nature structural & molecular biology

Degradation of cellular contents can occur via the proteasome or lysosome systems[1]. There are a number of pathways that deliver substrates for degradation to either system. Macroautophagy (commonly referred to as autophagy) is one pathway that facilitates the degradation of long-lived proteins, damaged organelles and infectious pathogens, resulting in the clearance of toxic materials and increased nutrient availability in the cell[2]. Because of these substrates, autophagy has been implicated in a number of physiological and pathological processes including development, pathogen infection, neurodegeneration and cancer[3].

During autophagy, a number of protein complexes orchestrate the formation of a lipid bilayer (termed phagophore or preautophagosome), which, upon maturation, engulfs cytoplasmic materials and forms the autophagosome. The eventual fusion of autophagosomes with lysosomes results in degradation of the autophagosome contents and recycling of nutrients back into the cytoplasm. Key players in these events include a family of ubiquitin-like proteins, such as LC3, which are involved in phagophore maturation and possibly cargo selection[4–7]. Membrane targeting of LC3 is essential for autophagy and requires a series of ubiquitin-like conjugation events that lead to the conjugation of cytosolic LC3 (LC3-I) to the membrane-bound, phosphatidylethanolamine-conjugated form (LC3-II)[4]. These events are catalyzed by autophagy-related proteins (or ATG proteins) with E1-, E2- and E3-like enzymatic activities, termed ATG7, ATG3 and ATG5-ATG12, respectively. Formation of the ATG5-ATG12 conjugate (herein referred to as ATG5-12) is also catalyzed by similar conjugation events but requires a distinct E2-like enzyme termed ATG10 (ref. 8). In addition to its possible role as an E3-like enzyme during LC3-II formation, the ATG5-12 conjugate has been shown to form a large protein complex with ATG16L1, which is thought to be required to specify the site of LC3 conjugation during autophagy[9]. Depletion of any of ATG5, ATG16L1 or ATG7 completely abolishes autophagosome formation[10–12], whereas depletion of ATG3 results in defective maturation of the phagophore structure[13]. LC3 can also be recruited to single-membrane structures, for example during phagocytosis or entotic cell clearance[14–16]. In these cases, the core machinery used during LC3-II formation is also required.

A number of upstream signaling complexes can regulate autophagy, including the Vps34 and ULK1 complexes. The Vps34 complex comprises a number of proteins, including the Vps34 lipid kinase, p150, Beclin and ATG14, and is essential for phagophore formation and proper recruitment of ATG proteins to the phagophore[17,

previously uncharacterized interaction between the ULK1 complex and the ATG5 complex is required for ULK1 complex–dependent, but not ULK1 complex–independent, autophagy processes.

RESULTS

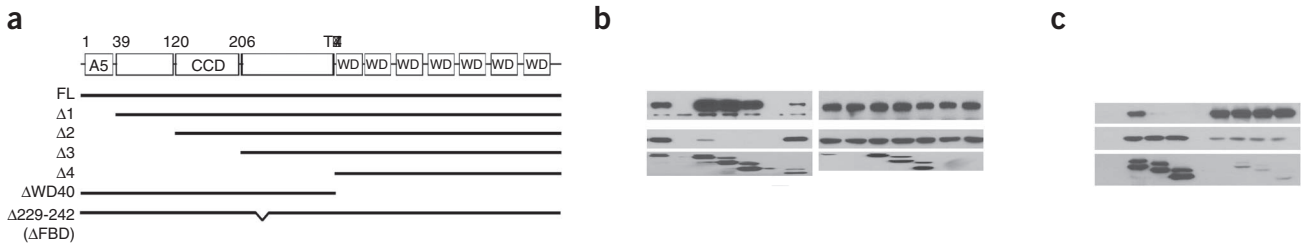
Pre-autophagosomes accumulate in the absence of ATG3

To study the molecular events during formation of the pre-autophagosome structures, we separated membrane-bound proteins from cytosolic proteins by subcellular fractionation. Cytosolic LC3 (LC3-I) was separated from membrane-bound LC3 (LC3-II) in wild-type mouse embryonic fibroblasts (MEFs; **Fig. 1a**). However, using this method, we scarcely detected the accumulation of upstream ATG proteins, such as ATG16L1, in the membrane fraction of wild-type MEFs even when autophagy was induced by amino acid deprivation (**Fig. 1b**). This suggests that the recruitment of upstream ATG proteins to pre-autophagosome structures may occur transiently. Previous studies suggest that in the absence of ATG3, pre-autophagosome structures are unable to mature into autophagosomes and therefore accumulate in the cell, probably leading to stabilization of upstream autophagy events that are transient in ATG3-expressing cells[13,21]. Therefore, we tested the subcellular localization of ATG16L1 in *Atg3*$^{-/-}$ MEFs. Notably, ATG16L1 accumulated in the membrane fraction of these cells (**Fig. 1b**). We detected no increase in ATG16L1 recruitment to the membrane fraction after 2 h of amino acid starvation in *Atg3*$^{-/-}$ cells, in agreement with previous studies[21]. Similarly, in immunofluorescence analyses we found that structures containing ATG16L1, which colocalized with endogenous ATG5, were detectable even under nutrient-rich conditions in the absence of ATG3 (**Fig. 1c**).

Identification of FIP200 as an ATG16L1-interacting partner

Next we sought to identify previously uncharacterized protein-protein interactions of membrane-bound ATG proteins in *Atg3*$^{-/-}$ cells using ATG14 and ATG16L1 as baits. For this, we used tandem affinity purification to purify ATG14 and ATG16L1 complexes followed by SDS-PAGE analysis and silver staining (**Fig. 2a**). We observed prominent bands in the ATG14 sample that correspond to known ATG14 interacting partners, including Vps34 and p150, confirming that functional autophagy complexes can be purified by this method. In the case of ATG16L1 purification, we detected a distinct band of a molecular weight greater than 170 kDa in addition to the ATG5-12 conjugate, the known binding partner of ATG16L1. Mass spectrometric analysis revealed the identity of this band as FIP200, an essential component of the ULK1 complex. Previously, the ULK1 complex has been proposed to be functionally linked to the ATG5 complex, but how these two

residues 1–335 deleted) was defective in binding to FIP200. These results suggest that the interaction between ATG16L1 and FIP200 is mediated



complex–independent manner that correlates with an increase in cellular ammonia levels and lack of mTORC1 inhibition[27,28]. We confirmed this using ULK1 and ULK2 double-knockout MEFs, in which glucose starvation induced formation of LC3-II that was susceptible to lysosomal degradation (**Fig. 6a**). We also observed that glucose starvation did not induce ULK1 dephosphorylation on residues that are dephosphorylated during amino acid starvation[29] (**Fig. 6b**). Notably, when we reconstituted *Atg16l1*$^{-/-}$ cells with full-length ATG16L1 or ATG16L1ΔFBD, we observed comparable conversion of LC3-I to LC3-II upon glucose starvation, whereas the absence of ATG16L1 expression completely abolished LC3 conjugation (**Fig. 6c**). This indicates that ATG16L1ΔFBD is fully functional during such ULK1 complex–independent autophagy. Similarly, we observed comparable formation of GFP-LC3 punctate structures in cells expressing full-length ATG16L1 or ATG16L1ΔFBD (**Fig. 6d**). Overall, these analyses suggest that, unlike ULK1 complex–dependent autophagy induced by amino acid starvation, ULK1 complex–independent autophagy induced by glucose starvation does not require FIP200 binding to ATG16L1.

DISCUSSION

In this study we offer evidence for a direct interaction between the ULK1 complex component FIP200 and the ATG5 complex component ATG16L1. The finding that this interaction is specifically required for ULK1 complex–dependent autophagy provides mechanistic insights into how the ULK1 complex communicates with other ATG complexes such as the ATG5 complex. The ATG5 complex (including its component ATG16L1) belongs to the essential core autophagy machinery, whereas the ULK1 complex seems to mediate autophagy induced by only certain specific triggers. The LC3 conjugation reaction is intact in cells with genetic deletion of ULK1 (refs. 30,31), and LC3-II formation takes place at basal levels in cells depleted of ULK1 complex components[22,32]. Moreover, the ULK1 complex, but not the ATG5 complex, is dispensable for glucose starvation–induced autophagy[27].

It would be interesting to consider whether ATG16L1 might be a convergence point mediating other upstream autophagy signals, in addition to signals through ULK1. It is evident, however, that the FBD region of ATG16L1 is not necessarily responsible for all other signals because ATG16L1ΔFBD is functionally intact during glucose starvation–induced autophagy. Another recognizable structure in ATG16L1 is the C-terminal WD40 repeats, a versatile protein-protein interaction domain that is not present in yeast ATG16. Although not required for autophagy triggered by amino acid starvation, it is possible that this region mediates certain other autophagy signals. If so, then the C-terminal region of ATG16L1 enables the mammalian autophagy pathway to sense more diverse and complex signals compared to its yeast counterpart. However, it is also possible that the WD40 repeats in mammalian ATG16L1 are relevant only to non-autophagy processes[10,33]. In light of this, it would be useful to explore the potential pathological role of the Crohn's disease–associated ATG16L1 mutation (T300A), which lies within the WD40 repeats, in both autophagy-related and non–autophagy-related processes[34,35].

A detailed comparison of ATG16L1 with its homolog ATG16L2 should also shed light on the function of ATG16L1 during autophagy. Unlike ATG16L1, ATG16L2 is unable to support autophagy or localize to the phagophore structures despite its ability to bind ATG5, self-oligomerize and form a large protein complex with the ATG5-12 complex[26]. ATG16L1 proteins from various vertebrate species all have highly conserved FBD regions, whereas ATG16L2 lacks this domain. Consistent with this, we found ATG16L2 did not interact with FIP200. However, whether the lack of FBD renders ATG16L2 inactive in autophagy is unclear, as ATG16L1 with its FBD deleted can still mediate ULK1 complex–independent autophagy. In addition, neither yeast nor *Caenorhabditis elegans* ATG16 have this domain (there is no true FIP200 homolog in these organisms either). Furthermore, previous *in vitro* biochemical studies suggest that yeast ATG16 is not required for the E3-like activity of ATG5-12 during the conjugation of ATG8 (the yeast homolog of LC3) to phosphatidylethanolamine[36]. Thus, in mammalian cells, the difference between ATG16L1 and ATG16L2 in autophagy does not seem to be due to their differential influence on the E3-like enzymatic activity of the ATG5 complex. The exact structural and biochemical mechanism that renders ATG16L1 but not ATG16L2 an essential functional component in autophagy has yet to be defined.

In conclusion, this study has uncovered a previously uncharacterized functional interaction between two upstream ATG complexes and has demonstrated that ATG16L1 is not only an essential structural component of the ATG5 complex but also a signaling protein that can mediate specific upstream signals during autophagy, such as those transduced by the ULK1 complex.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

ACKNOWLEDGMENTS

We thank S. Akira (Osaka University) for *Atg16l1*$^{-/-}$ MEFs, M. Komatsu (Tokyo Metropolitan Institute of Medical Science) for *Atg3*$^{-/-}$ MEFs, N. Mizushima (Tokyo Medical and Dental University) for *Atg5*$^{-/-}$ MEFs, C. Thompson (Memorial Sloan Ketteringomp C14(958(t32)-4(o8427)9(D)-18(e)-12t)-2(t)-6(5112(s)-12(,)-12(

AUTHOR CONTRIBUTIONS
N.G. and X.J. designed the study and wrote the paper; N.G. and O.F. performed the experiments; N.G., O.F., M.O. and X.J. analyzed the data.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

Published online at http://www.nature.com/doifinder/10.1038/nsmb.2475.
Reprints and permissions information is available online at http://www.nature.com/reprints/index.html.

1. Ciechanover, A. Proteolysis: from the lysosome to ubiquitin and the proteasome. *Nat. Rev. Mol. Cell Biol.* **6**, 79–87 (2005).
2. Wirawan, E., Vanden Berghe, T., Lippens, S., Agostinis, P. & Vandenabeele, P. Autophagy: for better or for worse. *Cell Res.* **22**, 43–61 (2012).
3. Levine, B. & Kroemer, G. Autophagy in the pathogenesis of disease. *Cell* **132**, 27–42 (2008).
4. Geng, J. & Klionsky, D.J. The Atg8 and Atg12 ubiquitin-like conjugation systems in macroautophagy. 'Protein modifications: beyond the usual suspects' review series. *EMBO Rep.* **9**, 859–864 (2008).
5. Bjørkøy, G. *et al.* p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death. *J. Cell Biol.* **171**, 603–614 (2005).
6. Pankiv, S. *et al.* p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. *J. Biol. Chem.* **282**, 24131–24145 (2007).
7. Kraft, C., Peter, M. & Hofmann, K. Selective autophagy: ubiquitin-mediated recognition and beyond. *Nat. Cell Biol.* **12**, 836–841 (2010).
8. Nemoto, T. *et al.* The mouse APG10 homologue, an E2-like enzyme for Apg12p conjugation, facilitates MAP-LC3 modification. *J. Biol. Chem.* **278**, 39517–39526 (2003).
9. Fujita, N. *et al.* The Atg16L complex specifies the site of LC3 lipidation for membrane biogenesis in autophagy. *Mol. Biol. Cell* **19**, 2092–2100 (2008).
10. Saitoh, T. *et al.* Loss of the autophagy protein Atg16L1 enhances endotoxin-induced IL-1β production. *Nature* **456**, 264–268 (2008).
11. Mizushima, N. *et al.* Dissection of autophagosome formation using Apg5-deficient mouse embryonic stem cells. *J. Cell Biol.* **152**, 657–668 (2001).
12. Komatsu, M. *et al.* Impairment of starvation-induced and constitutive autophagy in Atg7-deficient mice. *J. Cell Biol.* **169**, 425–434 (2005).
13. Sou, Y.S. *et al.* The Atg8 conjugation system is indispensable for proper development of autophagic isolation membranes in mice. *Mol. Biol. Cell* **19**, 4762–4775 (2008).
14. Florey, O., Kim, S.E., Sandoval, C.P., Haynes, C.M. & Overholtzer, M. Autophagy machinery mediates macroendocytic processing and entotic cell death by targeting single membranes. *Nat. Cell Biol.* **13**, 1335–1343 (2011).
15. Martinez, J. *et al.* Microtubule-associated protein 1 light chain 3 alpha (LC3)-associated phagocytosis is required for the efficient clearance of dead cells. *Proc. Natl. Acad. Sci. USA* **108**, 17396–17401 (2011).
16. Florey, O. & Overholtzer, M. Autophagy proteins in macroendocytic engulfment. *Trends Cell Biol.* **22**, 374–380 (2012).
17. Itakura, E., Kishi, C., Inoue, K. & Mizushima, N. Beclin 1 forms two distinct phosphatidylinositol 3-kinase complexes with mammalian Atg14 and UVRAG. *Mol. Biol. Cell* **19**, 5360–5372 (2008).
18. Zhong, Y. *et al.* Distinct regulation of autophagic activity by Atg14L and Rubicon associated with Beclin 1-phosphatidylinositol-3-kinase complex. *Nat. Cell Biol.* **11**, 468–476 (2009).
19. Ganley, I.G. *et al.* ULK1.ATG13.FIP200 complex mediates mTOR signaling and is essential for autophagy. *J. Biol. Chem.* **284**, 12297–12305 (2009).
20. Jung, C.H. *et al.* ULK-Atg13–FIP200 complexes mediate mTOR signaling to the autophagy machinery. *Mol. Biol. Cell* **20**, 1992–2003 (2009).
21. Itakura, E. & Mizushima, N. Characterization of autophagosome formation site by a hierarchical analysis of mammalian Atg proteins. *Autophagy* **6**, 764–776 (2010).
22. Hara, T. *et al.* FIP200, a ULK-interacting protein, is required for autophagosome formation in mammalian cells. *J. Cell Biol.* **181**, 497–510 (2008).
23. Mizushima, N. *et al.* Mouse Apg16L, a novel WD-repeat protein, targets to the autophagic isolation membrane with the Apg12-Apg5 conjugate. *J. Cell Sci.* **116**, 1679–1688 (2003).
24. Fujioka, Y., Noda, N.N., Nakatogawa, H., Ohsumi, Y. & Inagaki, F. Dimeric coiled-coil structure of *Saccharomyces cerevisiae* Atg16 and its functional significance in autophagy. *J. Biol. Chem.* **285**, 1508–1515 (2010).
25. Matsushita, M. *et al.* Structure of Atg5Atg16, a complex essential for autophagy. *J. Biol. Chem.* **282**, 6763–6772 (2007).
26. Ishibashi, K. *et al.* Atg16L2, a novel isoform of mammalian Atg16L that is not essential for canonical autophagy despite forming an Atg12–5-16L2 complex. *Autophagy* **7**, 1500–1513 (2011).
27. Cheong, H., Lindsten, T., Wu, J., Lu, C. & Thompson, C.B. Ammonia-induced autophagy is independent of ULK1/ULK2 kinases. *Proc. Natl. Acad. Sci. USA* **108**, 11121–11126 (2011).
28. Eng, C.H., Yu, K., Lucas, J., White, E. & Abraham, R.T. Ammonia derived from glutaminolysis is a diffusible regulator of autophagy. *Sci. Signal.* **3**, ra31 (2010).
29. Shang, L. *et al.* Nutrient starvation elicits an acute autophagic response mediated by Ulk1 dephosphorylation and its subsequent dissociation from AMPK. *Proc. Natl. Acad. Sci. USA* **108**, 4788–4793 (2011).
30. Alers, S. *et al.* Atg13 and FIP200 act independently of Ulk1 and Ulk2 in autophagy induction. *Autophagy* **7**, 1423–1433 (2011).
31. Kundu, M. *et al.* Ulk1 plays a critical role in the autophagic clearance of mitochondria and ribosomes during reticulocyte maturation. *Blood* **112**, 1493–1502 (2008).
32. Wei, H. *et al.* Suppression of autophagy by FIP200 deletion inhibits mammary tumorigenesis. *Genes Dev.* **25**, 1510–1527 (2011).
33. Fujita, N. *et al.* Differential involvement of Atg16L1 in Crohn disease and canonical autophagy: analysis of the organization of the Atg16L1 complex in fibroblasts. *J. Biol. Chem.* **284**, 32602–32609 (2009).
34. Rioux, J.D. *et al.* Genome-wide association study identifies new susceptibility loci for Crohn disease and implicates autophagy in disease pathogenesis. *Nat. Genet.* **39**, 596–604 (2007).
35. Hampe, J. *et al.* A genome-wide association scan of nonsynonymous SNPs identifies a susceptibility variant for Crohn disease in ATG16L1. *Nat. Genet.* **39**, 207–211 (2007).
36. Hanada, T. *et al.* The Atg12-Atg5 conjugate has a novel E3-like activity for protein lipidation in autophagy. *J. Biol. Chem.* **282**, 37298–37302 (2007).