Plasma membrane contributes to the formation of pre-autophagosomal structures

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Autophagy is a catabolic process in which lysosomes degrade cytoplasmic contents transported in double-membraned autophagosomes. Autophagosomes are formed by the elongation and fusion of phagophores, which derive from pre-autophagosomal structures. The membrane origins of autophagosomes are unclear and may involve multiple sources, including the endoplasmic reticulum and mitochondria. Here we show in mammalian cells that the heavy chain of clathrin interacts with Atg16L1 and is involved in the formation of Atg16L1-positive early autophagosome precursors. Atg16L1 associated with clathrin-coated structures, and inhibition of clathrin-mediated internalization decreased the formation of both Atg16L1-positive precursors and mature autophagosomes. We demonstrated that the plasma membrane contributes directly to the formation of early Atg16L1-positive autophagosome precursors. This may be particularly important during periods of increased autophagosome formation, because the plasma membrane may serve as a large membrane reservoir that allows periods of autophagosome synthesis at levels many-fold higher than under basal conditions, without compromising other processes.

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Clathrin-mediated endocytosis regulates early stages of autophagosome formation

Having shown that clathrin heavy chain interacted with the autophagosome precursor marker, Atg16L1, we next tested whether early steps in clathrin-mediated endocytosis regulated the initial stages of autophagosome formation. (This question needs to be distinguished from the distinct process in which fully formed, mature (Atg16L1-negative) autophagosomes fuse with early and late endosomes to form the hybrid organelles called amphisomes, a step that seems to be important for subsequent autophagosome–lysosome fusion\textsuperscript{[10–12].} We first tested the effect of knockdown of some key components involved in early endocytosis on autophagosome formation. Changes in the steady-state levels of LC3-II can be altered as a result of formation and/or degradation. To assess changes in LC3-II (autophagosome) formation, one can assess LC3-II levels in the presence of saturating concentrations of bafilomycin A1 (BafA1). Because BafA1 inhibits LC3-II degradation and autophagosome–lysosome fusion, differences in LC3-II levels in response to par

To provide further support for the role of clathrin in autophagosome formation, given its additional effects that impair autophagosome maturation, we studied the effects of clathrin heavy-chain knockdown in HeLa cells stably expressing an mRFP–GFP–LC3 construct (mRFP–GFP–LC3) (Fig. 2a; Supplementary Information, Fig. S1d–f). Epsin is an accessory protein to clathrin that induces membrane curvature, and AP2 and AP1 are clathrin adaptor proteins at the plasma membrane and Golgi, respectively. We noted that the effects of clathrin heavy-chain knockdown on LC3-II levels in the absence of BafA1 varied—this is probably because clathrin knockdown decreases LC3-II formation, which will decrease LC3-II levels in the absence of BafA1, but clathrin knockdown also increases autophagosome–lysosome fusion and LC3-II degradation\textsuperscript{[20–22]} (see also below), which will increase LC3-II levels in the absence of BafA1; the net effect will depend on the relative kinetics of the two systems, which may vary according to the efficiency of knockdown or the different treatment conditions used\textsuperscript{[23,24].} Our impression was that the effects of clathrin knockdown on synthesis were more pronounced with more effective knockdowns—for example, two rounds of knockdown for 5 days. Such strategies are known to be required for effective clathrin knockdown, owing to its long half-life\textsuperscript{[25].}

To provide further support for the role of clathrin in autophagosome formation, given its additional effects that impair autophagosome maturation, we studied the effects of clathrin heavy-chain knockdown in HeLa cells stably expressing an mRFP–GFP–LC3 construct (mRFP and GFP are monomeric red fluorescent protein and green fluorescent protein, respectively)\textsuperscript{[26,27].} Because the two fluorescent proteins

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Figure 1 Atg16L1 interacts with clathrin heavy chain. (a) HeLa cell lysates were immunoprecipitated with Atg16L1 antibody (Atg16L1) or no antibody (No Ab) in control, and subjected to SDS–PAGE; proteins were stained with SimplyBlue safe stain (Invitrogen) in accordance with the manufacturer’s protocol. Bands indicated in the box were cut out and digested with trypsin, and identified by MALDI–TOF MS. HC, heavy chain. (b) HeLa cells transfected for 24 h with control vector, or wild-type or different deletion mutants (residues 2–77, 2–275 and 232–607) of Flag–Atg16L1 were immunoprecipitated with anti-Flag antibody (Atg16L1) and immunoblotted with anti-clathrin, anti-Flag and anti-Atg5 antibodies. Clathrin interacts with the N terminus of Atg16L1, in a similar manner to Atg12–Atg5. Total lysates were run alongside as controls for protein input. FL, full-length. (c) HeLa cell lysates were immunoprecipitated (IP) with Atg16L1 antibody (+Ab) or no antibody (no Ab) and immunoblotted with anti-clathrin, anti-AP2 and anti-Atg16L1 antibodies. Total cell lysates (TL) were also blotted for input controls. Endogenous Atg16L1 interacts with clathrin heavy chain and AP2. (d) HeLa cells transfected for 24 h with Flag–Atg16L1 and increasing concentrations of Atg5 were immunoprecipitated (IP) with anti-Flag antibody (Atg16L1) and immunoblotted with anti-clathrin, anti-Flag and anti-Atg5 antibodies. Atg5 overexpression neither interferes with nor increases clathrin–Atg16L1 binding. Uncropped images of blots are shown in Supplementary Information, Fig. S9.
have different pKᵢ values, this construct can be used as a probe for autophagosome maturation. At physiological pH — that is, in newly formed autophagosomes — both proteins are stable, leading to both red and green fluorescence. On acidification — that is, fusion with the lysosome — green fluorescence is rapidly lost because of the high pKᵢ of GFP, and only red fluorescence remains. Consistent with our LC3-II blotting data, clathrin heavy-chain knockdown resulted in an increase in the numbers of autophagosomes (vesicles positive for both red and green) and a decrease in the numbers of autolysosomes (red positive, green negative) in the absence of BafA1 (as a result of decreased delivery of autophagosomes to lysosomes), but resulted in a decrease in the number of autophagosomes (about 30%) in the presence of BafA1 (because of impaired autophagosome formation) (Fig. 2c). As we saw with clathrin heavy chain knockdown, AP2 knockdown also decreased autophagosome formation with this assay, whereas AP1 knockdown had no effect (Supplementary Information, Fig. S1g).
The effects of knockdown of clathrin heavy chain, epsin or AP2 on autophagosome formation (LC3-II levels under BafA1 conditions) were also obvious under conditions of autophagy induction (treatment with trehalose or starvation (Supplementary Information, Fig. S2a–c)), in which these knockdowns all decreased new autophagosome formation by about 30% (Supplementary Information, Fig. S2a–c). Consistent with our LC3 western blot data, we also observed a decrease in the proportion of cells with more than 50 endogenous LC3 vesicles under starvation conditions.

Figure 3 Influence of clathrin-mediated endocytosis on Atg16L1-positive autophagosome precursors. (a–d) HeLa cells transfected for 72 h with control, epsin 1, clathrin heavy chain, AP2 or AP1 siRNA were treated with HBSS (to induce autophagy) for 6 h, after which they were fixed and immunostained for endogenous Atg16L1. Representative cells for the control and epsin 1 siRNA are in a, and percentages of HeLa cells with Atg16L1 vesicles are quantified in b (for epsin 1), c (for clathrin) and d (for AP1 and AP2). Three asterisks, *P < 0.0001. Scale bars, 10 μm. (Note that we used single siRNAs for all experiments and the effects were confirmed with two independent sequences for clathrin heavy chain). *n = 600 cells.

(e, f) HeLa cells transfected for 24 h with Flag-tagged wild-type Atg16L1 or the Atg16L1 deletion mutants 2–275 Atg16L1 or 232–607 Atg16L1 were fixed and immunostained with anti-Flag antibody (e); the proportions of cells with Flag–Atg16L1 vesicles are represented in f. Representative images of Atg16L1 vesicles with the different mutant constructs are shown in e. The large boxes in each panel are higher magnifications of regions in the smaller boxes. Three asterisks, *P < 0.0001; n.s., not significant. *n = 100 cells. (g–i) HeLa cells transfected with control, epsin 1, clathrin heavy chain, AP2 or AP1 siRNA, as above, were transfected with GFP–Atg16L1 and tomato LC3 for a further 24 h, after which the cells were fixed. g shows representative cells for control, epsin 1 and clathrin siRNA. The percentage of GFP–Atg16L1 (green) that co-localized (yellow, marked by arrows) with tomato LC3 vesicles (red) is quantified as shown in the graph in h (control, epsin 1 and clathrin siRNA) and i (control, AP2 and AP1 siRNA). Three asterisks, *P < 0.0001. *n = 21 cells. Scale bars, 10 μm. All error bars represent s.e.m.
on knockdown of clathrin heavy chain or AP2 (Fig. 2d). Similarly, when transfected with tomato LC3 we also saw a decrease in the proportion of cells with more than 50 LC3 vesicles in trehalose-treated cells with knockdown of clathrin heavy chain or epsin, or in untreated cells with AP2 knockdown (Supplementary Information, Fig. S3a–c).

The block in autophagic flux with knockdown of clathrin heavy chain, epsin or AP2 was also correlated with an increase in aggregation of a model aggregate–prone protein, huntingtin exon 1 with 74 polyglutamine repeats (Q74)19–21, which is a potent autophagy substrate18,22,23 (Supplementary Information, Fig. S3d, e), shows epsin 1 and AP2 data; the clathrin data were published previously11); because aggregation is a concentration-dependent phenomenon, the proportion of cells with these aggregates increases with a wide range of autophagy inhibitors and decreases with autophagy inducers19,22–24. We also found that knockdown of clathrin or AP2 increased the levels of endogenous p62, another autophagy substrate (Supplementary Information, Fig. S3f, g). Clathrin knockdown also significantly increased the proportion of cells with endogenous p62 aggregates (Supplementary Information, Fig. S3g). However, because autophagic substrates will accumulate if there is either impaired autophagosome formation and/or decreased delivery of autophagosomes to lysosomes, these substrate accumulation assays cannot distinguish between these scenarios.

Influence of clathrin-mediated endocytosis on Atg16L1-positive autophagosome precursors

Because the knockdown of clathrin heavy chain, epsin or AP2 inhibited the formation of new autophagosomes, we next examined their effects on the formation of autophagosome precursors, as revealed by Atg16L1-positive structures6,8. Endogenous Atg16L1 shows a punctate staining pattern on induction of autophagy (for example, by starvation or by treatment with rapamycin or trehalose), corresponding to an increase in the formation of precursor structures (Fig. 3a; Supplementary Information, Fig. S4a, b; and data not shown). Furthermore, endogenous Atg16L1 vesicle numbers were not increased by BafA1 (Supplementary Information, Fig. S4c), suggesting that this readout is not affected by perturbations that impair the lysosomal degradation of mature autophagosomes. We observed that knockdown of clathrin heavy chain, epsin or AP2 (but not AP1) resulted in a roughly 30% decrease in the formation of Atg16L1 vesicles under starvation conditions, suggesting a block in the formation of pre-autophagosomal structures (Fig. 3a–d; Supplementary Information, Fig. S4a, b). Knockdown of clathrin heavy chain, epsin or AP2 also decreased the numbers of Atg16L1 structures associated with overexpressed Atg16L1 (data not shown; note that we found that low levels of Atg16L1 overexpression (400–750 ng per six-well plate) did not inhibit autophagosome formation, as has been reported for high-level viral overexpression6). We found that the N-terminal deletion mutant of Atg16L1 (residues 232–607) that did not interact with clathrin formed significantly fewer vesicles, in comparison with the wild-type protein or C-terminal deletion mutant that can bind clathrin (residues 2–275) (Fig. 3e, f).

During the autophagosome formation process, LC3-II is recruited onto the Atg16L1-positive pre-autophagosomal structures, which ultimately form autophagosomes, at which point the Atg16L1 complex is removed and recycled. We next performed co-localization experiments...
Figure 5 Atg16L1 vesicles co-localize with vesicles labelled with cholera toxin subunit B. (a) HeLa cells transfected for 24 h with GFP–Atg16L1 (green) and CFP–LC3 (blue) were incubated with Alexa Fluor-555-conjugated cholera toxin subunit B (red) for 15 min at 4 °C (allowing toxin to bind to the plasma membrane). Then cells were incubated at 37 °C (allowing internalization of cholera toxin) for 10 min and fixed for confocal analysis. Vesicles positive for both Atg16L1 and cholera toxin are yellow (also see high-magnification images at the right). Note that the small Atg16L1 vesicles co-localizing with cholera toxin are negative for LC3 (as marked with yellow arrows), and the Atg16L1 vesicles co-localizing with both cholera toxin and LC3 (marked with blue arrows) are shown in the magnified panels at the right. Scale bar, 10 μm. (b) HeLa cells transfected for 24 h with GFP–Atg16L1 (green) were incubated with Alexa Fluor-555-conjugated cholera toxin subunit B (red) as in a, after which they were fixed, immunostained for endogenous (end.) EEA1 (blue) and analysed by confocal microscopy. Vesicles positive for Atg16L1 and cholera toxin B are yellow (white arrows) and vesicles positive for both EEA1 and cholera toxin are purple (blue arrows) and are shown in the high-magnification images in the middle. The graph shows percentage co-localization. Cho. tox., cholera toxin. n = 20 cells. Scale bar, 10 μm. (c) HeLa cells treated with HBSS for 6 h were incubated with Alexa Fluor-555-labelled cholera toxin subunit B (red) as in a. Cells were then fixed, and immunostained for endogenous Atg16L1 (green) and endogenous EEA1 (blue). Cells were analysed as in b; the graph shows quantification of the results. Scale bar, 10 μm. Arrows indicate Atg16L1–cholera toxin co-localization; arrowheads indicate EEA1–cholera toxin co-localization. n = 20 cells. (d) HeLa cells treated with HBSS for 6 h were incubated with Alexa Fluor-555-labelled cholera toxin subunit B (red) as in a. Cells were then fixed and immunostained for endogenous Atg5 (green) or endogenous Atg12 (green). Co-localization of the Atg5 (n = 18 cells) or Atg12 vesicles (n = 39 cells) with cholera toxin is shown in yellow (arrows) and is quantified in the graph. Scale bar, 10 μm. All error bars represent s.e.m.
with GFP–Atg16L1 and tomato LC3 vesicles, to assess the progression of autophagosome formation from early pre-autophagosomal structures (Atg16L1-positive, LC3-negative) to maturing autophagosomes (Atg16L1-positive, LC3-positive). We found that knockdown of clathrin heavy chain, epsin or AP2 decreased the co-localization of GFP–Atg16L1 vesicles with tomato LC3 vesicles (Fig. 3g–i), which suggests that these proteins probably regulate the formation of autophagosomes at a very early stage in the pathway.

**Plasma membrane contributes to autophagosome precursors**

During our analyses of pre-autophagosomal structure formation, we often found Atg16L1 vesicles close to the plasma membrane (Fig. 4a).
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This observation was supported by total internal reflection fluorescence (TIRF) microscopy, which enables selective visualization of thin regions, such as the plasma membrane (and the cytoplasmic area immediately beneath it). TIRF microscopy with GFP–Atg16L1 and mRFP-tagged glycosphatidylinositol (GPI) protein (found in small microdomains at the plasma membrane) revealed many Atg16L1 vesicles (Fig. 4b) that also co-localized significantly with mRFP–GPI structures (Fig. 4b), suggesting that the GFP–Atg16L1 vesicles were either at the plasma membrane or very close to it. Indeed, immunogold electron microscopic analysis of GFP–Atg16L1–transfected cells showed Atg16L1 labelling on clathrin-coated vesicle profiles near the plasma membrane (Fig. 4c, untreated panels). These data, together with our earlier observation that autophagosome formation is impaired on inhibition of very early endocytic trafficking, suggested that plasma membrane might be a source of membrane for the pre-autophagosomal structures.

We next used different assays to assess the role of the plasma membrane as a membrane contributor for autophagosome initiation. We used the cholera toxin subunit B internalization assay to assess the role of the plasma membrane in autophagosome initiation. We incubated the cells with Alexa Fluor-555-conjugated cholera toxin subunit B (red) at 4 °C, followed by further incubation for 10 min at 37 °C, after which the cells were fixed for confocal analysis (Fig. 7b). The co-localization (yellow) of GFP–Atg16L1 (green) with cholera toxin (red) in control or clathrin heavy-chain knockdown is shown and is quantified in the graphs in c. n = 25 cells. Three asterisks, P = 0.0002 for clathrin heavy-chain knockdown, and P < 0.0001 for AP2 knockdown. All error bars represent s.e.m. Scale bar, 5 μm.

**Figure 7** Plasma membrane contributes to Atg16L1-positive autophagosome precursors. (a) HeLa cells transfected for 24 h with GFP–Atg16L1 (green) were incubated with CellMask Orange plasma membrane stain for 5 min at 37 °C, after which they were imaged immediately (in an incubated chamber at 37 °C). A time series after fusion of a CellMask Orange vesicle (red) with a GFP–Atg16L1 vesicle (green) is shown. Scale bar, 5 μm. A higher-magnification image showing co-localization (yellow) of GFP–Atg16L1 vesicle (green) with CellMask Orange-positive vesicle (red), indicated by arrows, is also shown in the top and bottom panels. (b, c) HeLa cells transfected with control, clathrin heavy chain, AP1 or AP2 siRNA for 72 h were subsequently transfected with GFP–Atg16L1 (green) together with the siRNA for the next 24 h. The cells were then incubated for 15 min with Alexa Fluor-555-conjugated cholera toxin subunit B (red) at 4 °C, followed by further incubation for 10 min at 37 °C, after which the cells were fixed for confocal analysis (b). The co-localization (yellow) of GFP–Atg16L1 (green) with cholera toxin (red) in control or clathrin heavy-chain knockdown is shown and is quantified in the graphs in c. n = 25 cells. Three asterisks, P = 0.0002 for clathrin heavy-chain knockdown, and P < 0.0001 for AP2 knockdown. All error bars represent s.e.m. Scale bar, 5 μm.
**Figure 8** Effect of endocytic vesicle scission on phagophore formation. (a) HeLa cells transfected with two rounds of control, clathrin heavy chain or AP2 siRNA for 4 days were collected for immunoprecipitation (IP) with anti-Atg16L1 antibody. Western blot for total lysate (bottom) and IP (top) were performed with anti-Atg16L1 and anti-clathrin antibodies. Note the decrease in Atg16L1–clathrin interaction with AP2 knockdown. The graph shows quantification of the Atg16L1–clathrin or Atg16L1–AP2 with knockdown of control, clathrin heavy chain or AP2 from two independent experiments. (b) HeLa cells transfected for 24 h with Flag-tagged wild-type Atg16L1, GFP–PLC(PH) together with empty vector (control) or dominant-negative dynamin II mutant (K44A dynamin; DN-Dyn) were fixed and analysed by confocal microscopy. Note the co-localization of Atg16L1 with PLC(PH) at the plasma membrane; this is quantified in the graph at the right. Asterisk, $P < 0.01$. $n = 20$ cells. Scale bars, 5 μm. DAPI, 4ʹ,6-diamidino-2-phenylindole. (c) HeLa cells treated for 5 h with HBSS alone or with HBSS containing 80 μM dynasore were collected for immunoprecipitation (IP) with anti-Atg16L1 antibody. Western blot analyses for total lysate (TL) and IP were performed with anti-Atg16L1 antibody and anti-clathrin antibodies. Note the strong Atg16L1–clathrin interaction with dynasore treatment. The graph shows quantification from two independent experiments. All error bars represent s.e.m. d, Representation of the contribution of the plasma membrane to autophagosome precursor formation. Cholera toxin is internalized by clathrin-dependent and clathrin-independent endocytosis. Cholrin knockdown significantly decreases cholera toxin uptake. Clathrin-coated vesicles (CCV) budding immediately from the plasma membrane (EEA1-negative) are precursors to early endosomes (EE) and/or late endosomes or multivesicular bodies (LE/MVB) budding directly from the plasma membrane (EEA1-positive). Previous studies showed that delivery of fully formed autophagosomes to lysosomes requires fusion of such autophagosomes with early or late endosomes or multivesicular bodies (LE/MVB) to form amphisomes, which are Atg16L1-negative, LC3-positive and positive for endosomal markers. We show here that inhibition of clathrin-dependent internalization inhibits the formation of early Atg16L1-positive precursors that mature to form phagophores and, later, autophagosomes. These Atg16L1 vesicles were positive for other early autophagosomal markers (Atg5 and Atg12) but not for early endosomal markers (EEA1, AP2). AP1 is a clathrin adaptor at the plasma membrane, whereas AP1 localizes to the trans-Golgi network and endosomes. Uncropped images of blots are shown in Supplementary Information, Fig. S9.
whether the plasma membrane fused with GFP–Atg16L1 vesicles, as revealed by live-cell or fixed-cell microscopy and double-labelling immunogold electron microscopy. This assay relies on the binding of cholera toxin to the outside of the plasma membrane and its subsequent internalization to track whether the plasma membrane is contributing to early precursor structures. Internalization of cholera toxin into HeLa cells is significantly attenuated by inhibition of clathrin-mediated endocytosis. At very early time points, immediately after incubation with the cholera toxin, we observed clear labelling of the plasma membrane, and vesicles immediately internalizing from the plasma membrane were often fused with GFP–Atg16L1 vesicles close to the plasma membrane (Fig. 5a; Supplementary Information, Fig. S5a, b; Supplementary Information, Movie S1). We observed many GFP–Atg16L1 vesicles close to the cell surface that co-localized with vesicles positive for cholera toxin (Fig. 5a, b; Supplementary Information, Fig. S5a, b). We noted that many of the small GFP–Atg16L1 vesicles close to the plasma membrane were LC3-negative (Fig. 5a, yellow arrows on the merged panel), suggesting that they were very early precursors that later acquired the characteristics of phagophores when they were also LC3-positive (Fig. 5a, blue arrows on the merged panel). The number of GFP–Atg16L1 vesicles decreased on treatment with 3-methyladenine, which blocks autophagosome formation (Supplementary Information, Fig. S5c). The GFP–Atg16L1 vesicles co-localized with other early autophagosomal markers such as Atg5 and Atg12 (but did not co-localize with ER (BIP) or Golgi (GM130) markers; Supplementary Information, Figs S5d and S6a–c), and also did not co-localize with EEA1, an early endosomal marker (Fig. 5b). This suggests that the GFP–Atg16L1-positive structures are very early autophagosome precursors that are associated with endocytic vesicles that have recently budded from the plasma membrane. These endocytic vesicles are probably not early endosomes (which are EEA1-positive) but are more analogous to the endocytic vesicle precursors that precede endosomes. We observed similar results when we performed the cholera toxin internalization assay with endogenous Atg16L1 (Fig. 5c), GFP–Atg16L1 stable cells (Supplementary Information, Fig. S7a, b), endogenous (Fig. 5d) or overexpressed (Supplementary Information, Fig. S7c) Atg5, or endogenous Atg12 (Fig. 5d). We found that vesicles containing 2–275 Atg16L1 (which binds clathrin) co-localized with cholera toxin vesicles, similar to wild-type Atg16L1, whereas this was not true of 232–607 Atg16L1 (which cannot bind clathrin) (even though the co-localization of cholera toxin and EEA1 was similar for all three Atg16L1 constructs) (Fig. 6a; Supplementary Information, Fig. S7d). Immunogold electron microscopy also showed internalized vesicles close to the plasma membrane labelled with both cholera toxin and GFP–Atg16L1 (Fig. 6b; Supplementary Information, Fig. S7e) supporting the immunofluorescence data. Similar results to the cholera toxin internalization assay were observed when we used two other plasma membrane markers, the slowly internalizing dye CellMask Orange (Fig. 7a) or the fast internalizing dye FM4–64 (Supplementary Information, Fig. S7f); both showed that vesicles derived from the plasma membrane often fused with GFP–Atg16L1 vesicles near the plasma membrane.

Knockdown of clathrin heavy chain or AP2 significantly decreased the co-localization of Atg16L1-positive vesicles with cholera-toxin-labelled vesicles (Fig. 7b, c), which is consistent with our earlier data showing that knockdown of clathrin heavy chain or AP2 inhibited autophagosome formation. (We tested clathrin-mediated endocytosis in our system by performing transferrin-internalization assays and found that the knockdown conditions for clathrin heavy chain and AP2 that we used significantly impaired clathrin-dependent endocytosis (Supplementary Information, Fig. S8a).) The reason why the effects of knockdown of clathrin heavy chain or AP2 might not be even stronger than those we observed is that non-clathrin-mediated endocytosis routes also contribute to the uptake of cholera toxin, and it is possible that these may also regulate autophagosome formation. In addition, it is difficult to obtain strong functional knockdown with these proteins.

Here we have identified a direct contribution of the plasma membrane to the formation of pre-autophagosomal structures by means of clathrin-mediated internalization of vesicles derived from the plasma membrane. Our data suggest that this is mediated through the interaction of Atg16L1 and clathrin heavy chain. Because AP2 also interacted with Atg16L1 (Fig. 1c), we performed immunoprecipitation assays in cells by using knockdown of clathrin heavy chain or AP2. We observed a decrease in the Atg16L1–clathrin interaction with AP2 knockdown (but no change in the Atg16L1–AP2 interaction with clathrin-heavy chain knockdown), suggesting that AP2 may mediate the Atg16L1–clathrin interaction (Fig. 8a). This is not surprising, because AP2 links clathrin to the membrane and also mediates its interactions with other key endocytic proteins. It remains to be established exactly how Atg16L1 is specifically targeted to clathrin-coated vesicles. Because AP2 knockdown did not completely abolish the Atg16L1–clathrin interaction, it is possible that other adaptor/accessory proteins might be involved in mediating the Atg16L1–clathrin interaction, but the identification of this interaction is beyond the scope of this paper.

**Effect of endocytic vesicle scission on phagophore formation**

For further characterization of the importance of endocytosis in phagophore formation, we inhibited the scission of endocytic vesicles with dominant-negative dynamin II (K44A dynamin II) and observed a decrease in the number of Atg16L1 vesicles, similar to that observed with knockdown of clathrin heavy chain or AP2 (Supplementary Information, Fig. S8b). Dominant-negative dynamin treatment of cells analysed for cholera toxin–Atg16L1 vesicle co-localization resulted in the formation of tubular morphologies with the cholera toxin, consistent with previous findings, and Atg16L1 was seen on these structures (Supplementary Information, Fig. S8c). The dominant-negative dynamin II also redistributed Atg16L1 close to the inner surface of the plasma membrane, as judged by significantly enhanced Atg16L1–phosphatidylinositol 4,5-biphosphate (PtdIns(4,5)P₂) co-localization observed by confocal microscopy (Fig. 8b; Supplementary Information, Fig. S8d). PtdIns(4,5)P₂ is a well-recognized plasma membrane lipid that is frequently used as a plasma membrane marker, and the pleckstrin homology domain of phospholipase Cδ fused to GFP (PLCδ–GFP) is routinely used to recognize PtdIns(4,5)P₂ in this context. Consistent with these observations, dynasore, a cell-permeable dynamin inhibitor, caused a marked increase in Atg16L1–clathrin interaction (Fig. 8c), which was correlated with a marked accumulation of Atg16L1 in clathrin-coated structures close to the plasma membrane at the electron microscopic level (Fig. 4c). It is therefore likely that Atg16L1 localizes to the clathrin-coated endocytic vesicles close to the plasma membrane before scission, and our data are compatible with a requirement for vesicle scission for phagophore maturation.
DISCUSSION
The membrane origin of autophagosomes is one of the critical questions in the autophagy field. Although contributions from multiple membrane sources are widely acknowledged to be a likely possibility, the mechanism by which they give rise to mature autophagosomes is largely unknown. Our data suggest that plasma membrane contributes to early autophagosome precursor structures, a phenomenon that is dependent on the association of Atg16L1-positive vesicles with the plasma membrane through Atg16L1–AP2/clathrin heavy-chain interactions. Subsequent scission of the Atg16L1/clathrin/AP2-associated structures, leading to the formation of early endosomal-like intermediates, is a crucial step that enables the liberation and maturation of these Atg16L1 vesicles into autophagosomes. These autophagosome precursors that we see may be membrane structures that precede the phagophores, and these represent an even earlier stage in autophagosome assembly (Fig. 8d). The ability of plasma membrane to contribute to autophagosome formation may be particularly important during periods of increased autophagy, because the large surface area of the plasma membrane may serve as a large membrane reservoir that allows cells periods of autophagosome synthesis at levels many-fold higher than under basal conditions, without compromising other processes.

METHODS
Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturecellbiology/

Note: Supplementary Information is available on the Nature Cell Biology website.

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AUTHOR CONTRIBUTIONS
B.R., K.M., L.J. and D.C.R. designed and analysed the experiments. B.R., K.M. and L.J. performed the experiments. C.P. performed all the immunogold electron microscopy analysis. B.R. and D.C.R. wrote the manuscript. D.C.R. supervised the project.

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METHODS

Mammalian cell culture. HeLa cells were grown at 37 °C in DMEM medium (Sigma) supplemented with 10% FBS, 100 U ml⁻¹ penicillin/streptomycin, 2 mM l-glutamine and 1 mM sodium pyruvate, under 5% CO₂. HeLa cells stably expressing GFP-mRFP-LC3 were cultured in DMEM containing 10% FBS, 2 mM l-glutamine, 100 U ml⁻¹ Penicillin/Streptomycin and 500 µg ml⁻¹ G418 (Sigma)⁻¹. The cells were seeded at (1–2) × 10⁴ per well in six-well plates, and transfection was performed with LipofectAMINE (for DNA) or LipofectAMINE 2000 (for siRNA and double transfections with DNA and siRNA) reagents (Invitrogen), using the manufacturer's protocol. Predesigned single siRNAs were ordered from Applied Biosystems (siRNA IDs: clathrin heavy chain, 107565 and s22326; epsin 1, s26714; AP2-A1, 16708; AP1-μ1, 138606).

Immunocytochemistry. Immunocytochemistry was performed in HeLa cells fixed with 4% paraformaldehyde or methanol (for endogenous Atg16L1, endogenous Atg5 and endogenous Atg12 staining). Primary antibodies were as follows: anti-haemagglutinin (anti-HA, 1:1,000) (Covance), anti-EEA1 (1:200; Abcam, Cell Signalling), anti-Atg12 (1:250; Cell Signalling), anti-Atg5 (1:200; Sigma), anti-Atg16L1 (1:500; MBL International, CosmoBio), anti-gm130 (1:250; BD Biosciences), anti-grp78 (BiP, Abcam) and anti-Flag M2 (1:500; Sigma Aldrich). Co-localization analysis was performed on confocal images with the Zeiss TIRF 3 system using an αPlan-Fluar 100× 1.4 numerical aperture oil-immersion lens under the control of the AxioVision software. Laser lines at 488 and 561 nm were used for GFP–Atg16L1 and mRFP–GFP excitation, respectively.

Western blot analysis. Western blot analysis was performed using standard techniques with an ECL enhanced chemiluminescence detection kit (GE Healthcare) or with direct infrared fluorescence detection on an Odyssey Infrared Imaging System. The primary antibodies used include anti-Epsin (1:1,000; Santa Cruz Biotechnology), anti-clathrin (1:1,000; BD Bioscience), anti-Atg16L1 (1:1,000; MBL International Corporation), anti-actin (1:5,000; Sigma Aldrich), anti-tubulin (1:5,000; Sigma Aldrich), anti-Flag (1:5,000; Sigma Aldrich), anti-AP1-μ1 (1:3,000; Abcam), anti-AP1-A1 (1:1,000; Abcam), anti-LC3 (1:4,000; Novus Biologicals). Immunoprecipitation experiments were performed with standard protocols. For dynasore experiments, cells were treated with 80 µM dynasore (Sigma) for 5 h before collection of cells for immunoprecipitation.

Immunogold electron microscopy. HeLa cells transfected with GFP–Atg16L1 for 24 h were incubated for 15 min with 2.5 µg ml⁻¹ HRP–cholera toxin subunit B (Molecular Probes) at 4 °C, after which they were incubated for 10 min at 37 °C. For the dynasore experiment, cells were treated with 50 µM dynasore (Sigma) for 4 h before fixation. The cells were then fixed with a mixture of 2% paraformaldehyde and 0.2% glutaraldehyde in PBS for 2 h, at room temperature. Cells were then prepared for ultrathin cryosectioning and the immunogold labelled in accordance with a previously described protocol. In brief, fixed cells were washed once in PBS/0.02 M glycine, after which cells were scraped into 12% gelatin in PBS and embedded in the same solution. The cell-gelatin was cut into 1-mm blocks, infiltrated with 2.3 M sucrose at 4 °C, mounted on aluminium pins and frozen in liquid nitrogen. Ultrathin cryosections were picked up in a mixture of 50% sucrose and 50% methylcellulose and incubated with anti-HRP or anti-clathrin and anti-GFP and revealed with 10-nm and 15-nm protein A gold (Utrecht).

Transferrin uptake assay. Cells were collected and resuspended in ice-cold serum-free CO₂-independent medium (SFM) containing 10 mg ml⁻¹ BSA and centrifuged for 2 min at 1,200g and 4 °C, after which they were resuspended in 300 µl of SFM/BSA containing Alexa-488 transferrin (Molecular Probes) and incubated on ice for 5 min for prebinding. They were then incubated for 5 min at 37 °C to allow internalization. The cells were chilled on ice, centrifuged and washed once with 700 µl SFM/BSA. The pellet was then resuspended in 300 µl of acid wash solution (0.1 M glycine, 150 mM NaCl, pH 3), incubated for 4 min on ice, centrifuged, and the process repeated. The pellet was then resuspended in chilled PBS/BSA and analysed by fluorescence-activated cell sorting.

Statistics. Significance levels for comparisons between groups were determined by t-tests, repeated-measures or factorial analysis of variance with STATVIEW software, v. 4.53 (Abacus Concepts).

Figure S1 A. HeLa cells transfected with wild-type or T300A mutant of Flag-Atg16L1 for 24 h were immunoprecipitated (IP) with anti-Flag antibody (Atg16L1) and immunoblotted with anti-clathrin and anti-Flag antibodies. Clathrin bound to a similar extent to both wild-type and T300A Atg16L1. TL – total cell lysate. B, C. HeLa cells grown in normal media or HBSS for 6 h were either left untreated or treated with bafilomycin A1 (BafA1) for the last 4 h before the cells were collected for western blot analysis. LC3-II to actin ratio under the different conditions is shown in the graph. D, E, F. HeLa cells, transfected with two rounds of control (Cont), epsin 1 (Eps), AP2 or AP1 siRNAs for 5 d, were subjected to western blot analysis for anti-epsin, anti-clathrin-heavy chain, anti-AP2, anti-AP1 and anti-actin antibodies to test efficiency of the knockdown. G. HeLa cells stably expressing GFP-mRFP-LC3 were transfected with two rounds of control, AP2 or AP1 siRNA for 5 d during which they were either left untreated (UT) or treated with Bafilomycin A1 (BafA1) for the last 15 h. Cells were then fixed and analysed on a fluorescent microscope. Quantification of autophagic vacuoles (AV) or autolysosomes (AL) per cell in the different conditions is shown in the graph.
**Figure S2 A.** HeLa cells transfected with two rounds of control, epsin 1 or clathrin-heavy chain siRNA for 5 d grown with or without 100 mM trehalose (to induce autophagy) for the last 15 h, were either left untreated or treated with Bafilomycin A1 (+/- BafA1) for the final 4 h, after which they were lysed for western blot analysis with anti-LC3 and anti-tubulin antibodies. Quantification of this blot is shown in the bottom. B. Similar experiments as in A were performed with control, AP1 or AP2 siRNA and the ratio of LC3-II to tubulin upon control, epsin, clathrin-heavy chain, AP2 or AP1 knockdown, with, or without Bafilomycin A1, under trehalose treatment conditions is quantitated from three independent experiments (two for epsin) and represented in the graph. ** - p<0.001, *** - p<0.0001. No statistics were done for epsin as only two independent experiments were done. C. Similar LC3-II analyses as in A were performed with control, clathrin-heavy chain or AP2 knockdown except that the cells were grown in Hank’s balanced salt solution (instead of trehalose) with or without BafA1 for 4 h before collecting them for western blot analysis. Quantitation of LC3-II/tubulin for this experiment is represented in the graph in panel C. NS - not significant, *** - p<0.0001.
Figure S3 A. A representative image of HeLa cells transfected with tomato-LC3 is shown. An example of tomato-LC3 transfected cell with >50 LC3 vesicles is marked by an arrow. B. HeLa cells transfected with control, epsin 1 or clathrin-heavy chain siRNA for 72 h were transfected with tomato-LC3 (along with siRNA) for a further 24 h with or without trehalose. Cells were fixed and percentage of LC3-transfected cells with more than 50 LC3-vesicles were counted. *** - p<0.0001. C. HeLa cells transfected with control, AP1 or AP2 siRNA for 72 h were then transfected with tomato-LC3 for the last 24h, after which they were fixed. Quantitation of LC3-positive cells with more than 50 vesicles is shown in the graph. *** - p<0.0001, NS- not significant. D, E. HeLa cells transfected with control, epsin 1 or AP2 siRNA for 48 h were transfected with HA-tagged huntingtin exon 1 with 74 polyglutamine repeats (Q74) for a further 24 h after which they were fixed, and immunostained with anti-HA antibody. A cell with aggregates is marked by an arrow and a transfected cell without aggregates can also be seen, to illustrate the aggregates. The proportions of Q74-transfected cells with aggregates were scored and represented in the graphs. *** - p<0.0001, NS- not significant. F. HeLa cells, transfected with two rounds of control (Cont), clathrin-heavy chain (Cla), AP2 or AP1 siRNAs for 5 d, were subjected to western blot analysis for anti-p62 and anti-actin is shown. Quantitation of band intensities from three independent experiments is on the right. NS- not significant, ** - p<0.001, *** - p<0.0001. G. Similar knockdown experiment as in F was performed with control or clathrin-heavy chain and at the end of the knockdown the cells were fixed and immunostained for endogenous p62. Quantification of endogenous p62 aggregates in control v clathrin (Clat) knockdown cells is depicted in the graph. *** - p<0.0001.
**Figure S4.** A. HeLa cells transfected with control or epsin 1 siRNA for 72 h were treated with Hanks balanced salt solution (to induce autophagy) for 6 h, after which they were fixed and immunostained for endogenous Atg16L1. An inverted image of the confocal image presented in Fig. 3A is shown. Some of the Atg16L1 vesicles (black dots) are marked with arrows. B. HeLa cells, transfected with two rounds of control (Cont) or clathrin-heavy chain (Clat) siRNAs for 5 d were treated with or without (untreated) rapamycin for 15 h before fixation and immunostaining with anti-Atg16L1. Proportion of cells with Atg16L1 vesicles (red dots, arrows in rapamycin panel) upon control or clathrin-heavy chain knockdown is shown in the graph. *** - p<0.0001. C. HeLa cells transfected with GFP-Atg16L1 for 24 h were either left untreated or treated with bafilomycin A1 (BafA1) for the last 15 h. Cells were then fixed and analysed on cellomics arrayscan. Quantification of vesicle number and vesicle size under untreated or with BafA1 is represented in the graph.
Figure S5 A. HeLa cells transfected with GFP-tagged Atg16 for 24 h were incubated with Alexa fluor 555-labelled cholera toxin-subunit B (red) for 15 minutes at 4°C (to allow toxin binding to the plasma membrane). The cells were then shifted to 37°C (to allow cholera toxin internalization) and observed under a live cell confocal microscope. Imaging was performed at 1 frame per 10 seconds. Arrows indicate colocalization between Atg16 and cholera toxin (in yellow). A magnified image of a single panel is showed in B. Scale bar 10 μm. C. HeLa cells transfected with GFP-Atg16L for 24 h were treated with or without 3-methyladenine (3MA) for the last 15 h, fixed and the number of GFP-Atg16L vesicles per cell were quantitated and depicted in the graph. D. HeLa cells transfected with GFP-Atg16L for 24 h were fixed and immunoprobed for endogenous Atg12 or endogenous Atg5. Co-localisation of Atg16L1 (green) with Atg5 (red) or Atg12 (red) is shown in yellow and marked with arrows.
**Figure S6 A.** HeLa cells transfected for 24 h with Flag-Atg16L1 (blue) and GFP-Atg5 (green) were incubated with Alexa fluor-555 conjugated cholera toxin-subunit B (red) for 15 minutes at 4°C (to allow toxin binding to the plasma membrane). The cells were then incubated at 37°C (which allows cholera toxin internalization) for 10 minutes and fixed for confocal analysis. Co-localisation of vesicles positive for Atg16L1, Atg5 and cholera toxin is marked with arrows in the higher magnification images. **B.** Similar experiment as in A was performed with the conjugation deficient Atg5 mutant, GFP-K130R Atg5 and observed co-localisation of vesicles positive for Atg16L1, K130R Atg5 and cholera toxin (arrows in magnified panel). **C.** HeLa cells transfected with GFP-Atg16L for 24 h were fixed and immunostained with anti-BiP or anti-GM130. No co-localisation was observed with Atg16L1 and BiP or GM130.
Figure S7 A. HeLa cells transfected for 72 h with GFP-Atg16L1 were selected with 600 μg/ml G418 for two weeks, after which the cells were treated with or without HBSS to visualize GFP-Atg16L1 vesicles. Note that they are not clonal lines but a mixed population of cells stably expressing GFP-Atg16L1. The cells treated with HBSS were incubated with Alexa fluor 555-labelled cholera toxin-subunit B (red) for 15 minutes at 4°C. The cells were then incubated at 37°C for 10 minutes after which they were fixed for confocal analysis. Co-localisation (yellow) of GFP-Atg16L1 vesicles (green) with cholera toxin (red) is shown in the magnified image on the right. Scale bar 10 μm. B. HeLa cells stably expressing Atg16L1 were treated with HBSS or HBSS+3-methyl adenine and the number of Atg16L1 vesicles per cell was counted as shown in the graph. C. HeLa cells transfected with GFP-Atg5 (green) and CFP-LC3 (blue) for 24h were incubated with Alexa fluor 555-labelled cholera toxin-subunit B for 15 minutes at 4°C, after which they were incubated at 37°C for 10 minutes, fixed and analysed by confocal microscopy. The co-localisation of GFP-Atg5 (green) with cholera toxin (red) is marked by yellow arrows, GFP-Atg5 (green) with cholera toxin (red) and LC3 (blue) is marked by blue arrow and the LC3-vesicle is marked with a pink arrow on the magnified image on the far right panel. The quantitation of the co-localisation events is shown in the graph. Scale bar 10 μm. D. HeLa cells transfected for 24h with Flag-tagged wild-type Atg16L1 or the Atg16L1 deletion mutants - 2-275 or 232-607 Atg16L1 were fixed and imunostained with anti-Flag antibody before confocal analysis. Co-localisation of wild-type or 2-275 Atg16L1 (red) with GFP-Atg5 (green) is marked by arrows in the inset and quantitated as shown in the graph. HeLa cells transfected for 24 h with Flag-tagged wild-type Atg16L1 or the Atg16L1 deletion mutants - 2-275 or 232-607 Atg16L1 were incubated with HRP-conjugated cholera toxin-subunit B for 15 minutes at 4°C, after which they were incubated at 37°C for 10 minutes, fixed and immunostained for EEA1. Different co-localisation events are depicted in the graph. NS – not significant, ** - p<0.001. E. HeLa cells transfected with GFP-Atg16L1 for 24 h were incubated with HRP-labelled cholera toxin-subunit B (red) for 15 minutes at 4°C after which they were then shifted to 37°C for 10 minutes and processed for immuno-gold EM with anti-GFP antibody (10nm gold particles) and anti-HRP antibody (15nm gold particles). Scale bar – 100nm. F. HeLa cells transfected for 24 h with GFP-Atg16L1 (green), were incubated with FM4-64 for 1 minute at 4°C. The cells were then imaged immediately after shifting to 37°C. A time series following fusion of a FM4-64 vesicle (red) with GFP-Atg16L1 vesicle (green) is shown. This set of stills one second apart was captured within 5 minutes of switching to 37°C. A higher magnification image showing co-localisation (yellow) of GFP-Atg16L1 vesicle (green) with FM4-64 vesicle (red) is in the bottom panel. PM – plasma membrane. Scale bar – 10μm.
**Figure S8** A. HeLa cells transfected with two rounds of control (Cont), clathrin-heavy chain (Clat), AP1 or AP2 siRNA were subjected to Alexa 488-labelled transferrin internalisation assay and the amount of fluorescent-transferrin internalised under different knockdown conditions is measured on FACS and presented in the graph. NS – not significant, ** - p<0.001.

B, C. HeLa cells transfected for 24 h with Flag-tagged wild-type Atg16L1 together with empty vector (top panels) or dominant-negative dynamin-II mutant (K44A Dyn; bottom panels) were incubated with Alexa fluor 555-labelled cholera toxin-subunit B (red) for 15 minutes at 4°C after which they were incubated at 37°C for 10 minutes and fixed for confocal analysis. Note the tubular staining pattern with cholera toxin in cells expressing K44A-Dyn and also Atg16L1 co-localisation with these structures. Quantitation of Flag-Atg16L1 vesicles in control cells and in cells expressing K44A-Dynamin (DN-Dyn) is shown in the graph. * - p<0.01.

D. HeLa cells transfected for 24 h with Flag-tagged wild-type Atg16L1, GFP-PLC(PH) together with empty vector (top panels) or dominant-negative dynamin-II mutant (K44A Dyn; bottom panels) were fixed and analysed on the confocal microscope. Note the co-localisation of Atg16L1 with PLC(PH) at the plasma membrane which is quantified in the graph. * - p<0.01.
**Movie S1** HeLa cells transfected with GFP-tagged Atg16 for 24 h were incubated with Alexa fluor 555-labelled cholera toxin-subunit B (red) for 15 minutes at 4°C (to allow toxin binding to the plasma membrane). The cells were then shifted to 37°C (to allow cholera toxin internalization) and observed under a live cell confocal microscope. Imaging was performed at 1 frame per 10 seconds.