SHORT TAKE

Intact endothelial autophagy is required to maintain vascular lipid homeostasis

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Summary

**The physiological role of autophagic flux within the vascular
endothelial layer remains poorly understood. Here, we show that
in primary endothelial cells, oxidized and native LDL stimulates
autophagosome formation. Moreover, by both confocal and elec-
tron microscopy, excess native or modified LDL appears to be
engulfed within autophagic structures. Transient knockdown of the
essential autophagy gene ATG7 resulted in higher levels of intra-
cellular ^{125}I-LDL and oxidized LDL (OxLDL) accumulation, suggesting
that in endothelial cells, autophagy may represent an important
mechanism to regulate excess, exogenous lipids. The physiological
importance of these observations was assessed using mice contain-
ing a conditional deletion of ATG7 within the endothelium. Follow-
ing acute intravenous infusion of fluorescently labeled OxLDL, mice
lacking endothelial expression of ATG7 demonstrated prolonged
retention of OxLDL within the retinal pigment epithelium (RPE) and
choroidal endothelium of the eye. In a chronic model of lipid excess,
we analyzed atherosclerotic burden in ApoE$^{-/-}$ mice with or without
endothelial autophagic flux. The absence of endothelial autophagy
markedly increased atherosclerotic burden. Thus, in both an acute
and chronic *in vivo* model, endothelial autophagy appears critically
important in limiting lipid accumulation within the vessel wall. As
such, strategies that stimulate autophagy, or prevent the age-
dependent decline in autophagic flux, might be particularly bene-
ficial in treating atherosclerotic vascular disease.**
Key words: autophagy; lipids; atherosclerosis; mouse.

Cardiovascular disease, driven in part by the accumulation of modified
lipids within the vessel wall, represents the leading cause of death in
developed nations (Go *et al.*, 2013). Considerable attention and study

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has been directed at the molecular consequences following the
subendothelial deposition of lipids. These consequences include
the recruitment of inflammatory cells and the subsequent engulfment
of this deposited subendothelial lipid by resident macrophages
(Chinetti-Gbaguidi *et al.*, 2014; Randolph, 2014). In contrast, relatively
little is known regarding the steps proximal to lipid deposition and what
regulatory role, if any, the endothelium might play in this process.

While evidence is limited for the case of the endothelium, in other cell
types it appears that autophagy may play a critical role in maintaining
overall lipid homeostasis. For instance, mice bearing ATG5-deficient
macrophages appear to exhibit increased plaque formation when bred
with pro-atherogenic mouse strains (Liao *et al.*, 2012; Razani *et al.*,
2012). While the basis for this increased plaque burden is undoubtedly
complex, evidence suggests that macrophage-specific ATG5 deletion
results in impaired lipophagy (Sergin & Razani, 2014). Lipophagy refers
to the specific degradation of lipids by the autophagic machinery. This
concept was perhaps first described in the liver where genetic disruption
of macroautophagy led to the accumulation of lipid droplets (Singh
et al., 2009). These initial observations have been extended and
lipophagy now appears to be important in maintaining lipid homeostasis
in a diverse set of cell types from neurons to fibroblasts (Singh *et al.*,
2009; Liu & Czaja, 2013; Settembre & Ballabio, 2014). Here, we
demonstrate an important role for endothelial autophagy in maintaining
vascular lipid homeostasis.

It has been previously observed that when endothelial cells in culture
are exposed to OxLDL they respond with an increase in autophagosomes
(Nowicki *et al.*, 2007; Zhang *et al.*, 2010; Muller *et al.*, 2011; Menghini
et al., 2014). In general, autophagic flux can be assessed by simultane-
ously analyzing the levels of LC3-II, which labels autophagosome
membranes, and measuring the levels of p62, a protein cleared via
autophagic degradation. The combination of increased LC3-II along with
decreased p62 levels is consistent with an overall increase in autophagic
flux. Consistent with past observations, following exposure of human
umbilical vein endothelial cells (HUVECs) to OxLDL, the level of LC3-I to
LC3-II conversion was increased (Fig. 1A,B). As we have previously
described (Torisu *et al.*, 2013), siRNA -mediated knockdown of ATG7
was effective in reducing ATG7 expression in HUVECs (Fig. S1A,B,
Supporting information), and endothelial cells with reduced ATG7
expression had markedly impaired capacity to generate LC3-II before or
after OxLDL exposure (Fig. 1A,B). The level of LC3-II following OxLDL
exposure was further enhanced by the presence of the lysosomal
inhibitor chloroquine (Fig. 1C). In contrast, levels of p62 were not as
markedly affected after OxLDL exposure (Fig. 1A and Fig. S1C) raising
the possibility, as previously noted, of alteration in lysosomal function
after OxLDL exposure (Jerome, 2010). When primary endothelial cells
were electroporated with a plasmid encoding GFP-LC3 and subsequently
exposed to fluorescently labeled OxLDL, we noted an increase in LC3 dot
formation, again consistent with an increase in autophagosomes
(Fig. S1D,E). Interestingly, the OxLDL within endothelial cells was often
surrounded by GFP-LC3 positive structures whose appearance was
consistent with autophagosomes (Fig. 1D). These observations sug-
gested that following uptake of OxLDL, lipids may be directly or indirectly
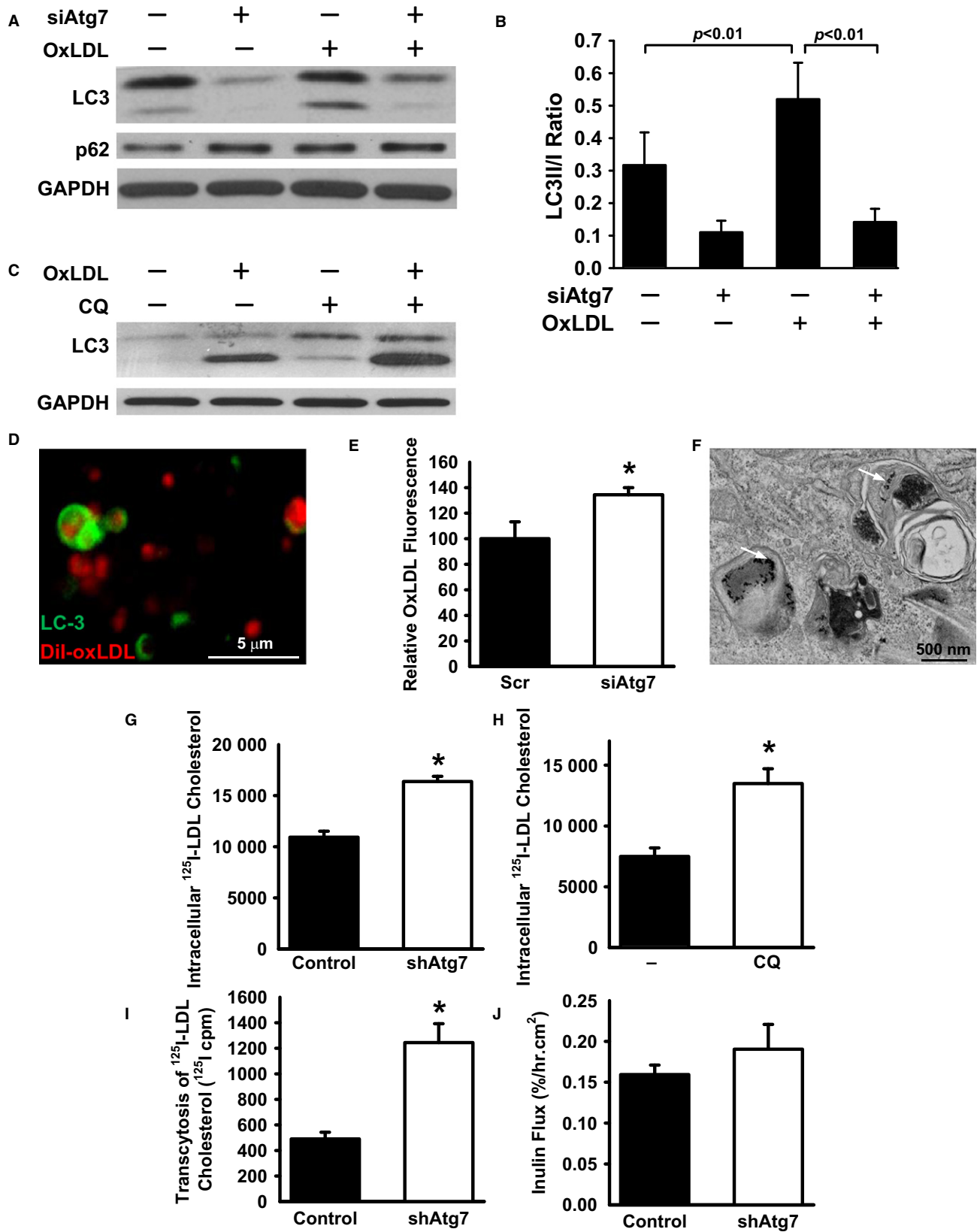
trafficked into autophagosomes. Moreover, given the growing evidence that intracellular lipids can be degraded by lysosomal lipases (Singh & Cuervo, 2012; Liu & Czaja, 2013; Settembre & Ballabio, 2014), intact endothelial autophagy might modulate levels of OxLDL accumulation. Consistent with this hypothesis, OxLDL accumulated at higher levels in endothelial cells lacking an intact autophagy machinery (Fig. 1E).

Native LDL is thought to be taken up by receptor-mediated endocytosis and delivered to lysosomes through the endosomal pathway. It has been appreciated for some time that genetic deficiencies in lysosomal lipase impairs cholesterol hydrolysis leading to intracellular lipid accumulation (Goldstein *et al.*, 1975). Increasing evidence also suggests that endosomal and autophagosomal pathways are closely connected and share common machinery (Rusten *et al.*, 2012; Hyttinen *et al.*, 2013). Electron micrographs of HUVECs incubated with gold-labeled native LDL identified gold-labeled particles within double membrane autophagosomal structures (Fig. 1F). Similar to what we observed following OxLDL treatment, native LDL also appeared to increase the number of autophagosomes (Fig. S1F,G) and to be in close contact with GFP-LC3 positive structures (Fig. S1H). As we have previously described, we next used lentivirus-mediated shRNAs to stably knockdown ATG7 expression (Torisu *et al.*, 2013). We noted that ^{125}I-LDL cholesterol accumulated to a higher level in cells with reduced Atg7 (Fig. 1G). Increased ^{125}I-LDL cholesterol accumulation was also observed following treatment of endothelial cells with the autophagy and lysosomal inhibitor chloroquine (Fig. 1H). This increase was not due to any apparent alterations in levels of the LDL receptor of surface binding of ^{125}I-LDL cholesterol (Fig. S1I,J).

The deposition of native or modified lipids in the sub-endothelial space is thought to contribute to vascular disease. To begin to model this phenomenon in vitro, we analyzed the transcytosis of ^{125}I-LDL cholesterol in confluent monolayers of HUVECs following control or ATG7 knockdown. As noted in Fig. 1I, following a 24-h incubation, there was evidence for increased ^{125}I-LDL transcytosis across ATG7-deficient endothelial monolayers. In contrast, Atg7 knockdown did not affect the permeability to inulin (Fig. 1J). The differences in transcytosis rates may simply reflect higher rates of accumulation of ^{125}I-LDL cholesterol in ATG7 knockdown cells (Fig. 1G) or perhaps may reflect recent observations directly linking autophagy to barrier function (Nighot *et al.*, 2015).

While a number of sophisticated in vitro assays have been developed for assessing endothelial barrier function (Wegener & Seebach, 2014), we sought to assess a potentially more physiologically relevant model by analyzing mice with a conditional endothelial deletion of ATG7 (Torisu *et al.*, 2013). We took advantage of the rich vascular plexus in the eye and the previously described transport of lipids from the choroidal blood vessels to the retinal pigment epithelium (RPE) (Picard *et al.*, 2010). To assess whether the acute handling of modified lipids depends on intact endothelial autophagy, we injected control mice (WT/WT; VE-Cadherin Cre) or Atg7^{endo} mice (fl/fl;VE-Cadherin Cre) mice with fluorescently labeled OxLDL (dil-OxLDL).We next analyzed the retinas of mice 24 and 48 h after OxLDL injection by confocal microscopy. Deposits of dil-OxLDL were evident in the mouse retinal pigment epithelium (RPE) and the choroidal endothelium beneath the RPE. Twenty-four hours post injection, dil-OxLDL uptake in the mouse eye was similar in Atg7^{endo}

and control mice (data not shown). However, 48 h after dil-OxLDL administration, the fluorescent tagged dil-OxLDL was retained at higher levels within the RPE and choroidal endothelium of the Atg7^{endo} mice than in wild-type tissue (Fig. 2A,B). Quantification of dil-OxLDL fluorescence confirmed the increased persistence of dil-OxLDL in Atg7^{endo} mice (Fig. 2C, $n = 6$ eyes per group, $P < 0.0001$ by two-tailed t-test). Thus, in this acute model of modified LDL exposure, autophagy-deficient mice demonstrate increased and persistent lipid accumulation.

We next sought to assess a more chronic model of lipid exposure by crossing our control and Atg7^{endo} mice into an ApoE$^{-/-}$ background. The presence or absence of endothelial ATG7 did not significantly alter serum lipid or glucose levels, nor did it alter overall body weight or body composition (Table S1, Supporting information). In contrast, consistent with altered lipid handling, we observed increased en face Oil Red O-positive staining in high-fat-fed ApoE$^{-/-}$Atg7^{endo} mice (Fig. 2D). These differences were maintained when mice were analyzed at 8 weeks of age (e.g., 4 weeks of high-fat diet), 16 weeks of age (12 weeks of high-fat diet) or 24 weeks of age (20 weeks of high-fat diet; Fig. 2E–G). A similar analysis of cross-sectional plaque area in the aortic root was also consistent with the observation that high-fat-fed ApoE$^{-/-}$Atg7^{endo} mice had increased atherosclerotic lesion size when compared with control animals (Fig. 2H,I). Preliminary analysis of the plaque composition revealed a trend for increased necrotic area in the ApoE$^{-/-}$Atg7^{endo} mice (Fig. S2, Supporting information).

Taken together, our results demonstrate an important role for endothelial autophagy in maintaining vascular lipid homeostasis. Further work is needed to better define how autophagy modulates the intracellular fate of engulfed lipids (Fig. S1K). Nonetheless, the ability of endothelial cells to target excessive native or modified lipids for autophagy-dependent degradation appears to be an important mechanism to limit atherosclerotic plaque formation. Our in vitro results suggest that knockdown of ATG7 or treatment with chloroquine can increase endothelial lipid accumulation. Given that chloroquine works as an inhibitor of lysosomal processes, the most likely explanation is that similar to what has been described in other tissues; endothelial cells rely, at least in part, on autophagosomal-mediated delivery of lipids to the lysosome for degradation. In this context, it may be relevant that the gene lysosomal acid lipase A (LIPA) has been recently identified in genome-wide studies as a potential susceptibility locus for atherosclerotic disease (Wild *et al.*, 2011; Vargas-Alarcon *et al.*, 2013). In addition, it is of potential interest that a number of genes associated with lipid metabolism have also been linked to age-related retinal diseases (Black & Clark, 2015).

Given that autophagic flux is believed to decline as a function of age (Cuervo, 2008), the age-dependent decline in endothelial autophagy might contribute to the sharp rise in age-related cardiovascular disease. Interestingly, pharmacological efforts that augment autophagy appear
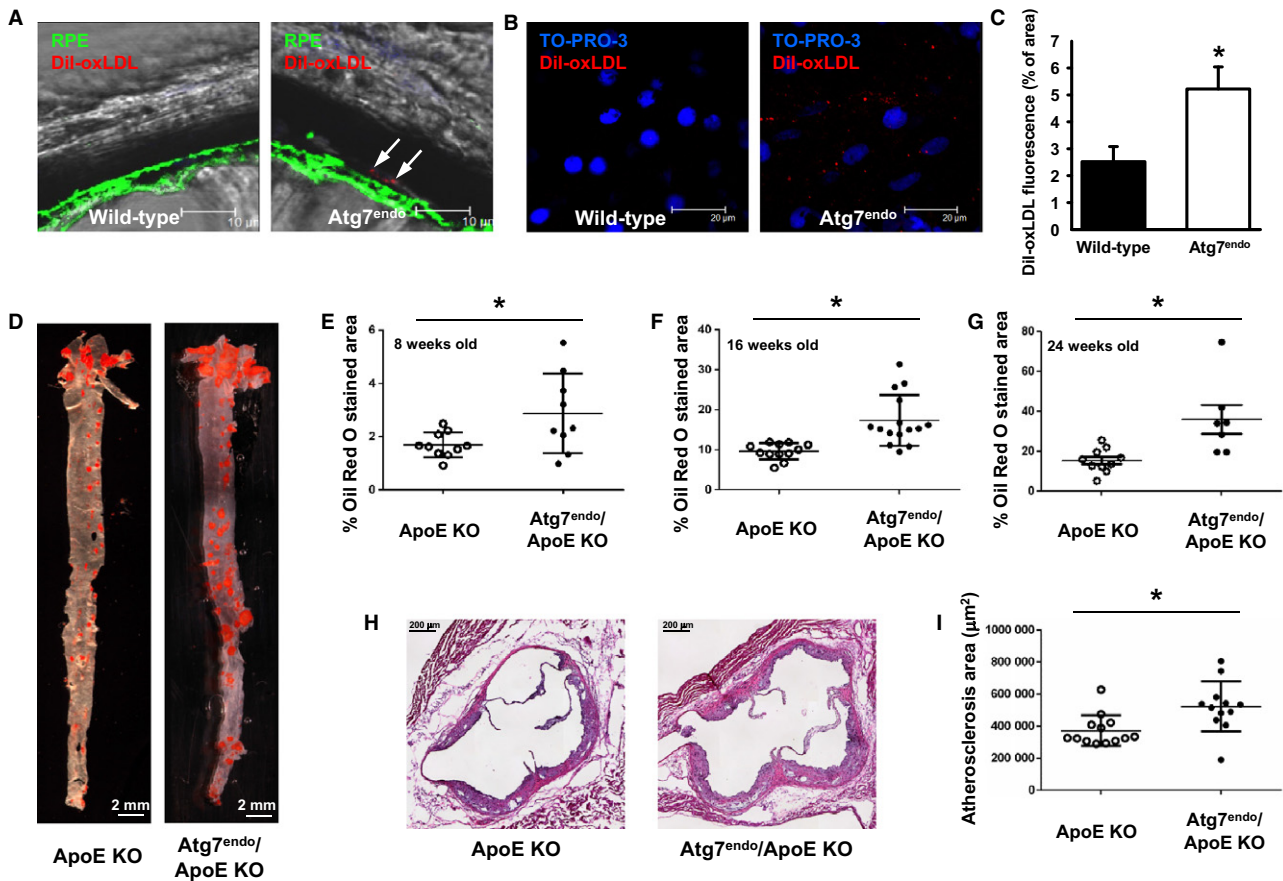
Fig. 2 Autophagy regulates *in vivo* vascular lipid deposition. (A) Deposition of fluorescently labeled OxLDL in the retina of control (WT/WT; VE-cadherin Cre) or Atg7[endo] mice 48 h after injection. Arrows represent retained dil-OxLDL particles deposited sub-RPE, at the level of the RPE basal membrane. (B) Representative whole mount images from Atg7[endo] mice demonstrating accumulation of fluorescent particles (presumptive vesicles) within binucleated RPE cells and in the adjacent extracellular matrix and endothelium of the choriocapillaris. (C) Quantification of labeled OxLDL in the retina of control or Atg7[endo] mice (*n* = 6 eyes per group) 48 h after infusion, *$P < 0.05$ by two-tailed unpaired *t*-test. (D) Representative Oil Red O stained aortas from control (WT/WT VE-cadherin Cre/ApoE$^{-/-}$ abbreviated as ApoE KO) or Atg7[endo]/ApoE KO (fl/fl VE-Cadherin Cre/ApoE$^{-/-}$) mice. (E) Quantification of Oil Red O staining at 8 weeks (*n* = 10 ApoE KO and *n* = 9 Atg7[endo]/ApoE KO mice, *$P < 0.05$ two-tailed unpaired t-test with Welch's correction). (F) 16 weeks (*n* = 12 ApoE KO and *n* = 15 Atg7[endo]ApoE KO mice, *$P < 0.01$ by two-tailed unpaired t-test with Welch's correction) and (G) 24 weeks of age (*n* = 10 ApoE KO and *n* = 7 Atg7[endo]/ApoE KO mice, *$P < 0.03$ by two-tailed unpaired *t*-test with Welch's correction). (H) Micrographs of the aortic root at 16 weeks of age. (I) Quantification of plaque area at 16 weeks between the two genotypes (*n* = 13 ApoE KO and *n* = 12 Atg7[endo]/ApoEKO mice, *$P < 0.01$ by two-tailed unpaired *t*-test with Welch's correction).

to reverse some of the properties of mouse and human arterial aging (LaRocca *et al.*, 2012). Previous studies have also suggested that the atherosclerotic plaque is enriched for autophagosomes (Martinet & De Meyer, 2009). In addition, recent mouse genetic studies have implicated the role of autophagy in macrophage foam cell formation (Muller *et al.*, 2011; Ouimet *et al.*, 2011; Le Guezennec *et al.*, 2012; Razani *et al.*, 2012; Sergin & Razani, 2014). Our results complement these observations and suggest that similar to what was observed in macrophages, endothelial autophagy is important in limiting atherosclerotic progression. It is tempting to speculate that enhancing autophagy may therefore be a beneficial strategy to reduce the rate of age-dependent cardiovascular disease. Such speculation is supported by previous observations including that inhibition of mTOR (a kinase known to function as a negative regulator of autophagy) with pharmacological agents such as rapamaycin appears to inhibit atherosclerosis in a number of different animal models (Castro *et al.*, 2004; Pakala *et al.*, 2005; Mueller *et al.*, 2008). Similarly, strategies such as calorie restriction, that are known to elevate autophagic flux, appear to reduce cardiovascular disease in both mice and humans (Guo *et al.*, 2002; Fontana *et al.*, 2004). Manipulation

of vascular autophagy might therefore be an attractive therapeutic target to potentially limit atherosclerotic disease independent of serum lipid values.

Acknowledgments

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Fig. S1. Characterization of the role of autophagy in endothelial lipid homeostasis.

Fig. S2. The role of autophagy in ApoE KO mouse atherosclerotic plaque formation.

Table S1. Serum chemistry and body weight.

Data S1. Experimental procedures.