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REVIEW

Beclin 1-Vps34 complex architecture: Understanding the nuts and bolts of therapeutic targets

Deanna H. Morris[1], Calvin K. Yip[2], Yi Shi[3], Brian T. Chait[3], Qing Jun Wang (✉)[1,4,5]

[1] *Department of Molecular and Cellular Biochemistry, University of Kentucky, Lexington, KY 40536, USA*
[2] *Department of Biochemistry and Molecular Biology, The University of British Columbia, Vancouver, BC V6T1Z3, Canada*
[3] *Laboratory of Mass Spectrometry and Gaseous Ion Chemistry, The Rockefeller University, New York, NY 10065, USA*
[4] *Department of Toxicology and Cancer Biology, University of Kentucky, Lexington, KY 40536, USA*
[5] *Markey Cancer Center, University of Kentucky, Lexington, KY 40536, USA*

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Abstract **Autophagy is an important lysosomal degradation pathway that aids in the maintenance of cellular homeostasis by breaking down and recycling intracellular contents. Dysregulation of autophagy is linked to a growing number of human diseases. The Beclin 1-Vps34 protein-protein interaction network is critical for autophagy regulation and is therefore essential to cellular integrity. Manipulation of autophagy, in particular via modulation of the action of the Beclin 1-Vps34 complexes, is considered a promising route to combat autophagy-related diseases. Here we summarize recent findings on the core components and structural architecture of the Beclin 1-Vps34 complexes, and how these findings provide valuable insights into the molecular mechanisms that underlie the multiple functions of these complexes and for devising therapeutic strategies.**

Keywords Beclin 1, Vps34, Nrbf2, complex, structure, CX-MS, EM, inhibitor, drug design

Introduction

Autophagy and its molecular machinery

Autophagy is an intracellular degradation process which, often initiated by stressors, functions to breakdown and recycle damaged organelles, long-lived or aggregated proteins, and pathogens. This key function of autophagy is evolutionarily conserved from yeast to higher eukaryotes, and many of the autophagy-related (*Atg*) yeast genes have mammalian orthologs (Nakatogawa et al., 2009; Mizushima et al., 2011). In mammals, there are three pathways for autophagic recycling: microautophagy (Kunz et al., 2004; Uttenweiler et al., 2007), chaperone-mediated autophagy (Massey et al., 2006), and macroautophagy (Suzuki and Ohsumi, 2007; Itakura et al., 2012). Microautophagy and chaperone-mediated autophagy eliminate cytoplasmic materials by directly depositing cargoes into late endosomes or lysosomes, whereas macroautophagy involves the formation of unique double-membraned autophagosomes that later fuse with late endosomes or lysosomes to recycle cellular molecules and organelles. Of the three pathways, the majority of the research to date has been focused on macroautophagy (hereafter called autophagy), which is the emphasis of this review.

The autophagic process is composed of a series of sequential steps: induction/cargo recognition, phagophore formation, vesicle expansion and completion, autophagosome-lysosome fusion, cargo degradation, and recycling of essential components (Fig. 1). This process is orchestrated by a myriad of autophagy proteins, which form functional multiprotein complexes: the Ulk1 complex, the Beclin 1-Vps34 complexes, the Atg2-WIPI2 complex, and both Atg12-Atg5·Atg16L1 and LC3-phosphotidylethanolamine (PE) ubiquitin-like conjugation systems (Chew and Yip, 2014; Hurley and Schulman, 2014). In particular, assembly of the Beclin 1-Vps34 multiprotein complexes that control both autophagosome biogenesis and maturation is crucial to cellular maintenance. Functional anomalies (whether instigated by genetic deficiencies or signaling defects) in the molecular components of the Beclin 1-Vps34 complexes

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Correspondence: Qing Jun Wang
E-mail: qingjun.wang@uky.edu

cause disruption in cellular homeostasis, and are associated with many human diseases, including but not limited to infection, aging, neurodegeneration, and cancer (Choi et al., 2013) (see more discussion below). Therefore, understanding 1) the entirety of the Beclin 1-Vps34 complex components including the structure of each component, 2) the molecular architecture of the protein complexes as a whole including how the protein complex components interact and assemble, and 3) how compromise of these protein complexes causes specific pathologies, is at the forefront of much autophagy research.

Beclin 1-Vps34 complexes in infection

There are numerous infectious diseases linked to the autophagy process. Studies show that as an important self-defense mechanism, host cells can clear intracellular pathogens (e.g., bacteria and viruses) through xenophagy, a specific type of cargo-selective autophagy; conversely, pathogens can also target the autophagic machinery, including the Beclin 1-Vps34 complex components, to invade cells and enhance their virulence (Kudchodkar and Levine , 2009; Münz, 2011; Arroyo et al., 2014).

Beclin 1 has been shown to be critical for immunity. Multiple viral proteins, including α-herpes simplex virus 1 (HSV-1)-encoded neurovirulence factor ICP34.5 (Orvedahl, 2007) and the γ-herpesvirus Bcl-2 homologs (i.e., KSHV v-Bcl-2 and murine γ-HV68 M11) (Pattingre et al., 2005; Ku et al., 2008; Sinha et al., 2008), bind Beclin 1 and prevent autophagosome formation. The human immunodeficiency virus (HIV) type-1 Nef protein (Kyei et al., 2009) and influenza A M2 protein (Gannagé et al., 2009) bind Beclin 1 and block autophagosome maturation. HIV-1 infection also downregulates Beclin 1 at the transcriptional level and

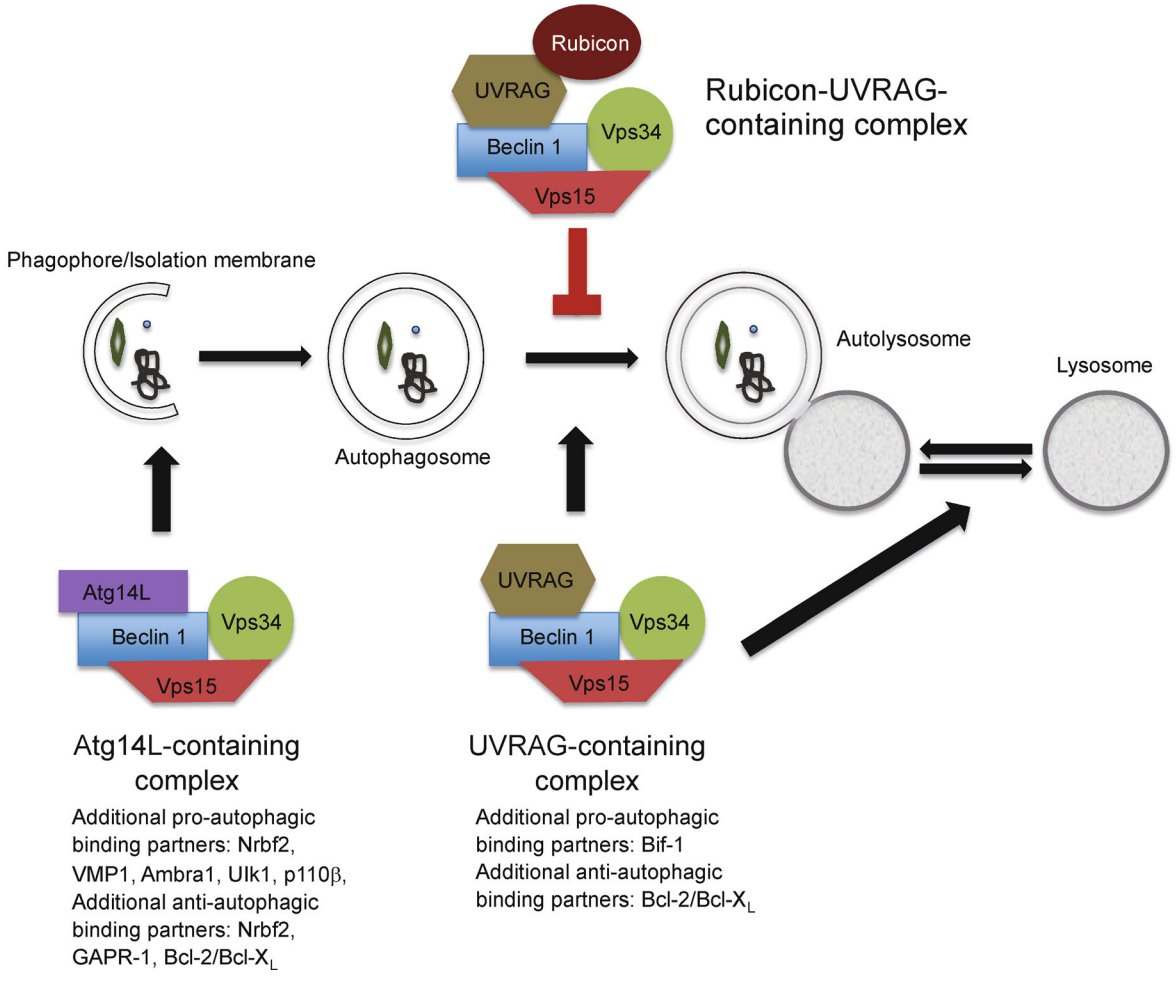


Figure 1 A schematic of the autophagy pathway with the involvement of the Beclin 1-Vps34 class III PI(3)K complexes identified. The core class III PI(3)K complexes consist of Beclin 1, Vps34, and Vps15. The Atg14L-containing Beclin 1-Vps34 complex marks the sites at which the phagophores/isolation membranes form to enclose the cytoplasmic contents to be degraded. The phagophores subsequently elongate and seal off to form mature autophagosomes. Autophagosome maturation, particularly autophagosome fusion with the lysosome, is facilitated by the UVRAG-containing Beclin 1-Vps34 complex. The inner membranes of the autophagosomes and their contents are degraded and recycled to maintain cellular homeostasis. The interaction of Rubicon with the UVRAG-containing Beclin 1-Vps34 complex blocks the fusion between autophagosomes and lysosomes. In addition, the UVRAG-containing Beclin 1-Vps34 complex also regulates the tubule scission step during autophagic lysosome reformation.

inhibits autophagy (Zhou and Spector, 2008). Despite its susceptibility to be hijacked by viruses, Beclin 1 can promote innate antimicrobial immune responses by directly interacting with cyclic GMP-AMP synthetase (cGAS).This interaction suppresses cyclic GMP-AMP production and releases the inhibitory action of RUN domain and cysteine-rich domain containing, Beclin 1-interacting protein (Rubicon (Matsunaga et al., 2009; Zhong et al., 2009)) on the UV-irradiation resistance-associated gene (UVRAG (Liang et al., 2006))-containing Beclin 1-Vps34 complex, thus allowing for induction of autophagic removal of cytosolic pathogen DNA (Liang et al., 2014). Moreover, Beclin 1 is important for immune cell development, as Beclin 1 deficiency results in dramatic depletion of T and B cell precursors (Arsov et al., 2008, 2011).

Other components of the Beclin 1-Vps34 complexes may also be important for fine-tuning the immune response. For example, Rubicon is utilized by the Kaposi's sarcoma-associated herpesvirus (KSHV) to inhibit autophagosome maturation; specifically, KSHV promotes the interaction of Rubicon with the UVRAG-containing Beclin 1-Vps34 complex, thus blocking Vps34 catalytic activity (Liang et al., 2013). Rubicon is also shown to function as a regulator of the NADPH-oxidase-containing phagocytosis complex (Yang et al., 2012a) and the CARD9-containing signaling complex (Yang et al., 2012b), connecting this Beclin 1-interacting protein to the innate immune response against various microbial infections.

Beclin 1-Vps34 complexes in aging and neurodegeneration

Autophagy can be induced by starvation. Coincidentally, dietary restriction is the most effective means to decelerate aging (Bergamini et al., 2007; Cuervo, 2008). Studies show that rapamycin, which induces autophagy through inhibition of mammalian target of rapamycin complex 1 (mTORC1), prolongs lifespan in mice (Harrison et al., 2009); therefore, dietary restriction, which inhibits mTORC1, may be critical for decelerating aging also through inducing autophagy.

Specifically, Beclin 1 and its interacting proteins are important in aging and age-related neurodegenerative diseases such as Huntington's disease (HD), Alzheimer's disease (AD), Parkinson's disease (PD), and Amyotrophic lateral sclerosis (ALS). Inactivation of *bec-1*, the *Caenorhabditis elegance* ortholog of *Becn1* (the gene encoding Beclin 1), reversed the lifespan extension of the insulin-like tyrosine kinase receptor *daf-2* loss-of-function mutation (Meléndez et al., 2003). Beclin 1 levels decline with age in the human brain (Shibata et al., 2006), consistent with the ideas that 1) reduced Beclin 1 levels lead to a decline in autophagic activity and 2) declined autophagic activity is probably an important factor contributing to aging (Terman, 1995; Brunk and Terman, 2002; Terman et al., 2006). Moreover, Beclin 1 is recruited to the cytoplasmic Huntingtin (Htt) inclusions in the brain of the R6/2 HD mouse model and accumulation of mutant Htt is highly sensitive to decreased Beclin 1 levels, suggesting that accumulation of mutant Htt in the aged brain is likely a consequence of age-dependent reduction of Beclin 1 levels and autophagic activity (Shibata et al., 2006). It is also reported that a decline in Beclin 1 expression in the brains of AD patients can result in reduced Vps34 protein levels, leading to neurotoxic accumulation of autophagosomes as well as impaired amyloid precursor protein (APP) processing and turnover (Pickford et al., 2008; Jaeger et al., 2010). Microglia isolated from AD brains also show significantly reduced Beclin 1 levels, which may lead to impaired retromer trafficking and receptor-mediated phagocytosis, contributing to AD pathology (Lucin et al., 2013). Furthermore, PINK1, a key neuroprotective protein in PD, interacts with Beclin 1 to promote both basal and starvation-induced autophagy (Arena et al., 2013). Recent studies also reveal two roles for Beclin 1, through its interaction with another PD protein, PARK2, in the translocation of PARK2 to mitochondria and the initiation of mitophagy prior to formation of autophagosomes (Choubey et al., 2014). This study reported additional interactions of PARK2 with the Beclin 1-interacting proteins Vps34 and autophagy/Beclin 1 regulator 1 (AMBRA1), which are enhanced upon treating cells with carbonyl cyanide m-chlorophenyl hydrazone (CCCP) (Choubey et al., 2014). Interestingly, an earlier study showed that lentivirus-mediated overexpression of Beclin 1 induced autophagy, and reduced the accumulation of abnormal α-synuclein aggregates and related neurodegenerative pathology in α-synuclein models of PD (Spencer et al., 2009). In addition, Beclin 1 is associated with ALS; however, in this context, Beclin 1 reduction protects against ALS development (Nassif et al., 2014).

Beclin 1-Vps34 complexes in cancer

Mono-allelic loss of *BECN1* in 40%-75% of sporadic human breast, ovarian, and prostate cancers was first reported in 1999, linking autophagy deficiency to cancer (Aita et al., 1999; Liang et al., 1999). Subsequently, mouse genetic studies show that heterozygous disruption of *Becn1* increased the frequency of spontaneous tumors (e.g., mammary hyperplasia, liver and lung carcinomas, and lymphomas), implicating a haploin-sufficient tumor suppressor function of Beclin 1 (Qu et al., 2003; Yue et al., 2003). However, with the improvement of human tumor sequencing and gene expression databases, a recent study reveals close proximity of *BECN1* to the *BRAC1* gene on chromosome 17q21, and the presence of deletions encompassing both *BRCA1* and *BECN1*, or deletions of only *BRCA1*, but not deletions of only *BECN1*, in breast and ovarian cancers, challenging Beclin 1's tumor suppressor role (Laddha et al., 2014). In addition, a study using tissue-specific *Atg5* and *Atg7* knockout mice shows that autophagy deficiency can lead to benign tumors in liver, but not in other tissues (Takamura et al., 2011). These new findings leave the mechanism underlying

Beclin 1's role as a tumor suppressor under contention. Further data focusing on the direct impact of Beclin 1 loss in a tissue specific manner, notably in breast, ovarian, and prostate, is necessary to corroborate its context-dependent role as a tumor suppressor. However, the connections between *UVRAG* mutation and colon/gastric cancers remain uncontested (Liang et al., 2006; Kim et al., 2008), and a recent human genetic study draws a new link between *Nrbf2* and breast cancer (Darabi et al., 2015), relating the Beclin 1 interactome to cancer.

Synopsis of this review

Pharmacological modulators of autophagy that are currently in clinical trials are limited to sirolimus (mTOR inhibitor) and hydroxychloroquine (ClinicalTrials.gov), with the target of hydroxychloroquine yet to be identified. As summarized in the subsections "Beclin 1-Vps34 complexes in infection", "Beclin 1-Vps34 complexes in aging and neurodegeneration" and "Beclin 1-Vps34 complexes in cancer", the functionality and regulation of the Beclin 1-Vps34 complexes are important in autophagy-related pathologies. Therefore, the Beclin 1-Vps34 complexes provide promising targets for therapeutics to treat these autophagy-related diseases. Structure-based drug design is essential for successful creation of highly potent and target-specific drugs, examples of which include the design of small-molecule inhibitors targeting three major classes of antiapoptotic proteins— anti-apoptotic B cell lymphoma 2 (Bcl-2), inhibitor of apoptosis proteins (IAPs), and murine double-minute 2 (MDM2) (Martell et al., 2013; Bai and Wang, 2014). This review covers a growing number of studies that determine the Beclin 1 interactome and elucidate the structural architecture of the Beclin 1-Vps34 complexes, employing techniques such as affinity purification followed by mass spectrometry, nuclear magnetic resonance (NMR), X-ray crystallography, crosslinking mass spectrometry (CX-MS), and electron microscopy (EM). This review also takes an in-depth look at the most recently identified Beclin 1-Vps34 complex component, Nrbf2, the high resolution structures of the Beclin 1-Vps34 complex core components, and the architecture of the Beclin 1-Vps34 complexes. Lastly, this review uses an example of the most recent breakthrough in structure-based design for inhibitors of the Vps34 catalytic site to illustrate how understanding the identity, function, structure of each component of the Beclin 1-Vps34 complexes, as well as the architecture of the Beclin 1-Vps34 complexes as a whole, can facilitate the design of pharmacological interventions that specifically target autophagy.

Beclin 1 interactome

Core components

In mammalian autophagy, phosphatidylinositol 3-phosphate (PtdIns(3)P) production is essential to recruit effectors for autophagosome formation (Simonsen et al., 2004; Axe et al., 2008; Polson et al., 2010; Fan et al., 2011; Dooley et al., 2014) and autophagosome/endosome maturation (Pankiv et al., 2010; Sun et al., 2010, 2011). PtdIns(3)P is produced primarily by the class III phosphatidylinositide-3-kinase (PI(3)K) Vps34 and its levels are also affected by class II PI(3)K and phosphoinositide 3-phosphatases (e.g., Jumpy (Vergne et al., 2009) and MTMR3 (Taguchi-Atarashi et al., 2010)). The class III PI(3)K complexes consist of three core subunits: Beclin 1 (the yeast *Atg6/Vps30* ortholog), Vps34, and Vps15 (Volinia et al., 1995; Kihara et al., 2001). All Beclin 1 forms complexes with Vps34, whereas a considerable portion of Vps34 does not interact with Beclin 1, Atg14L or UVRAG (Kihara et al., 2001; Kim et al., 2013). The Beclin 1-Vps34-Vps15 core complex regulates the autophagy pathway as well as the endosomal pathway, depending on additional components (Zhong et al., 2009; Thoresen et al., 2010; Zhong et al., 2014). Loss of Beclin 1 destabilizes the Beclin 1-Vps34-Vps15 core complex and leads to an impairment in Vps34 activity, autophagic flux, and endocytic trafficking (Zhong et al., 2009; Thoresen et al., 2010; McKnight et al., 2014; Zhong et al., 2014). Beclin 1 significantly enhances the interaction between UVRAG and Rubicon, the two additional class III PI(3)K complex components that regulate the endosomal pathway (Zhong et al., 2009; Thoresen et al., 2010). It is also reported that depletion of Vps15 function results in a reduction in Vps34 and Beclin 1 protein levels, indicating a dependency of the stability of the core complex on Vps15 (Thoresen et al., 2010).

Two complexes, distinct functions

The yeast Atg6/Vps30-Vps34-Vps15 core complex forms two distinct complexes and functions in autophagy and vacuolar enzyme sorting, by interacting with the adapter proteins Atg14 and Vps38, respectively (Kihara et al., 2001). Likewise, the mammalian Beclin 1-Vps34-Vps15 core complex forms at least two complexes with distinct functions. One Beclin 1-Vps34 complex contains Atg14L (Atg14-like, the yeast *Atg14* ortholog, also named Atg14 or Barkor for Beclin-1-associated autophagy-related key regulator) which positively regulates autophagy by promoting autophagosome formation (Itakura et al., 2008; Sun et al., 2008; Matsunaga et al., 2009; Zhong et al., 2009). In particular, Atg14L localizes to the endoplasmic reticulum (ER) (Matsunaga et al., 2010), more specifically to the ER-mitochondria contact sites (Hamasaki et al., 2013), in a process dependent on the Ulk1 complex (Itakura and Mizushima, 2010; Matsunaga et al., 2010) and the ER SNARE, syntaxin 17 (Hamasaki et al., 2013). ER-localized Atg14L then recruits the core Beclin 1-Vps34-Vps15 complex to the autophagosome initiation sites to generate PtdIns(3)P (Itakura and Mizushima, 2010; Matsunaga et al., 2010). Atg14L is also required for phosphorylation of Beclin 1 by AMP-activated protein kinase

(AMPK) and this phosphorylation event is necessary for optimal autophagic activity (Fogel et al., 2013; Kim et al., 2013). Only very recently, Atg14L was reported to function beyond autophagosome biogenesis: Atg14L can self-associate, bind to the syntaxin 17-synaptosomal-associated protein, 29 kDa (SNAP29) complex, and promote autophagosome-endolysosome fusion (Diao et al., 2015).

The second Beclin 1-Vps34 protein complex contains UVRAG, which shows 10% identity and 32% similarity to yeast *Vps38* (Itakura et al., 2008). The binding of UVRAG and ATG14L to the core complex is mutually exclusive (Itakura et al., 2008; Sun et al., 2008; Matsunaga et al., 2009; Zhong et al., 2014). In association with the core complex, UVRAG promotes autophagosome maturation (Itakura et al., 2008; Liang et al., 2008; Kim et al., 2015). In particular, UVRAG interacts with class C Vps and Rab7 to promote fusion of autophagosomes with late endosomes/lysosomes (Liang et al., 2008).The interaction of UVRAG with the C-Vps tethering complex, which accelerates autophagosome maturation and endocytic vesicle trafficking, occurs independent of Beclin 1 (Liang et al., 2008; McKnight et al., 2014). In contrast, the negative autophagy regulator Rubicon binds UVRAG when S498 of UVRAG is phosphorylated by mTORC1, inhibiting autophagosome maturation (Matsunaga et al., 2009; Zhong et al., 2009; Kim et al., 2015). Despite consensus on the role of UVRAG in endosomal trafficking and autophagosome maturation, as well as the recent exciting findings of autolysosome tubule scission (Munson et al., 2015), insulin sensing and organismal glucose homeostasis regulation (Nemazanyy et al., 2015) by the UVRAG-containing Beclin 1-Vps34 complex, it remains under debate whether the UVRAG-containing Beclin 1-Vps34 complex plays a role in autophagosome formation. For example, some evidence shows that UVRAG associates with Bif-1 and Beclin 1 to promote autophagosome formation (Liang et al., 2006; Takahashi et al., 2007); conversely, other groups were unable to detect autophagy impairment upon UVRAG deficiency (Itakura et al., 2008; Takáts et al., 2014).

Collectively, the Atg14L-containing and UVRAG-containing class III PI(3)K complexes appear to diverge the Beclin 1-Vps34-Vps15 core complex for autophagy and endocytic trafficking, respectively. It will be interesting to see if the association of Beclin 1 with UVRAG is attenuated and Beclin 1 is switched to Atg14L-containing class III PI(3)K complex when cells undergo environmental changes that induce autophagy.

Nrbf2: A novel component of the Atg14L-containing Beclin 1-Vps34 complex

Recent affinity purification- and mass spectrometry-based proteomic analyses of the mammalian autophagy protein-protein interaction network confirmed previously reported binding partners of the Beclin 1-Vps34-Vps15 core complex, and revealed a novel component of this complex, nuclear receptor binding factor 2 (Nrbf2, also named COPR2 for comodulator of proliferator-activated receptor (PPAR) and retinoid X receptor (RXR) isoform 2) (Behrends et al., 2010; Cao et al., 2014; Lu et al., 2014; Zhong et al., 2014). Nrbf2 was originally characterized as a regulator of nuclear receptors including PPARα, retinoic acid receptor (RAR), and RXRα (Yasumo et al., 2000; Flores et al., 2004) without any known function in autophagy.

The interaction of Nrbf2 with the Beclin 1-Vps34-Vps15 core complex is mediated by Atg14L, revealing a functional role for cytoplasmic Nrbf2 (Lu et al., 2014; Zhong et al., 2014). Nrbf2 was found to have either no (Cao et al., 2014; Zhong et al., 2014) or weak (Behrends et al., 2010; Lu et al., 2014) interaction with UVRAG. These results place the Nrbf2 protein primarily as a component of the Atg14L-containing class III PI(3)K complex that regulates autophagosome biogenesis; this inference was confirmed by the colocalization of Nrbf2 with FIP200, Ulk1, and Atg5 (Zhong et al., 2014), as well as the requirement of full length Nrbf2 for WIPI2-positive phagophore formation (Lu et al., 2014). Detailed protein-protein interaction analyses using immunoprecipitation and Western blot analyses further revealed that Nrbf2 is important for linking the Beclin 1-Atg14L to theVps34-Vps15 sub-complexes (Lu et al., 2014; Zhong et al., 2014). In addition to the mammalian Nrbf2 studies, an independent study in yeast revealed that the protein encoded by *Atg38*, the presumptive *Nrbf2* ortholog, is a component of the yeast Atg6/Vps30-Vps34-Vps15 complex, interacts with Atg14, but not Vps38, links the Atg6/Vps30-Atg14 to the Vps34-Vps15 subcomplexes, and colocalizes with Atg17 (Araki et al., 2013). Taken together, these results suggest that the protein-protein interactions and cellular localization of Atg38/Nrbf2 during autophagy is evolutionarily conserved.

Despite the consensus on the protein-protein interactions that involve Atg38/Nrbf2 and on the colocalization of Atg38/Nrbf2 with pre-autophagosomal structures (PAS)/isolation membranes, the functional significance of Atg38/Nrbf2 as a component of the Atg14/Atg14L-containing class III PI(3)K complex in autophagy control is under debate. In the human RPE-1 cell line, Nrbf2 siRNA treatment resulted in increased autophagic flux (as monitored by p62 levels, LC3II in the absence versus presence of a lysosomal inhibitor, and long-lived protein degradation), and increased total cellular PtdIns (3)P levels (Zhong et al., 2014). In contrast, *Nrbf2*$^{-/-}$ mouse embryonic fibroblast (MEF) cells (Lu et al., 2014), HEK293A cells stably expressing GFP-LC3 (Cao et al., 2014), Nrbf2 siRNA treated U2OS GFP-LC3 cells (Behrends et al., 2010), and *Atg38Δ* and *Atg38Δ GFP-Atg14* yeast cells (Araki et al., 2013) showed defective autophagy. *Nrbf2*$^{-/-}$ MEF cells also showed defective Atg14L-associated Vps34 activity (Lu et al., 2014). These discrepancies are presumably rooted in the context-dependency of the Beclin 1-Vps34 protein-protein interaction network (Zhong et al., 2014).

Other Beclin 1-interacting proteins

Beclin 1 is known to interact with additional protein partners to elicit specific autophagic responses, including but not limited to negative regulators (e.g., Bcl-2, Bcl-X_L and Golgi-associated plant pathogenesis-related protein 1 (GAPR-1, also called GLIPR2 for glioma pathogenesis-related protein 2)) and positive regulators (e.g., Vacuole membrane protein 1 (VMP1), Ambra1, Dapper1, class IA PI(3)K p110β, and serine-threonine kinase 38 (STK38)). The anti-apoptotic Bcl-2 family members interact with Beclin 1 and block Beclin 1 interaction with the other components of the Beclin 1-Vps34 complexes, leading to inhibition of autophagy and reduction in UVRAG-mediated endocytic trafficking (Pattingre et al., 2005; Maiuri et al., 2007; Noble et al., 2008). Phosphorylation of Beclin 1 at residue T119 in its Bcl-2 homology (BH3) domain by death-associated protein kinase (DAP-kinase) (Zalckvar et al., 2009), and phosphorylation of Bcl-2 at residues T69, S70, and S87 of the non-structured loop by c-Jun N-terminal protein kinase 1 (JNK-1) (Wei et al., 2008) independently disrupt the interaction between Beclin 1 and Bcl-2 and enhance autophagic activity in the cells. Moreover, the epidermal growth factor receptor (EGFR) tyrosine kinase binds to Beclin 1, leading to its phosphorylation at residues Y229, Y233 and Y352, which in turn enhances Beclin 1 interaction with Bcl-2 to inhibit autophagic activity (Wei et al., 2013). Similarly, GAPR-1, which binds a region (residues 267 – 284) of the evolutionarily conserved domain (ECD) of Beclin 1, also negatively regulates autophagy by tethering Beclin 1 to the Golgi apparatus (Shoji-Kawata et al., 2013).

VMP1 was originally discovered in pancreatic tissues undergoing pancreatitis-induced autophagy, where VMP1 expression results in autophagosome formation (Ropolo et al., 2007). Studies show that VMP1, via its C-terminal Atg domain, directly binds the BH3 domain of Beclin 1, promoting dissociation of Bcl-2 from Beclin 1; these activities result in the Beclin1-Vps34 complex formation and autophagy (Ropolo et al., 2007; Molejon et al., 2013).

Ambra1, an essential protein for neural tube development, is not only required for starvation-induced autophagy (McKnight et al., 2012), but also involved in mitochondrial dynamics and degradation (Strappazzon et al., 2011, 2014; Van Humbeeck et al., 2011). Ambra1 binds Beclin 1 and serves its pro-autophagic role by assisting in the regulation of the Beclin 1-Vps34 complexes (Fimia et al., 2007). Through its association with Ulk1 kinase and the E3 ligase TRAF6, Ambra1 localizes the Beclin 1-Vps34-Vps15 core complex to omegasomes (Nazio et al., 2013). In this transient association, Ulk1 phosphorylates Ambra1 and Beclin 1; phosphorylated Ambra1 promotes the stability and ubiquitinylation of Ulk1 by TRAF6, leading to autophagy induction (Nazio et al., 2013). In addition, under normal cellular homeostatic conditions, Ambra1 is a substrate of mTORC1, resulting in inhibition of Ambra1's pro-autophagic effects (Nazio et al., 2013).

Dapper1, a Dishevelled-interacting protein known as a modulator of Wnt signaling through promoting lysosomal degradation of Dishevelled, was recently found to bind Atg14L and Beclin 1, enhancing Atg14L-containing Beclin 1-Vps34 complex formation, Vps34 activity, autophagosome biogenesis, and autophagic flux (Ma et al., 2014). Loss of *Dpr1*, the gene encoding Dapper1, specifically in the mouse CNS resulted in motor coordination impairment and CNS pathologies resembling autophagy-deficient mice (Ma et al., 2014).

Besides its canonical role in inhibiting autophagy via the Akt-mTORC1 axis, p110β binds the autophagy-promoting Atg14L-containing Beclin 1-Vps34 complex and facilitates the generation of cellular PtdIns(3)P (Dou et al., 2010). This complex also contains Rab5 (Ravikumar et al., 2008) and acts as a molecular sensor for growth factor availability (Dou et al., 2013).

Last but not least, most recently, STK38 has been reported to bind to Beclin 1 and promote autophagosome biogenesis through enhancing the autophagy-inducing, exocyst component Exo84-RalB interaction and recruitment of the Atg14L-containing Beclin 1-Vps34 complex to Exo84 (Bodemann et al., 2011; Joffre et al., 2015).

In short, Beclin 1 has been found to interact with a growing number of binding partners, which either promote autophagy/endocytosis or inhibit autophagy. Future research into the molecular mechanisms surrounding these interacting partners will provide more insights into the roles that the Beclin 1-Vps34 complexes play in regulating different cellular trafficking pathways under various nutrient and stress conditions.

Structures of the Beclin 1-Vps34 complex components

The domains of the Beclin 1-Vps34 complex components are largely identified (Fig. 2). However, despite significant efforts toward resolving structures of the Beclin 1-Vps34 complex components, to date high resolution structures are available only for the Beclin 1 BH3, coiled-coil (CCD), and ECD (or BARA for β-α repeated, autophagy-specific) domains, Nrbf2 microtubule interacting and trafficking (MIT) domain (PDB database entry only), Vps34 helical and catalytic (HELCAT) domains, and Vps15 WD-40 domain (see below and summarized in Table 1 and Fig. 3).

Beclin 1 structure

Beclin 1, first identified as a Bcl-2-interacting protein (Liang et al., 1998), is composed of 450 amino acids for human and 448 amino acids for mouse. Beclin 1 serves as a platform molecule for the class III PI(3)K complexes by protein-protein interactions through three domains: the BH3 domain (corresponding to residues 107–127 for human) (Feng et al.,

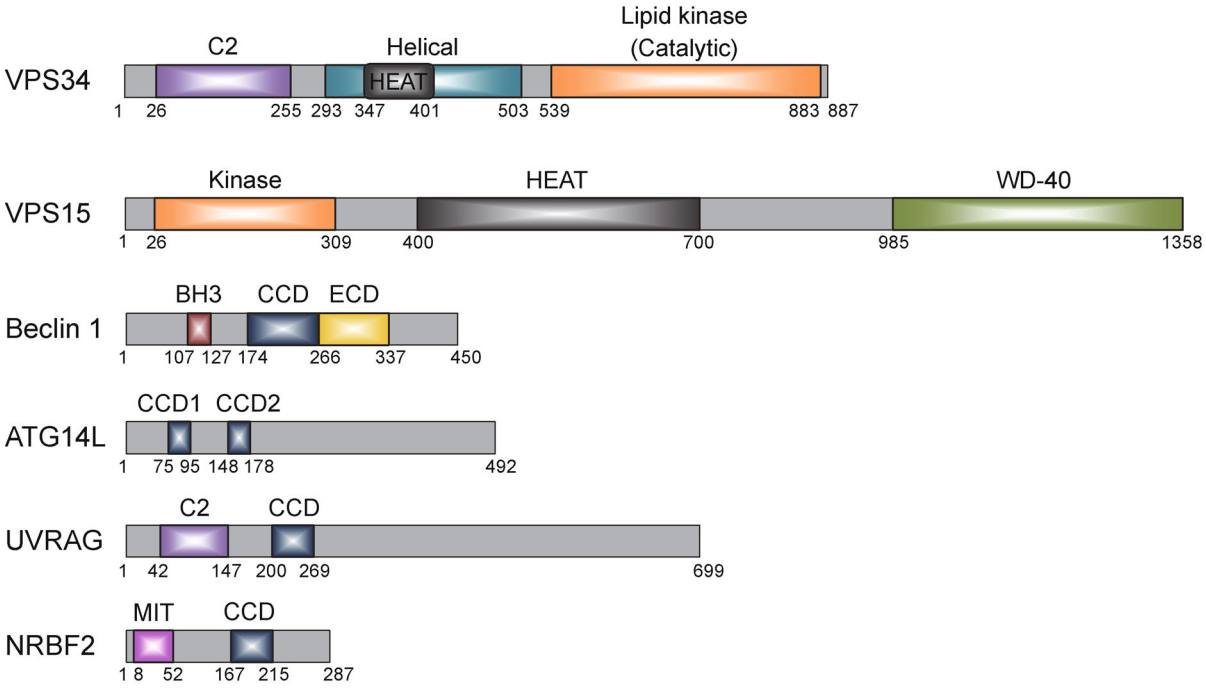


Figure 2 Domain structures of the proteins associated with the human Beclin 1-VPS34 complexes. Abbreviations: BH3, Bcl-2 homology 3; CCD, coiled-coil domain; ECD, evolutionarily conserved domain; MIT, microtubule-interacting and trafficking.

Table 1 Summary of reported structures for Beclin 1-Vps34 complex components

Component	Domain	Species	Residues in the constructs	Detection assay	Reference	PDB code
Beclin 1	BH3 domain	*H. sapiens*	107–135	X-ray crystallography	Oberstein et al., 2007	2P1L
		H. sapiens	104–131	NMR	Feng et al., 2007	2PON
		M. musculus	106–124	X-ray crystallography	Ku et al., 2008	3BL2*
		H. sapiens	105–130	X-ray crystallography	Sinha et al., 2008	3DVU*
		H. sapiens	107–130	X-ray crystallography	Su et al., 2014	4MI8*
	CCD	*R. norvegicus*	174–266	X-ray crystallography	Li et al., 2012	3Q8T
	ECD/BARA	*S. cerevisiae*	BARA 320–539	X-ray crystallography	Noda et al., 2012	3VP7
		H. sapiens	ECD 248–450	X-ray crystallography	Huang et al., 2012	4DDP
Vps34	C2 domain					
	Helical domain & Lipid kinase domain	*D. melanogaster*	258–949	X-ray crystallography	Miller et al., 2010	2X6H, 2X6F**, 2X6I**, 2X6J**, 2X6K**
		H. sapiens	293–887	X-ray crystallography	Dowdle et al., 2014	4PH4**
		H. sapiens	282–879	X-ray crystallography	Ronan et al., 2014	4OYS**
		H. sapiens	282–879	X-ray crystallography	Pasquier et al., 2015	4UWF**, 4UWG**, 4UWH**, 4UWK**, 4UWL**
Vps15	Kinase domain					
	HEAT domain					
	WD-40 domain	*S. cerevisiae*	1027–1454	X-ray crystallography	Heenan et al., 2009	3GRE
Nrbf2	MIT	*M. musculus*	1–97	NMR	Suetake et al., 2015	2CRB
	CCD					

Notes:
*Co-crystal structure in complex with γ-herpesvirus 68 M11, the viral Bcl-2 homolog.

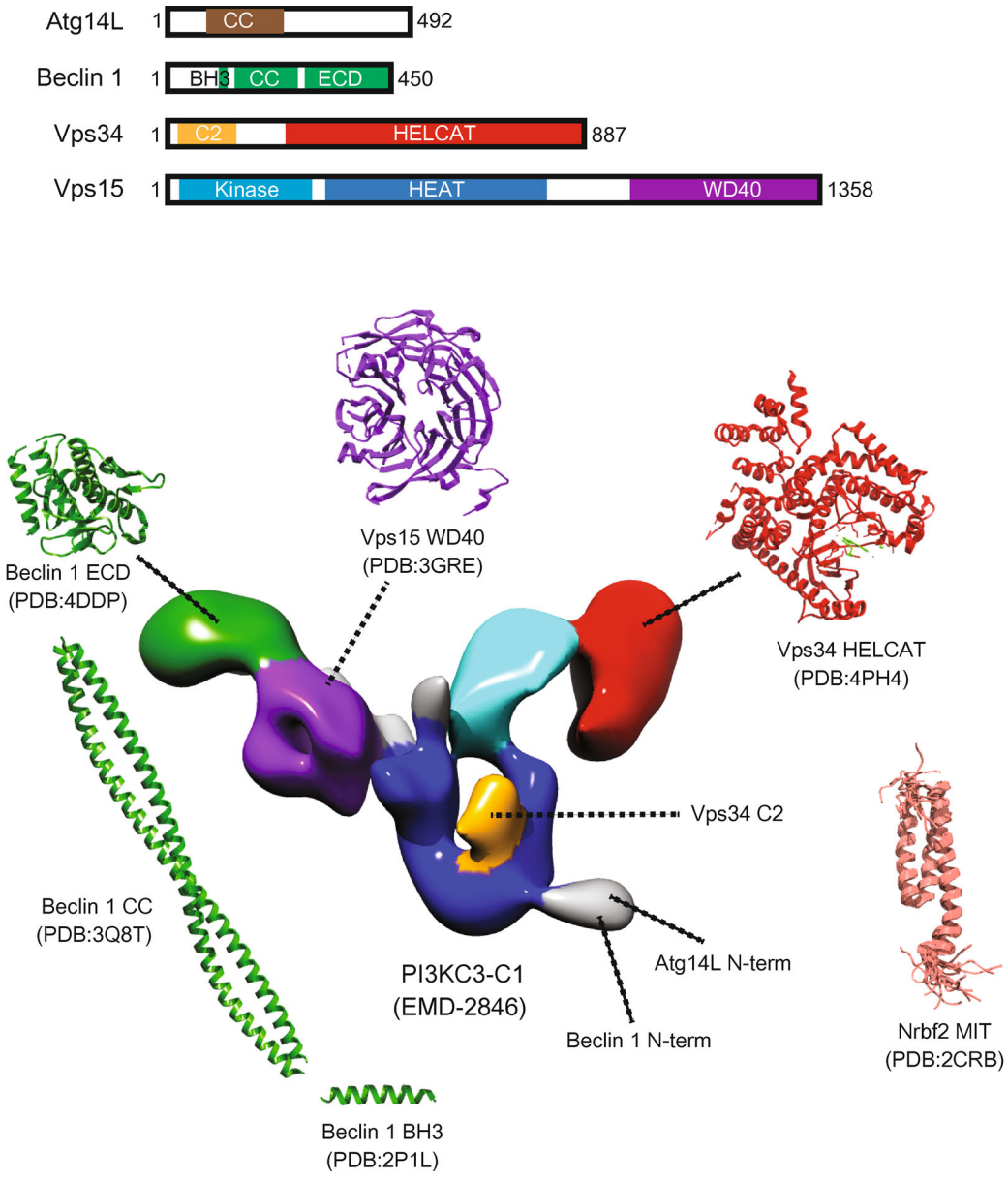


Figure 3 Currently available structural information of the Atg14L-containing, autophagic Beclin 1-Vps34 class III PI(3)K complex I (PI3KC3-C1). PI3KC3-C1 consists of four components: Atg14L, Beclin 1, Vps34, and Vps15. The V-shaped 3D reconstruction of human PI3KC3-C1 was determined by single-particle EM (EMDB: EMD-2846). Vps15 is thought to be the scaffolding subunit and the locations of its protein kinase (cyan), HEAT (blue), and WD-40 (purple) domains are colored in the EM-based 3D model. Thus far, the only available high-resolution structural information for this protein came from crystallographic analysis of yeast Vps15 WD-40 domain (PDB: 3GRE, residues 1031–1454). The catalytic subunit of PI3KC3-C1 is Vps34, which produces PtdIns(3)P at the autophagosome, endosome and lysosome membranes. The crystal structure of the helical and catalytic domains ("HELCAT") of human VPS34 in complex with the selective inhibitor PIK-III has been recently determined (PDB:4PH4, residues 293-871 in red, inhibitor in green). Beclin1, which plays a central role in regulating the activity of PI3KC-C1, contains three functional domains: the N-terminal BH3 domain (PDB: 2P1L, residues 107–135 for human), the central CCD (PDB: 3Q8T, residues 144–269 for rat), and the ECD (PDB: 4DDP, residues 248-450 for human). The approximate locations of the Beclin 1 ECD in the context of the full complex, as determined by EM-based labeling, is colored in green in the 3D model. The positions of Vps34 C2 domain and the N-termini of Atg14L and Beclin 1 are colored in orange and gray, respectively. Nrbf2, although recently shown to be a subunit of the PI3KC-C1, was not included in the single-particle EM study. However, the structure of the N-terminal MIT domain of mouse Nrbf2 has previously been determined by NMR spectroscopy (PDB: 2CRB, residues 4-86).

2007; Oberstein et al., 2007), the CCD (residues 174–266 for rat; residues 187–319 for *Saccharomyces cerevisiae*) (Li et al., 2012; Noda et al., 2012), and the C-terminal evolutionarily conserved domain (ECD, residues 244–337 for human) or the β-α repeated, autophagy-specific (BARA) domain (residues 320 – 557 for *S. cerevisiae* Atg6/Vps30) (Furuya

et al., 2005; Oberstein et al., 2007; Huang et al., 2012; Noda et al., 2012).

The short N-terminal BH3 motif of Beclin 1 is known to bind Bcl-2 family proteins, including cellular Bcl-2 (Pattingre et al., 2005; Maiuri et al., 2007) and Bcl-X_L (Feng et al., 2007; Maiuri et al., 2007; Oberstein et al., 2007; Su et al., 2014), KSHV v-Bcl-2 (Pattingre et al., 2005), and the γ-herpesvirus 68 Bcl-2 homolog M11 (Ku et al., 2008; Sinha et al., 2008; Su et al., 2014). The Beclin 1 BH3 domain forms an amphipathic α-helix and fits into a hydrophobic pocket in the Bcl-X_L, as revealed by both X-ray crystallography (Oberstein et al., 2007) and NMR spectroscopy (Feng et al., 2007). Important residues in Beclin 1 BH3 domain for binding to the Bcl-2 family proteins include L110 and F121 for mouse Beclin 1 (Ku et al., 2008) and correspondingly L112 and F123 for human Beclin 1 (Sinha et al., 2008) as well as G120 and D121 for human Beclin 1 (Su et al., 2014). Su and colleagues also identified a selective Beclin 1 BH3 domain-derived, inhibitory peptide that attenuates the M11-mediated downregulation of autophagy without binding to cellular Bcl-2 homologs (Su et al., 2014). As mentioned before in the subsection "Other Beclin 1-interacting proteins", phosphorylation of Beclin 1 in its BH3 domain at T119 by DAP-kinase (Zalckvar et al., 2009) and phosphorylation of the unstructured loop of Bcl-2 at residues T69, S70, and S87 by JNK-1 (Wei et al., 2008) independently disrupt the interaction between Beclin 1 and Bcl-2 and enhance autophagic activity in the cells; and the EGFR tyrosine kinase binds to Beclin 1 and phosphorylates Beclin 1 at residues Y229, Y233 and Y352, leading to enhanced Beclin 1 interaction with Bcl-2 and inhibition of autophagic activity (Wei et al., 2013). These studies provide the molecular mechanisms by which autophagy can beregulated by the Bcl-2 family proteins, which are important for understanding the crosstalks between apoptosis and autophagy as well as how viruses can subvert host autophagy for infection.

Interactions with factors other than Bcl-2 family proteins at the Beclin 1 BH3 domain also impact autophagy regulation. For example, binding of the VMP1 C-terminal Atg domain at the Beclin 1 BH3 domain releases Beclin 1 from Bcl-2 to form the Beclin1-Vps34 complexes and induce autophagy (Ropolo et al., 2007; Molejon et al., 2013). The interaction between Ambra1 and Beclin 1 also occurs via the BH3 domain region (residues 141 – 150) (Strappazzon et al., 2011), although Ambra 1 is capable of interacting with Bcl-2 independent of Beclin 1. It is thought that when cells are faced with acute stresses, Ambra1 disassociates from Bcl-2 and subsequently binds to Beclin 1 to promote autophagy. The exact structural context behind the Ambra1-Beclin 1 interaction awaits further investigation.

The CCD is a common structural motif for hydrophobic protein-protein interactions. Beclin 1, Atg14L and UVRAG all possess CCD (Liang et al., 2006; Sun et al., 2008; Matsunaga et al., 2009; Zhong et al., 2009; Li et al., 2012). Beclin 1 is known to self-associate (Matsunaga et al., 2009). Recent X-ray crystallographic analysis revealed that the Beclin 1 CCD (rat, residues 174–266) consists of a dimeric anti-parallel coiled-coil, interacting in a head-to-tail fashion with an unstable interface (Li et al., 2012). The Beclin 1 CCD is also necessary for assembly of the Atg14L- (Sun et al., 2008; Matsunaga et al., 2009; Zhong et al., 2009; Li et al., 2012) and UVRAG-containing (Liang et al., 2006; Matsunaga et al., 2009; Li et al., 2012) Beclin 1-Vps34 complexes that regulate autophagic and endocytic pathways, respectively. The homodimeric Beclin 1 is metastable, and the Beclin 1 CCD interface allows division of the two Beclin 1 molecules, and assembly of the more stable Beclin 1-Atg14L or Beclin 1-UVRAG heterodimers. The Beclin 1-UVRAG interaction is stronger than the Beclin 1-Atg14L interaction, as shown by *in vitro* assays and cell culture analysis (Li et al., 2012). It is thought that the functionally inactive Beclin 1 homodimers serve as a reservoir for new Beclin 1-Vps34 complex formation when required by changes in cellular conditions. The structure of the Beclin 1 CCD revealed a population of polar or charged residues in the hydrophobic interface of Beclin 1 dimers that render the Beclin 1 homodimers unstable and promote stable interactions between Beclin 1 and Atg14L/UVRAG (Li et al., 2012). However, the mechanism by which Atg14L or UVRAG manipulates these residues to form stable coiled-coil assemblies is still unclear. Much will be deduced about the molecular mechanism of these specific interactions when the structure of each Beclin 1-Vps34 complex is revealed.

The ECD is located at the C-terminal half of Beclin 1 without sequence homology to any known structure; nonetheless, this region of the protein is indispensable for Beclin 1 functionality (Huang et al., 2012). Recent studies examining the crystal structure of the C-terminal region of Beclin 1 that encompasses the ECD led to the identification of a novel class of membrane binding domain (Huang et al., 2012). This stable domain consists of three superimposable internal repeats, each comprised of a pair of short β-strands followed by an α-helix. The three α-helices form a helical bundle, surrounded by three β-sheets, six loops and an N-terminal α-helix (Huang et al., 2012). Specifically, hydrophobic protrusion in the surface loop L4, consisting of three aromatic residues (F359, F360, and W361), facilitates Beclin 1 binding to lipid membranes enriched in cardiolipin. Although the mechanism and significance of cardiolipin preference are not understood, this aromatic hydrophobic finger is necessary for the insertion of the protein into lipid membranes and the resultant deformation of the liposomes that resembles membrane curvature. Mutations that disrupt this aromatic finger compromise omegasome formation and autophagy (Huang et al., 2012). It will be interesting to see if the Beclin 1 ECD aromatic finger residues facilitate interaction with endosomal membranes. It is speculated that the Beclin 1 ECD hydrophobic finger is masked as a result of Beclin 1 homodimerization, and that inhibition is relieved upon Beclin 1-Atg14L/UVRAG heterodimerization (Huang et al., 2012).

In contrast, mutations in the hydrophobic finger did not cause drastic alteration in the Beclin 1-Atg14L/UVRAG/Vps34 interactions. Adjacent to the aromatic finger in the Beclin 1 ECD is a deep cleft, but the functionality of this feature remains unclear.

Interestingly, studies examining the C-terminal region of the yeast Atg6/Vps30 protein revealed a globular fold comprised of three β-sheet-α-helix repeats (Noda et al., 2012) that are similar to the mammalian Beclin 1 ECD structure; this finding, along with sequence homology, suggests an evolutionarily conserved feature of this domain (Huang et al., 2012; Noda et al., 2012). However, the aromatic finger residues found in mammalian Beclin 1 are not conserved in yeast Atg6/Vps30 (F430, R431, and K432 in our own alignment and in (Noda et al., 2012)). The lack of hydrophobic finger in yeast Atg6/Vps30 also poses the question as to whether and how Atg6/Vps30 binds membranes. Truncation analysis of Atg6/Vps30 C-terminal domain determined that, like Beclin 1 ECD, Atg6/Vps30 ECD is not required for the assembly of either Atg14- or Vps38-containing Atg6/Vps30-Vps34 complex; rather it is required for targeting the Atg14-containing Atg6/Vps30-Vps34 complex to the PAS, but is not necessary for Vps38-mediated vacuolar protein sorting (Noda et al., 2012). Thus, the yeast Atg6/Vps30 C-terminal domain is termed BARA domain, short for β-α repeated, autophagy-specific domain. It will be interesting to see if the Beclin 1 ECD is dispensable for endocytic trafficking.

Vps34 structure

Vps34 was originally identified in yeast as part of the PI(3)K complex, responsible for control of intracellular protein trafficking (Herman and Emr, 1990; Schu et al., 1993), with substrate specificity for Ptdlns but not PtdIns4P or Ptdlns(4,5) P2 (Schu et al., 1993). Human Vps34 was subsequently cloned and found to specifically phosphorylate Ptdlns to PtdIns(3)P (Volinia et al., 1995). Common to the catalytic subunits of other PI(3)Ks, Vps34 is composed of an N-terminal C2 domain (residues 1–255), a middle helical domain (residues 293–530), and a C-terminal catalytic kinase domain (residues 533–887) (Backer, 2008). Vps34 interacts with the membrane via the C2 domain, as well as the extreme C-terminal helix that is also required for lipid kinase activity in both yeast (Budovskaya et al., 2002) and human (Siddhanta et al., 1998; Miller et al., 2010). It is thought that the C2 domain binds Beclin 1 (Liang et al., 2006), although it has no role in regulating enzyme catalytic activity (Miller et al., 2010). The Vps34 C-terminal kα11 and kα12 helices bind Vps15 (Miller et al., 2010). The last 11 amino acids at the C terminus of Vps34 are required for PI(3)K activity, although they are not required for binding to Vps15 (Budovskaya et al., 2002).

The crystal structure of *Drosophila melanogaster* Vps34 (*Dm*Vps34) HELCAT domains (i.e., Δ1-257), either alone or in complex with inhibitors, shows a compact unit with a regulatory C-terminal helix as well as a solenoid helical domain packed against a classical bilobal catalytic domain characteristic of protein kinases (Miller et al., 2010). This crystal structure features 1) a hook-shaped, completely ordered, phosphoinositide binding loop (activation loop) for recognizing PtdIns, 2) a phosphate binding loop (P-loop) for binding ATP, and 3) a catalytic loop that catalyzes the transfer of the ATP γ-phosphate to the 3-hydroxyl of PtdIns (Miller et al., 2010). The crystal structures of the human Vps34 HELCAT domains were later determined in the apo form and in the presence of newly developed Vps34 inhibitors (Dowdle et al., 2014; Ronan et al., 2014; Pasquier et al., 2015).

Mutational analysis of the *Dm*Vps34 activation loop key residues proposed to bind phosphoinositide head group (e.g., K833, P832 and Y826; corresponding to human K771, P770 and Y764, respectively) shows either significantly impaired enzyme activity or complete inactivity. The Vps34 catalytic loop contains a conserved DRH motif (*Dm* 805-807; *Hs* 743-745) and two aspartic acid residues (*Dm* 805 and 823; *Hs*743 and 761), all of which are important for the enzymatic reaction. Interestingly, deletion or mutations (e.g., H879A and W885A) of the C-terminal helix greatly diminish ATPase activity in the presence of the PtdIns:PS vesicles, whereas deletion or mutations (e.g., W885A and Y884A) of the C-terminal helix or mutations in the activation loop (e.g., K771A) increase basal ATPase activity in the absence of vesicles, suggesting dual roles of the C-terminal helix in activation (on the membrane) and auto-inhibition (off the membrane) (Miller et al., 2010). These results are confirmed by the structure which reveals that the C-terminal helix blocks the catalytic loop when Vps34 is not associated with lipid membrane, but upon association with lipid membrane, the obstruction is displaced, allowing for Vps34 catalytic activity.

The ATP binding site of Vps34 is unique in regions critical to inhibitor binding, such as the P-loop, hinge region and gatekeeper amino acids (Ronan et al., 2014). The Vps34 ATP binding pocket is significantly smaller in volume than that of other PI(3)Ks (e.g., the class I p110γ), as a result of distinct structural characteristics of Vps34 including the inward-curling P loop. Also unique to Vps34, a bulky residue in the P loop (*Hs* F612; *Dm* F673) packs against the aromatic hinge residue (*Hs* F684; *Dm* Y746), likely leading to a more rigid and constrained ATP binding pocket in Vps34 as compared to in other Class I PI(3)Ks. As a result, the smaller and more rigid and constrained ATP binding pocket of Vps34 restricts the binding of typical PI(3)K inhibitors, making it very difficult to develop potent and high-affinity inhibitors specific for Vps34.

The structure of Vps34 (either apo-protein or in complex with 3-methyladenine (3-MA) or any of the new Vps34 inhibitors) provides a better understanding of the Vps34 ATP binding pocket, leading to successful structure-based design of improved (high affinity and highly specific) compounds

targeting the Vps34 activity (see the subsection "Drug design directly targeting Vps34") (Dowdle et al., 2014; Ronan et al., 2014; Pasquier et al., 2015). In addition, the unique C-terminal helix may also represent a target for specific, non-ATP-competitive Vps34 inhibitors.

Vps15 structure

Vps15 was originally identified in yeast as a 160 kDa protein serine/threonine kinase associated with Vps34 (Herman et al., 1991a, 1991b). The membrane localization of Vps34 and activation of Vps34 lipid kinase activity are dependent on the Vps15 serine/threonine kinase activity (Stack et al., 1993). Similarly, human Vps15 (formerly called p150, 166 kDa) associates with Vps34 in an approximately equimolar ratio (Volinia et al., 1995) and markedly increases the i*n vitro* Vps34 lipid kinase activity (Panaretou et al., 1997; Yan et al., 2009).

Experimental data from yeast (Kihara et al., 2001), fly (Lindmo et al., 2008), and human (Yan et al., 2009) suggest that Vps15 plays a role in Vps34-dependent autophagy. Notably, regulation of Vps34 activity by autophagy-related proteins and by nutrients requires the presence of Vps15 (Yan et al., 2009). In particular, Vps15 enhances the binding of Beclin 1/UVRAG to Vps34, as well as Beclin 1/UVRAG-stimulated or amino acid/glucose-sensitive Vps34 activity; and Beclin 1/UVRAG enhances binding of Vps15 to Vps34 without increasing the total Vps34 and Vps15 levels (Yan et al., 2009).

Human Vps15 consists of an N-terminal serine/threonine kinase domain (residues 11–262), a C-terminal WD-40 domain (residues 1000–1300), and an intermediate domain (residues 400–700) containing several HEAT repeats that connect the kinase and WD-40 domains (Panaretou et al., 1997; Heenan et al., 2009). Moreover, both yeast Vps15 (Herman et al., 1991a, 1991b) and humanVps15 (Panaretou et al., 1997) are myristoylated at their N-termini ((M)$G_1A_2Q_3L_4S_5L_6$ (yeast) and (M)$G_1N_2Q_3L_4A_5G_6$ (human)). Myristoylation anchorsVps15 to the target membranes such as endosomal membranes and isolation membranes (or pre-autophagosomal site (PAS) in yeast), facilitates translocation of Vps34 to these membranes, and activates the essential PI (3)K activity of Vps34 (Yan et al., 2009). The WD-40 domain of Vps15 binds activated Rab5 to target the Vps34-Vps15 sub-complex to early endosomes (Christoforidis et al., 1999; Murray et al., 2002). The WD-40 domain of Vps15 also binds Rab7 and the PtdIns3P phosphatases myotubularin 1 and 2 (MTM1 and MTM2) mutual exclusively on late endosomes (Cao et al., 2007, 2008).

The crystal structure of the *S. cerevisiae* Vps15 WD-40 domain features a seven-bladed propeller resembling that of typical Gβ proteins (Heenan et al., 2009). The WD-40 domain serves as a scaffold to assemble proteins such as Atg14. Co-immunoprecipitation studies using full length and truncation mutants of Vps15 show that the WD-40 domain alone and the kinase domain alone pull down Atg14 (Heenan et al., 2009). In addition, mutagenesis studies show that the kinase domain together with the intermediate domain of Vps15, but neither the WD-40 domain alone nor the WD-40 domain together with the intermediate domain, partially retain vacuolar sorting of carboxypeptidase Y (Heenan et al., 2009); however, putative substrates of the kinase domain of Vps15 have yet to be identified.

Beclin 1-Vps34 complex architecture

Understanding how protein complexes come together to regulate cellular processes is beneficial for development of potent and improved therapies to control diseases which these complexes influence. However, isolation of endogenous multi-protein complexes is technically challenging due to the dynamic nature, low abundance, and heterogeneity associated with such assemblies. Here we summarize the current understanding of Beclin 1-Vps34 complex architecture using affinity purification (AP) in combination with Western blot analysis, direction interaction detection by *in vitro* binding assays, crosslinking mass spectrometry, and single-particle electron microscopy.

Affinity purification in combination with Western blot analysis

Beclin 1-Vps34 complex architecture has been assessed by affinity purification in combination with Western blot analysis, which examines pairwise protein-protein interactions (summarized in Table 2). Most data indicate that the binding of Beclin 1/Atg6 to Atg14L/Atg14 and UVRAG/Vps38 is mutually exclusive (Kihara et al., 2001; Sun et al., 2008; Itakura et al., 2008; Matsunaga et al., 2009; Zhong et al., 2014), suggesting that there are two major Beclin 1-Vps34 complexes.

Genetic knockout or transient/stable knockdown of one component of the Beclin 1-Vps34 complex has been a widely used experimental approach to determine the protein component(s) that are critical for complex assembly and stability. For example, this approach was used to demonstrate that Beclin 1, Vps34 or Vps15 stabilizes both Atg14L and UVRAG; Beclin 1 stability depends on Vps34, Vps15, Atg14L, UVRAG and Nrbf2, while the stabilities of Vps34 and Vps15 depend on each other (Kihara et al., 2001; Itakura et al., 2008; Thoresen et al., 2010; Zhong et al., 2014).

Moreover, this approach has also been utilized to show that in RPE-1 cells, Beclin 1 is required for Vps34→Vps15 and Vps34→Nrbf2 interactions, where the arrows point from bait to prey; Atg14L is required for Nrbf2→Vps34, Nrbf2→Vps15, and Beclin 1↔Nrbf2 interactions; and Nrbf2 is required for Atg14↔Vps34, Atg14L→Vps15, and Vps34→Beclin 1 interactions (Zhong et al., 2014). Several of these requirements for intermolecular interactions within the Beclin

Table 2 Summary of reported interactions among the Beclin 1-Vps34 complex key components including Beclin 1, Vps34, Vps15, Atg14L, UVRAG and Nrbf2

Component #1	Domain of Component #1	Component #2	Direct interaction	Detection assay	Reference	Confirmed by CX-MS (Shi et al., 2015)	Consistent with EM (Baskaran et al., 2015)
Beclin 1 (Atg6/Vps 30 in yeast)	Full length (h, 1–450)	Beclin 1	Direct interaction	Y2H	Matsunaga et al., 2009		
		Vps34	–	AP	Kihara et al., 2001; Matsunaga et al., 2009; Furuya et al., 2005; Liang et al., 2006; Sun et al., 2008; Yan et al., 2009; Zhong et al., 2009, 2014; Behrenda et al., 2010; Araki et al., 2013; Cao et al., 2014; Lu et al., 2014		
		Vps15	–	AP	Kihara et al., 2001; Itakura et al., 2008; Sun et al., 2008; Matsunaga et al., 2009; Zhong et al., 2009, 2014; Behrenda et al., 2010; Lu et al., 2014		
		Atg14L	Direct interaction	AP, Y2H	Kihara et al., 2001; Kametaka et al., 1998; Itakura et al., 2008; Sun et al., 2008; Matsunaga et al., 2009; Zhong et al., 2009, 2014; Behrenda et al., 2010; Lu et al., 2014		
		UVRAG/Vps38	Direct interaction	AP, Y2H	Kihara et al., 2001; Liang et al., 2006; Itakura et al., 2008; Sun et al., 2008; Matsunaga et al., 2009; Zhong et al., 2009, 2014; Behrenda et al., 2010; Lu et al., 2014		
		Nrbf2	–	AP	Zhong et al., 2009, 2014; Behrenda et al., 2010; Araki et al., 2013; Cao et al., 2014; Lu et al., 2014		
	BH3 domain						
	CCD	Beclin 1	Direct interaction	AP, analytical ultracentrifugation, *in vitro* binding, bimolecular fluorescence complementation assay	Li et al., 2012		
		Vps34	–	AP	Liang et al., 2006	Y	Y
		Atg14L	Direct interaction	AP, analytical ultracentrifugation, *in vitro* binding	Sun et al., 2008; Matsunaga et al., 2009; Zhong et al., 2009; Li et al., 2012	Y	
		UVRAG	Direct interaction	AP, analytical ultracentrifugation, *in vitro* binding	Liang et al., 2006; Sun et al., 2008; Matsunaga et al., 2009; Li et al., 2012	Y	
	ECD/BARA	Atg14L	–	AP	Zhong et al, 2009	Y	
		Vps34	–	AP	Furuya et al., 2005; Liang et al., 2006 Noda et al., 2012		

(*Continued*)

Component #1	Domain of Component #1	Component #2	Direct interaction	Detection assay	Reference	Confirmed by CX-MS (Shi et al., 2015)	Consistent with EM (Baskaran et al., 2015)
Vps34	Full length (1–887)	Beclin1	–	AP, *in vitro* binding	Kihara et al., 2001; Matsunaga et al., 2009; Furuya et al., 2005; Liang et al., 2006; Sun et al., 2008; Yan et al., 2009; Zhong et al., 2009, 2014; Behrenda et al., 2010; Araki et al., 2013; Cao et al., 2014; Lu et al., 2014		
		Vps15	Direct interaction	AP, crosslinking	Stack et al., 1993, 1995; Volinia et al., 1995; Panaretou et al., 1997; Matsunaga et al., 2009; Yan et al., 2009; Zhong et al., 2014; Behrenda et al., 2010; Araki et al., 2013; Cao et al., 2014;		
		Atg14L	–	AP	Kihara et al., 2001; Sun et al., 2008; Itakura et al., 2008; Matsunaga et al., 2009; Behrenda et al., 2010; Araki et al., 2013; Cao et al., 2014; Zhong et al., 2014;		
		UVRAG	–	AP	Itakura et al., 2008; Matsunaga et al., 2009; Yan et al., 2009; Behrenda et al., 2010; Araki et al., 2013; Cao et al., 2014; Zhong et al., 2014;		
		Nrbf2	–	AP	Behrenda et al., 2010; Araki et al., 2013; Cao et al., 2014; Lu et al., 2014; Zhong et al., 2014		
	C2 domain	Beclin 1	–	AP	Liang et al., 2006; Itakura et al., 2008	Y	Y
		Atg14L	–	AP	Itakura et al., 2008	Y	Y
		UVRAG	–	AP	Itakura et al., 2008	Y	Y
	Helical domain						
	Lipid kinase domain	Vps15	–	Y2H, functional assays (e.g., CPY sorting or maturation, PI(3)P levels)	(yeast) Volinia et al., 1995		Y
Vps15	Full length (1–1358)	Beclin 1	–	AP	Kihara et al., 2001; Itakura et al., 2008; Sun et al., 2008; Matsunaga et al., 2009; Zhong et al., 2009, 2014; Behrends et al., 2010		
		Vps34	Direct interaction	AP, Y2H, crosslinking	Volinia et al., 1995; Panaretou et al., 1997; Matsunaga et al., 2009; Yan et al., 2009; Behrends et al., 2010; Cao et al., 2014; Zhong et al., 2014 (yeast) Stack et al., 1993; Stack et al., 1995; Budovskaya et al., 2002; Matsunaga et al., 2009; Araki et al., 2013		
		Atg14L	–	AP	Itakura et al., 2008; Matsunaga et al., 2009; Behrends et al., 2010; Zhong et al., 2014		
		UVRAG	–	AP	Behrends et al., 2010		
		Nrbf2	Direct interaction	AP, *in vitro* binding	Behrends et al., 2010; Araki et al., 2013; Cao et al., 2014; Lu et al., 2014; Zhong et al., 2014		
	Kinase domain	Vps34	Direct interaction	Y2H	(yeast) Budovskaya et al., 2002		Y

(*Continued*)

Component #1	Domain of Component #1	Component #2	Direct interaction	Detection assay	Reference	Confirmed by CX-MS (Shi et al., 2015)	Consistent with EM (Baskaran et al., 2015)
				Crosslinking	(yeast) Stack et al., 1995		
		Atg14	–	AP	(yeast) Heenan et al., 2009		
	HEAT domain	Vps34	–	Y2H	(yeast) Budovskaya et al., 2002		
	WD-40 domain	Atg14	–	AP	(yeast) Heenan et al., 2009		
		Nrbf2	–	*In vitro* binding	Cao et al., 2014		
Atg14L (Atg14 in yeast)	Full length (1–492)	Beclin 1	Direct interaction	AP, Y2H	Kametaka et al., 1998; Kihara et al., 2001; Itakura et al., 2008; Sun et al., 2008; Matsunaga et al., 2009; Zhong et al., 2009, 2014; Behrends et al., 2010;		
		Vps34	–	AP	Kihara et al., 2001; Itakura et al., 2008; Sun et al., 2008; Matsunaga et al., 2009; Behrends et al., 2010; Araki et al., 2013; Cao et al., 2014; Zhong et al., 2014		
		Vps15	–	AP	Itakura et al., 2008; Matsunaga et al., 2009; Behrends et al., 2010; Zhong et al., 2014		
		Nrbf2	Direct interaction	AP, *in vitro* binding	Behrends et al., 2010; Araki et al., 2013; Cao et al., 2014; Lu et al., 2014; Zhong et al., 2014		
	Zinc finger						
	CCD1 (75–95)	Beclin 1	–	AP	Zhong et al., 2009; Lu et al., 2014		Y
		Vps34	–	AP	Zhong et al., 2009		
		Nrbf2	–	AP	Lu et al., 2014; Zhong et al., 2014		
	CCD2 (148–178)	Beclin 1	–	AP	Zhong et al., 2009; Lu et al., 2014	Y	
	CCD (71–184 or 88–178)	Beclin 1	Direct interaction	AP, analytical ultracentrifugation, *in vitro* binding	Itakura et al., 2008; Sun et al., 2008; Matsunaga et al., 2009; Li et al., 2012	Y	
		Vps34	–	AP	Itakura et al., 2008	Y	
UVRAG (Vps38 in yeast)	Full length (1–698)	Beclin 1	Direct interaction	AP, Y2H	Kihara et al., 2001; Liang et al., 2006; Itakura et al., 2008; Sun et al., 2008; Matsunaga et al., 2009; Zhong et al., 2009, 2014; Behrends et al., 2010;		
		Vps34	–	AP	Itakura et al., 2008; Matsunaga et al., 2009; Yan et al., 2009; Behrends et al., 2010; Araki et al., 2013; Cao et al., 2014; Zhong et al., 2014		
		Vps15	–	AP	Matsunaga et al., 2009; Behrends et al., 2010		
		Nrbf2	–	–	only shown in (Lu et al., 2014)		
	C2 domain						
	CCD	Beclin 1	Direct interaction	AP, analytical ultracentrifugation, *in vitro*	Liang et al., 2006; Sun et al., 2008; Li et al., 2012	Y	
		Vps34	–	binding		Y	

(*Continued*)

Component #1	Domain of Component #1	Component #2	Direct interaction	Detection assay	Reference	Confirmed by CX-MS (Shi et al., 2015)	Consistent with EM (Baskaran et al., 2015)
Nrbf2 (Atg38 in yeast)	Full length (1–287)	Beclin 1	Weak direct	AP, Y2H (weak)	Zhong et al., 2009; Behrends et al., 2010; Araki et al., 2013; Cao et al., 2014; Lu et al., 2014; Zhong et al., 2014		
		Vps34	Weak direct	AP, Y2H (weak)	Behrends et al., 2010; Araki et al., 2013; Cao et al., 2014; Lu et al., 2014; Zhong et al., 2014		
		Vps15	Direct interaction	AP, *in vitro* binding	Araki et al., 2013; Cao et al., 2014; Lu et al., 2014; Zhong et al., 2014		
		Atg14L	Direct interaction	AP, *in vitro* binding, Y2H	Behrends et al., 2010; Araki et al., 2013; Cao et al., 2014; Lu et al., 2014; Zhong et al., 2014		
		UVRAG	–	AP	only shown in (Lu et al., 2014)		
		Nrbf2/Atg38	Direct interaction	AP, Y2H	Araki et al., 2013; Zhong et al., 2014		
	MIT domain (8–52) Yeast 1–80	Beclin 1	–	AP	Zhong et al., 2014		
		Atg14L	–	AP	Lu et al., 2014		
		Atg14	Direct interaction	Y2H	Araki et al., 2013		
	Yeast 1–120	Atg14	Direct interaction	Y2H, *in vitro* binding	Araki et al., 2013		
		Vps34	Direct interaction	Y2H, *in vitro* binding	Araki et al., 2013		
	CCD (167–215)	Nrbf2	Direct interaction	AP, Y2H	Araki et al., 2013; Zhong et al., 2014		
	Or yeast 121–226	Vps34	Direct interaction	Y2H	Araki et al., 2013		

Notes:
· Abbreviations: AP – affinity purification; Y2H – yeast two hybrid; CX-MS – crosslinking mass spectrometry; EM – single-particle electron microscopy; Y – Yes.
· Mutual exclusiveness of Atg14L and UVRAG was reported in binding to Beclin 1 (Sun et al., 2008; Li et al., 2012), Nrbf2 (Cao et al., 2014; Zhong et al., 2014), or binding to each other (Itakura et al., 2008; Sun et al., 2008; Matsunaga et al., 2009; Li et al., 2012; Lu et al., 2014; Zhong et al., 2014). Ectopically expressed Atg14L and UVRAG were co-IP'ed (Zhong et al., 2009) .
· Residue numbers follow selected references, as they are not exactly the same in different references.

1-Vps34 complexes were confirmed in *Nrbf2*$^{-/-}$ MEF cells and mouse brain models: e.g., Atg14L is required for the Beclin 1→Nrbf2 interaction, and Nrbf2 is required for the Atg14↔Vps34, Atg14L→Vps15, Vps34→Vps15, and Vps34→Beclin 1 interactions, and the total and Atg14L-linked Vps34 activities (Lu et al., 2014). Moreover, the regulation of Vps34 kinase activity by Nrbf2 requires Vps15 (Lu et al., 2014). Similarly, in yeast, Atg14 is required for Atg38 to bind Vps34, Vps15 and Atg6/Vps30, and Atg38 is required for Atg14 to bind Vps34 and Vps15, but not Atg6/Vps30 (Araki et al., 2013). In addition, in yeast, Vps38 is required for Atg6/Vps30 to interact with Vps34 and Vps15 (Kihara et al., 2001). Taken together, these studies suggest that Atg38/Nrbf2 is an integral structural component that brings Vps34-Vps15 and Beclin 1-Atg14L (or Atg6/Vps30-Atg14 in yeast) sub-complexes together.

Another experimental approach to assess Beclin 1-Vps34 complex architecture by affinity purification in combination with Western blot analysis involves utilizing ectopically expressed, tagged full length and truncation mutants of Beclin 1 complex components to identify protein domains critical for complex assembly (summarized in Table 2). Using this approach, it has been shown that the Beclin 1 CCD (residues 144-269) is sufficient to bind to UVRAG and Vps34 (Liang et al., 2006; Matsunaga et al., 2009), and the Beclin 1 CCD is both sufficient and necessary to bind to Atg14L (Sun et al., 2008; Matsunaga et al., 2009; Zhong et al., 2009). In a similar fashion, the Beclin 1 ECD domain (residues 244-337) has been shown to be required for the Beclin 1-Vps34 interaction and Beclin 1-associated Vps34 lipid kinase activity (Furuya et al., 2005; Liang et al., 2006).

For Vps34, the Vps34 N-terminal C2 domain (residues 1-282) is sufficient to bind to Beclin1 (Liang et al., 2006), and this domain also interacts with Atg14L and UVRAG (Itakura et al., 2008). The WD-40 domain (residues 1027–1454) of Vps15 alone and the kinase domain (residues 1–294) alone pull down Atg14 in yeast (Heenan et al., 2009). Endogenous immunoprecipitation with anti-Vps15 antibody further shows that point mutations in the Vps15 kinase domain (e.g.,D165R and E200R) greatly weaken the Vps34-Vps15 association (Stack et al., 1995). In addition, both the WD-40 domain and the other regions of Vps15 bind to Nrbf2 (Cao et al., 2014).

Both UVRAG and Atg14L utilize their CCD to bind to the Beclin 1-Vps34-Vps15 core complex. The UVRAG CCD (residues 200-269) is sufficient and necessary to bind to Beclin 1 (Liang et al., 2006). Deletion of the first or both CCD domains of Atg14L abolished the Atg14L-Nrbf2 interaction (Lu et al., 2014; Zhong et al., 2014). In agreement with this finding, Atg38 can only be pulled down by full-length yeast Atg14, but not truncated mutants, suggesting that a rather large portion of Atg14 is necessary for its binding to Atg38 (Araki et al., 2013). In addition, deletion of Atg14L CCD (residues 71–184) abolished both the Atg14L-Beclin 1 and Atg14L-Vps34 interactions without compromising Atg14L localization to the isolation membranes. However, Atg14L ΔCCD mutant could not restore autophagy in Atg14L siRNA-treated cells. These result suggest that Atg14L CCD is required for autophagy but not for Atg14L isolation membrane translocation (Itakura et al., 2008; Sun et al., 2008; Matsunaga et al., 2009; Zhong et al., 2009).

Nrbf2 contains an N-terminal MIT domain (residues 8-52). MIT domains are known to form an asymmetric three-helix bundle involved in protein-protein interactions, and are conserved among Vps4 (Obita et al., 2007; Stuchell-Brereton et al., 2007; Kieffer et al., 2008), spastin (Ciccarelli et al., 2003), katanin, and a sorting nexin, which may play a role in intracellular trafficking (Phillips et al., 2001). The Nrbf2 MIT domain is required for Nrbf2-Atg14L, Atg14L-Vps34, and Atg14L-Vps15 interactions, the total and Atg14L-linked Vps34 activities, and the formation of WIPI2-associated phagophores upon autophagy induction (Lu et al., 2014). Moreover, both Nrbf2 MIT domainand CCD (residues 168-209) facilitate Vps34-Vps15 interactions and Vps15-enhanced Vps34 activity (Lu et al., 2014). In addition, Atg38/Nrbf2 appears to form a homodimer via the CCD, facilitating the stability of the Atg14L/Atg14-containing complex (Araki et al., 2013; Zhong et al., 2014).

Direct interaction detection

Co-immunoprecipitation of two proteins from cell cultures or tissues does not necessarily guarantee direct interactions between these components. Thus, recombinantly purified or *in vitro* translated proteins are often used to determine if two proteins directly bind to one another. Protein pairs within the Beclin 1-Vps34 complexes for which direct interactions have been reported include: Vps34 (via helices kα11 and kα12)-Vps15 (Miller et al., 2010), Vps34 (via C2 domain)-Beclin 1 (Miller et al., 2010), Vps15 (via either WD-40 domain alone or other regions)-Nrbf2 (Cao et al., 2014), Beclin 1 (via CCD)-Beclin 1 (via CCD) (Li et al., 2012), Beclin 1 (via CCD)-Atg14L (via CCD) (Li et al., 2012), Beclin 1 (via CCD)-UVRAG (via CCD) (Liang et al., 2006; Li et al., 2012), Atg14L-Nrbf2 (Lu et al., 2014), and Atg38 (via residues 1–120)-Vps34/Vps15 (Araki et al., 2013) (summarized in Table 2). Interestingly, Beclin 1 forms, via its CCD, a metastable homodimer that readily dissociates to form more stable heterodimers through the CCDs of Atg14L and UVRAG (Li et al., 2012). Beclin 1 mutants defective in homodimer formation (e.g., L178A/L259A, L178A/L192A, and L178A/L196A) retain WT capability for the Beclin 1-Atg14L or Beclin 1-UVRAG heterodimer formation; whereas Beclin 1 mutants that form "stabilized" homodimers (e.g., E189L/A255L or E189L/A217L/E224L/A255L) show impaired interaction (Li et al., 2012).

Yeast two hybrid (Y2H) is another widely-used experimental approach to identify direct interactions. For example, both the N-terminalprotein kinase domain (residues 1–117 or longer) and the HEAT domain (which contains three tandem repeatsof about 39 amino acids each) of Vps15 are required

for interaction with Vps34 (via a 28-amino acid region encompassing residues 837-864) (Budovskaya et al., 2002). Y2H also demonstrates Beclin 1 homodimer formation as well as direct Beclin 1-Atg14L and Beclin 1-UVRAG interactions (Matsunaga et al., 2009). Y2H further shows that in yeast, Atg38 self-associates via residues 120-226 and this self-association is required for the association between the Vps34-Vps15 and Atg14-Atg6/Vps30 sub-complexes. Additionally, the Atg38 MIT (residues 1-80) domain directly binds Atg14, Atg38 weakly binds Atg6/Vps30 and Vps34, and the Atg38-Vps34 binding is independent of the MIT domain (Araki et al., 2013) (Table 2).

Crosslinking mass spectrometry (CX-MS)

Crosslinking with dithiobis (succinimidylpropionate) (DSP) followed by immunoprecipitation with anti-Vps15 antibody has previously been used to demonstrate Vps34-Vps15 interaction (Stack et al., 1993) and impairment of this interaction due to point mutations in the Vps15 kinase domain, e.g., D165R and E200R (Stack et al., 1995).

We recently developed a novel and robust proteomic strategy that provide the requisite sensitivity for hybrid structural dissection of large, multi-subunit native complexes (Shi et al., 2015). This strategy utilizes engineered, ultra-high affinity GFP V_HH nanobodies for efficient and pristine affinity capture of native complexes, and features direct on-bead crosslinking (by amine-specific disuccinimidyl suberate crosslinker) of the affinity captures followed by bottom-up proteomics and high-resolution MS to identify the cross-linked peptides. These cross-linked peptides are used as “chemical rulers” for measuring inter-atomic distances to determine the architectures of the complexes (Shi et al., 2015). We applied this technology to study the molecular architecture of the Beclin 1-Vps34 complexes from a single *Becn1-EGFP/+* transgenic mouse liver. Our study revealed multiple crosslinks between the Beclin 1 CCD (residues 142-267) and Vps34 N-terminal C2 domain (residues 1-255), including Beclin1(201)-Vps34(36), Beclin1(204)-Vps34(33), Vps34(36)-Beclin1(204), and Beclin1(204)-Vps34(29) (Shi et al., 2015). This crosslinking information enriches the limited structural information that is currently available on these native complexes.

Besides confirming previously identified interacting domains (Table 2), the CX-MS strategy is also useful for identifying the proximities of specific cross-linked amino acids within and between the various subunits comprising the protein complexes. The novel intermolecular crosslinks that we identified include Beclin1(52)-UVRAG(217), Vps15 (280)-Vps34(287), Vps34(287)-Nrbf2(85), Vps15(1028)-Atg14L(343), Vps15(1028)-UVRAG(405), UVRAG(401)-Vps15(1028), Vps15(982)-UVRAG(395), UVRAG(395)-Vps15(982), Vps15(993)-Atg14L(184), Vps15(801)-Vps34 (79), Vps15(816)-Vps34(92), Vps15(888)-Vps34(33), and Vps15(951)-Vps34(180) (Shi et al., 2015). These findings suggest that the Vps34 C2 domain and a region between the HEAT and the C-terminal WD-40 domains of Vps15 are brought into close proximity in the fully assembled complex. The possibility of direct interaction between the Vps34 N-terminal C2 domain and the region between the HEAT and WD-40 domains of Vps15 provides additional possible contact points between these two subunits, which differs from the observation in yeast, in which the C-terminal lipid kinase domain of Vps34 interacts with kinase-HEAT domains of Vps15 (Budovskaya et al., 2002). The architecture of native mouse Beclin 1-Vps34 complexes revealed by CX-MS are in agreement with the human recombinant complexes solved by single particle electron microscopy (EM, see the next subsection "Single-particle electron microscopy and hydrogen-deuterium exchange").

Interestingly, the mapped crosslinks show that Atg14L and UVRAG may competitively interact for the same lysine residues on the Beclin 1 (at residue 235, in the CCD), Vps34 (at residues 29 and 209, in the C2 domain), and Vps15 (at residue 1028) triad. These crosslinks include UVRAG (284, in the CCD)-Beclin 1(235), Beclin 1(235)-Atg14L(161 and 164, in the CCD), Atg14L(136)-Vps34(29), Vps34(29)-UVRAG(255, in the CCD), UVRAG(266, in the CCD)-Vps34(29), Vps34(209)-Atg14L(136), UVRAG(266)-Vps34 (209), Vps15(1028)-Atg14L(343), Vps15(1028)-UVRAG (405), and UVRAG(401)-Vps15(1028). Although the Beclin 1 CCD is known to bind Atg14L and UVRAG in a mutually exclusive manner, neither the Vps34 N-terminal C2 domain or the C terminus of Vps15 have been previously reported to bind Beclin 1 CCD mutual exclusively, except that the Vps34 C2 domain was shown to interact with both Atg14L and UVRAG (Itakura et al., 2008).

Single-particle electron microscopy (EM) and hydrogen-deuterium exchange

Recent single-particle EM and hydrogen-deuterium exchange studies provide the first view on the overall architecture and dynamics of the Atg14L- and UVRAG-containing Beclin 1-Vps34 complexes in their fully functional states (Baskaran et al., 2015). A three dimensional reconstruction of the Atg14L-containing Beclin 1-Vps34 complex was obtained at 28 Å resolution, which shows a V-shaped, loosely connected, elongated structure. Complementary EM-based N- and C-terminal maltose binding protein (MBP) localization studies enabled the mapping of the positions of the subunit termini in the complex. Additional docking analysis of known crystal structures or homology models onto the density permitted generation of a three-dimensional model of the complex (Baskaran et al., 2015) (Fig. 3). In this model, Vps15 is positioned throughout the V-shaped structure and appears to organize the complex. The Vps15 N terminus is located near the C terminus of Vps34 in the right arm of the V, the Vps15 C-terminal WD-40 domain forms a donut shaped region in the left arm of the V, and the HEAT repeat domain forms an

arch-shaped region at the junction of the V, where the Vps34 N-terminal C2 domain, Beclin 1 N terminus, and Atg14L N terminus appear near the junction. The close proximity of the Beclin 1 and Atg14L N-termini suggests that the Atg14L and Beclin 1 coiled coils are parallel to each other, in contrast to the previously reported antiparallel coiled-coil for the Beclin 1 homodimer (Li et al., 2012). Beclin 1 is positioned along the left arm of the V, with its C-terminal BARA domain (or ECD) at the tip of the V and above the Vps15 WD-40 domain. Two long, narrow tubes of electron density (the first one located between the Beclin 1 BARA and Vps15 WD-40 domains; the second one located between the Vps15 WD-40 and HEAT domains) are presumed to account for the coiled-coil dimer of Atg14L and Beclin 1. This assumption is consistent with the newly identified contacts in our CX-MS study (i.e., Vps15 (801)-Vps34(79), Vps15(816)-Vps34(92), Vps15(888)-Vps34(33), and Vps15(951)-Vps34(180)) (see the subsection "Crosslinking mass spectrometry (CX-MS)") (Shi et al., 2015). These new data suggest that the Vps34 C2 domain interacts with the Beclin 1 and Atg14L CCDs and with the region between the HEAT and WD-40 domains of Vps15 (Table 2).

A single-particle EM structure of the UVRAG-containing Beclin 1-Vps34 complex was also obtained, which shows essentially identical overall conformation and architecture to those of the Atg14L-containing Beclin 1-Vps34 complex, with an additional electron density for the UVRAG N-terminal C2 domain near the junction of the V and the Atg14L N terminus (Baskaran et al., 2015). The newly identified crosslinks in our CX-MS study (i.e., Vps15(1028)-Atg14L (343), Vps15(1028)-UVRAG(405), UVRAG(401)-Vps15 (1028)) (see the subsection "Crosslinking mass spectrometry (CX-MS)") (Shi et al., 2015) further suggest that both Atg14L and UVRAG, like Beclin 1, also align the left arm of the V, likely "glued" to Beclin 1 by parallel CCDs. In addition, the newly identified Vps34(287)-Nrbf2(85) crosslink in our CX-MS study suggests that Nrbf2 is located at the region between the C2 and helical domains of Vps34 (likely where the UVRAG C2 domain is), which is consistent with the reports that Nrbf2 and UVRAG are also mutually exclusive (Araki et al., 2013; Cao et al., 2014; Zhong et al., 2014).

The dynamics of the full complex was analyzed by hydrogen deuterium exchange with MS readout (HDX-MS), which revealed a conserved 20-residue ordered region between the C2 and helical domains of Vps34 (i.e., residues 218-237, termed the C2-Helix Internal Linker (CHIL) motif) (Baskaran et al., 2015). It is postulated that this linker provides an anchor for a limited displacement of the Vps34 HELCAT domains. Furthermore, HDX-MS data also show that both the linker regions between the Vps34 C2 and helical domains (e. g., around residue 287) and between the Vps15 HEAT and WD-40 domains are highly disordered, consistent with lack of electron density in these regions. Structurally homogeneous subsets of EM images show Vps15 to pivot at the base of the V structure, between the WD-40 domain and the C terminus of the HEAT repeat. This, assisted by additional flexibility between the HEAT and kinase domain, allows movement of the Vps15 kinase domain relative to the WD-40 domain. The exact role that this movement plays in regulating the Beclin 1-Vps34 complex functions is unclear, although the Beclin 1 N terminus is in close proximity to the pivot point. In addition, our CX-MS results show crosslinks between this pivot point to Atg14L and UVRAG, i.e., Vps15(982)-UVRAG(395), UVRAG(395)-Vps15(982), and Vps15(993)-Atg14L(184). As the N-termini of Beclin 1 and Atg14L appear to be a convergent region for signaling inputs (e.g., phosphorylation by Ulk1 and AMPK, binding to Bcl-2, and ER-targeting), it is possible that the pivot point of the V receives signals while the right arm of the V transmits signals through the aforementioned intra-complex long range allosteric movement.

Results from single-particle EM and HDX-MS analyses of the reconstituted Atg14- and UVRAG-containing Beclin 1-Vps34 complexes (Baskaran et al., 2015), together with those from CX-MS analysis of native Beclin 1-Vps34 complexes (Shi et al., 2015), collectively lead to a model of subunit architecture and their dynamics (see Fig. 3 for the architecture of the Atg14L-containing Beclin 1-Vps34 complex). These approaches provide exciting and valuable ways for better analyzing multi-protein complexes like the Beclin 1-Vps34 complexes. These approaches, which allow analysis of endogenous protein complexes, permit modeling of structures that contain disordered or flexible regions, which is problematic for most other structural analysis platforms. Resolving protein complexes by these approaches will be useful in the design of drugs totarget these complexes more effectively.

Drug design targeting the Beclin 1-Vps34 complexes

Strong evidence linking autophagy to many human diseases suggests that the autophagy pathway is an important target for drug intervention. Therapeutic possibilities that aim atthe Beclin 1-Vps34 complexes may prove beneficial in the treatment of these diseases. Once a promising drug target is identified, structure-based design is often utilized for novel drug discovery during the hit-to-lead stage (Martell et al., 2013). Moreover, structure-based design has also been shown to be critical for developing new generation of drugs that overcome drug resistance (Martell et al., 2013). One structure-based design strategy proven very successful is the targeting of catalytic domains of enzymes, ion channels, or receptors. For example, STI571 (imatinib; Gleevec, Novartis, Basel, Switzerland), a competitive inhibitor at the ATP binding site of the oncogenic BCR-ABL, was developed through structure-based design in combination with *in vitro* screening for tyrosine kinase inhibitors (Druker et al., 1996). Additional structure-based design led to development of second generation BCR-ABL inhibitors (e.g., dasatinib and

nilotinib) that inhibit imatinib-resistant mutant BCR-ABL (Shah et al., 2004; Burgess et al., 2005). A second structure-based design strategy targets allosteric sites that are independent of the enzyme-actives ite, but cause conformational changes to alter enzyme function. A third design strategy targets protein-protein interactions, which has recently been shown to successfully influence previously non-druggable proteins or pathways due to hot spots or adaptability of the targeted interfaces. This type of drugs induce biochemical perturbations, including post-translational modification, allosteric transition, protein degradation or *de novo* protein synthesis, that can change protein-protein interactions (Michnick et al., 2007). Examples of structure-based drug design using this strategy include the small molecule BH3 mimetics (e.g., ABT-737) which competitively displace the pro-apoptotic Bcl-2 family proteins from binding to the anti-apoptotic Bcl-2 family proteins and lead to apoptosis, the SMAC mimetics which bind to anti-apoptotic proteins (e.g., XIAP, cIAP1 and cIAP2) and cause the release of apoptotic SMAC and caspases, and the MDM2 antagonists (e.g., Nutlin-3) which mimick the p53 residues for MDM2 binding and competitively displace p53 from the MDM2 binding site, promoting p53 stabilization and activation, and apoptosis in cancer cells with wild type p53 (Bai and Wang, 2014).

Phosphorylation of PtdIns to generate PtdIns(3)P is the primary function of Vps34 thus the Beclin 1-Vps34 complexes. Structure-based drug design can target the Vps34 PI(3)K activity either directly at the Vps34 catalytic site or indirectly via modulating the Beclin 1-Vps34 complex stability and architecture.

Drug design directly targeting Vps34

Vps34 is the sole class III lipid kinase, and it is known to regulate many cellular processes, including vesicular trafficking in the endosomal/lysosomal system (Schu et al., 1993; Stack et al., 1995), autophagy (Kihara et al., 2001), nutrient sensing in the mTOR pathway (Byfield et al., 2005; Nobukuni et al., 2005; Gulati et al., 2008; Xu et al., 2011; Yoon et al., 2011; Zhou et al., 2011; Jaber et al., 2012), and trimeric G-protein signaling to mitogen-activated protein kinase (MAPK) (Slessareva et al., 2006). Wortmannin (Powis et al., 1994), 3-MA (Seglen and Gordon, 1982; Blommaart et al., 1997) and LY294002 (Vlahos et al., 1994) are commonly used to inhibit Vps34 PI(3)K activity, even though they also inhibit class I PI(3)K (Wu et al., 2010).

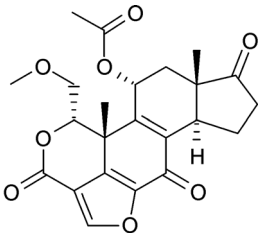
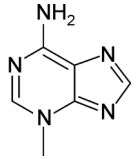
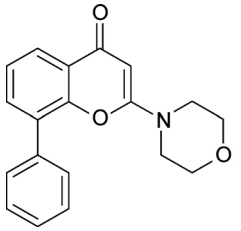
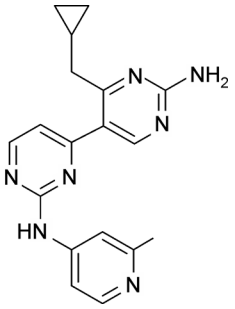
With the crystal structure of *Drosophila* Vps34 in place (Miller et al., 2010), development of Vps34 inhibitors to modulate Vps34 PI(3)K activity is at the forefront of much biomedical research. Recently, multiple research groups generated potent and highly selective small molecular inhibitors of Vps34 (summarized in Table 3), including Vps34-IN1 (IC_{50} = 25 nM) (Bago et al., 2014), PIK-III (IC_{50} = 18 nM) (Dowdle et al., 2014), SAR405 (IC_{50} = 1.2 nM) (Ronan et al., 2014), and compound 31 (IC_{50} = 2 nM) (Pasquier et al., 2015). These compounds are specific to Vps34 and few off-target effects have been observed against other PI(3)K isoforms or other lipid kinases (Bago et al., 2014; Dowdle et al., 2014; Pasquier et al., 2015; Ronan et al., 2014).

The development of these new Vps34 inhibitors subsequently led to the identification of novel Vps34 targets and clarification of the kinase-dependent biological functions of Vps34 (Bago et al., 2014; Dowdle et al., 2014; Pasquier et al., 2015; Ronan et al., 2014). The catalytic function of Vps34 remained elusive until a recent report by Bago et al. (2014). Utilizing the specific Vps34 inhibitor Vps34-IN1, Bago and colleagues showed that Vps34 controls the activity of SGK3 (serum- and glucocorticoid-regulated protein kinase 3), a PtdIns(3)P effector, by targeting SGK3 to the endosome where it is subsequently phosphorylated (Bago et al., 2014). In a second study, using the specific Vps34 inhibitor PIK-III, Dowdle and colleagues revealed a novel autophagy substrate, NCOA4, which is required for ferritin degradation in lysosomes and for iron homeostasis (Dowdle et al., 2014). A third ATP-competitive inhibitor of Vps34, SAR405, was reported to block starvation- and the ATP-competitive mTOR inhibitor AZD8055-induced autophagy by preventing autophagosome formation, as monitored by GFP-LC3 puncta and LC3II levels in the presence of hydroxychloroquine (Ronan et al., 2014). SAR405 was also reported to block vesicle trafficking, as shown by swollen LysoTracker- and Lamp1-positive late endosomes/lysosomes as well as impaired cathepsin D maturation without altering cellular uptake of dextran (Ronan et al., 2014). Further investigations are necessary to determine if inhibition of Vps34 PI(3)K activity utilizing these inhibitors will provide a useful therapeutic strategy for clinical use, either alone or in combination with other therapeutic agents. For example, evidence reveals a role for Vps34 in mTOR activation (Byfield et al., 2005; Nobukuni et al., 2005; Gulati et al., 2008; Xu et al., 2011; Yoon et al., 2011; Zhou et al., 2011; Jaber et al., 2012), providing the potential to combine a Vps34 inhibitor with a mTOR inhibitor to better combat disease (Ronan et al., 2014).

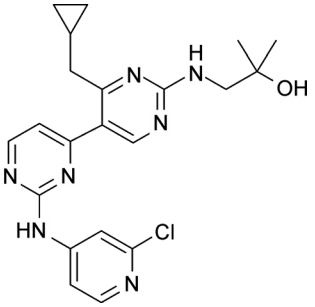
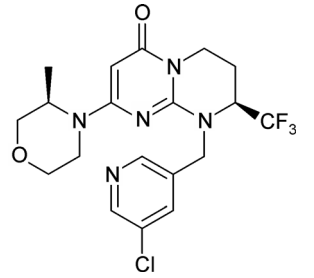
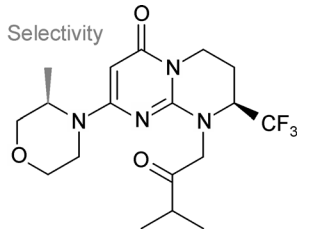
Drug design targeting the stability of the Beclin 1-Vps34 complexes

Besides directly targeting the Vps34 lipid kinase activity as described above, drugs can be designed to allosterically impair the Vps34 PI(3)K activity. One such approach is to modulate the stability of the Beclin 1-Vps34 complex. For example, spautin-1 administration is shown to block the activities of the ubiquitin-specific peptidase 10 and 13 (i.e., USP10 and USP13, which promote the de-ubiquitinylation and stability of Beclin 1), thereby leading to degradation of Beclin 1 and the Beclin 1-Vps34 complexes thus inhibition of autophagy (Liu et al., 2011).

Table 3 Summary of reported Vps34 inhibitors

Vps34 inhibitor	Molecular structure	IC50 (*in vitro*)	IC50 (*in vivo*)	IC50 for other lipid kinases	IC50 for protein kinases	Reference	Patent by
Wortmannin		1.8 – 4.0 nM	6 μM by counting GFP-2xFYVE puncta; 30 nM by overall proteolysis in rat hepatocytes	Wortmannin and its analogues are not inhibitors of Ptdlns-4-kinase (at conc. up to 32 μM).	Wortmannin and its analogues are not inhibitors of protein kinase C (at conc. up to 22 μM), c-Src tyrosine kinase (at conc. up to 5.5 μM), and phosphoinositide-specific phospholipase C (at conc. up to 0.27 mM).	Dowdle et al., 2014; Ronan et al., 2014; Blommaart et al., 1997; Powis et al., 1994	
3-methyladenine (3-MA)		~ 1 mM (at 50 μM ATP)	616 μM by counting GFP-2xFYVE puncta; 5 mM 3- MA inhibited 60% proteolysis in hepatocytes.			Dowdle et al., 2014; Blommaart et al., 1997; Seglen and Gordon, 1982	
LY294002 (2-(4-morpholinyl)-8-phenylchromone)		1.4 – 4.2 μM	10 μM by overall proteolysis in rat hepatocytes			Ronan et al., 2014; Blommaart et al., 1997; Vlahos et al., 1994	
PIK-III		18 nM	55 μM by counting GFP-2xFYVE puncta;	>1 μM for PI(3)Kα, PI(3)Kβ, PI(3)Kγ, PI(3)Kδ, PI4Kβ.	>9.1 μM for mTOR	Dowdle et al., 2014	Novartis

(*Continued*)

Vps34 inhibitor	Molecular structure	IC50 (*in vitro*)	IC50 (*in vivo*)	IC50 for other lipid kinases	IC50 for protein kinases	Reference	Patent by
VPS34-IN1 (1-[{2-[(2-chloropyridin-4yl) amino]-4'-(cyclopropyl-methyl)-[4,5'-bipyrimi-din]-2'-yl}amino]-2-methylpropan-2-ol) (CAS registry number 1383716-33-3)		25 nM	100 nM by counting GFP-2xFYVE puncta; 100 nM ~ 1 μM by SGK3 activity and T-loop/hydrophobi c motif phosphorylation	>1 μM for other lipid kinases: in the Dundee panel (19 lipid kinases, includes class I PI3Ks), the AstraZeneca panel (8 lipid kinases, includes class I) and ProQuinase panel (13 lipid kinases, includes class I and class II PI3Ks); did not significantly inhibit any of the lipid kinases tested including class I (p110α, p110β p110γ and p110δ) and all three members of the class II (PI3KC2α, PI3KC2β, PI3KC2γ) PI3Ks.	1 μM did not significantly inhibit the activity of any of the protein kinases in the Dundee panel (140 kinases) and the ProQinase panel (300 kinases)	Bago et al., 2014	Novartis (WO 2012085-815A1)
SAR405 ((8s)-9-[(5-Chloranylpyridin-3-yl)methyl]-2-[(3r)-3-Methylmorpholin-4-yl]-8-(Trifluoromethyl)-6,7,8,9a-Tetrahydro-3h-Pyrimido[1,2-A]pyrimidin-4-One) (CAS registry number1523406-39-4)		1.2 nM (K_D= 1.5 μM)	27 nM by counting GFP-2xFYVE puncta; 419 nM by counting % of cells with GFP-LC3 dots in response to starvation	*In vitro*:>10 μM for class I PI3Ks (p110α, p110β, p110γ and p110δ) and class II PI3Ks (PI3KC2α, PI3KC2β and PI3KC2γ); *In vivo*: 1 μM leads to 39%, 68%, and 63% inhibition of p110α, p110β and p110δ, respectively.	*In vitro*:>10 μM for mTOR; *In vivo*: 1 μM leads to 52% inhibition of SMG1, but not any other protein kinases. 10 μM did not affect Akt, S6, p53, and phosphorylation.	Ronan et al., 2014	Sanofi
Compound 31 ((2S)-8-[(3R)-3-Methylmorpholin-4-yl]-1-(3-methyl-2-oxobutyl)-2-(trifluoromethyl)-3, 4-dihydro-2Hpyrimido [1,2-a]pyrimidin-6-one)		2 nM (K_D= 2.7±0.9 nM)	82 nM by counting GFP-2xFYVE puncta;	*In vitro*:>2 μM for class I PI3Ks (p110α, p110β, p110γ and p110δ); and>10 μM for class II PI3Ks (PI3KC2α and PI3KC2γ);	*In vitro*:>10 μM for mTOR; *In vivo*: 8.5 μM for Akt (@Ser473);	Pasquier et al., 2015	Sanofi

Drug design targeting protein-protein interactions within the Beclin 1-Vps34 complexes

A common approach to alter Vps34 activity is to modulate the Beclin 1-Vps34 protein complex architecture, such as the availability of Beclin 1 for complex formation. A general mechanism of autophagy regulation repeatedly emerging from protein-protein interaction studies is the sequestration of Beclin 1 by negative regulators until autophagy induction signals are received. These negative regulators include Bcl-2 (Pattingre et al., 2005; Maiuri et al., 2007), Bcl-X_L(Feng et al., 2007; Maiuri et al., 2007; Oberstein et al., 2007), M11 (Ku et al., 2008; Sinha et al., 2008), and GAPR-1 (Shoji-Kawata et al., 2013).

Viral interference with the autophagic process prevents the antimicrobial host defense mechanism, and allows the virus to flourish. In some cases, virulence gene products block autophagy initiation through their interaction with Beclin 1 (Kudchodkar and Levine, 2009; Levine et al., 2011). Mounting evidence has already suggested that development of pharmacological agents aimed at increasing autophagy for treatment of bacterial, parasitic and viral infections may prove to be an effective therapeutic strategy. In these instances, BH3 mimetics disrupt the inhibitory interaction between the BH3 domain of Beclin 1 and Bcl-2/Bcl-X_L, leading to Beclin 1-dependent allosteric activation of the pro-autophagic lipid kinase Vps34, ultimately leading to autophagy induction (Maiuri et al., 2007). Similarly, Tat-Beclin 1 peptide disrupts the inhibitory interaction between GAPR-1 and Beclin 1, leading to Beclin 1-dependent Vps34 activation and autophagy induction. These processes have been shown to decrease the *in vitro* replication of several pathogens, including HIV-1, Sindbis virus, chikungunya virus, West Nile virus, and the intracellular bacterium, *Listeria monocytogenes*, and have reduced mortality in mice infected with chikungunya or West Nile virus (Shoji-Kawata et al., 2013).

In other cases, bacteria exploit the host autophagy initiation proteins, e.g., Ulk1, Beclin 1, Atg14L, and Vps34, to survive (Starr et al., 2012). In these instances, inhibitors that target either the pathogen's interaction withthe Beclin 1-Vps34 complexes, or individual proteins that comprise the complexes, might attenuate the pathogen's attack on the autophagy process. In addition, post-translational modifications of Beclin 1-Vps34 complex components (e.g., Beclin 1, Vps34, Atg14L) also modulate Beclin 1 complex architecture so as to regulate autophagy.

Challenges

Despite intense interest in targeting the Beclin 1-Vps34 complexes and autophagy for therapeutics, challenges remain for such drug design. For example, the Beclin 1-Vps34 complex components, structure, and architecture, as well as the dynamics and regulatory mechanisms of the complexes have not been fully elucidated. In addition, because Beclin 1-Vps34 complexes have multiple functions in autophagosome biogenesis, autophagosome maturation, and trafficking, the design of drugs that specifically target just one of the functions is likely to be challenging. One strategy to cope with this difficulty is to design drugs that specifically target interactions between Atg14L (or UVRAG) and the Beclin 1-Vps34-Vps15 core complex. Moreover, although counting GFP-2xFYVE puncta is a commonly used readout for PtdIns (3)P production, a more autophagy-specific readout (e.g., counting GFP-DFCP1 or GFP-WIPI2 puncta) may be needed for monitoring Atg14L-containing Beclin 1-Vps34 complex activity and for structure-based design in combination with high throughput screen.

There are also challenges that are common for targeting autophagy. For example, therapeutic success is contingent on the timing of the treatment, as cancer development goes through two phases with distinct autophagy contributions. During tumorigenesis, autophagy eliminates oncogenic abnormalities (Takamura et al., 2011), thus needs to be upregulated to eliminate cancer cells; however at later stages, tumor cells activate autophagy as a survival mechanism, therefore blockage of autophagy is important to suppress tumor growth and metastasis (Mathew and White, 2011; Karsli-Uzunbas et al., 2014). As most cancer treatment is aimed at pre-existing tumors, use of an autophagy inhibitor in combination with chemotherapeutic agents and ionizing radiation might constitute a more promising therapeutic strategy. To date, drugs aimed at specifically inhibiting the Beclin 1-Vps34 complexes do not exist in the clinic. Studies that test the newly developed Vps34 inhibitors should be valuable. Caution is also necessary when designing drugs to target autophagy, as they might possess autophagy-independent effects. Due to autophagy's important role in controlling infection and immunity, drugs that inhibit autophagy might cause adverse secondary symptoms.

As a deeper understanding of the role of autophagy in disease unfolds, it is expected that the protein interactions that regulate the relevant process will be more fully elucidated. Manipulation of these autophagy proteins (e.g., the Beclin 1-Vps34 complex components) or their gene expression has the potential to combat human diseases. With specific protein-protein interactions known and the development of more powerful computational capabilities, including protein-protein docking studies, the structural basis required to attain optimal drug design for complex protein-protein networks like the Beclin 1-interactome is coming into view (Vakser, 2014). With evolving technologies, development of synthetic peptides or small molecule drugs that selectively target specific binding/modification sites on the proteins mentioned in this review could prove beneficial in therapies that either up- or down-regulate autophagy.

While this review was in proof, Roger Williams group reported the 4.4 angstrome crystal structure of the yeast Vps38-containing Atg6/Vps30-Vps34 complex (Rostislavleva et al., 2015). This new work provides exciting atomic-

resolution insights into the architectures of the Beclin 1-Vps34 complexes and future structure-based drug design.

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Compliance with ethics guidlines

Deanna Morris, Calvin Yip, Yi Shi, Brian Chait, and Qingjun Wang declare that they have no conclict of interest. This manuscript is a review article and does not involve a research protocol requiring approval by the relevant institutional review board or ethics committee.

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