Autophagy Promotes Focal Adhesion Disassembly and Cell Motility of Metastatic Tumor Cells through the Direct Interaction of Paxillin with LC3

Graphical Abstract

Highlights

- Autophagy is required for the migration and invasion of metastatic tumor cells
- Autophagy promotes the degradation of paxillin and focal adhesion turnover
- Paxillin interacts with LC3B through a conserved LIR in a Src-regulated manner
- Autophagy is required for Src-regulated tumor cell motility

Authors

Marina N. Sharifi, Erin E. Mowers, Lauren E. Drake, ..., Marta Zamora, Stephanie Mui, Kay F. Macleod

Correspondence

kmacleod@uchicago.edu

In Brief

Sharifi et al. describe a role for autophagy in focal adhesion turnover and cell motility mediated by the interaction of processed LC3 with paxillin in a manner regulated by oncogenic Src. These studies suggest potential therapeutic value in targeting autophagy to inhibit metastatic dissemination.
Autophagy Promotes Focal Adhesion Disassembly and Cell Motility of Metastatic Tumor Cells through the Direct Interaction of Paxillin with LC3

Marina N. Sharifi,1,2,3,7 Erin E. Mowers,1,4,7 Lauren E. Drake,1,5 Chris Collier,1 Hong Chen,1 Marta Zamora,6 Stephanie Mui,1,2 and Kay F. Macleod1,2,*

1The Ben May Department for Cancer Research, University of Chicago, Chicago, IL 60637, USA
2Committee on Cancer Biology, University of Chicago, Chicago, IL 60637, USA
3Medical Scientist Training Program, University of Chicago, Chicago, IL 60637, USA
4Interdisciplinary Scientist Training Program, University of Chicago, Chicago, IL 60637, USA
5Committee on Molecular Pathology, University of Chicago, Chicago, IL 60637, USA
6Department of Radiology, University of Chicago, Chicago, IL 60637, USA
7Co-first author
*Correspondence: kmacleod@uchicago.edu
http://dx.doi.org/10.1016/j.celrep.2016.04.065

SUMMARY

Autophagy is a conserved catabolic process that plays a housekeeping role in eliminating protein aggregates and organelles and is activated during nutrient deprivation to generate metabolites and energy. Autophagy plays a significant role in tumorigenesis, although opposing context-dependent functions of autophagy in cancer have complicated efforts to target autophagy for therapeutic purposes. We demonstrate that autophagy inhibition reduces tumor cell migration and invasion in vitro and attenuates metastasis in vivo. Numerous abnormally large focal adhesions (FAs) accumulate in autophagy-deficient tumor cells, reflecting a role for autophagy in FA disassembly through targeted degradation of paxillin. We demonstrate that paxillin interacts with processed LC3 through a conserved LIR motif in the amino-terminal end of paxillin and that this interaction is regulated by oncogenic SRC activity. Together, these data establish a function for autophagy in FA turnover, tumor cell motility, and metastasis.

INTRODUCTION

Macro-autophagy (hereinafter termed autophagy) is a catabolic process important for the degradation of damaged organelles and protein aggregates, as well as the intracellular recycling of metabolites (Kroemer et al., 2010; Mizushima and Komatsu, 2011) that are used by tumor cells to survive nutrient stress, hypoxia, and cytotoxic therapies (Amaravadi et al., 2011; Kimmelman, 2011; White, 2012). Consequently, autophagy has emerged as a potential therapeutic target (Amaravadi et al., 2011). However, such efforts are complicated by the growing realization that autophagy plays opposing roles in tumorigenesis, likely influenced by the stage of progression, driving oncogene and tissue type (Galluzzi et al., 2015). Mouse models support a tumor-suppressive role for autophagy in the early stages of tumorigenesis by promoting genome stability and limiting necrosis and inflammation (Degenhardt et al., 2006; Karantza-Wadsworth et al., 2007; Mathew et al., 2009). Conversely, autophagy is required for malignant progression (Guo et al., 2013; Karsli-Uzunbas et al., 2014; Rao et al., 2014; Rosenfeldt et al., 2013; Strohecker et al., 2013). Consistently, clinical studies have associated increased staining for the autophagy marker LC3 with melanoma metastases and with node positivity and decreased overall survival in human breast cancer (Lazova et al., 2012), whereas overexpression of the key autophagy regulator Beclin1 is linked to reduced latency of melanoma metastasis (Giatromanolaki et al., 2011).

Here, we report that autophagy is required for the motility and invasion of highly metastatic tumor cells due to a function for autophagy in promoting focal adhesion (FA) turnover. The key FA protein paxillin is degraded by autophagy, and paxillin is targeted to the autophagosome through the Src-regulated interaction of LC3 with a conserved LC3-interacting region (LIR) in paxillin. These results broaden our understanding of the multifaceted role of autophagy in tumor progression and indicate that inhibiting autophagy may be an effective approach to blocking metastatic dissemination in the clinic.

RESULTS

Knockdown of Atg5 or Atg7 in Metastatic Mammary Tumor Cells Inhibits Autophagy without Inhibiting Cell Growth

To explore the role of autophagy in metastasis, we used the 4T1 orthotopic mouse mammary tumor model, in which 4T1 murine mammary carcinoma cells implanted into the mammary fat pads of syngeneic BALB/c mice form primary tumors that spontaneously metastasize to the lung and liver (Aslakson and Miller,
We inhibited autophagy in 4T1 cells through stable, short-hairpin (sh)-RNA (shRNA)-mediated knockdown of either Atg5 or Atg7, two autophagy-regulatory proteins required for autophagosome formation, in accordance with published guidelines (Staskiewicz et al., 2013). 4T1 clones stably expressing shAtg5 exhibited no detectable Atg5 protein in the presence or absence of the autophagy inhibitor bafilomycin A1 (bafA1) (Figure 1A). As expected, knockdown of Atg5 also resulted in the absence of the Atg5-12 conjugate (Figure S1A). Although parental cells exhibited robust autophagic flux with bafA1 treatment, as measured by increased autophagosome marker LC3B-II, shAtg5 clones failed to do so either in the presence of bafA1 (Figures 1C and 1D) or under hypoxia (Figure S1B). Similar results were obtained upon Atg7 knockdown (Figures 1B, 1C, and 1E).

Quantification of lung metastases demonstrated a statistically significant reduction (p < 0.0001) in mice bearing autophagy-deficient cells (Figure 1F), demonstrating the lack of autophagy in these tumors. Given the change in the morphology of the autophagy-deficient cells, we proceeded to examine whether inhibition of autophagy altered primary tumor growth. Injection of engineered 4T1 tumor cells into the mammary fat pads of syngeneic BALB/c female mice produced palpable mammary tumors within 2 weeks and large tumors within 4 weeks. LC3B-positive punctae/autophagosomes were readily visible in control tumors at 3 weeks but were absent in Atg5- and Atg7-deficient tumors (Figure 2A), indicating that autophagy was inhibited. However, consistent with in vitro data (Figure 1I), autophagy-deficient tumors did not exhibit reduced growth in vivo (Figure 2B), and there was no significant difference in the Ki-67 index (Figure 2C) or TUNEL staining (Figure 2D) between the autophagy-deficient and control tumors. Taken together, these data indicate that 4T1 tumor cells did not depend on autophagy for tumor cell proliferation or survival in vivo.

Although primary tumor growth was unaffected, inhibition of autophagy markedly reduced lung and liver metastases (Figures 2E and 2F). Numerous macro-metastases were evident in lung and liver sections from mice with parental and scrambled control cells but not shAtg5, cells (Figure 1G, red arrows). Despite this marked inhibition of autophagy in Atg5- and Atg7-deficient 4T1 cells, there was no significant decrease in cell growth (Figures 1H and 1I) or viability, compared with parental and scrambled shRNA cells, even when deprived of glucose or oxygen (Figure 1J). Thus, although metastatic 4T1 tumor cells exhibit high levels of autophagic flux, inhibition of autophagy did not limit cell growth in vitro.

### Autophagy Is Required for Metastasis of Tumor Cells from the Mammary Gland to the Lung and Liver

Injection of engineered 4T1 tumor cells into the mammary fat pads of syngeneic BALB/c female mice produced palpable mammary tumors within 2 weeks and large tumors within 4 weeks. LC3B-positive punctae/autophagosomes were readily visible in control tumors at 3 weeks but were absent in Atg5- and Atg7-deficient tumors (Figure 2A), indicating that autophagy was inhibited. However, consistent with in vitro data (Figure 1I), autophagy-deficient tumors did not exhibit reduced growth in vivo (Figure 2B), and there was no significant difference in the Ki-67 index (Figure 2C) or TUNEL staining (Figure 2D) between the autophagy-deficient and control tumors. Taken together, these data indicate that 4T1 tumor cells do not require autophagy for tumor cell proliferation or survival in vivo.

Although primary tumor growth was unaffected, inhibition of autophagy markedly reduced lung and liver metastases (Figures 2E and 2F). Numerous macro-metastases were evident in lung and liver sections from mice with parental and scrambled control tumors but not mice with autophagy-deficient tumors (Figure 2E). Quantification of lung metastases demonstrated a statistically significant reduction (p < 0.0001) in mice bearing autophagy-deficient tumors relative to parental or control tumors (Figure 2E). Although parental tumors were treated with hydroxychloroquine (HCQ), an Food and Drug Administration (FDA)-approved inhibitor of autophagy with the same mechanism of action as bafA1 (Klionsky et al., 2016). As with genetic inhibition of autophagy, HCQ treatment did not alter primary tumor growth (Figure S2A) but significantly reduced lung metastasis (p < 0.0001) (Figure 2G). Together, these results demonstrate that autophagy is required for spontaneous metastasis in the 4T1 in vivo model.

The known functions of autophagy in promoting cell survival during extracellular matrix (ECM) detachment, growth factor withdrawal, and nutrient deprivation (Fung et al., 2008; Kuma et al., 2004) are believed to promote progression following escape from the primary tumor. Indeed, autophagy is required for tumor cell survival in the bloodstream during hepatocellular carcinoma metastasis (Peng et al., 2013). To investigate whether autophagy is required at later stages of metastasis in the 4T1 model, engineered tumor cells were injected directly into the circulation via the tail vein, bypassing earlier steps in the metastatic cascade. After 2 weeks, autophagy-deficient tumor cells formed as many lung metastases as parental and control cells (Figures S2B and S2C), indicating that autophagy is not required in this model for tumor cell survival in the circulation or metastatic outgrowth at secondary sites. This is consistent with our finding that autophagy is not required for 4T1 tumor cell proliferation or survival in vitro (Figures 1G and 1H), or for primary tumors in vivo (Figures 2B–2D), and indicates that reduced metastasis of autophagy-deficient tumors (Figures 2E and 2F) was due to failure to escape from the primary tumor.

### Autophagy Is Required for Tumor Cell Motility In Vitro

Consistent with the observed defect in spontaneous metastasis in vivo, autophagy-deficient 4T1 cells exhibited markedly decreased migration and invasion through collagen compared with control cells (Figures 3A and 3B). Inhibition of autophagy with chloroquine (CQ) also inhibited the motility of 4T1 cells (Figure 3C). Inhibition of autophagy in the metastatic MDA-MB-231 human breast cancer (Figures S3A–S3C) and B16.F10 mouse melanoma (Figures S3D and S3E) cell lines produced similar effects. Furthermore, autophagy-deficient 4T1 cells exhibited markedly altered morphology in time-lapse differential interference contrast (DIC) imaging (Movies S1, S2, and S3). Unlike control cells, autophagy-deficient cells did not spread out (Figure 3D) or form protrusions (Figure 3E). Together, these results indicate that autophagy is required for metastatic tumor cell migration, invasion, and spreading in vitro.

Given the change in the morphology of the autophagy-deficient cells, which could be consistent with a transition to a more epithelial cellular program, as well as recent work suggesting that the autophagy adaptor protein p62/Sqstm1 regulates Twist degradation (Qiang et al., 2014), we assessed Twist and E-cadherin protein levels in autophagy-deficient cells but found no difference relative to autophagy-competent cells (Figure S3F). Furthermore, because cell motility is driven by ATP, the production of which can be supported by autophagy, we also examined whether the motility defects of autophagy-deficient cells were due to insufficient ATP levels. As expected, Atg5 knockdown reduced cellular ATP levels. However, although treatment with methyl pyruvate restored ATP levels (Figure S3G), it failed to rescue cell invasion (Figure S3H) or protrusion formation (Figure S3I). Thus, the defective motility of autophagy-deficient tumor cells was not due to reduced ATP levels.
Figure 1. Autophagy-Deficient 4T1 Cells Do Not Exhibit Altered Growth Rate

(A) Western blot for Atg5 in parental, scrambled shRNA and two Atg5 shRNA-expressing 4T1 clones in the presence or absence of 100 nM bafA1 (4 hr).

(B) Detection of Atg7 by IP followed by western blotting in parental, control, and Atg7 shRNA cells. IB, immunoblot.

(C) Densitometric quantification of LC3B-II in parental and scrambled Atg5 and Atg7 shRNA cells ± bafA1 (**p < 0.001; NS, not significant). Data indicate mean ± SEM; n = 3.

(D and E) Representative western blot for LC3B-I and II in Atg5- and Atg7-deficient cells ± bafA1.

(F) IF for endogenous LC3.

(G) Electron microscopy of autophagy-competent (left) and -deficient (right) 4T1 cells.

(H and I) Cell growth in complete medium over 96 hr. Data indicate mean ± SEM; n = 3.

(J) Cell viability after 24 hr in complete medium lacking glucose or at 1% O2 assessed by propidium iodide (PI) exclusion. Data indicate mean ± SEM; n = 2. NS, not significant.
Figure 2. Autophagy Inhibition Reduces Metastasis to the Lung and Liver

(A) Immunohistochemistry for LC3B in control (i), Atg5-deficient (ii), and Atg7-deficient (iii) 4T1 primary tumors at 3 weeks. Scr, scrambled.

(B) Orthotopic tumor volume of parental and scrambled Atg5, and Atg7 shRNA tumors over time \( p = 0.2106 \), repeated-measures ANOVA. Data indicate mean ± SEM; \( n = 8 \).

(C and D) Quantification of nuclear proliferation marker Ki-67 (C) and TUNEL staining (D) of tumors after 4 weeks. Data indicate mean ± SEM, \( n = 6 \). NS, not significant.

(E) Representative H&E-stained sections of lungs and livers of mice with parental and scrambled Atg5 and Atg7 shRNA tumors 4 weeks post-orthotopic injection.

(F) Mean number of lung metastases in mice with control or autophagy-deficient tumors at 4 weeks \( p < 0.0001 \), one-way ANOVA. Data indicate mean ± SEM; \( n = 15 \) (parental), \( n = 20 \) (scrambled shRNA), \( n = 18 \) (Atg5 shRNA-1 and -2), and \( n = 8 \) (Atg7 (shRNA-1 and -2). NS, not significant.

(G) Mean number of lung metastases at 4 weeks in mice with parental tumors that received PBS or 60 mg/kg HCQ i.p. every 3 days following tumor implantation \( p < 0.0001 \), one-way ANOVA. Data indicate mean ± SEM; \( n = 8 \) per group.
FA Disassembly Is Impaired in Autophagy-Deficient Tumor Cells

FAs are large macromolecular complexes that link the actin cytoskeleton to the ECM to provide traction, and properly regulated FA dynamics are critical for cell migration (Parsons et al., 2010; Ridley, 2011; Webb et al., 2002). IF for the FA proteins paxillin and zyxin revealed an increase in both FA size and number in autophagy-deficient cells (Figures 4A–4C). Although paxillin is recruited early to nascent FAs (Laukaitis et al., 2001), zyxin is found only in mature FAs (Zaidel-Bar et al., 2003), indicating that the more numerous and larger FAs in the autophagy-deficient cells were mature. This phenomenon was replicated upon bafA1 treatment of both parental 4T1 and B16.F10 cells (Figures S4B and S4C). As with the morphologic changes (Figure 3E), the alteration in FAs was density independent (Figure 4A; Figure S4A). The FA abnormalities were observed in autophagy-deficient cells grown on glass, fibronectin, or collagen I (Figure S4D), indicating that defects in cell spreading and FA accumulation were not due to altered interaction with or modulation by the cellular substratum.

To determine whether the FA abnormalities in autophagy-deficient tumor cells were the result of altered FA dynamics, we assessed FA assembly and disassembly rates using time-lapse imaging of EGFP-paxillin FAs in control and Atg5-deficient 4T1 cells (Figure 4D; Movies S4 and S5). EGFP-paxillin incorporation into, and disappearance from, FAs was linear on a semilogarithmic plot of fluorescence intensity over time, as described previously (Webb et al., 2004), allowing us to calculate assembly and disassembly rate constants (Figure 4E). Although Atg5-deficient cells exhibited no difference in FA assembly rates relative to control cells, the disassembly rate was significantly reduced; FAs disassembled completely within 15 min in control cells (Figure 4D, top panel, arrows) but required >30 min in Atg5-deficient cells (Figure 4E, bottom panel, arrows). Therefore, inhibition of autophagy increases FA size and number due to impaired FA disassembly.

Aberrant FAs in Autophagy-Deficient Tumor Cells Are Associated with Paxillin Accumulation

Cells lacking FAK (Ilić et al., 1995), catalytically active Src (Fincham and Frame, 1998), or paxillin (Hagel et al., 2002) exhibit decreased spreading and migration associated with increased FA number and size and exhibit impaired FA disassembly (Webb et al., 2004), similar to our observations in autophagy-deficient 4T1s. Although we did not detect any changes in FAK or Src protein levels or phosphorylation (Figures S5A and S5B), we observed a marked increase in total paxillin protein levels in autophagy-deficient 4T1 cells (Figures 5A and 5B). BafA1 increased paxillin protein levels in parental, but not autophagy-deficient, 4T1 cells (Figures 5A–5C) as well as in MDA-MB-231
and B16.F10 cells (Figures 5D and 5E). Paxillin levels were also elevated in lysates from Atg5- and Atg7-deficient tumors relative to control primary 4T1 tumors (Figure 5F). Immunohistochemistry confirmed these increased paxillin levels in autophagy-deficient tumors (Figure 5G). Thus, autophagy inhibition is associated with paxillin accumulation in vitro and in vivo.

To determine whether increased paxillin levels underlie the cell motility and FA defects in autophagy-deficient 4T1s, we used small interfering RNA (siRNA) to knock down paxillin expression in autophagy-deficient 4T1 cells to levels similar to those in parental cells (Figure 5H). Reducing paxillin levels in autophagy-deficient cells normalized FA morphology (Figure 5I). Importantly, knockdown of paxillin and normalization of FAs rescued the motility of autophagy-deficient cells (Figures 5J and 5K). Together, these results demonstrate that inhibition of autophagy in metastatic tumor cells results in the accumulation of paxillin, thereby inhibiting FA disassembly, cell spreading, and cell motility.

**Paxillin Is Stabilized in Autophagy-Deficient Cells and Colocalizes with LC3**

Increased paxillin in autophagy-deficient cells was not due to increased paxillin mRNA levels (Figure S5C) but was associated with increased protein stability (Figure S5D), indicating that inhibition of autophagy reduces paxillin degradation. Paxillin can be degraded at the proteasome (Abou Zeid et al., 2006), and autophagy inhibition can reduce proteasomal activity (Korolchuk et al., 2009). However, proteasomal inhibition with MG132 or ALLN did not increase paxillin levels in control 4T1 cells (Figure S5E), and proteasomal degradation was not inhibited in autophagy-deficient 4T1 cells (Figure S5F). Thus, we investigated a direct role for autophagy in degrading paxillin.

By live-cell confocal imaging, we detected mApple-paxillin in punctate cytosolic structures that co-localized with the autophagosome marker EGFP-LC3B (Figure 6A; Figure S6A), confirming that paxillin colocalizes with autophagosomes in autophagy-competent 4T1 cells. EGFP-LC3B also co-localized with mApple-paxillin-positive FA structures (Figure 6B; Figure S6A), indicating that autophagosomes co-localize with FAs in parental cells, consistent with previous reports (Sandilands et al., 2012). In combination with our data demonstrating that both transient and permanent inhibition of autophagic flux...
increase paxillin levels, the colocalization of paxillin with autophagosomes strongly suggests that paxillin is degraded by autophagy. Immunohistochemistry revealed punctate paxillin staining in 4T1 tumors expressing scrambled, but not Atg5, shRNA (Figure S6B), consistent with the presence of paxillin at autophagosomes in vivo.

**Paxillin Interacts Directly with LC3**

Paxillin can be ubiquitinated (Didier et al., 2003), and cargo receptor proteins such as p62/Sqstm1 and Nbr1 are known to target ubiquitinated proteins to autophagosomes for degrada-

**Figure 5. Autophagy-Deficient 4T1 Cells Have Increased Paxillin**

(A and B) Western blot for paxillin in control and autophagy-deficient 4T1 cells ± 100 nM bafA1 (4 hr) to inhibit autolysosomal degradation. (C) Densitometric quantification of paxillin fold increase with bafA1 in parental and scrambled, Atg5, and Atg7 shRNA 4T1 cells (**p < 0.01; ***p < 0.001; NS, not significant). Data indicate mean ± SEM; n = 3.

(D and E) Western blot for paxillin ± bafA1 (4 hr) in MDA-MB-231 (D) and B16.F10 (E) cells. (F) Western blot for paxillin in lysates from parental and scrambled Atg5 and Atg7 shRNA expressing 4T1 primary tumors 3 weeks after orthotopic injection. (G) Paxillin immunohistochemistry in parental and scrambled Atg5 and Atg7 shRNA-expressing 4T1 primary tumor sections 3 weeks after orthotopic injection. Scale bars, 50 μm. (H) Western blot confirming paxillin knockdown in autophagy-deficient 4T1 cells. (I) Paxillin and zyxin IF in autophagy-deficient 4T1 cells treated with control or paxillin siRNA. (J and K) Quantification of the effect of siRNA-mediated paxillin knockdown in autophagy-deficient 4T1 cells on migration and invasion (**p < 0.01). Data indicate mean ± SEM; n = 3.

motion (Rogov et al., 2014); both have been implicated in the regulation of cell migration (Kenific et al., 2016; Qiang et al., 2014). However, neither paxillin accumulation (Figures S6C and S6D) nor reduced cell migration or invasion (Figures S6E and S6F) were observed in 4T1 cells knocked down for p62/Sqstm1 or Nbr1, indicating that neither protein is involved in paxillin degradation. Cytosolic proteins and macromolecular structures can also be targeted for autophagy via direct interaction with processed LC3 (Rogov et al., 2014).

Given that paxillin colocalizes with LC3B in the cytosol and at FAs (Figures 6A and 6B), we tested for an interaction between LC3B and paxillin. We successfully co-immunoprecipitated mApple-paxillin and trace levels of endogenous paxillin with EGFP-LC3B in both 4T1 (Figure 6D) and B16.F10 cells.
Figure 6. Paxillin Co-localizes with Autophagosomes

(A and B) 4T1 cells expressing mApple-paxillin and EGFP-LC3B were treated with bafA1, and live cells were imaged by confocal microscopy. 3D reconstruction reveals that mApple-paxillin colocalizes with EGFP-LC3B (arrows) (A) and that EGFP-LC3B colocalizes with FAs (arrows) (B).

(C) Alignment of human paxillin residues 37–43 (bottom) with the −3 to +4 residues of known tryptophan- and tyrosine-containing LIRs.

(F) Parental Scrambled LC3B LC3B

(G) Paxillin/Zyxin

(H) Fold change

I) Fold change

(legend continued on next page)

Cell Reports 15, 1660–1672, May 24, 2016 1667
(Figure 6E). Furthermore, an in vitro binding assay demonstrated that paxillin was pulled down with GST (glutathione S-transferase)-LC3B, but not GST, demonstrating that LC3B is able to directly bind paxillin in the absence of any adaptors (Figure S6H). Consistent with these results, shRNA-mediated knockdown of LC3B (Figure S6I) led to an accumulation of paxillin (Figure 6F), enlarged FAs (Figure 6G), and reduced cell motility (Figures 6H and 6I), phenocopying the effects of Atg5 and Atg7 deficiency. These data illustrate the requirement for a direct interaction between paxillin and LC3B-II to promote targeted degradation of paxillin by autophagy and FA disassembly.

**Defining a LIR Motif in Paxillin that Is Regulated by Src**

To determine whether the interaction of paxillin with LC3 requires the putative LIR motif, we generated a paxillin mutant in which the critical tyrosine at the +1 position of the putative LIR motif, we generated a paxillin mutant that paxillin by autophagy and FA disassembly.

Defining a LIR Motif in Paxillin that Is Regulated by Src

Consistent with these results, shRNA-mediated knockdown of LC3B (Figure S6I) led to an accumulation of paxillin (Figure 6F), enlarged FAs (Figure 6G), and reduced cell motility (Figures 6H and 6I), phenocopying the effects of Atg5 and Atg7 deficiency. These data illustrate the requirement for a direct interaction between paxillin and LC3B-II to promote targeted degradation of paxillin by autophagy and FA disassembly.

**Autophagic Degradation of Paxillin Is Required for Src-Stimulated Motility**

To determine whether the Src-regulated interaction of paxillin with LC3 underlies the motility defects of autophagy-deficient cells, we performed transwell migration assays. Although the expression of SrcY527 strongly stimulated the motility of control 4T1 cells, it failed to do so in autophagy-deficient shAtg5 cells (Figure 7E). Furthermore, the motility of cells expressing mApple-paxillin-WT was strongly stimulated by the co-expression of SrcY527F (Figure 7E), but cells expressing either the Y40A or QEIAAA mutants were defective for Src-stimulated motility (Figure 7F). These data demonstrate that stimulation of the interaction between paxillin and LC3 by oncogenic Src and the subsequent autophagic degradation of paxillin promote cellular migration.

In summary, our work identifies a role for autophagy in promoting metastatic tumor cell migration and invasion by degrading paxillin and promoting FA disassembly that is dependent on the Src-regulated interaction of paxillin with processed LC3B.

**DISCUSSION**

We have demonstrated that autophagy promotes cell spreading, migration, and invasion of highly metastatic tumor cells in vitro and is required for early steps in the metastatic cascade in the 4T1 mouse mammary tumor model in vivo, with similar effects observed in the MDA-MB-231 human breast cancer and B16.F10 melanoma cell lines. Our data establish that autophagy is required to promote FA disassembly by degrading the FA protein paxillin, that paxillin interacts directly with LC3B, and that the conserved amino-terminal LIR motif is critical to the interaction. Consistent with the knowledge that Y40 in the paxillin LIR motif is an established Src phosphorylation target, expression of constitutively active SrcY527F dramatically increased both the interaction of paxillin with LC3B and tumor cell motility. Furthermore, Atg5-deficient cells were refractory to the migration-stimulatory effects of SrcY527F, indicating that autophagy plays a critical role downstream of oncogenic Src in promoting FA disassembly through targeted degradation of paxillin. Interestingly, Src phosphorylation of a critical tyrosine in the LIR motif of FUNDC1 has been reported to modulate the interaction of this protein also with LC3B (Liu et al., 2012)

Likely due to the aggressive nature of the metastatic tumor cells examined here, we did not observe significant reductions in cell growth or viability in vitro (Figures 1I and 1J) or in vivo (Figure 2B) following autophagy inhibition. Importantly, although autophagy-deficient 4T1 cells were unable to metastasize successfully from an orthotopic site, they were able to colonize the lung when injected directly into the circulation, indicating that survival in the circulation and outgrowth in the lung were not impaired by loss of autophagy in this model. Thus, we have been able to separate a specific requirement for autophagy in...
cell motility during metastasis that is independent of effects on proliferation or cell viability.

The link between autophagy and paxillin was first suggested based on genetic interactions between Atg1 and paxillin in *D. melanogaster* (Chen et al., 2008). However, the degradation of paxillin is not the only mechanism by which autophagy can modulate FA dynamics. Treatment of patient-derived glioblastoma cells with phosphatidylinositol 3-kinase, an upstream regulator of autophagy, increased tumor cell motility through mitochondrial redistribution to the cortical cytoskeleton to promote FA turnover (Caino et al., 2015). Additionally, FIP200, a protein that forms part of the Ulk1/Atg13 pre-initiation complex (Hara et al., 2008), inhibits FAK autophosphorylation and cellular migration when overexpressed (Abbi et al., 2002). Finally, AMPK activation induces autophagy but reduces cell migration in an Ulk1- and FIP200-dependent manner in glioblastoma, prostate cancer cells, and normal fibroblasts (Caino et al., 2013). We did not observe any change in FAK autophosphorylation (Figure S5A) or FIP200 levels (data not shown) when autophagy was inhibited, but it is possible that alterations in autophagic flux could alter FIP200-FAK signaling in other systems not studied here.

Our work adds to a growing body of work linking autophagy to tumor cell motility, invasion, and metastasis (Kenific et al., 2016; Lock et al., 2014; Macintosh et al., 2012; Qiang et al., 2014). A recent study identified a critical role for autophagy in the secretion of matrix metalloproteinases (MMPs) and cytokines during RAS-driven invasion (Lock et al., 2014), and knockdown of Atg12 has been shown to inhibit invasion in a glioblastoma cell line (Macintosh et al., 2012). Overexpression of autophagy genes has been associated with the more aggressive mesenchymal subtype of primary glioblastoma, and the substrate adaptor p62/Sqstm1 and autophagic flux were required for the invasion and migration of glioblastoma stem-like cell lines (Galavotti et al., 2013). However, an autophagy-independent role for p62 in promoting cell migration by binding Twist and preventing its degradation has also been identified (Qiang et al., 2014). We observed that paxillin turnover by autophagy was independent of p62/Sqstm1...
(Figure S6C), indicating that autophagy can modulate cellular dynamics through both p62-independent and p62-dependent mechanisms. We also show that, in the highly metastatic tumor cells examined here, NBR1, a different cargo receptor protein implicated in the migration of Ras-expressing epithelial cells (Kenfic et al., 2016), does not stabilize paxillin (Figure S6D). However, we cannot rule out the possibility that adaptors other than p62/Sqstm1 and NBR1 may facilitate binding of paxillin to LC3B. Finally, the levels of Twist and E-cadherin were unchanged in autophagy-deficient 4T1 cells (Figure S3F), indicating that the motility defects of autophagy-deficient tumor cells here were not explained by the mechanisms reported in other studies (Kenfic et al., 2016; Jiang et al., 2014). This suggests that autophagy regulates cell motility through different mechanisms in different cell types and that the mechanism described here may be specific to highly metastatic and mesenchymal tumor cells. For the requirement for autophagy in the survival of dormant tumor cells in GIST (Gupta et al., 2010) and ovarian cancer (Lu et al., 2008) illustrates an additional role for autophagy at a later step in the metastatic cascade. Together with our work identifying a critical role for autophagy in FA disassembly through paxillin degradation and its requirement for escape from the primary tumor, these studies highlight the potential utility of inhibiting autophagy to block tumor metastasis.

EXPERIMENTAL PROCEDURES

Cell Culture

Cells were grown in DMEM/10% FBS/1% penicillin and streptomycin (Pen/Strep) (1% MEM-NEAA was added to 4T1 media). Mouse Atg7 (TRCN0000375444), p62 (TRCN0000098619), Nbr1 (TRCN00000238310), MAP1LC3B/Atg8 (TRCN00000120800), paxillin (TRCN00000305202), and non-targeting control shRNA (SHC002) and custom shRNA to human/mouse MAP1LC3B/Atg8 (TRCN0000120800), paxillin (clones 177 [western blot; WB] and 349 [IF]), E-cadherin (BD Biosciences); Twist (Abcam); α-GFP (Santa Cruz); and α-ubiquitin (Pierce). Cells were lysed in RIPA (1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 10 mM Tris-HCl [pH 8.0], 0.14 M NaCl) or NP40 buffer (LC3 WBs; 150 mM NaCl, 50 mM Tris-HCl [pH 7.5], 1 mM EDTA, 1% NP40 buffer) with protease and phosphatase inhibitors. Lysates were centrifuged at 16,300 × g for 15 min at 4°C; supernatants were separated by SDS-PAGE. After transfer to nitrocellulose or polyvinylidene fluoride (PVDF) (LC3), membranes were blocked in milk or BSA and incubated at 4°C overnight with primary antibody. Detection was performed by enhanced chemiluminescence after 2-hr incubation with horseradish-peroxidase (HRP)-conjugated secondary antibodies (Dako).

For colo3, cells were lysed in IP lysis buffer (150 mM NaCl, 50 mM Tris-HCl [pH 8], 5 mM EDTA, 5% glycerol, 1% triton, 25 mM NaF) supplemented with phosphatase and protease inhibitors (Roche). Lysates were pre-cleared 1 hr with binding control agarose beads and incubated overnight with GFP-Trap agarose beads (Chromotek) at 4°C. Beads were washed in IP lysis buffer, and bound protein was eluted.

For in vitro binding assays, GFP-Trap agarose beads (Chromotek) were incubated with 5 μg GST (Novus) or GST-LC3B (Enzo) in IP lysis buffer for 1 hr at 4°C, washed, and blocked with 5% BSA overnight. Subsequently, beads were washed and incubated 1 hr with 0.5 μg paxillin (Origene) at 4°C. After final washes, bound protein was eluted. All densitometry was performed on scanned films using ImageJ (NIH). Band intensities were normalized to the loading control, and normalized values are reported as fold change relative to the first lane.

IF

For LC3B staining, cells were fixed in 4% PFA, permeabilized in 100% MeOH at −20°C, and blocked in 2% fetal bovine serum (FBS)/1% goat serum/PBS. Otherwise, cells were fixed and permeabilized (3.7% PFA/PBS in 100 mM PIPES [pH 6.8], 10 mM EGTA, 1 mM MgCl2, and 0.2% Triton-X), washed in Tris-buffered saline (TBS)/0.1% Triton-X, and blocked in TBS/0.1% BSA. Primary antibodies were α-LC3 (Cell Signaling), α-Paxillin (BD Biosciences clone 349), and α-Zyxin (B71, Mary Beckerle, University of Utah). Cells were imaged with an Axiovert 200M wide-field fluorescence microscope (Zeiss). Image deconvolution was performed with Openlab software (PerkinElmer); all other image analysis was performed with ImageJ.

Quantification of FA Size and Number

After image deconvolution, an ImageJ macro was written to perform the following steps: one round of background subtraction followed by thresholding on FAs with the renyi entropy algorithm. The analyze-particles function was used to measure the number of FAs and total FA area. To confirm that

lanes, and livers were harvested at 4 weeks post-orthotopic injection, fixed in 10% neutral buffered formalin (NBF), embedded in paraffin, sectioned, and stained as described later. For tail vein injections, 5 × 102 cells in 100 μl were injected per 8-week-old female BALB/c mouse (Charles River Laboratories), and lungs were harvested at 2 weeks and processed as described earlier. ImageJ was used to segment and count lung metastases on eight HE-stained 25-μm serial lung sections per mouse for both spontaneous and experimental metastasis assays.

Immunohistochemistry

KI-67 quantification, TUNEL staining, and LC3B immunohistochemistry were performed as described previously (Rosenfeldt et al., 2012). Paxillin was detected in situ using a specific antibody (Santa Cruz, H-114, 1:80 dilution) and standard immunohistochemistry. Stained sections were digitized and quantified using a ScanScope XT automated slide scanning system and Spectrum Plus image analysis software (Aperio). For Ki-67, a tuned nuclear quantification v9 algorithm was used to report DAB+ nuclei as a percentage of total (hematoxylin-stained) nuclei on ten random non-necrotic 0.25-mm2 sections per slide. For TUNEL, a tuned color deconvolution v9 algorithm was used to report DAB+ area as a percentage of total tissue area for each section.

Western Blots and IP Analyses

Primary antibodies were α-LC3, α-Atg5 (Novus); α-Atg7, α-β-actin (Sigma); α-Atg12, α-Nbr1 (Cell Signaling); α-paxillin (clones 177 [western blot; WB] and 349 [IF]), E-cadherin (BD Biosciences); Twist (Abcam); α-GFP (Santa Cruz); and α-ubiquitin (Pierce). Bound protein was eluted.

For coIPs, cells were lysed in IP lysis buffer (150 mM NaCl, 50 mM Tris-HCl [pH 8], 5 mM EDTA, 5% glycerol, 1% triton, 25 mM NaF) supplemented with phosphatase and protease inhibitors (Roche). Lysates were pre-cleared 1 hr with binding control agarose beads and incubated overnight with GFP-Trap agarose beads (Chromotek) at 4°C. Beads were washed in IP lysis buffer, and bound protein was eluted.

For in vitro binding assays, GFP-Trap agarose beads (Chromotek) were incubated with 5 μg GST (Novus) or GST-LC3B (Enzo) in IP lysis buffer for 1 hr at 4°C, washed, and blocked with 5% BSA overnight. Subsequently, beads were washed and incubated 1 hr with 0.5 μg paxillin (Origene) at 4°C. After final washes, bound protein was eluted. All densitometry was performed on scanned films using ImageJ (NIH). Band intensities were normalized to the loading control, and normalized values are reported as fold change relative to the first lane.

IF

For LC3B staining, cells were fixed in 4% PFA, permeabilized in 100% MeOH at −20°C, and blocked in 2% fetal bovine serum (FBS)/1% goat serum/PBS. Otherwise, cells were fixed and permeabilized (3.7% PFA/PBS in 100 mM PIPES [pH 6.8], 10 mM EGTA, 1 mM MgCl2, and 0.2% Triton-X), washed in Tris-buffered saline (TBS)/0.1% Triton-X, and blocked in TBS/0.1% BSA. Primary antibodies were α-LC3 (Cell Signaling), α-Paxillin (BD Biosciences clone 349), and α-Zyxin (B71, Mary Beckerle, University of Utah). Cells were imaged with an Axiovert 200M wide-field fluorescence microscope (Zeiss). Image deconvolution was performed with Openlab software (PerkinElmer); all other image analysis was performed with ImageJ.

Quantification of FA Size and Number

After image deconvolution, an ImageJ macro was written to perform the following steps: one round of background subtraction followed by thresholding on FAs with the renyi entropy algorithm. The analyze-particles function was used to measure the number of FAs and total FA area. To confirm that
this accurately represented the FAs in the original image, the particles analyzed were manually compared to the original. For each experiment, 10–12 images/120–150 cells per sample were analyzed.

Live-Cell Microscopy and Quantification of FA Dynamics
Timelapse DIC was performed with an Olympus LCV110U VivaView microscope system. 3D cell-depth imaging was performed using a Zeiss LSM780 microscope. Total intern al fluorescence reflection (TIRF) imaging of EGFP-paxillin FAs was performed with a Leica TIRFM superresolution microscope system (evanescent wave depth, 80 nm; images acquired every 60 s for 30 min). No significant photobleaching occurred. The fluorescent intensity of individual EGFP-paxillin FAs over time on background-subtracted images was determined using ImageJ. Semilogarithmic plots of fluorescent intensity as a function of time were linear for both assembly and disassembly of EGFP-paxillin FAs, and rate constants were calculated from the slopes. Average rate constants were determined from 10–15 FAs from five cells for each sample. Confocal imaging of mApple-paxillin and EGFP-LC3B was performed on an Olympus DSU Spinning Disk confocal microscope. Colocalization was analyzed on background-subtracted Z-stacks (0.5-μm slices) in ImageJ using the the JACoP object-based method (Bothe and Cordelieres, 2008) with 10–15 images (10–25 cells) per condition per experiment.

Statistics
Data were analyzed with GraphPad Prism. Significance was determined by unpaired Student’s t test for two-group comparisons and one-way ANOVA for comparisons of more than two groups. A Tukey post hoc test was used to identify significant pairwise differences when a one-way ANOVA identified a significant difference between means. Significance for tumor growth curves was determined by repeated-measures two-sample ANOVA.

SUPPLEMENTAL INFORMATION
Supplemental Information includes seven figures and five movies and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2016.04.065.

AUTHOR CONTRIBUTIONS

ACKNOWLEDGMENTS
We thank Mary Beckerie (University of Utah) for the Zyxin antibody (B71); Rick Horwitz (University of Virginia) for the EGFP-paxillin expression plasmid; Sara Courtneidge (OHSU) for the pSG5-SRC52 plasmid; Fred Miller (Wayne State University) for 4T1 tumor cells; Thomas Gajewski (University of Chicago) for B16.F10 melanoma cells; and Ana Pasapera (NIH NHLBI) for the paxillin IP protocol. This work is supported by NIH grant RO1 CA162405 (to K.F.M.), the University of Chicago Cancer Center Support Grant (P30 CA014599).

Received: June 18, 2014
Revised: February 26, 2016
Accepted: April 16, 2016
Published: May 12, 2016

REFERENCES


Fincham, V.J., and Frame, M.C. (1998). The catalytic activity of Src is dispensable for translocation to focal adhesions but controls the turnover of these structures during cell motility. EMBO J. 17, 81–92.


