

Class III PI3K Vps34 plays an essential role in autophagy and in heart and liver function

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A critical regulator of autophagy is the Class III PI3K Vps34 (also called PIK3C3). Although Vps34 is known to play an essential role in autophagy in yeast, its role in mammals remains elusive. To elucidate the physiological function of Vps34 and to determine its precise role in autophagy, we have generated Vps34^{fl/fl} mice, in which expression of Cre recombinase results in a deletion of exon 4 of Vps34 and a frame shift causing a deletion of 755 of the 887 amino acids of Vps34. Acute ablation of Vps34 in MEFs upon adenoviral Cre infection results in a diminishment of localized generation of phosphatidylinositol 3-phosphate and blockade of both endocytic and autophagic degradation. Starvation-induced autophagosome formation is blocked in both Vps34-null MEFs and liver. Liver-specific Albumin-Cre;Vps34^{fl/fl} mice developed hepatomegaly and hepatic steatosis, and impaired protein turnover. Ablation of Vps34 in the heart of muscle creatine kinase-Cre;Vps34^{fl/fl} mice led to cardiomegaly and decreased contractility. In addition, while amino acid-stimulated mTOR activation was suppressed in the absence of Vps34, the steady-state level of mTOR signaling was not affected in Vps34-null MEFs, liver, or cardiomyocytes. Taken together, our results indicate that Vps34 plays an essential role in regulating functional autophagy and is indispensable for normal liver and heart function.

LC3 | SQSTM1/p62 | 3-MA | epidermal growth factor receptor | transferrin

Macroautophagy (referred to as autophagy hereafter) is a dynamic membrane trafficking process that involves the delivery of intracellular content to lysosomes for degradation. A fully executed autophagy includes the formation of double-membraned autophagosomes, the fusion of autophagosomes to late endosomes/lysosomes, and the digestion of the enclosed content by lysosomal hydrolases. Autophagy is constantly maintained at the basal level and is up-regulated in response to stress conditions, such as nutrient and energy limitation, hypoxia, and DNA damage. Autophagy is necessary for cellular and tissue homeostasis, by eliminating damaged organelles and misfolded proteins, and its dysregulation is implicated in developmental defects and numerous diseases (1–5).

Vps34 is the Class III phosphoinositide 3-kinase (PI3K) that phosphorylates phosphatidylinositol to generate phosphatidylinositol 3-phosphate [PI(3)P], a phospholipid central for membrane trafficking processes. The localized production of PI(3)P acts to recruit effector proteins containing FYVE or PX domains that control membrane docking and fusion during the formation of internal vesicles (6). Vps34 is the only PI3K identified in yeast thus far, and its essential role in vacuolar protein delivery was initially described through yeast genetics studies (7). In metazoans, Vps34 has been found to play a critical role in endocytosis, autophagy, and TOR activation (8, 9). The essential role of Vps34 in autophagy has been established largely through the use of the pharmacological inhibitors wortmannin and 3-methyladenine (3-MA), which have been used to suppress autophagy in many studies in the literature. However, because of the lack of

specificities of these PI3K inhibitors, the precise role of Vps34 in autophagy remains unclear. It was only recently that the physiological role of mammalian Vps34 has been investigated in mouse gene knockout studies (10, 11). Surprisingly, it was reported that although genetic deletion of Vps34 in sensory neurons leads to disruption of the endocytic pathway, the autophagic pathway is still intact in these cells, raising the question as to whether Vps34 is necessary for autophagy in mammals (10).

In the present study, we have generated conditional Vps34 gene knockout mice. Through tissue-specific expression of the Cre recombinase, Vps34 is deleted in the liver or heart, two organs where autophagy is known to play physiological roles. We have also generated Vps34^{fl/fl} murine embryonic fibroblasts (MEFs), in which Vps34 can be acutely deleted through infection with adenoviral Cre. We report here that ablation of Vps34 leads to a blockade of autophagy in MEFs, liver, and heart, and that Vps34 plays an essential role in maintaining normal liver and heart functions.

Results

Generation of Vps34 Conditional Knockout Mice. Vps34 (PIK3C3) conditional knockout mice (Vps34^{fl/fl}) were generated with exon 4 of the Vps34 gene flanked by loxP sites (Fig. 1A and Fig. S1A). When exposed to Cre recombinase, exon 4 is excised, causing a frame-shift mutation leading to the deletion of 755 of the 887 amino acids of Vps34. The Vps34^{fl/fl} mice were bred to muscle creatine kinase (Mck)-Cre or albumin (Alb)-Cre mice to generate tissue-specific knockouts in heart and liver, respectively, to study the effect of Vps34 loss in these organs.

Characterization of Vps34-Null MEFs. Mouse embryonic fibroblasts (MEFs) were generated from day 13–16 embryos of Vps34^{+/fl} matings. MEFs with each of the possible genotypes were immortalized with SV40 large T antigen. Expression of Cre recombinase resulted in virtually complete loss of Vps34 protein as shown by immunoblotting (Fig. 2A). Vps34^{+/+} MEFs infected with adenoviral Cre or Vps34^{fl/fl} MEFs infected with control adenovirus were used as controls for possible effects of flox or Cre expression. No obvious differences between the two groups of MEFs were observed. Vps34-null MEFs displayed a drastic proliferative defect compared with the wild-type control (Fig. S1B). By day 6 after Cre addition, large translucent vacuoles appeared in the Vps34-null MEFs (Fig. 1B). These vacuoles

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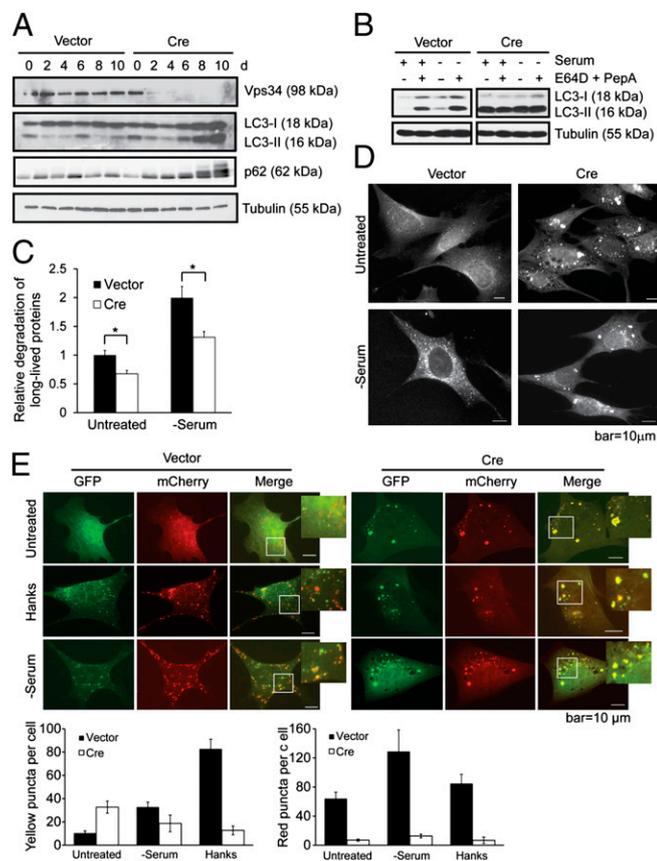


Fig. 2. Autophagy flux is impaired in *Vps34*^{fl/fl} MEFs. (A) *Vps34*^{fl/fl} MEFs were infected with control or Cre virus. Cell lysates were collected at indicated time points after infection and probed for Vps34, LC3, and p62. The fluctuation of the levels of LC3 and p62 in control cells may be due to the change of confluency during passaging. (B) *Vps34*^{fl/fl} MEFs were infected with vector control or Cre. At 7 d after infection, cells were left untreated or serum deprived for 6 h, with or without the addition of lysosomal protease inhibitors E64D (10 μ g/mL) and PepA (10 μ g/mL). (C) MEFs were labeled with ¹⁴C-valine for 24 h and left untreated or cultured in serum-free medium for 6 h. Degradation of long-lived proteins was measured. Average of three independent experiments \pm SEM is shown. **P* < 0.05. (D) *Vps34*^{fl/fl} MEFs infected with vector or Cre virus were cultured in serum-free medium for 6 h. Cells were stained for LC3 for immunofluorescence analysis. Note that although serum deprivation induces LC3 puncta in vector control cells, LC3 forms large-sized structures upon Cre infection, with no further increase upon serum starvation. (E) Vector and Cre-infected *Vps34*^{fl/fl} MEFs were transfected with mCherry-GFP-LC3. Forty-eight hours after transfection, cells were starved in Hanks buffer or serum-free medium. Cells were observed under a deconvolution microscope. Representative images are shown. The average numbers of yellow or red puncta were obtained from three countings. Note although nutrient starvation induces both autophagosomes (yellow) and autolysosomes (red) in wild-type cells, both inductions are inhibited in *Vps34*-null cells.

Vps34-null cells, the gold particles tend to form aggregates that were not associated with membrane structures, indicating that the large-sized LC3-positive structures are not autophagosomes and are instead part of protein aggregates (Fig. S3B).

To further assess whether functional autophagic flux is inhibited in *Vps34*-null cells, we used the mCherry-GFP-LC3 construct. Owing to the more stable nature of mCherry compared with GFP in the acidified compartment, functional autophagic flux can be determined by the appearance of more red puncta (15). In wild-type MEFs, a number of red puncta are observed under fed conditions, representing the basal level autophagy (Fig. 2E). Upon nutrient starvation, the numbers of both yellow (as a result

of merged green and red signals) and red puncta increased, indicating an increase in autophagosomes and autolysosomes, respectively. In contrast, large-sized yellow aggregates were observed in both fed and nutrient-starved conditions in *Vps34*-null MEFs, and few red puncta were observed under either fed or starved conditions (Fig. 2E), suggesting that both autophagosome formation and autolysosome function are blocked in *Vps34*-null cells.

Ablation of *Vps34* Leads to Diminished Localized PI(3)P Production and Autophagosome Formation. It is believed that the localized production of PI(3)P is essential for autophagy. In yeast, *Vps34* is the sole PI3K responsible for PI(3)P production (9, 16). However, in metazoans, PI(3)P may be produced via *Vps34*-independent mechanisms, including dephosphorylation of PtdIns (3,4,5)P₃ by PtdIns 5-phosphatases and 4-phosphatase (17) or directly through the Class II PI3K (18). To observe PI(3)P generation, the PI(3)P binding domain FYVE conjugated to GFP (GFP-2xFYVE) was expressed, which forms puncta in wild-type cells (Fig. 3A). In sharp contrast, the formation of FYVE puncta was virtually absent in *Vps34*-null MEFs (Fig. 3A), indicating the lack of localized production of PI(3)P. Similarly, the PI3K activity associated with *Vps34* or Beclin 1 immunoprecipitates was substantially lower in *Vps34*-null MEFs than in wild-type cells (Fig. S4).

Because intracellular PI(3)P has been described to play critical roles for both endocytosis and autophagy, we examined the autophagic pool of PI(3)P by determining the localization of GFP-DFCP1 (double FYVE domain-containing protein 1), which localizes to omegasomes in a PI(3)P-dependent manner upon autophagy initiation (19). Although serum starvation induced the punctate pattern of GFP-DFCP1 in wild-type cells, it failed to do so in *Vps34*-null cells (Fig. 3B). Moreover, although serum starvation induced Atg12 punctate formation and partial colocalization with GFP-DFCP1 in wild-type cells, Atg12 remained diffused in *Vps34*-null cells (Fig. 3B). These results, together with the observations that cargo-containing autophagosomes were absent in *Vps34*-null MEFs and livers (Figs. 1C and 4F and Fig. S3B), indicate that early autophagosome formation is blocked in *Vps34*-null cells. Interestingly, *Vps34* has been implicated in regulating Atg5-Atg12 conjugation (20), which complexes with Atg16L and serves as an E3-like enzyme for LC3 conjugation to PE. Although an increase in free Atg5 was observed, the level of conjugated Atg5-Atg12 was not significantly affected in *Vps34*-null cells (Fig. S5). This finding is consistent with the observation that LC3 lipidation can occur in the absence of *Vps34* (Figs. 2A and B and 4A), indicating that Atg5-Atg12 conjugation is independent of *Vps34*.

***Vps34* Is Essential for Acute mTOR Activation by Amino Acids but Not for Steady-State mTOR Signaling.** In addition to regulation of membrane trafficking, an undefined function of *Vps34* is the activation of TOR signaling. We examined mTOR activation in MEFs upon amino acid stimulation. Activation of mTOR as assessed by phospho-S6 and phospho-4EBP1 was compromised in *Vps34*-null MEFs (Fig. 3C), indicating that *Vps34* plays an essential role in mediating amino acid activation of mTOR. Although this finding is in agreement with studies using *Vps34* RNAi silencing (21–23), we also noticed that at the steady state, mTOR activation in MEFs cultured in complete medium was not affected by *Vps34* deficiency (Fig. 3C). No apparent defect in mTOR signaling was observed in the *Vps34*-null liver under either fed or fasted conditions (Fig. 3D). Similarly, mTOR signaling was not affected by the lack of *Vps34* in isolated cardiomyocytes cultured in complete medium (Fig. 3E). Therefore, although *Vps34* plays an important role in mTOR activation upon acute amino acid stimulation, it is dispensable for mTOR signaling at the steady state.

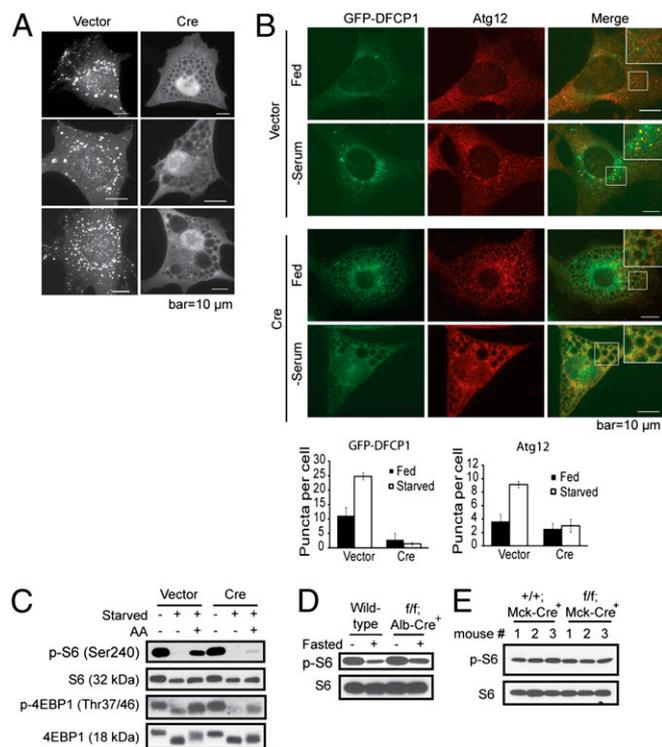


Fig. 3. Localized production of PI(3)P, autophagosome formation, and mTOR signaling are impaired in the absence of Vps34. (A) Ablation of Vps34 leads to diminished PI(3)P production. Vps34^{fl/fl} MEFs stably expressing GFP-YFVE were infected with vector control or Cre. Three representative cells from each group are shown. Note the lack of YFVE puncta in Cre-infected MEFs, indicating the absence of PI(3)P. (B) Serum starvation fails to induce DFCP1 and Atg12 puncta in Vps34-null cells. Vps34^{fl/fl} MEFs were infected with vector control or Cre virus for 5 d. Cells were transiently transfected with GFP-DFCP1. Forty-eight hours after transfection, cells were fixed and immunofluorescence for Atg12 was performed. Representative images are shown. The average numbers of yellow or red puncta were determined from eight countings and are shown with SEM. Note that upon serum withdrawal, vector control cells exhibit colocalization of DFCP1 and Atg12 puncta. In contrast, no puncta formation was observed for either DFCP1 or Atg12 in the Vps34-null cells. (C) Amino acid-stimulated mTOR activation is compromised in the absence of Vps34. Vps34^{fl/fl} MEFs infected with vector or Cre were starved and stimulated for 30 min with 2× MEM amino acids. mTOR signaling was measured by the levels of phospho-S6 and phospho-4EBP1. (D and E) Steady-state mTOR signaling is not affected by the loss of Vps34. Whole liver lysates from wild-type and Vps34^{fl/fl}; Alb-Cre⁺ mice (D) and lysates from cardiomyocytes isolated from wild-type and Vps34^{fl/fl}; Mck-Cre⁺ mice (E) were probed for phospho-S6 and total S6.

Vps34 Plays an Essential Role in the Liver. To study the effect of Vps34 deletion in vivo, we bred Vps34^{fl/fl} animals with Alb-Cre mice, in which expression of Cre is under the control of the albumin promoter, resulting in deletion of floxed genes specifically in the liver by 6 wk of age (24). Successful deletion of Vps34 was confirmed in hepatocytes isolated from 8-wk-old mice (Fig. 4A). Littermates with either Vps34^{fl/fl}; Alb-Cre⁻ or Vps34^{+/+}; Alb-Cre⁺ genotypes were used as controls for the possible effect of either floxed Vps34 or Cre, and did not show apparent differences, hence are both referred to as wild-type mice. In addition, heterozygous Vps34^{+/-}; Alb-Cre⁺ mice were indistinguishable from wild-type mice. Noticeably, liver-specific Vps34^{-/-} mice were smaller than wild-type mice and exhibited enlarged and pale livers (Fig. 4B). Histological analysis revealed the presence of intracellular vacuolation, and Oil Red O staining indicated an accumulation of lipids in Vps34^{-/-} livers (Fig. 4C). Additionally, Periodic Acid Schiff (PAS) staining revealed a lack of glycogen

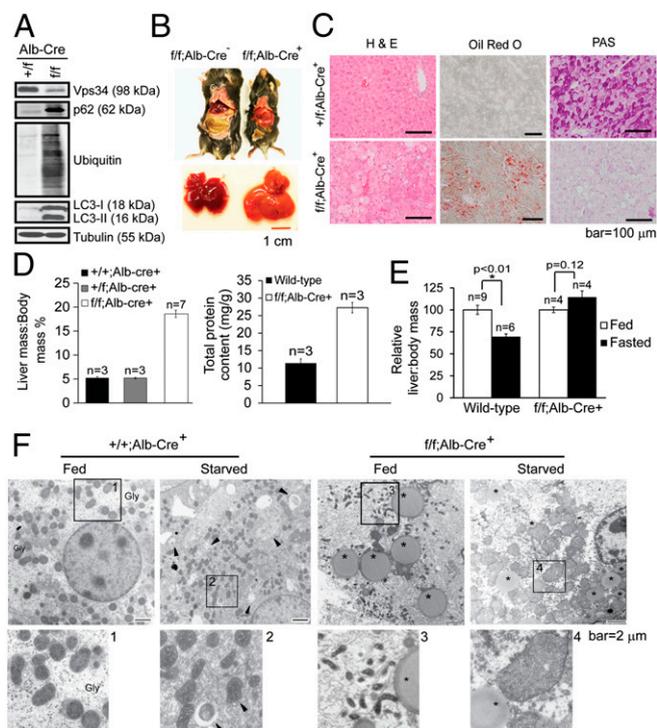


Fig. 4. Ablation of Vps34 in the liver leads to hepatomegaly and steatosis. (A) Lysates from primary hepatocytes with indicated genotypes were probed with indicated antibodies. Note the accumulation of p62, LC3-I/II, and polyubiquitinated proteins in Vps34-null hepatocytes, indicative of blocked autophagic protein degradation. (B) Gross anatomical views of representative mice and livers with indicated genotypes. (C) Livers isolated from mice with indicated genotypes were stained with hematoxylin and eosin (H&E), Oil Red O, or Periodic Acid Schiff (PAS). Representative images are shown. (D) The mass of livers with indicated genotypes was measured and expressed relative to body mass (Left). Total protein content of wild-type and Vps34-null livers was quantified and expressed relative to the body weight (Right). (E) Wild-type and Vps34^{fl/fl}; Alb-Cre⁺ mice were fasted for 24 h. Liver/body mass ratio was determined and expressed as normalized to that of respective fed animals. Note the difference between fed and fasted Vps34-null livers is statistically insignificant. (F) Electron micrographic images of livers from fed and 24 h fasted Vps34^{fl/fl}; Alb-Cre⁻ and Vps34^{fl/fl}; Alb-Cre⁺ mice. Higher magnification views of boxed areas are numbered and shown in Lower. Gly, glycogen area; arrowheads point to autophagosomes; asterisks denote lipid droplets. Note the lack of glycogen deposition, accumulation of lipid droplets, and smaller mitochondria in fed Vps34-null livers. Starved Vps34-null livers are devoid of autophagosomes but are noted to have swollen mitochondria.

deposits (Fig. 4C). Both liver mass and liver protein content of Alb-Cre⁺; Vps34^{fl/fl} mice were significantly higher than that of wild-type mice (Fig. 4D). After 24 h of fasting, wild-type mice showed a decrease of liver mass of ~30%, whereas no significant liver weight change was detected in Vps34^{fl/fl}; Alb-Cre⁺ mice (Fig. 4E). Increased levels of p62, LC3-I/II, and polyubiquitinated proteins were observed in isolated hepatocytes (Fig. 4A) and in Vps34-null total liver lysates (Fig. S6A). By EM, Vps34-null livers revealed deficient hyaloplasmic glycogen deposits and the presence of large lipid droplets (Fig. 4F), consistent with the PAS and Oil Red O staining results (Fig. 4C). In certain hepatocytes in the Vps34-null liver, large-sized vacuoles representing swollen endosomes/lysosomes were observed, similar to what was seen in the Vps34-null MEFs (compare Fig. S6B and Fig. 1C). Interestingly, a large number of small-sized mitochondria was observed in the Vps34^{-/-} livers under fed conditions (Fig. 4F, box 3), suggesting a possible defect in mitochondrial fusion. Moreover, although a 24-h fast induced autophagosomes in wild-

type liver, Vps34-null liver displayed virtually no autophagosomes and many swollen mitochondria (Fig. 4F).

To further study the involvement of Vps34 in liver autophagy, Vps34^{fl/fl} mice were bred with GFP-LC3 transgenic mice (25), then with the Alb-Cre mice. As expected, a diffused pattern of GFP-LC3 was observed in the wild-type liver. A 24-h fast caused the formation of GFP-LC3 puncta in the wild-type liver, representative of starvation-induced autophagy (Fig. S6C). However, in the Vps34^{-/-} livers, large GFP-LC3 aggregates were observed (Fig. S6C), similar to those in Vps34^{-/-} MEFs (Fig. 2D and E and Fig. S3). The amount and pattern of GFP-LC3 aggregates appeared unaffected by fasting (Fig. S6C). The majority of the Alb-Cre;Vps34^{fl/fl} mice died within 1 yr (Fig. S6D). Taken together, these results indicate that functional autophagy flux is blocked in Vps34^{-/-} livers. The appearance of hepatomegaly, hepatic steatosis, increased liver protein content, lack of glycogen deposition, and increased fatality are highly consistent with the phenotype observed in the autophagy-deficient Atg7^{-/-} and Atg5^{-/-} livers (26–28).

Vps34 Plays an Essential Role in the Heart. Autophagy also plays an important role in the heart. As such, Vps34^{fl/fl} mice were bred to Mck-Cre mice (29) to delete Vps34 in cardiomyocytes (Fig. 5F and Fig. S7A). Gross anatomical analysis of Mck-Cre;Vps34^{fl/fl} hearts revealed marked cardiomegaly (Fig. 5A) and increased heart to body weight ratio (Fig. 5B). Echocardiography of the animals in vivo demonstrated an increase in left ventricular wall thickness and mass, and decreased cardiac contractility with lower ejection fraction and fractional shortening (Fig. 5C and Table 1). Although they appeared normal at birth, Mck-Cre;Vps34^{fl/fl} mice died between 5 and 13 wk of age (Fig. 5D). Electron microscopy revealed disorganized mitochondria and Z-lines in Vps34^{-/-} hearts, and an increased number of small-sized mitochondria and enlarged vacuoles (Fig. 5E). Consistent

Table 1. Echocardiographic measurements

Measurement	Wild-type	Vps34 ^{fl/fl} ;Cre ⁺
LVEDD, mm	3.17 ± 0.06	2.93 ± 0.18
LVESD, mm	1.58 ± 0.03	2.03 ± 0.14
SWT, mm	0.13 ± 0.01	0.43 ± 0.13
PWT, mm	0.96 ± 0.03	0.94 ± 0.16
FS, %	50.0 ± 3.4	30.1 ± 3.4*
EF, %	82.3 ± 3.2	57.7 ± 5.2*
LV mass, mg	34.8 ± 4.5	49.8 ± 6.4*

EF, ejection fraction; FS, fractional shortening; LV, Left ventricular; LVEDD, LV end-diastolic diameter; LVESD, LV end-systolic diameter; PWT, posterior wall thickness; SWT, septal wall thickness. Values are mean ± SEM. *Significantly different from the control group, $P < 0.05$, Student's t test. $n = 4$ for control; $n = 6$ for Vps34^{fl/fl};Cre⁺.

with the Vps34-null MEFs and liver, Vps34^{-/-} hearts and cardiomyocytes displayed elevated levels of p62, LC3-I/II, and polyubiquitinated proteins (Fig. 5F and Fig. S7A), and the presence of large GFP-LC3 aggregates (Fig. S7B). These data indicate that Vps34 is necessary for autophagic flux in the heart, and that Vps34 ablation leads to heart failure and death.

Discussion

Class III PI3K Vps34 is the only PI3K that is evolutionarily conserved from yeast to mammals (30). It has been implicated as a critical regulator for a number of molecular and cellular events including endocytic trafficking, autophagy, and mTOR signaling. Its versatile molecular function has been attributed to its various binding partners and distinct subcellular localizations (31–33). Its importance is exemplified by the fact that germ-line loss of Vps34 leads to embryonic lethality (10). Although Vps34 has been shown to play an essential role in autophagy in yeast and *Drosophila* (34, 35), its precise molecular function in mammalian autophagy remained elusive. A recent loss-of-function study in mouse sensory neurons suggested that functional autophagy flux still exists upon Vps34 deletion (10). Using a conditional loss-of-function mouse model of Vps34, we have examined the role of Vps34 in MEFs, liver, and heart. In agreement with the literature, endocytic protein turnover was markedly deficient in Vps34-null cells (Fig. 1E). Importantly, marked accumulation of p62, LC3, and polyubiquitinated proteins was observed in both Vps34-null liver and heart. Acute deletion of Vps34 in MEFs resulted in blocked autophagic degradation as assessed by various biochemical assays. Furthermore, large GFP-LC3 aggregates were observed in Vps34-null cells and tissues, similar to what was reported (10). Characterization of autophagy flux clearly indicated the accumulation of large-sized LC3 structures results from abrogated flux rather than induction of the onset of autophagy. Therefore, the abnormalities that we observe in Vps34-null liver and heart such as hepatomegaly, hepatic steatosis, cardiac hypertrophy, and defective contractility can be at least partially explained by defective autophagy because these phenotypes were also observed in Atg7^{-/-} and Atg5^{-/-} animals (26, 27, 36). In comparison with the Atg5- and Atg7-deficient settings, the more severe phenotype caused by loss of Vps34 may be due to the loss of autophagy-independent functions such as endocytosis.

Although the precise role of Vps34 throughout the full autophagic process including formation of omegasomes, maturation of autophagosomes, and autophago-lysosome fusion still needs to be determined, our study indicates that the formation of functional autophagosomes is largely impaired in Vps34-null cells. This conclusion is supported by the lack of electron-dense material in intracellular vacuoles observed by EM (Figs. 1C and 4F and Fig. S3B) and by the failure of GFP-DFCP1 and Atg12 puncta formation (Fig. 3B). It is interesting to note that several phenomena that we observed in Vps34-null cells (accumulation

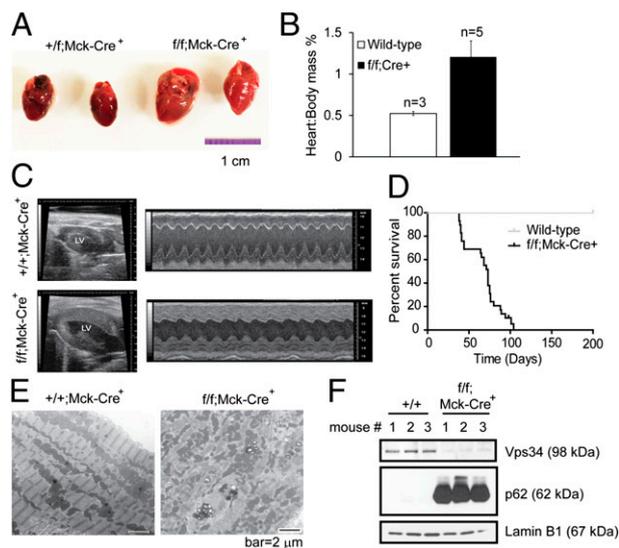


Fig. 5. Cardiac dysfunction in Mck-Cre;Vps34^{fl/fl} mice. (A) Hearts isolated from 6-wk-old Mck-Cre;Vps34^{fl/fl} and Mck-Cre;Vps34^{fl/fl} mice were photographed. (B) Hearts were weighed, and the heart mass is normalized to body mass. (C) Representative B-mode (left ventricle labeled) and M-mode echocardiographic images of control and Mck-Cre;Vps34^{fl/fl} mice. (D) Kaplan–Meier survival curve of Mck-Cre;Vps34^{fl/fl} mice. (E) Representative EM images of control and Mck-Cre;Vps34^{fl/fl} hearts. Note that wild-type heart section shows characteristic alignment of mitochondria and organization of Z-lines, whereas the Vps34-null tissue shows disorganized mitochondria and Z-lines. (F) Cardiomyocytes were isolated from control and Mck-Cre;Vps34^{fl/fl} hearts. Cell lysates were probed with indicated antibodies.

of LC3 aggregates and LC3 lipidation) are distinct from what is observed by using PI3K inhibitors such as 3-MA or wortmannin, suggesting Vps34-independent effects of these inhibitors in suppressing autophagy.

Our study also indicates a positive role of Vps34 in regulating the mTOR signaling in response to acute amino acid stimulation (Fig. 3C). This notion is in agreement with previous reports using RNAi and overexpression approaches (21–23), and in Vps34-null embryos (11), because the early stage embryo depends on nutrients rather than growth factors to activate mTOR (37). However, consistent with a study in *Drosophila* showing that TOR activity is not affected by loss of Vps34 in the whole larvae (35), we observe here that the steady-state mTOR activation is not affected by loss of Vps34 (Fig. 3D and E). Therefore, the effect of Vps34 on TOR may depend on the duration (acute vs. chronic) and/or type (nutrient vs. hormonal) of stimuli.

Our study suggests that cautions should be taken when using Vps34 inhibitors in research and in the clinic. The role of autophagy in the initiation, development, and maintenance of human cancers is under intensive investigation (2, 38–41). Pharmacological inhibitors of autophagy to be used in conjunction with traditional anti-cancer therapies are under investigation (42). Our study, together with other reports (10, 11), shows that complete ablation of Vps34 leads to profound cellular and organ damage. Hence, systemic use of Vps34 inhibitors as anticancer drugs may have serious side

effects. Molecules that dictate Vps34 for distinct cellular activities might be more viable drug targets.

Materials and Methods

Mice. Vps34 (Pik3c3) gene conditional knockout ES cells in the C57BL/6 background were purchased from Sanger Institute. See *SI Materials and Methods* for further details for the creation of Vps34^{fl/f} mice. GFP-LC3 transgenic mice were a gift from Noboru Mizushima (Tokyo Medical and Dental University, Tokyo, Japan) (25). Alb-Cre and Mck-Cre mice were purchased from the Jackson Laboratory. For fasting studies, mice were deprived of food while having free access to water. All mouse experiments were done in compliance with the Stony Brook University and University of Alabama at Birmingham Institutional Animal Care and Use Committee guidelines.

Statistics. Student's *t* test was used to compare the differences between two groups. Significance was judged when *P* < 0.05. Kaplan–Meier curves for mouse survival were constructed using the Log-rank (Mantel–Cox) test.

For other materials and methods, please see *SI Materials and Methods* for details.

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