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Journal
Nature Cell Biology, 17(1)

ISSN
1465-7392

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Publication Date
2015

DOI
10.1038/ncb3089

Ironing out VPS34 inhibition

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The class III phosphoinositide 3-kinase VPS34 regulates autophagosome formation. Three groups have developed VPS34 inhibitors and shown their utility in investigating and defining autophagic processes.

Macroautophagy (commonly called autophagy) is a lysosomal pathway that degrades and recycles diverse cytosolic components in response to nutrient deprivation and a wide array of other stresses. The pressing need for identification of autophagy-specific pharmacological inhibitors remains, as autophagy is a critical metabolic adaptation and homeostatic process in many cellular contexts and diseases, including neurodegeneration and cancer[1]. One protein that has attracted immense interest as a potential target to inhibit autophagy is the class III phosphoinositide 3-kinase (PI3K) vacuolar protein sorting 34 (VPS34), the most ancient and conserved PI3K isoform in eukaryotes that generates intracellular pools of phosphatidylinositol 3-phosphate (PtdIns(3)P)[2,3]. VPS34 forms two distinct complexes that localize either at pre-autophagosomal structures (VPS34–VPS15–ATG14–UVRAG–Beclin1) or at endosomes (VPS34–VPS15–UVRAG–Rab5–EEA1) (Fig. 1)[4]. VPS34 activity generates intracellular PtdIns(3)P, which is incorporated into the membranes of vesicular organelles, including autophagosomes and endosomes. This leads to the recruitment of adaptor proteins possessing PtdIns(3)P-binding FYVE and PX domains, thereby enabling diverse organelle functions including membrane trafficking and signal transduction with high spatiotemporal specificity[5]. Reporting in *Nature Cell Biology*, Dowdle *et al.* have identified the small molecule PIK-III as a pharmacological inhibitor of VPS34 and demonstrate its efficacy as an autophagy inhibitor[6]. Two additional groups report the development of distinct inhibitors of VPS34 (SAR405 and VPS34-IN1, respectively) that cause defects in autophagosome formation and endosomal trafficking[7,8]. All three VPS34 inhibitors are exquisitely selective for the class III PI3K VPS34 and not the closely related class I and class II PI3Ks.

Initial work using these three distinct inhibitors clearly demonstrates VPS34 control of autophagy and suggests important roles for VPS34 at endosomes. Dowdle *et al.* demonstrate that PIK-III treatment decreases autophagosome formation, leading to an accumulation of known substrates (NBR1, p62/SQSTM1, NDP52) that are normally degraded by the autophagy pathway[9]. The Pasquier group similarly shows that starvation-induced autophagy is completely abrogated by the VPS34 inhibitor SAR405 (ref. 7). Finally, the Alessi group delineates distinct effects for their VPS34 inhibitor VPS34-IN1 on endosome biogenesis[8]. These studies reveal an 80% loss in endosomal labelling following VPS34-IN1 treatment, measured using a GFP–FYVE fusion protein as a reporter of intracellular PtdIns(3)P production. Notably, cells still exhibit residual GFP–FYVE vesicular labelling, suggesting that VPS34-independent pathways may contribute to PtdIns(3)P generation and endosome formation. Additionally, the Alessi group demonstrates that VPS34 inhibition impacts the localization of serum- and glucocorticoid-regulated kinase-3 (SGK3), the only protein kinase known to interact specifically

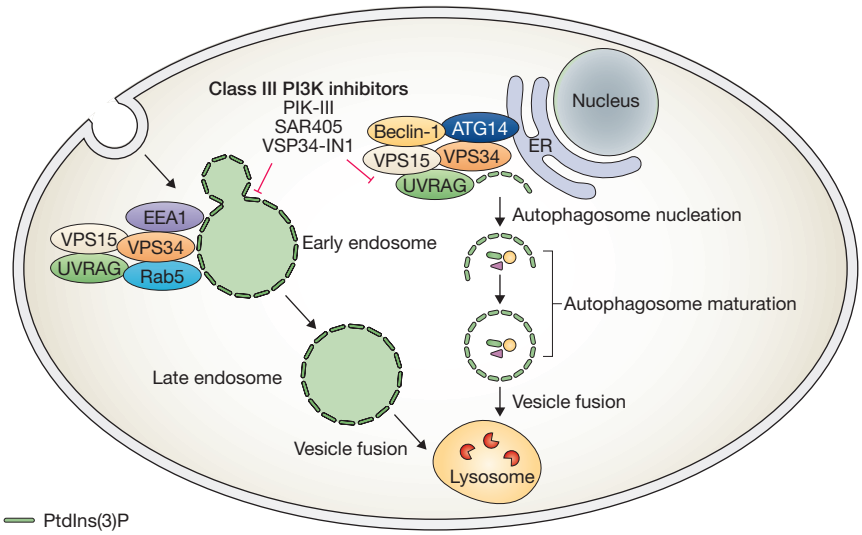

Figure 1 Functions of VPS34 in autophagy and endocytosis. VPS34 forms distinct complexes required for endocytosis (left) as well as for autophagosome nucleation and maturation (right). The resulting autophagosomes and endosomes, along with their vesicular contents, are commonly trafficked to the lysosome for degradation. VPS34 inhibitors (PIK-III, SAR405 and VPS34-IN1) prevent the generation of PtdIns(3)P essential for the proper formation and trafficking of both autophagosomes and endosomes, thereby inhibiting lysosomal degradation of vesicular contents via these two pathways.

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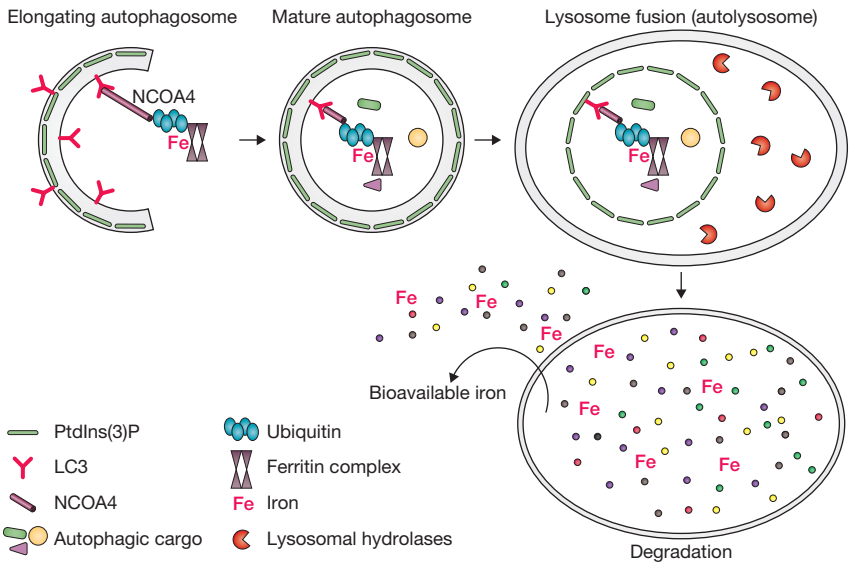

Figure 2 NCOA4 and selective degradation of ferritin complexes. NCOA4 is a selective autophagy receptor that binds iron-containing ferritin complexes and sequesters them into autophagosomes via protein–protein interactions with MAP1LC3 (LC3), a mammalian ATG8 isoform present on the developing autophagosome membrane. Upon autophagosome maturation and fusion with the lysosome, the sequestered NCOA4 and ferritin are degraded along with other cytosolic components, resulting in the release of bioavailable iron.

with PtdIns(3)P via its N-terminal PX domain, to EEA1-positive endosomes, resulting in defective SGK3 phosphorylation. Although the functional significance of VPS34 inhibition on endocytosis and SGK3 activity requires further investigation, these studies illustrate that pharmacological VPS34 inhibition has the potential to impact multiple PtdIns(3)P-dependent intracellular processes and reinforce that these agents should not be construed as being autophagy-specific.

Nevertheless, the importance of these VPS34 inhibitors as tools in autophagy research is poignantly illustrated through their utility in identifying putative autophagy substrates, both autophagy cargo receptors and autophagic targets. While initially viewed to be a non-selective bulk degradation pathway, autophagy is now appreciated as a selective process that targets specific proteins and organelles, via autophagy cargo receptors, for lysosomal degradation, termed 'selective autophagy'[9]. Several autophagic receptors, such as p62/SQSTM1, NBR1, OPTN and NDP52 have been identified using genetic ablation of autophagy-related genes (ATGs) or chemical lysosomal inhibitors disrupting autophagic degradation. Nevertheless, the identification of autophagic substrates has been a challenge due to the lack of specificity of pharmacological inhibitors and low overlap between datasets[10]. Accordingly, Dowdle *et al.* used quantitative liquid chromatography–mass spectrometry analysis (LC–MS) of cells treated with PIK-III to identify several autophagy substrates and candidate autophagic receptors. Because proteins targeted for autophagic degradation are commonly ubiquitinated[9], the authors took advantage of a proteomics strategy interrogating both diglycyl-enriched ubiquitinated peptides as well as the global proteome of cells treated with PIK-III. Using this approach, NCOA4 emerged as a top hit, second only to the known autophagy receptor NBR1. Interestingly, TMEM59, a protein shown to target endosomal membranes for autophagic degradation[11], was another top hit in the LC–MS analysis, suggesting potential interactions between endosomal and autophagic pathways.

Notably, a recent proteomics study by Mancias *et al.* similarly uncovered NCOA4 as an autophagosome-enriched protein[10]; hence, these experiments independently verify NCOA4 as a bona fide autophagy substrate. The authors demonstrate that NCOA4 protein accumulates in cells treated with PIK-III or the lysosomal inhibitor bafilomycin A, while NCOA4 mRNA remains unchanged. Furthermore, starved cells undergoing autophagy degrade NCOA4 in a time-dependent manner similar to known autophagy receptors. Although NCOA4 was originally hypothesized to be a co-activator of the nuclear androgen receptor (AR)[12], the discovery of its autolysosomal localization suggests otherwise. Hence, the authors utilized streptavidin-purified NCOA4 to reveal candidate cargos via unbiased LC–MS and identified ferritin heavy chain (FTH1) and ferritin light chain (FTL), the two principal components of a large multi-subunit complex capable of storing and chelating intracellular iron. In conditions of low iron bioavailability, these ferritin complexes are lysosomally degraded, giving rise to increased bioavailable iron. Although genetic studies suggested a requirement for autophagy in ferritin degradation[13,14], the precise mechanisms by which ferritin is targeted for autophagic degradation have been unclear until the recent study by Mancias *et al.* uncovering NCOA4 as a potential autophagy cargo receptor mediating ferritin turnover[10]. Dowdle *et al.* now corroborate and expand these findings. They demonstrate that FTH1 and FTL levels are decreased upon nutrient starvation in an FTH1-dependent manner. Notably, NCOA4, FTH1 and FTL all co-localize with LAMP2-positive autolysosomes but not with EEA1-positive endosomes. Upon deleting NCOA4 in DLD1 cells using clustered regularly interspaced short palindromic repeats (CRISPR) technology, FTH1 and FTL accumulate even under starvation conditions. Finally, treatment of NCOA4$^{-/-}$ cells with deferasirox (DFX), which promotes the autophagic degradation of ferritin complexes, does not promote the turnover of FTH1 and FTL. These results indicate that intracellular iron bioavailability is regulated by NCOA4-dependent autophagic degradation of ferritin complexes (Fig. 2).

Due to the requirement of iron in many biological reduction and oxidation reactions and the high toxicity of excess iron, pharmacological regulation of intracellular iron stores has important implications for diseases such as anaemia (iron deficiency) and haemochromatosis (excess iron). Bioavailable iron regulates haeme biogenesis, erythroid cell development and erythropoiesis. Furthermore, excess iron accumulation in a wide range of tissues can lead to oxidative damage, mediated by Fenton-like reactions that generate hydroxyl radicals and reactive oxygen species[15]. Dowdle *et al.* probe NCOA4 function *in vivo* by generating chimeric mice with CRISPR-deleted NCOA4 embryonic stem cells. These animals exhibit profound accumulation of intracellular iron deposits in macrophages of the splenic red pulp compared to wild-type chimeric animals. Hence, one can speculate that VPS34 inhibition

of NCOA4-mediated selective autophagic degradation of ferritin complexes will have an untold side effect *in vivo* — accumulation of iron deposits and reduced iron bioavailability.

Future *in vivo* studies are required to determine the potential of these novel VPS34 inhibitors as pharmaceutical agents and will undoubtedly uncover novel effects of autophagy inhibition in the context of human disease. Additionally, the use of VPS34 inhibitors in conjunction with specific class I PI3K inhibitors endows researchers with a strategy to study the poorly understood class II PI3Ks. Overall, the promise of specific, pharmacological inhibition of VPS34 has opened up exciting scientific possibilities that will undoubtedly expand our understanding of both autophagic and endocytic processes.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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Solving the centriole disengagement puzzle

Andrew M. Fry

The microcephaly protein, Cep215, contributes to the engagement of duplicated centrioles in interphase. Now two distinct pools of Cep215 at centrosomes are identified, one bound to Cep68 and the other to pericentrin. Plk1-mediated degradation of Cep68 and separase-mediated cleavage of pericentrin release both pools of Cep215, thereby promoting centriole disengagement.

Maintaining orderly progression through the centrosome cycle is key to human health. Centrosomes are the major site of microtubule nucleation and contribute to cell polarity, migration and division. As a result, defects in the centrosome cycle can cause many human diseases, with cancer as perhaps the most obvious associated pathology. The majority of tumour cells have excess centrosomes, promoting genome instability and invasion[1,2]. Furthermore, centrosome defects are implicated in a range of inherited genetic diseases, including ciliopathy syndromes and brain developmental disorders[3].

Human cells begin the cell cycle in G_1 with two centrosomes that are each composed of a single barrel-shaped centriole. These centrosomes sit close together in the cytoplasm with a proteinaceous linker tethering the proximal ends of the two centrioles. As cells progress through S phase and replicate their DNA, the two centrosomes duplicate, with each of the 'parental' centrioles giving birth to a new 'pro-centriole' that grows directly off, and remains tightly attached to, the sidewall of the parent. When cells progress through mitosis, both these connections are broken[4]. First, the proteinaceous linker between the parental centrioles is disassembled in early mitosis, in a process called centrosome disjunction. This facilitates the timely separation of centrosomes in prophase that is important for accurate chromosome segregation. Second, the tight association between pro-centrioles and their parents is released in late mitosis, in a process called centriole disengagement. This is essential to license centrosomes for the next round of duplication in the subsequent cell cycle.

Components of the proteinaceous tether that keeps the two centrosomes together during interphase have been identified, and include C-Nap1, rootletin, Cep68 and Cep215 (ref. 5). Moreover, regulatory mechanisms that promote centrosome disjunction, such as phosphorylation of C-Nap1 by Nek2, have been characterized[4]. In contrast, proteins required to link pro-centrioles to the parent centriole sidewall remain unknown and the nature of centriole engagement is unclear. Hence, the mechanism of centriole disengagement has proven a difficult puzzle to solve. In this issue of *Nature Cell Biology* Pagan and colleagues provide new insights into the process of centriole disengagement[6]. It also provides an explanation for how cells ensure timely progression through these crucial stages of the centrosome cycle.

The authors began by examining how Cep68 is regulated through the cell cycle. They found that Cep68 is degraded in early mitosis following phosphorylation at residue Ser-332 by the Plk1 mitotic kinase. This generates a phosphodegron that recruits the $SCF^{\beta TrCP}$ (Skp–Cullin–F-box protein) ubiquitin ligase and leads to proteasomal degradation of Cep68. Surprisingly though, despite Cep68 being a component of the inter-centriolar linker and essential for recruitment of rootletin to this structure, a non-degradable version of Cep68 (S332A) did not prevent linker disassembly or the execution — or even timing — of centrosome disjunction.

So what's the purpose of Cep68 degradation? The answer appears to lie in an unexpected connection between Cep68 and the second separation event, centriole disengagement. Identification of βTrCP as the ubiquitin ligase that targets Cep68 for degradation came from analysis of Flag–Cep68 binding proteins using the multidimensional protein identification (MudPIT) proteomics technique. Besides βTrCP, and indeed additional components of the SCF, this screen pulled out two other centrosome proteins that have been implicated in centriole disengagement: Cep215, also called

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