like protein X; OPTN, optineurin; Ub, ubiquitin.

In addition to protein aggregates, selective autophagy is an important mechanism for the degradation of organelles. For example, damaged and superfluous mitochondria are targeted to autophagosomes in a process termed mitophagy[24,25]. Numerous studies have led to the identification of mitophagy receptors such as Atg32 in yeast, as well as BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3), NIP3-like protein X (NIX) and FUN14 domain-containing protein 1 (FUNDC1) in mammals[26–33]. These receptors all possess an LIR motif to directly target mitochondria to autophagosomes. In addition, phosphorylation of these receptors is one mechanism to regulate mitophagy. For example, phosphorylation of Atg32 at residue serine 114 is necessary for its interaction with the adaptor protein Atg11, which recruits core ATGs to form autophagosomes that engulf mitochondria[34]. Similarly, the phosphorylation of serine residues flanking the LIR motif of BNIP3 promotes the binding of BNIP3 to LC3B and Golgi-associated ATPase enhancer of 16 kDa (GATE16; another Atg8 orthologue), thus facilitating mitophagy[35]. By contrast, dephosphorylation of the LIR of FUNDC1 is required to promote mitophagy during hypoxia[33].

Mammalian cells also utilize ubiquitin-dependent pathways to remove mitochondria. PTEN-induced putative kinase 1 (PINK1) is a mitochondrial protein kinase that accumulates on the outer membrane of depolarized mitochondria, which subsequently leads to the recruitment of the ubiquitin E3 ligase parkin and the polyubiquitylation of mitochondrial outer membrane proteins. Although p62 can recognize and cluster these ubiquitylated proteins, it remains unclear whether these aggregates are necessary for parkin-mediated mitophagy. Further details regarding the PINK1–parkin pathway in mitophagy are discussed elsewhere[25].

Molecular insight into the selective autophagic degradation of peroxisomes, termed pexophagy, originally came from studies of methylotrophic yeasts, such as *Pichia pastoris*. Two pexophagy receptors — Atg30 and Atg36 — have been identified in *P. pastoris* and

Methylotrophic yeasts
A genera of yeast that can only use methanol as the sole source of carbon and energy.

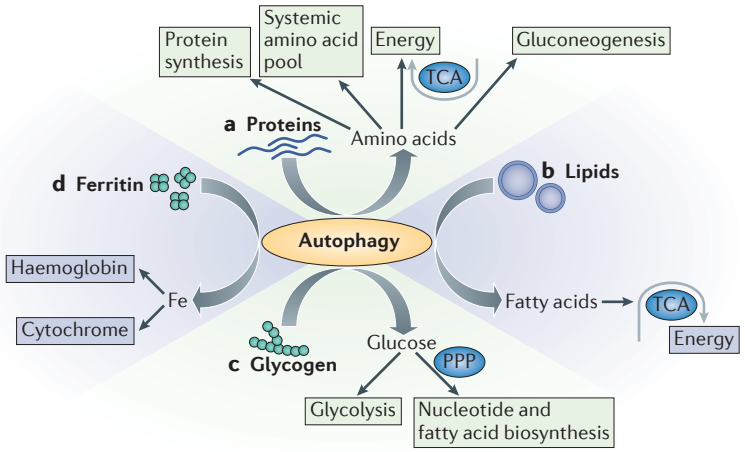

Figure 2 | **Autophagy-derived metabolites support diverse anabolic functions.** The autophagic degradation of proteins, lipids, glycogen and ferritin via autophagy enables multiple anabolic and biosynthetic pathways in cells. **a** | Under low nutrient conditions, autophagy-mediated protein catabolism results in the production of free amino acids that provide building blocks for protein synthesis and serve to maintain intracellular amino acid pools. These amino acids can also be converted to substrates that are utilized by the tricarboxylic acid (TCA) cycle for energy production or used as substrates for glucose production by gluconeogenesis. **b** | Fatty acids produced via lipophagy are converted into acetyl-CoA, which fuels the TCA cycle and energy production. **c** | Glycogen stores in liver and skeletal muscles are broken down to produce glucose that is utilized by glycolysis for energy (ATP) production as well as for the production of substrates, such as citrate, used for new lipid synthesis. In addition, glucose is diverted into the oxidative pentose phosphate pathway (PPP), a side branch of glycolysis important for nucleotide and fatty acid biosynthesis as well as the antioxidant response. **d** | Iron released from ferritin stores is utilized for the synthesis of metalloproteins such as haemoglobin and cytochromes.

Midbody
An intercellular bridge connecting the two dividing cells at the end of cytokinesis that functions to localize the site of abscission.

Midbody ring
A densely ubiquitylated ring-like macromolecular assembly of several proteins located at the midbody during the telomeric phase of cytokinesis.

mTOR–autophagy special coupling compartment
(TASCC). A recently discovered membrane compartment that is adjacent to Golgi apparatus. The TASCC is highly enriched for both mTOR and autolysosomes and promotes the synthesis of secretory proteins.

Glomerular podocytes
Highly specialized epithelial cells in kidney that are terminally differentiated and serve as an important component of the glomerular filtration barrier.

Saccharomyces cerevisiae, respectively[36,37]. In mammalian cells, pexophagy requires the autophagy cargo receptors p62 and NBR1, which recruit LC3-positive phagophores to monoubiquitylated peroxisomes[38,39].

In contrast to the turnover of mitochondria and peroxisomes, the precise role of selective autophagy receptors in the degradation of other organelles, such as ribosomes, nuclei and ER, remains unclear. However, notably, a recent study in *S. cerevisiae* reported that the degradation of ER is topologically equivalent to microautophagy but is not dependent on any genetic components of the core autophagy machinery or microautophagy machinery[40]. Selective autophagy can also target large macromolecules, such as lipids and iron complexes (see below), as well as intracellular pathogens (via a process termed xenophagy) and transient macromolecular structures within cells, such as the inflammasome, midbody and midbody ring[41–44].

Autophagy in energy metabolism

Although traditionally autophagy has been thought to break down cellular proteins during starvation, recent evidence suggests that it is active under basal, nutrient-rich conditions[45]. Furthermore, autophagy can mobilize diverse cellular energy stores such as carbohydrates, lipids and ferritin to replenish metabolites during both normal and stressed conditions (FIG. 2). In this section, we discuss how the diverse cellular constituents targeted via autophagy contribute to energy production and biosynthesis.

Protein catabolism. Early evidence that proteins are degraded by autophagy came from studies using hepatocytes isolated from perfused rat liver; these studies demonstrated that nutrient starvation increased the rate of protein degradation, accounting for ~4–5% of the total protein pool per hour versus 1.5% per hour under basal conditions[46,47]. More direct evidence that autophagy degrades proteins was gathered from multiple studies in yeast and mammals, in which the genetic ablation of autophagy decreased protein turnover[1]. These studies also showed that autophagy-mediated protein degradation sustains amino acid pools and protein synthesis in starving cells (FIG. 2). For example, in yeast, autophagy directly contributes to the intracellular amino acid pool because autophagy-incompetent cells (for example, Atg7-deficient mutants) are unable to restore amino acid pools following nitrogen starvation for just 3 hours[48]. Remarkably, the amino acids produced via autophagy are utilized for the synthesis of total proteins and probably the production of specific proteins, including Arg1p (arginino succinate synthetase for arginine biosynthesis) and heat shock protein 26 kDa (Hsp26p), both of which mitigate nitrogen depletion. Thus, the reduction in general protein synthesis due to diminished amino acid levels may further compromise the viability of autophagy-defective yeast mutants that are subject to prolonged nitrogen, carbon or sulfate starvation[48]. Autophagy also supports mitochondrial function in yeast cells by upregulating proteins involved in respiration and reactive oxygen species scavenging[49]. However, it is unclear whether autophagy-derived amino acid pools are used for the synthesis of these mitochondrial proteins that are crucial for yeast respiratory function.

Autophagy-derived amino acids are also important for enabling protein synthesis in mammalian cells. For instance, a defect in pre-implantation embryos was observed upon eliminating autophagy by genetically deleting *ATG5*; this defect was attributed to impaired protein recycling and protein synthesis owing to amino acid depletion[50]. Furthermore, in cells undergoing oncogene-induced senescence, autophagy contributes to amino acid flux via a specialized compartment termed the mTOR–autophagy special coupling compartment (TASCC), which is crucial for the enhanced synthesis of secretory proteins (BOX 2). Remarkably, TASCC formation has been observed in normal cells, such as glomerular podocytes, although the exact biological function of this compartment remains unclear[51]. Together, these studies illustrate how the catabolic functions of the autophagy pathway can be coupled to protein synthesis to sustain cellular function and viability.

In addition to maintaining protein synthesis, autophagy-mediated protein degradation is an important mechanism for producing energy (ATP) in response to stress (FIG. 2). Seminal studies showed that *Atg5*$^{-/-}$ and *Atg7*$^{-/-}$ neonatal mice exhibited reduced plasma levels of essential amino acids and branched-chain amino acids in different tissues[3,52]. This systemic insufficiency in branched-chain amino acids correlated with increased activity of AMP-activated protein kinase (AMPK)

Box 2 | **Autophagy and secretion**

Although autophagy is traditionally viewed as an autodigestive process, intriguing connections between the autophagy pathway and protein secretion are becoming increasingly appreciated. Studies have primarily focused on autophagy in unconventional secretion, a collection of processes through which certain proteins are secreted from cells either via direct trafficking from the endoplasmic reticulum (ER) to the plasma membrane in a Golgi-independent manner, or via the transport of cytoplasmic proteins lacking an amino-terminal ER signal sequence to the cell surface, completely bypassing the ER–Golgi route[126]. Autophagy-related proteins (ATGs) have been genetically implicated in the unconventional secretion of the acyl-CoA-binding protein Acb1 in yeast (AcbA in *Dictyostelium discoideum*), and inflammatory mediators such as interleukin-1β (IL-1β) and IL-18, the high mobility group protein B1 (HMGB1) and the integral membrane protein ΔF508 CFTR (cystic fibrosis transmembrane conductance regulator) in mammalian cells[127–131]. The unconventional secretion of these proteins is also dependent on Golgi membrane-binding proteins of the GRASP family in both yeast and mammals[129,130,132].

The molecular details of the unconventional secretion pathway are only beginning to emerge, and numerous questions remain unanswered regarding the role of autophagy. First, although genetic interconnections between ATGs and Grh1, the yeast GRASP orthologue, exist in *Saccharomyces cerevisiae*, recent research questions whether autophagy and Grh1 truly converge on a common secretory intermediate[133]. Second, although the early autophagosome-forming core machinery appears to be genetically required for unconventional secretion, it is unclear how the molecules to be secreted, such as Acb1 or CFTR, are actually incorporated into autophagosomes. Third, it is uncertain whether the autophagosomes are transported directly to the cell surface for secretion or whether they fuse with endocytic pathway components such as the multi-vesicular body (MVB). Because studies suggest that the exocytosis of MVBs can mediate the transport of intracellular cargo directly to the cell surface, the latter model seems the most plausible[134]. Indeed, early evidence suggests that the autophagy machinery may intersect with the endosomal sorting complexes required for transport (ESCRT) machinery to promote multiple MVB-associated functions, including exocytosis. For example, an ATG12–ATG3 complex interacts with the ESCRT-associated protein ALIX (also known as programmed cell death 6-interacting protein) to control MVB distribution, late endocytic trafficking and exosome biogenesis[135]. Finally, it remains obscure how secreted proteins potentially trafficking through autophagosomes are diverted away from lysosomes, or if autophagosome–lysosome fusion does occur, how the secretory cargo is protected from destruction via lysosomal hydrolytic enzymes.

In addition to unconventional secretion, components of the autophagy pathway have been genetically implicated in the regulated secretion of insulin, lysozymes, melanosomes, mast cell mediators and the contents of secretory lysosomes[136]. Furthermore, ATGs promote the efficient secretion of cytokines during oncogene-induced senescence and during cancer cell invasion[106,137]. Further dissecting the cellular mechanisms through which autophagy mediators facilitate these diverse secretory processes remains an important topic for future study.

in neonatal *Atg5*$^{-/-}$ mice, indicative of systemic ATP depletion. Because the metabolic products of branched-chain amino acids — acyl-CoA derivatives — are consumed by the tricarboxylic acid (TCA) cycle to create energy, autophagy-derived amino acids might be required to produce ATP during the neonatal starvation period.

Additional evidence that autophagy is important to produce amino acids came from studies using haematopoietic cell lines derived from *Bax*$^{-/-}$ *Bak*$^{-/-}$ mice[53]. These cell lines require interleukin-3 (IL-3) for the uptake of amino acids and nutrients from the external milieu. Upon IL-3 withdrawal, autophagy becomes crucial for energy production and cell viability; the concurrent inhibition of autophagy elicited a rapid decline in cellular ATP levels and profound cell death. However, both cell viability and ATP levels were restored upon the addition of methylpyruvate, a cell permeable TCA substrate; this result suggests that the amino acids released by autophagy in these cells are converted to substrates for energy production by mitochondria. Similar results were observed in primary mouse haematopoietic stem cells following metabolic stress due to cytokine withdrawal *in vitro* or nutrient starvation *in vivo*[54]. In addition, recent studies using liver-specific *Atg7*-null mice demonstrated that hepatic autophagy is critical for maintaining systemic blood glucose levels during fasting by converting autophagy-derived amino acids to glucose via hepatic gluconeogenesis[55]. Overall, these studies highlight the diverse functions of autophagy-derived amino acids in sustaining energy production via the TCA cycle and producing substrates that fuel gluconeogenesis.

The contributions of autophagy to tumour metabolism have been recently reviewed[56–58]. Similar to normal cells, tumour cell autophagy recycles macromolecules for amino acid synthesis and mitochondrial metabolism to support growth and survival[59]. Notably, a recent cellular proteomics study provided further insight into the role of autophagic proteolysis in tumour cell metabolism and survival. In Ras-mutant cancer cells subjected to metabolic stress, autophagy selectively targeted toxic or non-essential proteins for degradation, but excluded essential proteins that are necessary for maintaining autophagy and surviving stress. These results suggest that autophagy-mediated proteolysis remodels the proteome towards cellular pathways required for cell viability[60]. Finally, another major contribution of autophagy-mediated protein degradation is to remove aggregated or dysfunctional proteins to maintain the quality of intracellular proteins (BOX 1).

Transcriptional regulation of lipophagy. The discovery that intracellular lipid stores are degraded via autophagy has expanded our understanding of this catabolic process. During nutrient starvation, lipid droplets are hydrolysed to release free fatty acids for mitochondrial oxidation. The ability of autophagy to selectively degrade lipids is termed macrolipophagy (or lipophagy) and was first observed in hepatocytes[42]. Both *in vitro* and *in vivo* studies have demonstrated the accumulation of triglycerides and cholesterol when autophagy is genetically ablated[42]. Lipophagy also removes excess lipids to maintain basal lipid levels in liver cells (FIG. 3). The free fatty acids released by autophagy are used for β-oxidation and the TCA cycle to produce energy (FIG. 2). Importantly, acute challenge with a lipid stimulus negatively affects autophagic flux in cultured cells and mice, presumably because the increased intracellular lipid content diminishes autophagosome–endolysosomal fusion or suppresses the acidic and hydrolytic activity of lysosomes[61,62]. Hence, reduced hepatic macroautophagy due to excess lipid overload — for example, in obesity — exacerbates lipid accumulation *in vivo* and creates a vicious cycle that promotes hepatic ER stress and insulin resistance[63]. In support of this, reduced lipophagy in *Atg7* haploinsufficient mice is a possible cause of progression from obesity to diabetes because increased lipid toxicity promotes insulin resistance[64].

Gluconeogenesis
A process of glucose production by the metabolism of non-carbohydrate substrates such as pyruvate, lactate, oxaloacetate, glucogenic amino acids or fatty acids.

β-oxidation
The breakdown of fatty acids in the mitochondria into two carbon units of acetyl-CoA, which enter the citric acid cycle, and NADH and FADH2.

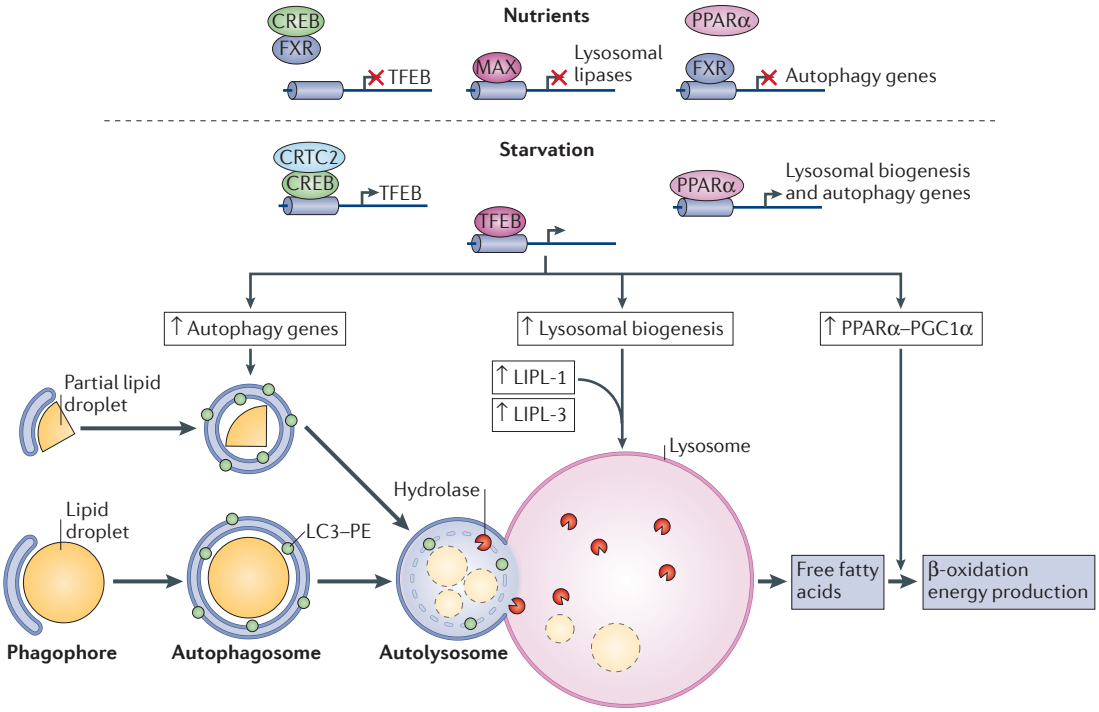

Figure 3 | **Transcriptional control of lipophagy.** During nutrient starvation, autophagy selectively degrades lipid droplets, a process termed lipophagy, by either sequestering a portion of larger droplets within LC3–phosphatidylethanolamine (LC3–PE)-bound phagophore membranes or by completely engulfing small lipid droplets. In the presence of nutrients, lipophagy is transcriptionally repressed by farnesoid X receptor (FXR), which inhibits the transcriptional activity of cAMP response element-binding protein (CREB) by disrupting the formation of a complex between CREB and its co-activator CRTC2; this complex is required for the transcription of TFEB (transcription factor EB), the protein product of which activates the transcription of autophagy and lysosomal biogenesis genes. FXR also competes with another nuclear receptor, peroxisome proliferator activator receptor-α (PPARα) for the binding sites in the promoter regions of autophagy genes. FXR specifically represses the transcription of autophagy genes; however, PPARα can activate the transcription of both autophagy and lysosomal biogenesis genes. Finally, in the presence of nutrients, MAX represses the transcription of lysosomal lipases. Upon starvation, FXR is inactivated, which allows PPARα to bind to the promoter of target genes and the CREB–CRTC2 complex to form and activate the transcription of TFEB, and consequently of TFEB target genes. Starvation also causes a switch from MAX-regulated transcription repression to TFEB-mediated transcription. MAX repressor is replaced by TFEB, which can further activate genes related to autophagy, lysosome biogenesis as well as the PPARα–PGC1α (PPARγ co-activator 1α) axis. Together, TFEB and PPARα support multiple processes favouring lipid catabolism, including autophagosome formation (by enhancing the expression of autophagy genes); lysosomal biogenesis to promote the degradation of autophagic cargo; the transcription of lysosomal lipases such as LIPL-1 and LIPL-3; and the activation of transcriptional programmes driving lipid catabolism in the cytosol (most notably, the TFEB-mediated activation of the PPARα–PGC1α axis).

Macrolipophagy is not limited to hepatocytes; it also occurs in fibroblasts, endothelial cells, lymphoblasts, dendritic cells, glial cells and neurons, suggesting that autophagic lipid degradation is important in diverse cell types[65–67]. Moreover, macrolipophagy has been reported in yeast, in which the degradation of lipid droplets is thought to support cell viability under carbon starvation. In yeast, the core autophagy proteins are involved in the degradation of lipid droplets and the process is distinct from other selective autophagy processes[68].

Before the discovery of lipophagy, the lysosomal compartment was principally thought to affect intracellular lipid pools by clearing lipoproteins endocytosed from the external environment. It is now evident that intracellular lipid droplets are broken down by the acidic lipases in lysosomes, a function that was previously attributed to cytosolic and ER lipases[69,70]. The importance of acidic lipases in lipophagy is further supported by recent studies in *Caenorhabditis elegans*, in which the transcriptional upregulation of the lysosomal lipases LIPL-1 and LIPL-3 was observed in response to starvation. Two transcription factors, MXL-3 and HLH-30, compete for binding to the promoter region of *lipl-1* and *lipl-3* genes; during nutrient starvation, HLH-30 overrides the transcriptional repressor MXL-3 to promote lipase expression[71]. Importantly, HLH-30 is the worm orthologue of the mammalian nutrient-sensitive transcription factor EB (TFEB), which simultaneously induces both autophagy and lysosomal genes during starvation[72]. Thus, the transcriptional activity of HLH-30 reinforces the upregulation of autophagy, lysosomal and lipolytic genes that are required for maintaining the supply of free fatty acids and glycerol for energy production[72]. Moreover, the deletion of *Tfeb* in mouse liver causes the accumulation of lipid droplets

after 24 hours of fasting, which is indicative of defects in intracellular lipid degradation[73]. Nevertheless, with regard to lipophagy, TFEB clearly possesses functions beyond its role as a master regulator of the autophagy–lysosomal axis; notably, TFEB also promotes the activation of peroxisome proliferator activator receptor-γ (PPARγ) co-activator 1α (PGC1α), a transcription factor that promotes lipid catabolism[73]. A complementary mechanism to regulate lipid degradation involves the nuclear receptor farnesoid X receptor (FXR; also known as the bile acid receptor), which competes with PPARα for binding to shared sites in the promoter regions of autophagy genes; when bound to the promoters of autophagy genes FXR represses their transcription in fed cells to inhibit lipophagy[74]. FXR can also inhibit the formation of a complex between the transcriptional activator cAMP response element-binding protein (CREB), which is active under conditions of fasting, and its co-activator CRTC2 to inhibit lipophagy in the liver of fed mice[75]. Collectively, these studies highlight the complexity of integrated transcriptional networks in fine-tuning lipophagy.

How lipids are selectively targeted for autophagy remains a topic of active investigation. Although LC3 may be directly recruited to lipid droplets to initiate the formation of a limiting membrane, this idea is controversial[76]. SNARE (SNAP receptor) proteins that have been implicated in both autophagosome biogenesis and lipid droplet fusion may also facilitate macrolipophagy[77]. The identity of the receptors targeting lipids to autophagosomes remains unclear, but recent research has indicated that dynamin 2, a GTPase involved in membrane scission, favours the degradation of hepatic lipid droplets by promoting the maturation of the autophagic compartment[78]. The disruption of dynamin 2 results in enlarged autolysosomes. These autolysosomes exhibit excessive tubular extensions that are associated with impaired autophagic lysosome reformation, a process that is critical for promoting the recycling and maintenance of the lysosomal pool in cells undergoing autophagy[78].

Autophagy in adipogenesis and energy balance. Adipose tissue is the primary storage organ for energy in the form of lipid droplets. Traditionally, adipose tissue is classified into two types: white adipose tissue (WAT) and brown adipose tissue (BAT). WAT, which consists of unilocular white adipocytes, is a major site of fat storage from which energy is mobilized in the form of triglycerides during nutrient deprivation. BAT is composed of specialized multilocular adipocytes that have higher numbers of mitochondria than unilocular white adipocytes; the multilocular adipocytes are involved in dissipating energy as heat during cold- or diet-induced thermogenesis by promoting the β-oxidation of fatty acids[79]. Recently, a third type of adipocyte was identified; called beige or brite adipocytes, these are brown-like adipocytes that are found in WAT[80]. These beige cells mimic white fat cells in the basal state, but function in a similar manner to brown fat cells by upregulating the expression of thermogenic genes in response to thermogenic stimuli. Interestingly, beige cells possess a gene expression pattern distinct from either white or brown fat cells[80].

In adipose tissue, autophagy appears to be crucial for the efficient differentiation of both white and brown adipocytes, rather than macrolipophagy. The role of autophagy in white adipogenesis was first demonstrated in primary mouse embryonic fibroblasts derived from *Atg5*-deficient mice[81]. These mouse embryonic fibroblasts failed to undergo adipogenesis and developed smaller-sized lipid droplets compared to control fibroblasts when stimulated to undergo differentiation. Similarly, autophagy inhibition in 3T3-L1 pre-adipocytes resulted in reduced levels of markers of white adipocyte differentiation and transcription factors that promote such differentiation along with decreased triglycerides[81]. *In vivo* studies using adipocyte-specific *Atg7*-deficient mice also showed that autophagy supports WAT differentiation[82,83]. Specifically, this deletion resulted in lean mice regardless of whether they had been fed a regular or high-fat diet. This phenotype was probably due to increased energy expenditure and lipid elimination via increased β-oxidation in adipocytes; in support of this hypothesis, white adipocytes contain more mitochondria, which is suggestive of 'browning' of WAT. Together, these studies demonstrate a key role for autophagy in WAT differentiation.

Similarly, the genetic ablation of autophagy in myogenic factor 5 (MYF5)-positive cells, a progenitor population that differentiates into skeletal muscle and BAT, resulted in profound defects in BAT differentiation and function[84]. Nevertheless, these animals displayed increased energy expenditure and raised body temperature compared to control mice, which correlated with the appearance of beige adipocyte features in the inguinal WAT, a subcutaneous adipose tissue. These mice also exhibited reduced skeletal muscle differentiation and glucose intolerance, suggesting that autophagy ablation in specific tissues can broadly affect systemic energy and glucose homeostasis.

Recent studies of mice in which autophagy was selectively inhibited in skeletal muscle further illustrate how autophagy ablation in specific tissue systemically affects lipid metabolism and energy homeostasis; these mice exhibit increased glucose tolerance and insulin sensitivity, reduced adiposity and resistance to diet-induced obesity[85]. This phenotype arises from an increase in dysfunctional mitochondria due to impaired mitophagy in muscle cells, which results in the increased expression of a stress-induced mitokine, fibroblast growth factor21 (FGF21). Intriguingly, increased FGF21 has systemic effects as it increases β-oxidation, lipolysis and the browning of WAT, thereby promoting energy expenditure. This study also demonstrated that mice with autophagic ablation in hepatocytes showed a remarkable resistance to high-fat-diet-induced hepatic steatosis and exhibited improved glucose tolerance. This effect is probably due to an increase in lipid catabolism driven by high levels of FGF21. This result differs from earlier reports showing that the absence of intact autophagy in hepatocytes leads to insulin resistance[63]. Overall, these studies reveal that the targeted inhibition of autophagy in one tissue may affect distant tissues by non-cell autonomous pathways; for example, the production of endocrine factors such as FGF21.

Hepatic steatosis
The accumulation of fat in the liver.

Further support for the idea that tissue-specific autophagy modulates broader systemic physiology comes from studies of hypothalamic neurons, in which autophagy has been implicated in controlling food intake and energy balance by regulating hypothalamic lipid metabolism[66,86]. Mice lacking ATG7 specifically in orexigenic agouti-related peptide (AgRP) hypothalamic neurons exhibit reduced body weight, total body fat and food intake in response to fasting[66]. This is because AgRP-specific *Atg7* deletion alters the levels of key neuropeptides in these hypothalamic neuronal populations, which is proposed to control appetite and energy homeostasis *in vivo*. Moreover, during nutrient starvation, normal hypothalamic cells take up more free fatty acids and accumulate more triglycerides, which induces hypothalamic autophagy; the resulting autophagy-derived intracellular lipids promote AgRP expression. Overall, these results illustrate how autophagy-regulated lipid homeostasis in a single hypothalamic neuronal population can affect energy balance in the entire animal.

Autophagy has also been examined in another nutrient-sensing hypothalamic neuron, the pro-opiomelanocortin (POMC) neuron, which produces anorexigenic neuropeptides that inhibit food intake and promote energy expenditure[87]. Multiple studies have demonstrated that *Atg7* deletion in POMC neurons causes increased body weight owing to hyperphagia[87–89]. However, more recent research argues against a general role for POMC neuron-specific autophagy in the control of energy homeostasis. In contrast to the effects induced by *Atg7* deficiency, the genetic deletion of either *Atg12* or *Atg5* in POMC neurons does not promote weight gain or adiposity in mice on a regular diet, even though these neurons exhibit robust and complete autophagy ablation[90]. Furthermore, upon consuming a high-fat diet, mice lacking ATG12 in POMC-positive neurons exhibit accelerated weight gain, adiposity and glucose intolerance, whereas mice lacking ATG5 in POMC-positive neurons do not. These recent studies suggest that individual ATGs may affect POMC neuronal function via non-autophagic mechanisms, rather than via the autophagy-dependent control of lipid metabolism[90]. Nevertheless, because autophagy regulates lipid and energy homoeostasis in numerous tissues, further understanding how autophagy impacts lipid homeostasis during obesity remains an important topic for future investigation.

Carbohydrate metabolism. Glycogen represents the major form of stored glucose in the liver; autophagic degradation of glycogen represents a crucial mechanism to maintain glucose homeostasis in response to increased demand for this carbohydrate[91,92] (FIG. 2). In the newborn, the plasma levels of the hormone glucagon increase in response to neonatal hypoglycaemia, resulting in the mobilization of glycogen stores in hepatocytes[93]. Glycogen exists in two principal intracellular pools: the cytosol and the autophagic vacuole. The phosphorolytic degradation of glycogen in the cytosol is mediated by the enzyme glycogen phosphorylase, whereas the hydrolytic degradation of this polysaccharide occurs via acid glucosidases located within lysosomes[46]. The autophagy-mediated lysosomal degradation of glycogen releases a non-phosphorylated form of glucose that can be rapidly utilized by starving neonatal cells; this type of glycogen degradation has also been reported in adult animals in both cardiomyocytes and skeletal muscles. Most notably, *in vivo* analysis of transgenic GFP-LC3 mice subjected to starvation indicates that there is an increase in autophagosome biogenesis in glycogen-rich, fast-twitch extensor digitorum longus muscle fibres in comparison to oxidative slow-twitch soleus muscles; this result suggests a link between autophagy regulation and glucose metabolism in adult muscle[94]. However, the importance of glycophagy (that is, the autophagic degradation of glycogen) in energy homeostasis in adults remains unclear. Interestingly, ATG7 deficiency in adult mice causes the accelerated depletion of glycogen from liver (the major organ for glycogen storage) during fasting but not under fed conditions[95].

Alterations in glycophagy have been associated with various genetic myopathies such as Pompe disease, Danon disease, infantile autophagic vacuolar myopathy and drug-induced vacuolar myopathies caused by treatment with chloroquine or hydroxychloroquine[96] (TABLE 1). Among these, Pompe disease has been extensively studied in the context of autophagy. Pompe disease is caused by a deficiency in lysosomal α-acid glucosidase (GAA), which results in the impaired degradation of glycogen as well as swollen, dysfunctional lysosomes, phenotypes that are most apparent in cardiac and skeletal muscles. A secondary phenotype of impaired GAA activity is defective autophagosome–lysosome fusion in the muscle fibres of patients with Pompe disease. This defect might interfere with the delivery of GAA into the lysosomal compartment, which has important implications for disease pathogenesis and the efficacy of recombinant enzymatic therapy in patients with Pompe disease[97–99]. Overall, despite the profound accumulation of glycogen-containing autophagic and lysosomal vesicles in skeletal myopathies, it remains uncertain whether impaired glycophagy functionally contributes to muscle dysfunction. However, genetic studies using a *Drosophila melanogaster* model of chloroquine-induced myopathy demonstrate that glycophagy in skeletal muscle is dependent on the core autophagy machinery[100]. Interestingly, this study also revealed that the efficient degradation of glycogen requires both enzymatic (phosphorolytic) and autophagic (hydrolytic) machineries[100]. However, an important unanswered question is whether the lysosomal degradation of glycogen is coordinated with the enzymatic degradation of glycogen via glycogen phosphorylase to maintain glucose levels. Such coordination is hinted at by a recent study in which *Atg7*-deficient adult mice showed accelerated glycogen mobilization during fasting[95].

In addition to its role in glycogen degradation, autophagy appears to have additional effects on glucose homeostasis in animals. For example, the suppression of autophagy in genetic and dietary models of obesity reduced blood glucose levels despite

Orexigenic
A stimulant (drug or hormone) that increases appetite.

Hyperphagia
An abnormal increase in appetite for the consumption of food, which is frequently associated with a defect in hypothalamic function.

Phosphorolytic degradation
The addition of a phosphate group to a substrate that initiates its cleavage.

Extensor digitorum longus muscle fibres
An example of a type II, fast-twitch muscle that has the ability to contract quickly and strongly but gets fatigued very rapidly.

Soleus muscles
An example of a type I, slow-twitch muscle that contains more mitochondria than type II, fast-twitch muscles; type I muscles contract for longer periods of time than type II muscles.

Pompe disease
Also called glycogen storage disease type II, Pompe disease is caused by a defect in lysosomal acid α-glucosidase.

Danon disease
A glycogen storage disease caused by a mutation in the gene encoding lysosome-associated membrane protein 2 (LAMP2).

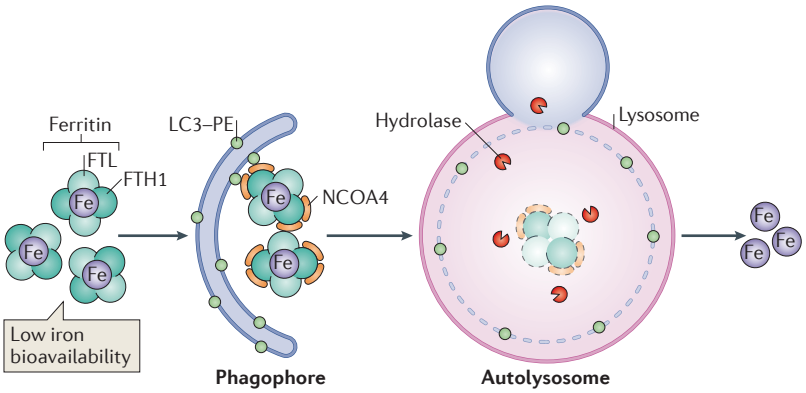

Figure 4 | **Ferritinophagy.** During conditions of low iron bioavailability in the cell, autophagy selectively degrades ferritin, an iron-containing protein complex, to mobilize bioavailable iron stores. This process is mediated by nuclear receptor co-activator 4 (NCOA4), an autophagy cargo receptor that binds ferritin heavy chain 1 (FTH1) and sequesters iron-containing ferritin complexes into autophagosomes by binding to microtubule-associated protein 1 light chain 3–phosphatidylethanolamine (LC3–PE) on the developing autophagosome membrane. Upon autophagosome maturation and fusion with the lysosome, both NCOA4 and ferritin are degraded, resulting in the release of bioavailable iron. FTL, ferritin light chain.

increased insulin resistance, possibly owing to impaired autophagy-mediated glycogen degradation[63]. Recent research using an inducible model of systemic *Atg7* ablation revealed a critical role for autophagy in the response of adult mice to starvation. Detailed metabolic analysis revealed that *Atg7* ablation causes a failure to mobilize serum-free fatty acids and initiates the rapid depletion of liver glycogen stores, resulting in low serum glucose levels and impaired metabolic fitness in starved animals[95]. Moreover, during exercise, autophagy is induced in cardiac and skeletal muscles, adipose tissue and pancreatic β-cells, which provides protection against glucose intolerance[101]. Similarly, in pancreatic β-cells, autophagy is important for maintaining glucose tolerance because mice lacking ATG7 in β-cells have reduced serum insulin levels and hyperglycaemia[102,103]. When these mice were fed a high-fat diet they exhibited reduced β-cell numbers and reduced insulin secretion[102]. In addition to crinophagy, a process involving the direct fusion of secretory granules with lysosomes, autophagy was thought to regulate the secretion of insulin-containing secretory granules to maintain stable insulin levels and thus indirectly contribute to glucose homeostasis[104]. Interestingly, a recent study has demonstrated that autophagy is suppressed in β-cells during starvation; starvation-induced nascent granule degradation occurs in these starved cells, resulting in a local increase of amino acids, thereby suppressing β-cell autophagy. Starvation-induced nascent granule degradation is positively regulated by inactive protein kinase D1 to suppress autophagy and prevent insulin release under nutrient-limiting conditions[105]. Finally, autophagy promotes glucose uptake and glycolytic flux in Ras-transformed mouse fibroblasts and breast cancer cells; however, it remains unclear whether autophagy similarly promotes intracellular glucose metabolism in oncogenic contexts beyond Ras[106].

Iron metabolism. The recent findings that autophagy promotes iron metabolism has further expanded our view of self-eating in mobilizing cellular nutrient stores (FIG. 2). Iron is required for countless biological processes, serving as a cofactor for several haem-containing and non-haem-containing proteins and enzymes. Excess iron is stored in ferritin, a large multisubunit molecule capable of chelating 2,000–2,500 atoms of iron per molecule[107]. Ferritin not only serves as the iron store for cells but also as a mechanism to prevent the generation of free radicals due to free iron. The regulation of iron levels is maintained in cells by a network of iron-dependent proteins; upon iron depletion, its bioavailability is maintained via release from ferritin. Initial studies using *Atg5*$^{-/-}$ fibroblasts revealed an important role for autophagy in ferritin degradation during iron depletion[108]. Subsequent ultrastructural studies in cells deficient in autophagy owing to the genetic deletion of either *FIP200* or *ATG9A* revealed the accumulation of ferritin cluster particles at the autophagosome formation site[109].

The mechanism by which ferritin is targeted for autophagic degradation has been clarified by two proteomic studies that identified nuclear receptor co-activator 4 (NCOA4) as a specific autophagy cargo receptor that binds ferritin and targets it for lysosomal degradation in a process termed "ferritinophagy". The newly identified function of NCOA4 as an autophagy cargo receptor is remarkably different from its originally identified roles as an androgen receptor co-activator[110]. Similar to known cargo receptors, NCOA4 is enriched in autophagosomes isolated from a human pancreatic cancer cell line[43]. In further support of its role as a receptor, NCOA4 was identified as an autophagy substrate when autophagy was inhibited using a highly selective chemical inhibitor of VPS34 (REF. 44). Biochemical studies indicated that NCOA4 interacts with the entire ferritin complex via ferritin heavy chain (FTH1) and colocalizes with ferritin in cells. Although NCOA4 lacks the canonical LIR motif found in other autophagy cargo receptors, the RNAi-mediated depletion of NCOA4 impaired the autophagic targeting and lysosomal-mediated degradation of ferritin, thereby implicating NCOA4 as a bona fide cargo receptor for ferritin turnover (FIG. 4). Moreover, the loss of NCOA4 reduced the level of bioavailable intracellular iron in cells subjected to iron depletion and led to the profound accumulation of iron in splenic macrophages *in vivo*[44]. Defects in ferritin turnover may cause the neurodegenerative disorder static encephalopathy of childhood with neurodegeneration in adults (SENDA), which is characterized by the accumulation of iron deposits in basal ganglia due to mutations in the autophagy gene WD repeat domain phosphoinositide-interacting protein 4 (*WDR45*)[111,112] (TABLE 1). Overall, these results highlight the importance of autophagy for iron homeostasis and bioavailability, especially during iron depletion.

Concluding remarks

Although autophagy is fundamentally regarded as a catabolic process, the studies overviewed here highlight the importance of this pathway in sustaining and even enabling anabolic pathways in certain settings. Indeed, autophagy-derived nutrients produced from the

Table 1 | **Effect of autophagy impairment in specific tissues and related pathologies**

Target tissue	Pathologies	Impaired autophagy type	Phenotype	Refs
Pancreatic β-cells, whole body (global haploinsuffiency)	Diabetes	Lipophagy, glycophagy, aggrephagy	Impaired insulin secretion, hyperglycaemia and accumulation of protein aggregates	64,102
Liver	Obesity	Lipophagy*, glycophagy	Insulin resistance, low blood glucose levels	63
Liver	Hepatic steatosis	Lipophagy	Increased lipid accumulation	42
Hypothalamus	Obesity	Hypothalamic lipophagy	Increased food intake, reduced energy expenditure	66,86,87
Skeletal, respiratory and cardiac muscle	Glycogen storage disease type II (Pompe disease)	Glycophagy	Accumulation of glycogen-filled lysosomes	138
Skeletal and cardiac muscle	Inherited myopathies	Glycophagy, aggrephagy	Accumulation of autophagosomes and glycogen	139
Liver	Deficiency in α1-antitrypsin	Aggrephagy	Accumulation of inclusions of insoluble enzyme α1-antitrypsin	121
Brain	Static encephalopathy in childhood with neurodegeneration in adulthood (SENDA)	Ferritinophagy	Iron accumulation in the globus pallidus and substantia nigra of the brain	111,112

*Current evidence suggests that the indicated autophagy type is impaired, but it has not been fully proven.

catabolic degradation of proteins, lipids, carbohydrates and ferritin all support diverse biosynthetic pathways, both during starvation and under basal conditions. Notably, because these anabolic functions are all intimately coupled to autophagy-mediated degradation in the lysosome, further understanding the regulatory mechanisms by which degradation products are exported out of the lysosomal lumen remains an important area for future study. In addition to its long-recognized role in autodigestion, non-canonical functions for the autophagy pathway in cellular synthetic functions are being increasingly appreciated, including, most notably, the autophagy-dependent control of protein secretion (BOX 2). As the autophagy-dependent secretion of cytokines and growth factors is likely to influence cell growth and function through non-cell-autonomous mechanisms, these findings have major biological implications. Hence, going forward, we will need to consider both autodigestive and non-canonical functions of autophagy to further ascertain how autophagy sustains core cellular and metabolic functions.

Further insight into how autophagy mobilizes various nutrient pools towards specific anabolic functions will also come from an increased understanding of selective autophagy in cells. Despite immense and exciting progress in this area of research, several aspects of selectivity remain unclear. For instance, the exact mechanisms directing the degradation of lipids in response to starvation must be further explored. Similarly, how glycogen pools are targeted by the autophagic machinery remains unknown, as does what determines the balance between the lysosomal and enzymatic degradation of glycogen. Finally, the recent discovery that NCOA4 is an autophagy receptor in the selective targeting of iron–ferritin complexes suggests that other selective targets for autophagy remain unidentified. Through the continued identification of new targets of autophagy and further dissection of the mechanisms of selectivity and metabolic pathways they support, we will gain new insight into how autophagy functions to sustain anabolism in both normal and diseased cells.

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Competing interests statement
The authors declare no competing interests.