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ATG2 and VPS13 proteins: Molecular highways transporting lipids to drive membrane expansion and organelle communication.

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Running title: ATG2 and VPS13 regulate lipid transfer.

Abbreviations:

- AMPK 5' AMP-activated protein kinase;
- ATG AuTophGy gene/protein;
- BPAN β -propeller protein-associated neurodegeneration ;
- ChAc Chorea-acanthocytosis;
- CVT Centriole to Vacuole;
- ER endoplasmic reticulum;
- ERMES Endoplasmic reticulum-mitochondrion encounter structure;
- EM Electron Microscopy;
- FIP200 200 KDa FAK Family Kinase-Interacting Protein;
- GABARAP GABA Type A Receptor-Associated Protein;
- GARP Golgi-associated retrograde protein;
- KO knockout;
- LAP LC3 associated phagocytosis;
- LANDO LC3-associated endocytosis;
- LD Lipid droplet;
- LC3 Microtubule associated protein Light chain 3;
- LIR LC3 interaction region;
- mTORC1 mammalian target of rapamycin complex 1;
- PA phosphatidic acid;
- PAS Pre-autophagosomal structure;
- PC Phosphatidylcholine;
- PE Phosphatidylethanolamine;

PI – phosphatidylinositol;

- PI3K phosphoinositide 3-kinase;
- PS phosphatidylserine;
- SAR selective autophagy receptor;
- UHRF1BP1/1L Ubiquitin Like With PHD And Ring Finger Domains 1 Binding Protein 1/1-Like;
- ULK1/1 Unc1-like kinase 1/2;
- VMP1 Vacuolar membrane protein 1;
- VPS Vacuolar Protein Sorting;
- WIPI WD-repeat protein Interacting with PhosphoInositides);
- WIR WIPI interaction region;

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Conflict of interest

The authors declare no conflicts of interest.

Abstract:

Communication between organelles is an essential process that helps maintain cellular homeostasis and organelle contact sites have emerged recently as crucial mediators of this communication. The emergence of a class of molecular bridges that span the inter-organelle gaps has now been shown to direct the flow of lipid traffic from one lipid bilayer to another. One of the keys components of these molecular bridges is the presence of an N-terminal Chorein/VPS13 domain. This is an evolutionarily conserved domain present in multiple proteins within the endocytic and autophagy trafficking pathways. Herein, we discuss the current state-of-the-art of this class of proteins, focusing on the role of these lipid transporters in the autophagy and endocytic pathways. We discuss the recent biochemical and structural advances that have highlighted the essential role Chorein-N domain containing ATG2 proteins play in driving the formation of the autophagosome and how lipids are transported from the endoplasmic reticulum to the growing phagophore. We also consider the VPS13 proteins, their role in organelle contacts and the endocytic pathway and highlight how disease-causing mutations disrupt these contact sites. Finally, we open the door to discuss other Chorein_N domain containing proteins, for instance UHRF1BP1/1L, their role in disease and look towards prokaryote examples of Chorein N-like domains. Taken together, recent advances have highlighted an exciting opportunity to delve deeper into inter-organelle communication and understand how lipids are transported between membrane bi-layers and how this process is disrupted in multiple diseases.

Introduction.

Trafficking pathways are often described as stand-alone processes that are highlighted in their own context. It is now clear that there is a great deal of connectivity between various components of these networks and what was once thought of as free-moving vesicles, are now known to be anchored at numerous points within the cell. The emergence of connections, or molecular bridges, between organelles and vesicles is becoming increasingly important especially in the

context of our understanding of how vesicles form, how content is transferred between organelles and how organelles or vesicles are transported throughout the cell.

Recently, the emergence of a class of proteins that contain a lipid-transfer domain to shuttle phospho-lipids between organelles and growing vesicles has significantly enhanced our understanding of these processes and highlighted the importance of these molecular bridges in the context of health and disease. In this review, we will highlight the recent advancements in this field, discuss the importance of these molecular bridges and highlight open questions. We will focus on several of the known mammalian proteins and their functions in the autophagy and endocytic pathways.

The Chorein_N domain.

At the heart of this emerging class of lipid transfer proteins lies the Chorein N domain (also known as VPS13 N-terminal domain). The Chorein N domain consists of approximately 100-150 amino acids and is often found at the N-termini of proteins [1-5]. Recent advances using biochemical and structural data have begun to shed some light on how these domains function at the molecular level[5-11]. Perhaps one of the most exciting and unexpected results was that the crystal structures of two families of Chorein N-domain containing proteins (ATG2 and VPS13) showed that they form channels that facilitate the flow of lipid moieties from one lipid bilayer to another [9, 12, 13]. The presence of Chorein N-domain containing proteins at organelle contact sites such as the endoplasmic reticulum (ER)-mitochondria, ER-lysosome and ER-phagophore (autophagosome) indicates that lipid transfer between organelles plays an important role in communication between these organelles [7, 13-24]. Indeed, the known Chorein N domaincontaining proteins are large (> 250kDa) and contain multiple domains that can span organelle contact sites [1-4, 15, 25, 26]. These recent advances have brought to the fore a method of organelle-to-organelle communication that has implications for organelle homeostasis in health and disease. In this review, we will focus on three families of proteins and their functions, known and unknown, in relation to the Chorein_N domain. These include the human VPS13 family, consisting of four members VPS13A-13D [2], ATG2 family (ATG2A & ATG2B) [16, 26, 27] and UHRF1BP1/1L [28-31]. All three of these protein families contain Chorein N domains at their ntermini and, particularly in the case of ATG2 and VPS13 families, have recently been characterized and the function of their Chorein N domains have been explored.

Autophagy core machinery and cargo selection.

Macroautophagy (henceforth autophagy) is the process of forming a double membrane vesicle, termed autophagosome, that surrounds specific cargo. The cargo is then transported to, and fuses with, the lysosome resulting in the degradation of the cargo and recycling of the building block components (amino acids and lipids) back into the cell. Autophagy therefore acts as both a quality control mechanism and a cellular recycling hub that allows cells and tissues to adapt to periods of stress and can aid their survival.

Autophagy is regulated by more than 30 core genes (ATG; Autophagy-related) and is directly controlled by the amino-acid-sensing mTORC1 complex (mammalian target of rapamycin complex 1) and the energy-sensing kinase AMPK (5' AMP-activated protein kinase). This ties the process tightly to the metabolic state of the cell. The formation of the double-membraned vesicle can originate at multiple sites within the cell including the ER [32, 33], golgi [34], outer

mitochondrial membrane [35] and plasma membrane [36]. There are several distinct phases that make up the lifecycle of an autophagosome; from birth (initiation), growth (elongation), maturation (closure) and turnover (fusion with the lysosome and degradation of the contents) (Figure 1A). However, all these steps serve to select specific cargo that the phagophore grows and surrounds, isolating it from the cell prior to lysosome-mediated degradation. Indeed, the autophagy pathway was initially thought to be non-specific and served to process bulk degradation of cytosolic contents during starvation. However, in the last decade, the term 'selective autophagy' has been coined to describe the specific targeting of complex structures for isolation, degradation and Typical substrates that have emerged include ubiquitinated-protein aggregates recycling. (aggrephagy), invading pathogens (xenophagy), damaged organelles (e.g., mitochondria, mitophagy) and swathes of membrane (such as ER; ER-phagy). These various types of autophagy are facilitated by specialised selective autophagy receptors (SARs). SARs interact directly with components of the autophagosome and the cargo simultaneously, allowing the autophagosome to grow and surround the cargo (and SAR) for degradation (Figure 1B). Some of the best characterised examples include p62/SQSTM1 (aggrephagy, xenophagy) [37, 38], NDP52 and OPTN (xenophagy) [39, 40], NBR1 (aggrephagy) [41], FAM134B (ER-phagy) [42] and Nix/BNIP3L and FUNDC1 (mitophagy) [43-45]. Interestingly, it has recently been shown that whilst starvation-induced autophagy is dependent on mTORC1 inactivation, selective autophagy can bypass this activation mechanism. For example, NDP52 can directly recruit and activate the ULK1 complex, normally inhibited by mTORC1, and drive the selective degradation of mitochondria, peroxisomes and intracellular pathogens such as Salmonella [46, 47]. Moreover, p62/SQSTM1 can recruit the ULK1 complex, through a direct interaction with FIP200 C-terminal region (CTR), to initiate phagophore formation at protein aggregate condensates [48]. The interaction of p62/SQSTM1 with FIP200 is mediated through a larger segment of p62/SQSTM1 that includes the LC3 interaction region (LIR), is phosphorylation dependent and where FIP200 and LC3 interact with p62/SQSTM1 this occurs in a mutually exclusive manner [48]. As our understanding of the process of selective autophagy expands, so does the number of receptors, their mechanisms of actions and the spatiotemporal regulation of the selective autophagy process. For an updated and comprehensive view of these receptors, see this recent review on the subject [49].

The formation of the autophagosome and subsequent fusion with the lysosome, where its contents and inner membrane are degraded and recycled, is regulated by multiple core ATG proteins. In mammals this consists of the initiation complex containing ULK1/2 (ATG1), ATG13 and FIP200 [50-53], a sensor for upstream nutrient signalling. The ULK1 complex subsequently

activates the phosphoinositide 3-kinase (PI3K) complex, consisting of Beclin1 (ATG6), PIK3C3 (VPS34), ATG14 and p150 [54]. This drives local PI3P production and the formation of the omegasome (the point of origin) of the autophagosome. The production of PI3P in this manner subsequently drives the recruitment of WIPI2 (WD-repeat protein Interacting with Phospholnositides family member 2), an essential PI3P effector during autophagosome biogenesis [55]. The elongation of the growing autophagosome (termed phagophore) is catalysed by a ubiquitin-like E1-E2-E3 conjugation system. ATG7 (E1) transfers the ubiquitin-like ATG8 proteins (LC3s and GABARAPs) and ATG12 to their E2-enzymes (ATG3 and ATG10 respectively). ATG12 is directly conjugated to ATG5, and the subsequent ATG5-ATG12 conjugate forms a complex with ATG16L1. WIPI2 recruits the E3-like ATG5-12-16L1 complex to the phagophore formation site [56, 57], which drives the attachment of ATG8 proteins directly onto phosphatidylethanolamine (PE) that is enriched in the phagophore membrane [58]. It is worth noting that recent data indicates that the ATG8s are dispensable for cargo recognition[59] and autophagosome biogenesis[60, 61] during selective autophagy. Surprisingly, GABARAPs, and not LC3s, are essential for the final fusion step with the lysosome [60, 61] in a HOPS complex and PLEKHM1 dependent manner [60-63]. In the absence of LC3/GABARAP proteins, the formation of the autophagosome around the cargo is driven by the clustering of SARs to localise FIP200 complex at sites of autophagosome biogenesis [46-48, 59] that can occur in an LC3-lipidation independent manner [59]. However, in the absence of the LC3/GABARAP isoforms, autophagosomes are smaller and form at a slower rate [61]. The ubiquitin-like conjugation reaction drives the expansion of the phagophore with the aid of the integral membrane protein ATG9, which is localized in highly mobile pools of ATG9 positive vesicles that help to deliver lipids and essential constituents, acting as seeds to define membrane contract sites and promote the expansion of the phagophore [64-66] and its eventual closure. In yeast, Atg9 establishes ER-phagophore contact sites through the recruitment of Atg2-Atg18 proteins[18].

Uncovering the role of ATG2 proteins in autophagosome biogenesis.

With regards to ATG protein function during autophagosome formation, perhaps the most elusive has been the family of ATG2 proteins. Despite being identified in yeast 20 years ago [16, 27], it is only recently that the ATG2 mechanism of action has been revealed both biochemically and structurally. Initial insights into the function of the ATG2 proteins, much like many of the ATGs, came from their genetic ablation in yeast. These studies revealed that yeast lacking *atg2* caused in a blockage of the autophagy/CVT pathway. An N-terminal mutant (atg2 G83E) showed a partial autophagy defect, compared to null and WT *atg2* cells [16, 27], indicating that the N-

terminus of ScAtg2 was needed for efficient autophagosome formation. In addition, the localisation of the ScAtg2 proteins was shown to be dependent on the PI3K complex, where *atg6 or atg14* null cells showed a dispersed ScAtg2 localisation [27], indicating a requirement for PI3P to maintain its localisation at the pre-autophagosomal structure (PAS). ScAtg2 was shown to be a direct interaction partner of ScAtg18 [67] and the PI3P-dependent localisation of ScAtg2 required an intact FRRG motif on ScAtg18, that when mutated resulted in loss of ScAtg2 on autophagic structures and a complete block in the autophagy pathway [68]. The interaction of ScAtg2 and ScAtg18 was shown to be constitutive and did not require PI3P [68], where the PI3P and ScAtg2 sites were distinct and separate [69].

In mammals, there are two isoforms of ATG2, ATG2A and ATG2B (Figure 2A), that are around 40% similar at an amino acid level. Like their yeast counterpart, human ATG2 proteins were shown to be essential for autophagosome formation, where depletion of both isoforms was required to block the autophagy pathway [26]. However, human ATG2A was unable to restore autophagic flux when reconstituted in $\triangle atg2$ yeast despite localizing to autophagy PAS structures [70]. Depletion of both ATG2A/B led to the accumulation of unclosed autophagic structures [26]. These were later shown, through electron microscopy (EM) ultrastructure studies, to be small, premature phagophores that were not closed [71]. However, it was unclear why loss of ATG2A/B in this context resulted in these structures. Much like ScAtg2, mammalian ATG2s interact with a PI3P-binding proteins related to ScAtg18. These are the WIPI family that has four members WIPI1, WIPI2, WIPI3 (WDR45L) and WIPI4 (WDR45), where WIPI1 and WIPI2 are the closest orthologues to ScAtg18 and WIPI2 is the essential PI3P effector during autophagosome biogenesis[56, 57]. Human ATG2A and ATG2B were shown to preferentially and constitutively interact with WIPI4 [72]. Recent crystal structure of the WIPI3/4 Interaction Region (W34IR) of ATG2A [73] confirmed the previous data implicating a conserved Y/HF motif that is critical for ATG2-WIPI3/4 interaction [11]. Interestingly, the region of WIPI3/4 that ATG2A/B interacts with, between blades 2 and 3, is the homologous region that was shown to be essential for WIPI2 interaction with ATG16L1 [56]. The helical WIPI2 interaction region (W2IR) of ATG6L1 binds in electropositive groove in WIPI2, whereas the extended W34IR of ATG2A binds in an electrostatically neutral grove of WIPI3[56]. This potentially explains why ATG2A cannot bind WIPI2 and ATG16L1 does not bind WIPI3/4. Interestingly, despite clear structural evidence of this interaction using cryo-EM [11, 74], the function of the ATG2A/B-WIPI4 interaction during autophagy is less well understood. Surprisingly, mutation of the W34IR on ATG2A does not impair autophagy flux [25, 75] and KO of WIPI4 alone does not impair autophagosome maturation, only when combined with WIPI3 KO [76]. Interestingly, mutations in WIPI4 result in β - propeller protein-associated neurodegeneration (BPAN) [76-78], potentially indicating a cell-type specific effect of WIPI4 on autophagy or an autophagy-independent role of WIPI4 in neurodegeneration.

ATG2 proteins act as a molecular bridge between the ER and growing autophagosome.

Perhaps some of the most significant breakthroughs regarding the function of the ATG2 proteins was the discovery that ATG2s can physically transport charged phospho-lipid moieties from one membrane to another driven by the Chorein N domain. The N-terminal (amino acids 1-198; HsATG2A) region of ATG2 proteins is essential for both autophagy flux and lipid droplet localisation [79] and is highly conserved domain present on all ATG2 isoforms as well as the closely related VPS13 protein family (see below). The lipid transfer activity of the Chorein N domain was shown for ScAtg2 (Figure 2B) [9] and mammalian ATG2A [7, 12] and ATG2B [80]. In all cases, ATG2 proteins can transfer negatively charged phospho-lipids directly between membranes in vitro [7, 12, 80, 81]. In particular, phosphatidylserine (PS) and phosphatidylethanolamine (PE) were target lipids to be transferred between membranes. At least in vitro, the WIPI4 interaction resulted in more tethering of vesicles and more efficient lipid transfer from between them [12]. Both PE and PS have been shown to be essential lipid components of autophagosomes that are required for the attachment of the ATG8 proteins (LC3s/GABARAPs) to the expanding membrane during both canonical and non-canonical autophagy such as LAP and LANDO [82-84]. This lipid transfer activity is also conserved in Drosophila melanogaster (Dm) Atg2. In DmAtg2 deficient flies, MS studies revealed that PS, phosphatidylinositol (PI) and phosphatidic acid (PA) accumulate on stalled phagophores; where phosphatidylcholine (PC) and PE were the dominant lipid species present in DmAtg2 wild-type phagophores [85]. This highlights the importance of the ATG2 proteins for the cellular lipid balance and functional phagophore/autophagosome formation. No such studies (to date) have looked at the effect on the loss of mammalian ATG2 isoforms and lipid balance on the various membrane structures and it would be insightful to understand how the loss of a single lipid transport affects the overall lipid balance in the cell and the downstream effect this has on signalling pathways. Other Chorein N domain containing proteins, VPS13 (Figure 2C) and UHRF1BP1/1L (Figure 2D) will be discussed in a later section.

Human ATG2A and ATG2B proteins exist as an extended rod shaped-structure, with the Chorein_N domain at one 'end' and the **W34IR** located at the other 'end' [11, 74] (**Figure 3A**). This rod-shaped structure has been proposed to act as a channel for the movement of lipids from

one membrane to another. It has been proposed that ATG2 can bind approximately 20 lipid moleties simultaneously [7] and transport them, bidirectionally, through an extended cavity circa 16nm long present in the rod-shaped structure of ATG2 proteins [7, 12] to ScAtg9 positive vesicles [64] and the phagophore [7, 9, 12, 80] to drive its expansion. This model of ATG2 lipid transport however presents a problem – ATG2s are not trans-membrane proteins and without aid, lipids would accumulate on the cytoplasmic membrane leaflet, starving the luminal leaflet and preventing phagophore expansion. This question was recently answered by a breakthrough defining the cryo-EM and crystal structure of ATG9, the only known transmembrane protein present at the early stages of autophagosome formation. The structure revealed that ATG9 forms a homo-trimer that forms distinct channels that traverse the membrane leaflet and facilitate the transport of phospholipids from cytosol to luminal leaflet [86, 87] (Figure 3). ATG9 was therefore redefined as a lipid scramblase. Lipid scramblases are a class of transmembrane proteins that can transfer lipids from one membrane leaflet to another in a bidirectional manner and without the need for ATP consumption. This contrasts with lipid translocases, flippases and floppases, which are unidirectional and utilize ATP [88, 89]. Importantly, it was shown that full-length ATG2A, but not the isolated Chorein N domain (1-345aa), could interact with ATG9A [90]. This mirrors data from yeast that shows that there are two amino acid patches located at the ScAtg2 C-terminus essential for the interaction between ScAtg2 and ScAtg9 [18]. Thus, lipids transferred via ATG2 to the growing phagophore will be flipped between the membrane leaflets by the scramblase ATG9. Additionally, two proteins that were already known to have a role in autophagy, TMEM41B [91-93] and VMP1 [94, 95], were identified as ER-resident lipid scramblases [96]. TMEM41B has been identified in several CRISPR/Cas9 screens and have highlighted its role in early autophagosome formation, where depletion leads to the formation of stalled immature phagophores[91-93]. In addition, VMP1(vacuole membrane protein1) an ER-resident multi-spanning membrane protein, was shown to be essential during early phases of autophagosome formation [94, 95]. VMP1 and TMEM41B (Figure 3B) were shown to form a physical complex in vitro and in vivo where overexpression of VMP1 could compensate and rescue autophagy flux in cells devoid of TMEM41B[92]. The molecular mechanisms by which these resident ER proteins contributed to phagophore formation, was recently revealed to act as lipid scramblases for essential lipids for phagophore formation namely PS, PC, cholesterol and PE [90, 96]. It is worth noting that VMP1 and TMEM41B can physically interact with the Chorein N domain of ATG2 [90] and that loss of either the scramblases VMP1, TMEM41B or ATG2A/ATG2B, results in a similar phenotype i.e. a build-up of small, immature phagophores and an increase in lipid droplet number and size [26, 71, 75, 91, 92, 94, 96]. The current model indicates that VMP1 and TMEM41B anchor the ATG2 N-terminus to the ER and supply the flow of lipids essential for phagophore formation to ATG2; this then transports them to the phagophore membrane. ATG9A, through an interaction at the C-terminus of ATG2, can distribute the phospholipids to the luminal membrane leaflet and drive the expansion of the phagophore (**Figure 3B**).

ATG2 is anchored at the ER membrane through the interactions with VMP1/TMEM41B at the N-terminus (Figure 3B), but what are the defining factors that aid the delivery of the lipids to the correct membrane? We know that ATG2 proteins not only associate with phagophores, but also lipid droplets and potentially regulate the flow of lipids to/from these compartments. How ATG2s specifically target phagophores for phospholipid transfer to grow and mature the autophagosome. Interestingly, it was shown that ATG2A N-terminal region (aa 1-198) was essential for autophagy, but dispensable for its localisation to lipid droplets[79]. Conversely, ATG2A C-terminus (aa 1830–1938) was essential for lipid droplet localisation, but dispensable for autophagy flux[79]. Moreover, a small amphipathic helix, which can insert into membranes, was essential for both lipid droplet localisation and autophagy flux [79]. This indicates that mammalian ATG2 proteins are capable of localizing to membrane structures independently of WIPI proteins. An important point to remember is that the ATG2-WIPI4 interaction is dispensable for autophagosome formation [25, 75], indicating that WIPI4 may only play a minor or stabilizing role in ATG2 recognition and/or tethering of phagophore membranes. Indeed, the major phospholipid WIPI4 recognizes is PI3P, which can be found extensively throughout the endosomal trafficking network on multiple membrane structures [97]. This would therefore not allow specific targeting of a recipient membrane for lipid transfer. However, one component of the autophagosomal membrane that is present from the early stages through to fusion with the lysosome, are the LC3/GABARAP family of ubiquitin-like proteins. LC3/GABARAPs are directly attached to the phospholipid moieties that ATG2s are responsible for transporting, namely PE and PS. Indeed, mammalian ATG2A and ATG2B were shown to directly interact with LC3/GABARAP proteins through a highly conserved LC3-interaction region (LIR) that surprisingly is only 30 amino acids N-terminal of the WIPI4-interaction region and showed a preference for GABARAP/GABARAPL1 interaction [75] (Figure 2A). Moreover, mutation of the ATG2-LIR abolished the interaction primarily with ATG8 proteins and blocked phagophore maturation in a similar manner to ATG2A/B double knock-out cells, but did not result in aberrant lipid droplet formation [75], indicating that the ATG2 interaction with GABARAP/L1, not WIPI4, is essential for phagophore formation (Figure **3B**). Given that GABARAP proteins are not essential for phagophore formation [60, 61], it is, as yet, unclear how this uncoupling of ATG2-GABARAP interaction blocks the expansion of the phagophore. One additional, and surprising, set of players in the phagophore expansion game are the ATG4 cysteine proteases. The role of the ATG4 enzymes during autophagy was thought to be to mediate the carboxyterminal cleavage of the ATG8 proteins and expose the C-terminal glycine for conjugation to PE [58, 98, 99] and catalyse their de-lipidation from autophagosomal membranes [99, 100]. However, recent evidence has shown that ATG4 enzymes drive the growth of the phagophore membrane by promoting ATG9-ATG2 ER-phagophore contact sites [101]. This shed a light on previous work showing that a catalytically inactive mutant of ATG4B, C74S, prevented the closure of autophagosomes [102] How ATG2, ATG4 and ATG9 may co-ordinate this process is not clear, however. These interactions between ATG2-ATG9-GABARAP proteins may therefore help define the correct membranes to transfer lipids to and stabilise these structures, where loss of one or more of these components prevents efficient lipid transfer and phagophore formation and/or size. However, despite these advances, we still do not understand what the on/off switches are for ATG2 'activity' in these circumstances and how the rate of lipid transfer is controlled? Is it like a tap that when open is like a flood gate of transfer or can this be fine-tuned, depending on the maturation stage of the phagophore/autophagosome, with rapid lipid transfer to grow and expand and slower towards completion?

VPS13 protein family.

Vacuolar protein sorting (VPS) family member 13 (VPS13) proteins were first identified approximately 20 years ago in a screen that identified mutations in a gene responsible for choreaacanthocytosis (ChAc, MIM 200150), which encoded the uncharacterised Chorein_N protein [1, 103]. Several highly similar Chorein N proteins were subsequently identified within the human genome with a high degree of similarity to yeast Vps13. These genes were reclassified as VPS13A (Chorein_N), VPS13B, VPS13C and VPS13D [2]. In addition to disease causing mutations in VPS13A (Chorein_N) VPS13B, VPS13C and VPS13D mutants have been linked to a wide variety of neurological conditions [104-109]. The VPS13 family of proteins are very large (>3000 aa) multi-domain proteins that share functional similarities between each other and with the autophagy ATG2 proteins. VPS13 proteins comprises of an N-terminal Chorein_N domain, VAB (VPS13 adaptor binding), ATG-related C-terminal (ATG-C) and a C-terminal PH domain and, in the case of VPS13D, a ubiquitin-associated (UBA) domain (Figure 2C). Like human ATG2 proteins, VPS13 orthologues are highly conserved with several organisms having multiple paralogues. For example, Drosophila melanogaster Vps13 has three paralogues most similar to human VPS13B, VPA13D and a combination of VPS13A/C; whereas Caenorhabditis elegans has two paralogues similar to VPS13D and VPS13A/C. This is like the ATG2 situation, with yeast, worms and fruit flies possessing a single Atg2 gene, and humans, mice and zebrafish have two paralogues, ATG2A and ATG2B.

A great deal of our understanding of Vps13 function has come from genetic studies of the yeast Vps13 gene. Studies have shown that loss of Vps13 in yeast resulted in retention of proteases and aminopeptidases in the trans-golgi network (TGN) and defects in vacuolar protein sorting [110, 111]. Additionally, yeast Vps13 was shown to function in the regulation of mitochondrial integrity [23], expansion of the prospore membrane during yeast sporulation [21]. In addition, Vps13 can function in parallel with the endoplasmic reticulum-mitochondrion encounter structure (ERMES) where it can partially compensate for loss of ERMES, a multi-subunit tethering complex linking the ER to mitochondria and facilitating lipid transfer between these compartments [112]. In addition to ER-mitochondrial contact sites, yeast Vps13 localizes to nucleus-vacuole junctions [23, 113] and mitochondria-endosome contacts [23]. Yeast Vps13 also localizes to single membrane sites such as peroxisomes [114] and Golgi [115]. This is mirrored by the mammalian proteins where VPS13A localizes to ER-mitochondria [13, 15, 116], ER-lipid droplet [13, 116] and mitochondria-endosome [15] contact sites. VPS13B localizes to golgi [109], endosomes [117] and acrosomes [118]. VPS13C localises to ER-endosome, ER-lysosome and ER-lipid droplet contact sites [13]; whereas VPS13D localises to Golgi, peroxisomes and mitochondria [17, 19, 20, 22]. Taken together, these studies highlight the widespread distribution of VPS13 proteins in endocytic and organelle tethering points.

One of the major breakthroughs in the field came from the crystallization of *Chaetomium* thermophilum Vps13 N-terminal Chorein N domain [13]. This domain shares a high degree of similarity to yeast Vps13, human VPS13 and the ATG2 N-terminal Chorein_N domain. The Vps13 crystal structure revealed a hydrophobic concave face suitable for the binding and mobilization of phospholipids by their fatty acyl chains [13]. A similar structure is observed in ScAtg2 Chorein N domain [9]. Cryo-EM reconstitution of yeast Vps13 (aa 1-1390) showed that not only does the Nterminal domain form a groove, but the 160kDa fragment formed a long channel lined with hydrophobic residues that could mobilize a number of fatty acids moleties, where mutation of residues along the channel could stall lipid transfer [6]. Similar to the ATG2 proteins, Vps13 can potentially transfer PS, PE and phosphatidic acid (PA) [13]. These studies, in combination with the related ATG2 data, suggest that VSP13 and ATG2 proteins serve as a related family of molecular bridges that can span organelles at specific contact sites and transfer lipids between compartments. Interestingly, VPS13 has also been linked with a role in the autophagy pathway. The *Dictyostelium discoideum* protein, TipC, a VPS13-related protein, was shown to be essential for efficient formation of autophagosomes [3] and depletion of human VPS13A in HeLa cells led to increased levels of lipidated GFP-LC3B that was attributed to a decrease in autophagy flux [3]. However, as the ER is an important point of autophagosome biogenesis, this defect in autophagy when VPS13A is depleted may be an indirect consequence of destabilized ER and mitochondrial morphology [116]. Moreover, in yeast lacking *vps13* it was shown that the protein was essential for the packing of the ER into autophagosomes [119]. Yet, it is unclear if the Vps13 proteins play a direct role in autophagy or whether their role is more indirect and their loss results in gross morphological changes at the sites of autophagosome biogenesis that negatively impact the process.

Organelle contact sites, where lipid transfer occurs, can be regulated by interaction partners. For example, the loss of VPS13D enhances ER-mitochondria interactions due to an increased stability of the tethering protein VAB [22]. Levels of VAB are in turn regulated by the interaction of VPS13D with VCP/p97, an ATPase responsible for the regulating protein degradation in a ubiquitin- and proteasome-dependent manner [22]. VPS13D was recently shown to traffic fatty acids from lipid droplets (LDs) to mitochondria at their contact sites where an interaction with the ESCRT-III component TSG101 was essential for this transfer [22]. Interestingly, VPS13D was identified in a screen, alongside TSG101 and Vps36, in a Drosophila RNAi screen to identify ubiquitin-binding proteins involved in cell size and autophagy phenotypes [120]. VPS13D was shown to have a ubiquitin-binding domain that was essential for regulating cell size and mitochondrial fission, but not in starvationinduced autophagy [120]. VPS13D is the only member of the VPS13 family shown to be essential for cell viability in human cell lines [121, 122] most likely due to its evolutionary conserved roles in regulating peroxisome biogenesis and mitochondrial morphology and turnover [14, 19, 20, 120, 123]. These new data shedding light on the structural and biochemical properties of lipid transfer domain containing proteins, have provided invaluable insights into the molecular basis of neurological disorders associated with mutations in the VPS13 family genes, such as movement ataxia in patients with VPS13D mutation [105, 106, 124].

Future perspectives: Existence of other potential Chorein_N like lipid transfer domains.

A great deal of our understanding of these lipid transfer proteins has arisen in the last 2-3 years, with multiple research groups advancing our knowledge through genetic, biochemical, and structural studies. We now have a strong foundation for developing essential aspects of lipid-based communication between organelles and key endocytic pathways. Application of this knowledge will be the key to unlocking other potential mediators of lipid transfer. For example, not only do VPS13 and ATG2 proteins contain the N-terminal Chorein_N domain, but another family

of proteins, namely UHRF1BP1/UHRF1BP1L, have as well. InterPro [125] domain searches show only the N-terminal Chorein_N domain as a recognized domain in these proteins (Figure **2D**). Indeed, UHRF1BP1 and UHRF1BP1L share only approximately 44% sequence homology, most of which is in the N-terminal region. Currently there are few functional studies regarding UHRF1BP1/1L proteins. Despite a lack of biological function, UHRF1BP1/1L have been identified as genetic risk loci for systemic lupus erythematosus [28, 30] and Parkinson's disease [29]. However, perhaps some insight into their function has come from mass spectrometry analysis of Rab GTPase interactomes. It was found that the Drosophila orthologue of UHRF1BP1 interacts with GTP-bound Rab5 [126]. In mammalian cells, a Rab interactome using a mitochondrial-based proximity labelling assay, where the GTPases were relocated to the mitochondrion, found that UHRF1BP1 potentially interacted with GTP-bound Rab7 and Rab10; whereas UHRF1BP1L interacted with Rab5, -6, -10 and Rab11 [127]. This is in line with VPS13 interacting with Rab GTPases. VPS13A was shown to interact with Rab7A [15] and VPS13B has been shown to interact directly with Rab6 [128]. Currently, no Rab GTPases have been shown to associate with ATG2 proteins despite a number being linked to the formation and maturation of the autophagosome (reviewed in [129]). UHRF1BP1L, also known as SHIP164, was shown to interact with syntaxin 6 and the Golgi-associated retrograde protein (GARP) complex and is potentially important for the trafficking in the early endosomal/retrograde system [31]. Taken together, there are still several questions as to the precise molecular functions of the UHRF1BP1/1L proteins, but like both VPS13 and ATG2 proteins, they are elegantly placed to facilitate lipid transfer within the endosomal trafficking network.

It appears that not only eukaryotes proteins containing lipid transfer domains, but some bacterial proteins also possess a Chorein_N-like domain that may have the ability to transfer lipids. A recent remote homology search has found that the *E.coli* proteins AsmA and TamB contain an N-terminal domain that is homologous to the Chorein_N domain. These bacterial proteins are anchored through an N-terminal transmembrane domain directly preceding the Chorein_N-like domain, making a pool of lipids for transfer readily accessible to these proteins [4]. These proteins protrude into the periplasmic space but interestingly, are not large enough to span the inter-membrane space of *E.coli*. Both AsmA and TamB would potentially require additional adaptor(s) to complete the lipid transfer from the inner to the outer membrane [4]. Taken together, the recently identified function of the Chorein_N domain coupled with the genetic, biochemical and structural studies of the proteins that possess them have given us critical insights into how organelles communicate with each other and how this process is disrupted in disease.

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Figure legends

Figure 1 Autophagosome biogenesis and destruction. The formation of the doublemembraned autophagosome is controlled by nutrient availability. This includes amino acids, energy (ATP levels) and can also be induced by cellular stresses. Alterations in any of these signals leads to the initiation of autophagosome formation with the early phagophore structure. The phagophore elongates and finally closes prior to fusion with the lysosome where the trapped material is broken down and recycled back to cells. This provides an adaptation and survival mechanism for the cell during periods of stress. (B) Cargo to be degraded can include intracellular pathogens, damaged organelles and protein aggregates. The cargo is recruited via interaction with autophagy receptor proteins that bind both the cargo and the ATG8 proteins (LC3/GABARAP families) which, in turn, are covalently attached to the phagophore membrane. This allows cargo to be sequestered and sealed into the autophagosome prior to its fusion with the lysosome.

Figure 2 Domain organisation of Chorein_N domain containing proteins. (A) Domain organisation of *Saccharomyces* cerevisiae (Sc)2, *Homo* sapien (Hs) ATG2A and ATG2B. The ATG2s contain the Chorein_N domain at the N-terminal, ATG2 C-terminal autophagy domain (ATG2-CAD) and an ATG-C-terminal domain. Human ATG2A and ATG2B also contain a conserved LC3 interaction region (LIR, orange) and a WIPI4 interaction region (WIR) located within 30 amino acids of each other. (**B**) Crystal structure of phosphatidylethanolamine (PE) bound to *Saccharomyces* pombe (Sp) 2 Chorein_N domain (PDB: 6A9J, generated in PyMol). α -helices (blue), β -sheets (orange) and loops (grey) are shown in cartoon (left) and surface (right) representation. The deep hydrophobic pocket is highlighted on the surface representation. (**C**) Domain architecture of mammalian VSP13 proteins. In addition to the Chorein_N domain, ATG-CAD and ATG_C domains, VPS13 proteins contain a VPS13 adaptor binding (VAB) domain, a Plekstrin homology domain (PH) and VPS13D has a Ubiquitin-associated (UBA) domain. (**D**) UHRF1BP1/1L contains only a Chorein_N domain, as assigned by InterPro.

Figure 3 Model of ATG2-mediated lipid transfer between membranes. (A) Cryo-electron microscopy (EM) structure (EMD-8899, generated by Jmol/JSmol) of human ATG2A (grey) bound to WIPI4 (green) with the approximate region of the Chorein_N domain highlighted (blue). **(B)** The N-terminal region of ATG2 (including the Chorein_N domain) is anchored at the ER (endoplasmic reticulum) through interactions with ER-resident scramblases VMP1 and/or TMEM41B which move phospho-lipids between bilayers in an ATP-independent manner. ATG2 subsequently transfers lipids such as phosphatidylethanolamine (PE; yellow) from the ER to the growing

phagophore where the membrane contact is stabilized by interactions with WIPI4 bound to PI3P(phosphatidyl-insotol-3-phosphate; red) on the phagophore, GABARAP proteins directly conjugated to PE and ATG9A at the phagophore side. ATG9A acts as a lipid scramblase to transfer lipids between membrane leaflets. ATG8 (LC3s/GABARAPs) proteins shown on the luminal side of the growing phagophore are conjugated to the PE and help target cargo and receptors for autophagy-mediated destruction.

Figure 1









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