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COMMENTARY

WIPI proteins: essential PtdIns3*P* effectors at the nascent autophagosome

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ABSTRACT

Autophagy is a pivotal cytoprotective process that secures cellular homeostasis, fulfills essential roles in development, immunity and defence against pathogens, and determines the lifespan of eukaryotic organisms. However, autophagy also crucially contributes to the development of age-related human pathologies, including cancer and neurodegeneration. Macroautophagy (hereafter referred to as autophagy) clears the cytoplasm by stochastic or specific cargo recognition and destruction, and is initiated and executed by autophagy related (ATG) proteins functioning in dynamical hierarchies to form autophagosomes. Autophagosomes sequester cytoplasmic cargo material, including proteins, lipids and organelles, and acquire acidic hydrolases from the lysosomal compartment for cargo degradation. Prerequisite and essential for autophagosome formation is the production of phosphatidylinositol 3-phosphate (PtdIns3*P*) by phosphatidylinositol 3-kinase class III (PI3KC3, also known as PIK3C3) in complex with beclin 1, p150 (also known as PIK3R4; Vps15 in yeast) and ATG14L. Members of the human WD-repeat protein interacting with phosphoinositides (WIPI) family play an important role in recognizing and decoding the PtdIns3*P* signal at the nascent autophagosome, and hence function as autophagy-specific PtdIns3*P*-binding effectors, similar to their ancestral yeast Atg18 homolog. The PtdIns3*P* effector function of human WIPI proteins appears to be compromised in cancer and neurodegeneration, and WIPI genes and proteins might present novel targets for rational therapies. Here, we summarize the current knowledge on the roles of the four human WIPI proteins, WIPI1–4, in autophagy.

This article is part of a Focus on Autophagosome biogenesis. For further reading, please see related articles: 'ERES: sites for autophagosome biogenesis and maturation?' by Jana Sanchez-Wandelmer et al. (*J. Cell Sci.* **128**, 185-192) and 'Membrane dynamics in autophagosome biogenesis' by Sven R. Carlsson and Anne Simonsen (*J. Cell Sci.* **128**, 193-205).

KEY WORDS: PI3P, PtdIns3*P*, WIPI, Autophagy, Phagophore

Introduction

Autophagy is a cytoprotective mechanism involving the degradation of proteins, lipids and organelles in the lysosomal compartment. By executing constitutive clearance of the cytoplasm and permitting the recycling of the degraded material, basal autophagy is important for cellular survival in all eukaryotes. The cytoplasmic material becomes sequestered in unique double-membraned vesicles called autophagosomes, either stochastically or by specific cargo recognition. Autophagosomes are formed by the elongation and closure of a membrane precursor, called the phagophore or isolation membrane (Fig. 1), and they acquire acidic hydrolases from lysosomes for cargo degradation. Upon starvation or a variety of cellular insults, autophagy is induced above the basal level to compensate for nutrient shortage and to provide monomeric constituents, such as amino acids, and energy for recycling processes (Choi et al., 2013; Feng et al., 2014; Jiang and Mizushima, 2014; Ohsumi, 2014; Shibutani and Yoshimori, 2014; Yang and Klionsky, 2010).

The formation of autophagosomes necessitates the concerted and sequential action of ATG proteins, originally identified in yeast (Itakura and Mizushima, 2010; Klionsky et al., 2003; Mizushima et al., 2011; Nakatogawa et al., 2009). ATG proteins are regulated by conserved nutrient and energy-dependent signaling cascades that crucially involve the mammalian target of rapamycin (mTOR) and AMP-activated protein kinase (AMPK) (Fig. 2C). Both mTOR and AMPK control the Unc-51-like kinases ULK1 and ULK2 (Meijer and Codogno, 2011; Mizushima, 2010; Russell et al., 2014). Nutrient availability activates protein synthesis and cell growth through the mTOR complex 1 (mTORC1), an essential switch towards anabolic pathways. When activated by growth factors through RHEB proteins, or by amino acids through RAG proteins (Dennis et al., 2011), mTORC1 inhibits autophagy by associating with and phosphorylating the autophagy-related protein ULK1, a serine/threonine-specific protein kinase that complexes with ATG13 and FIP200 (also known as RB1CC1) (Ganley et al., 2009; Hosokawa et al., 2009; Jung et al., 2009; Kim et al., 2011). By contrast, nutrient starvation permits autophagy initiation. AMPK, functioning as a switch towards catabolic pathways, activates autophagy in response to low cellular ATP levels either indirectly, through mTORC1 inactivation (Corradetti et al., 2004; Gwinn et al., 2008; Meijer and Dubbelhuis, 2004), or directly, through ULK1 phosphorylation (Egan et al., 2011; Kim et al., 2011).

Following ULK1 activation and subsequent ATG13 and FIP200 phosphorylation, ULK1 stimulates PtdIns3*P* production through beclin 1 phosphorylation, which leads to the activation of the phosphatidylinositol 3-kinase class III (PI3KC3, also known as PIK3C3) (Russell et al., 2013). Subsequently, PI3KC3 in complex with beclin 1, p150 (also known as PIK3R4; Vps15 in yeast) and ATG14L translocates to the initiation site for autophagosome formation, with ATG14L playing a crucial role

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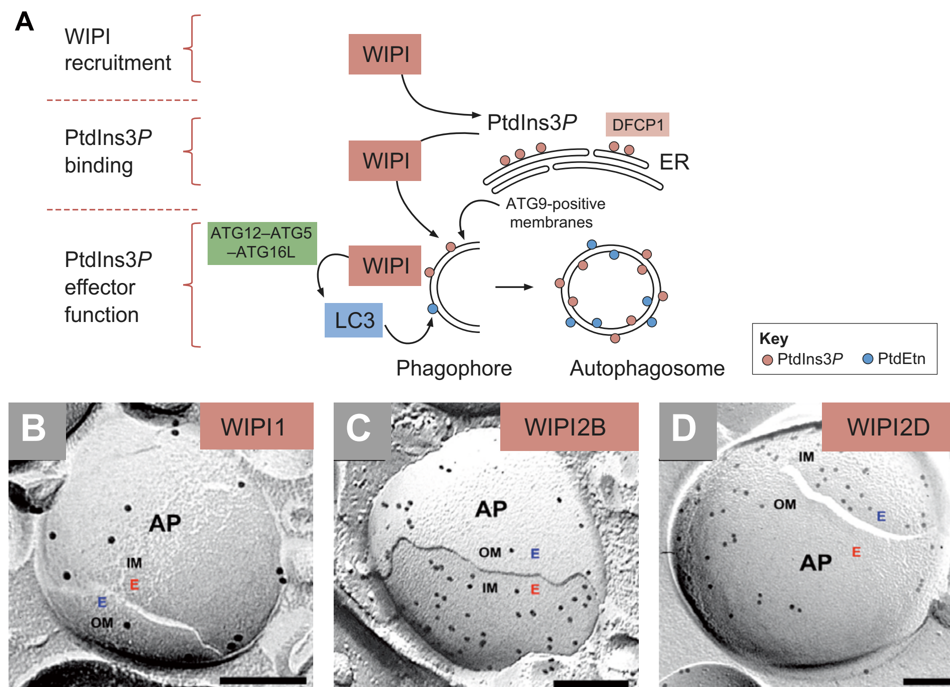

Fig. 1. Human WIPI proteins as essential PtdIns3*P* effectors at the nascent autophagosome. (A) Upon the initiation of autophagy, localized PtdIns3*P* production at the ER recruits human WIPI proteins, as shown for WIPI1 and WIPI2. PtdIns3*P* is also bound by DFCP1 with unknown consequences. ER dynamics permit the formation of the phagophore from omegasome or cradle-like ER structures and ATG9-positive membranes, by an as-yet-unknown mechanism. WIPI proteins, as shown for WIPI1, WIPI2 and WIPI4, localize to the phagophore. Here, WIPI2 recruits the ATG12–ATG5–ATG16L complex (highlighted in green) by direct binding to ATG16L1. Subsequently, LC3 is conjugated to PtdEtn. WIPI1 is also essential for LC3 lipidation, but the functional relationship between WIPI1 and WIPI2 is unknown. At the autophagosome, LC3 decorates the inner and outer membrane as it stays conjugated to PtdEtn. Proteins highlighted in red (WIPI, DFCP1) specifically bind to PtdIns3*P*. Cytosolic LC3 is highlighted in blue. (B–D) There is evidence that WIPI1 (B, endogenous WIPI1), WIPI2B (C, GFP–WIPI2B) and WIPI2D (D, GFP–WIPI2D) also become membrane proteins of the inner (IM) and outer (OM) autophagosomal membrane. AP, autophagosome; E, E-face (of note, the E- or P-face terminology with regard to the monolayers of the inner or outer autophagosomal membrane might change once the membrane origin of autophagosomes is identified). Scale bars: 200 μm. The images in B–D were reproduced from Proikas-Cezanne and Robenek, 2011, under the terms of the Creative Commons Attribution License (© 2011 The Authors Journal compilation, © 2011 Foundation for Cellular and Molecular Medicine/Blackwell Publishing Ltd). The schematic drawing of the endoplasmic reticulum was obtained from Motifolio.

in directing this PI3KC3 complex to the endoplasmic reticulum (ER) (Matsunaga et al., 2010). At the ER, the PI3KC3 complex stabilizes the ULK complex (Karanasios et al., 2013; Koyama-Honda et al., 2013). PI3KC3-mediated PtdIns3*P* production at the ER was identified by visualizing dynamic PtdIns3*P*-enriched ER structures called omegasomes (Axe et al., 2008) or cradles (Hayashi-Nishino et al., 2009). Omegasomes foster the formation of the phagophore, which is thought to form *de novo* by an as-yet-unknown mechanism (Roberts and Ktistakis, 2013; Simonsen and Stenmark, 2008). However, phagophore expansion is probably mediated by membrane uptake from endomembranes as well as from semiautonomous organelles (Lamb et al., 2013; Shibutani and Yoshimori, 2014).

Phagophore expansion requires two autophagosomal ubiquitin-like conjugation systems, the ATG12 and the LC3 (microtubule-associated protein 1A/1B-light chain 3) systems, both of which are necessary for the conjugation of LC3 to phosphatidylethanolamine (LC3–PtdEtn or LC3-II), a process that is referred to as LC3 lipidation (Hamasaki et al., 2013; Kabeya et al., 2000). Downstream of PtdIns3*P* production but prior to LC3 lipidation, the ATG16L1 complex, composed of ATG12 conjugated to ATG5 and associated with ATG16L (ATG12–ATG5–ATG16L) is recruited to the initiation site of autophagosome formation (Itakura and Mizushima, 2010), and functions as an E3-like ligase on the ATG3–LC3 conjugate to mediate the lipidation of LC3 (Fujita et al., 2008; Sakoh-Nakatogawa et al., 2013). LC3–PtdEtn, as a membrane protein of the nascent and mature autophagosome, fulfills at least three crucial functions: (1) it supports hemifusion events during phagophore expansion and closure, (2) it enables specific cargo recognition through binding to proteins harboring an LC3-interacting region (LIR) motif, and (3) it enables adaptor protein docking (Nakatogawa et al., 2008; Slobodkin and Elazar, 2013; Wild et al., 2014). The LC3-positive autophagosome sequesters cytoplasmic material and fuses with endosomes and lysosomes for cargo breakdown, and the degraded material is transported to the cytoplasm.

It is still not clear how autophagy is regulated during the succession of events from the production of ER-localized PtdIns3*P* to the *de novo* formation of PtdIns3*P*-enriched nascent autophagosomes (O'Farrell et al., 2013). Early studies made use of proteins harboring PtdIns3*P*-binding FYVE (conserved in Fab1, YOTB, Vac1 and EEA1) domains in order to visualize intracellular PtdIns3*P* on endosomes and nascent autophagosomes (Axe et al., 2008; Gillooly et al., 2000; Obara et al., 2008). By using the double FYVE domain-containing protein 1 (DFCP1, also known as ZFYVE1), the omegasome-like formation of the ER was identified in mammalian cells upon starvation, prior to phagophore formation (Axe et al., 2008). It has been suggested that, upon ER-localized PtdIns3*P* production by PI3KC3, DFCP1 binds to the ER through an interaction between its FYVE domain and PtdIns3*P*, rather than

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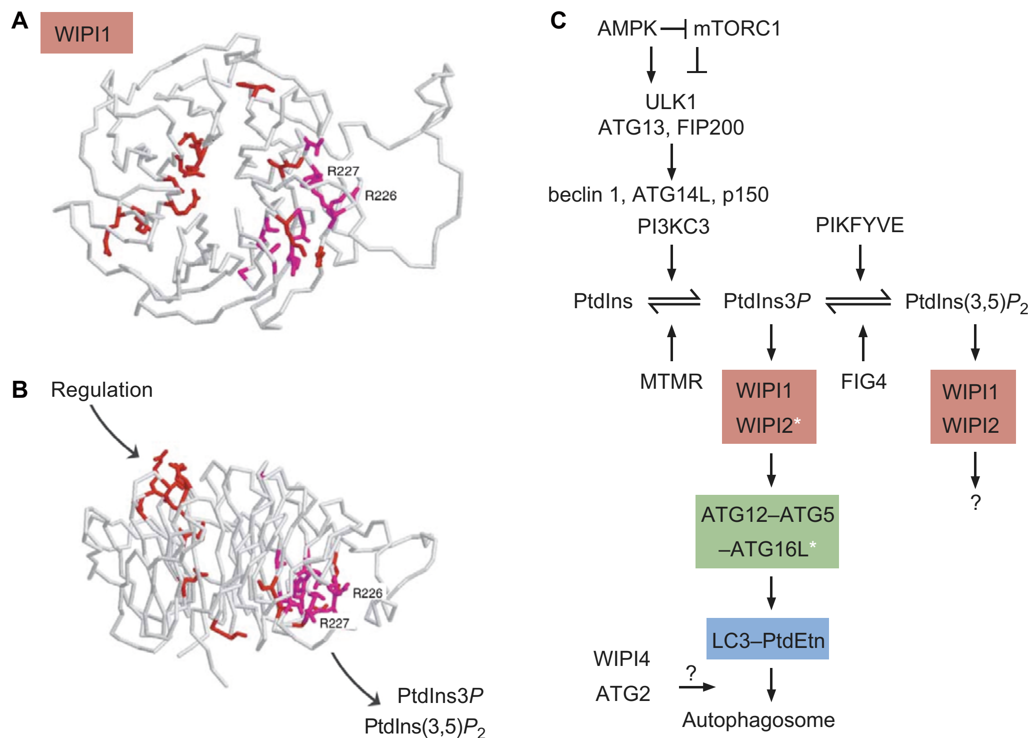

Fig. 2. WIPI proteins bind to PtdIns3*P* and PI(3,5)P_2 through a conserved cluster of residues within the β-propeller. (A) Conserved amino acids specific for the PROPPIN family have been identified and are depicted in the homology model of human WIPI1. Red, homologous residues; magenta, invariant residues. (B) The conserved residues cluster at opposite sides of the WIPI1 β-propeller. Homologous residues mediate multiple regulatory protein–protein interactions, such as the interaction between WIPI2 and ATG16L1 (indicated by asterisks in C), and invariant residues confer specific binding to PtdIns3*P* or PtdIns(3,5)P_2. (C) PtdIns3*P*-binding specificity is necessary for the functional contribution of WIPI proteins to autophagosome formation. AMPK activates ULK1 (in complex with ATG13 and FIP200) directly, by site-specific phosphorylation, and indirectly, by inhibiting mTORC1. Downstream of ULK1 activation, PI3KC3 (in complex with beclin 1, ATG14L and PIK3R4) produces PtdIns3*P*, which is subsequently bound by WIPI1 and WIPI2. WIPI1/2 binding to PtdIns3*P* is further controlled by members of the MTMR family, which hydrolyze PtdIns3*P* to PtdIns, and probably also by FIG4, which dephosphorylates PtdIns(3,5)P_2. Of note, WIPI1 and WIPI2 also bind specifically to PtdIns(3,5)P_2, the product of PIKFYVE activity, but the biological function of this binding is unknown. During autophagy initiation, WIPI1 and WIPI2 decode the PtdIns3*P* signal to confer LC3 lipidation (LC3–PtdEtn, LC3 conjugation to PtdEtn) through direct binding between WIPI2 and ATG16L (white asterisks) in complex with ATG12 conjugated to ATG5. WIPI4 bound to ATG2 is considered to function downstream of WIPI1/2 and LC3. The WIPI1 homology model (A,B) is reprinted by permission from Macmillan Publishers Ltd: Oncogene (Proikas-Cezanne et al., 2004), copyright 2004.

through an internal ER-targeting sequence in DFCP1 (Axe et al., 2008). Hence, DFCP1 can be considered as a PtdIns3*P* effector protein. However, the downstream consequences of this interaction remain unknown, because small interfering (si)RNA-mediated downregulation of DFCP1 does not appear to negatively affect autophagy (Axe et al., 2008; O'Farrell et al., 2013). Another FYVE-domain-containing protein, the autophagy-linked FYVE-protein (Alfy, also known as WDFY3), functions during the degradation of large cytoplasmic aggregates, which are characteristic of neurodegenerative diseases; however, Alfy does not appear to play a role during starvation-induced autophagy (Clausen et al., 2010; Filimonenko et al., 2010; Finley et al., 2003; Simonsen et al., 2004).

PROPPINs (β-propellers that bind phosphoinositides) are currently the only known PtdIns3*P*-binding proteins with conserved and essential PtdIns3*P* effector function in autophagy from yeast to human (Thumm et al., 2013). The human PROPPIN family members are called WD-repeat protein interacting with phosphoinositides (WIPI), and consist of the four members (WIPI1 to WIPI4) along with their splice variants (Proikas-Cezanne et al., 2004). Human WIPI proteins are considered to bridge PtdIns3*P* production and LC3 lipidation (Lamb et al., 2013). In this Commentary, we summarize the current status of knowledge on the four individual human WIPI members.

The human WIPI gene and protein family

The identification of the human WIPI genes was based on human liver cDNA library screening for novel p53 inhibitory factors (Waddell et al., 2001), leading to the identification of a partial 5′-truncated cDNA encoding human WIPI1 (WIPI1α) that localized to a region on human chromosome 17q known to be frequently imbalanced in human cancer (Box 1). On this basis, the four human WIPI members, WIPI1 to WIPI4, were identified through BLAST searching the human genome, and were then cloned and found to be ubiquitously expressed in normal human tissue, with high expression in skeletal muscle and heart (Proikas-Cezanne et al., 2004). Moreover, all WIPI genes were found to be aberrantly expressed in different human tumors (Box 1) (Proikas-Cezanne et al., 2004). WIPI1 gene expression is positively regulated by TFEB in liver (Settembre et al., 2013) and by PU.1 during neutrophil differentiation (Brigger et al., 2014), and is epigenetically repressed by the histone methyltransferase G9a (also known as EHMT2) (Artal-Martinez de Narvajas et al., 2013). Interestingly, upon starvation or activation of naive T cells, G9a dissociates from the WIPI1 promoter, leading to increased WIPI1 expression upon induction of autophagy (Artal-Martinez de Narvajas et al., 2013). WIPI2 gene expression has been found to be negatively regulated by ZKSCAN3, a master transcriptional repressor of autophagy and lysosome biogenesis (Chauhan et al., 2013).

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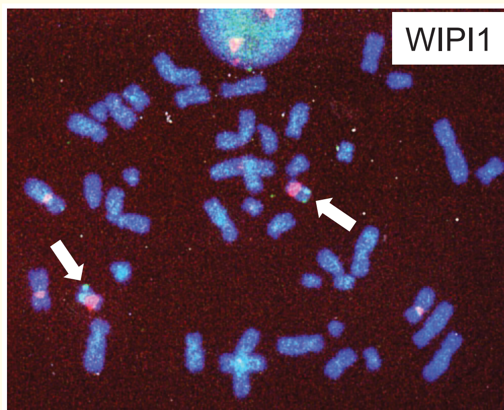
Box 1. WIPIs and cancer

Based on the initial observation that WIPI1 is encoded within a segment on the long arm of chromosome 17 [see the box figure, fluorescent *in situ* hybridization analysis localizing human WIPI1 to 17q24.3 (indicated by the arrows)], which is allelically imbalanced in a variety of tumors, subsequent WIPI gene expression analysis in matched human tumor samples suggested that there is aberrant expression of all human WIPI genes in different tumors (Proikas-Cezanne et al., 2004). Interestingly, aberrant expression of WIPI1 in 50% of human skin tumor samples and melanoma cell lines (Proikas-Cezanne et al., 2004) correlates with the high abundance of WIPI1 protein (http://www.proteinatlas.org/ENSG00000070540/cancer) and the identification of WIPI1 mutations in melanoma (Table 1). WIPI1 expression was also upregulated in 40% of cervical carcinoma patients. Moreover, non-silent mutations have been identified in all human WIPI members in large-scale genome analyses. In particular, three mutant WIPI1 and WIPI4 variants were found in analyses of lung cancer (Table 1).

In addition to the original report that beclin 1 induces autophagy and inhibits tumorigenesis (Liang et al., 1999), subsequent evidence showed that autophagy contributes to tumor development and therapy resistance (Liu and Ryan, 2012; White, 2012). Whether and how WIPI members contribute to tumorigenesis and malignancy, however, needs to be addressed in large-scale tumor patient analyses with regard to WIPI mutations, expression status and functional consequences for autophagy. Moreover, it needs to be determined whether WIPI mutations found in different tumors represent driver or passenger mutations. Nevertheless, in primary AML patient samples, a significantly repressed expression of WIPI1 was recently reported, in line with the functional dependence on WIPI-mediated autophagy during neutrophil differentiation (Brigger et al., 2014).



Box 2. Atg18 function in yeast autophagy

In yeast, three WIPI homologs have been identified: Atg18, Atg21 and Hsv2 (Barth et al., 2002; Barth and Thumm, 2001; Georgakopoulos et al., 2001; Guan et al., 2001). Atg18 has been reported to function in both autophagy and the cytoplasm-to-vacuole (Cvt) pathway (Barth and Thumm, 2001; Guan et al., 2001), and is predicted to fold into a seven-bladed β-propeller with six loops interconnecting the individual propeller blades (Dove et al., 2004). Atg18 specifically binds to both PtdIns3*P* and PtdIns(3,5)P_2 (Dove et al., 2004; Strømhaug et al., 2004) through a FRRG motif (Krick et al., 2006), with higher affinity for PtdIns(3,5)P_2 *in vitro* (Dove et al., 2004; Rieter et al., 2013). Functionally, the PtdIns3*P* effector activity of Atg18 was proposed to be essential for autophagy, whereas the PtdIns(3,5)P_2 effector activity was proposed to be required for vacuole morphology and inheritance, and retrograde transport (Efe et al., 2007). The essential PtdIns3*P*-effector function of Atg18 in *Saccharomyces cerevisiae* is interconnected with the function of Atg2 (Barth and Thumm, 2001; Shintani et al., 2001; Strømhaug et al., 2001; Wang et al., 2001), as Atg18 binds to Atg2, and the Atg18–Atg2 complex localizes to the autophagosomal membrane through the binding of Atg18 to PtdIns3*P* (Obara et al., 2008; Rieter et al., 2013; Suzuki et al., 2013; Suzuki et al., 2007). Thus, recruitment of Atg18–Atg2 depends on PtdIns3*P*, and as recently found, is facilitated by Atg1-mediated phosphorylation of Atg9 (Papinski et al., 2014). Vice versa, Atg9 cycling through the yeast pre-autophagosomal structure depends on Atg18 and Atg2 (Reggiori et al., 2004).

Yeast Atg21, which also binds to PtdIns3*P* and PtdIns(3,5)P_2 (Dove et al., 2004; Strømhaug et al., 2004), strictly functions in the Cvt pathway (Barth et al., 2002; Krick et al., 2008; Strømhaug et al., 2004). However, both Atg18 and Atg21 enable the recruitment of Atg8 (LC3) to the nascent autophagosome (Meiling-Wesse et al., 2004; Nair et al., 2010).

Hsv2 has been shown to function in micronucleophagy in *S. cerevisiae* (Krick et al., 2008). The protein structure of *K. lactis* Hsv2 was recently identified (Baskaran et al., 2012a; Krick et al., 2012), and the data confirmed the predicted seven-bladed β-propeller, as for yeast Atg18 (Dove et al., 2004) and human WIPI1 (Jeffries et al., 2004; Proikas-Cezanne et al., 2004). Furthermore, the two crucial arginines within the FRRG motif that have been identified in Atg18 (Dove et al., 2004; Krick et al., 2006) are situated in two different pockets of *K. lactis* Hsv2 and have been suggested to form two phospholipid-binding sites (Baskaran et al., 2012a; Krick et al., 2012). Mutation of the corresponding two phospholipid-binding sites in *S. cerevisiae* Atg18 revealed that the two sites are not redundant, but work cooperatively to confer the function of Atg18 in autophagy (Baskaran et al., 2012a). Moreover, the loop in blade 6 of *K. lactis* Hsv2, which is proposed to be responsible for membrane docking, was also found to be important for the autophagic function of Atg18 (Baskaran et al., 2012a).

In summary, Atg18, Atg21 and Hsv2 display different specificities in autophagic pathways and, furthermore, have been found to localize to the endosomal compartment in yeast (Krick et al., 2008). These data clearly indicate the need to search for further, non-autophagic functions of the human WIPI members as well as to dissect their different autophagic specificities.

As endogenous WIPI1 localizes in an autophagy-specific manner to cytoplasmic membranes that are positive for LC3, human WIPI1 was functionally described as a novel autophagy factor (Proikas-Cezanne et al., 2004), with a function related to that of ancestral yeast Atg18 (Barth and Thumm, 2001; Dove et al., 2004; Guan et al., 2001) in autophagy (Box 2). Furthermore, it was proposed that WIPI homologs in all eukaryotes share an evolutionarily conserved function in autophagy, which is compromised in human cancer (Proikas-Cezanne et al., 2004). Subsequent siRNA-mediated knockdown studies on human WIPI members have demonstrated an essential function in mammalian autophagy (Dooley et al., 2014; Gaugel et al., 2012; Liu and Ryan, 2012; Mauthe et al., 2011; Polson et al., 2010), as observed for WIPI homologs in lower eukaryotes, including *Drosophila melanogaster* (Scott et al., 2004), *Arabidopsis thaliana* (Xiong et al., 2005), *Caenorhabditis elegans* (Liu and Ryan, 2012) and *Dictyostelium discoideum* (Calvo-Garrido et al., 2014). In addition, further data point towards a crucial role of human WIPI members in cancer (Box 1) and other human pathologies, including neurodegeneration (Haack et al., 2012; Hayflick et al., 2013;

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Box 3. Atg-18 controls lifespan in *C. elegans*

Beside maintaining cellular homeostasis and securing cellular survival under conditions of stress, autophagy plays an important role in the regulation of lifespan and in aging and age-related diseases in multiple model organisms (Cuervo, 2008; Rubinsztein et al., 2011). Most of the signals regulating lifespan are connected to nutrient availability and metabolic activity, such as dietary restriction, mitochondrial respiration and the nutrient-sensing insulin–IGF-1 and TOR pathway (Gelino and Hansen, 2012). *C. elegans* carrying a mutation in the DAF-2 insulin-like receptor, live more than twice as long as wild-type nematodes (Kenyon et al., 1993) and exhibit increased autophagic activity, which is dependent on the ATG gene *bec-1* (beclin 1) (Meléndez et al., 2003). Subsequent studies using *C. elegans* have reinforced the idea that ATG genes are essential for long-lived phenotypes and that ATG mutants age faster and have shorter lives than wild-type nematodes (Hars et al., 2007; Meléndez et al., 2003; Tóth et al., 2008). However, autophagy might not always be beneficial for longevity, as RNAi-mediated downregulation of *unc-51* (ULK1 homolog) or *atg-9* (ATG9 homolog) actually increased the lifespan of *C. elegans* S6 kinase *rsks-1* mutants (Hashimoto et al., 2009).

C. elegans possesses two homologs of the human WIPI family: ATG-18 (a WIPI1/2 homolog) and EPG-6 (a WIPI3/4 homolog) (Proikas-Cezanne et al., 2004), both of which are necessary for proper autophagosome formation (Lu et al., 2011). Regarding lifespan regulation only the function of ATG-18 has been investigated so far, and the data demonstrate that the depletion of ATG-18 function decreased the lifespan of both wild-type and long-lived *daf-2* mutant nematodes (Hashimoto et al., 2009; Meléndez et al., 2003; Tóth et al., 2008). Moreover, *atg-18* mutant nematodes showed accelerated aging, represented by an earlier decline in locomotory activity and accumulation of lipofuscin granules when compared to that of wild-type nematodes (Hashimoto et al., 2009; Tóth et al., 2008). Furthermore, ATG-18 is also necessary for the increased lifespan observed upon the downregulation of LET-363, the *C. elegans* ortholog of TOR, the major inhibitor of autophagy (Tóth et al., 2008; Vellai et al., 2003). For future studies it will be interesting to investigate the role of EPG-6 in general lifespan regulation in *C. elegans*, as it has been shown that EPG-6-mutant L1 larvae have reduced lifespans in the absence of food (Lu et al., 2011).

Saitsu et al., 2013), phospholipidosis (Sawada et al., 2005) and cardiovascular diseases (Chasman et al., 2009). Moreover, WIPI members might contribute to human aging and longevity as ATG-18, the WIPI1/2 homolog in *C. elegans*, has been found to be essential for lifespan determination (Box 3).

The four human WIPI members were also described independently based on their sequence homology to yeast Atg18, and WIPI49 (WIPI1β) was shown to localize to the trans-Golgi and endosomal membranes in monkey cells (Jeffries et al., 2004). Importantly, GST-purified WIPI49 was shown to bind to PtdIns3*P*, phosphatidylinositol 5-phosphate (PtdIns5*P*) and phosphatidylinositol 3,5-bisphosphate [PtdIns(3,5)P_2], with a preference for PtdIns3*P* (Jeffries et al., 2004). Specific binding of human WIPI1 and also WIPI2 to these phosphoinositides with a preference towards PtdIns3*P* was confirmed in subsequent studies (Dooley et al., 2014; Gaugel et al., 2012; Polson et al., 2010; Proikas-Cezanne et al., 2007).

WIPI members fold into seven-bladed β-propeller proteins

Human WIPI proteins contain seven WD repeats that fold into an open 'Velcro'-arranged seven-bladed β-propeller, as predicted by homology modeling of human WIPI1 and as proposed for all WIPI homologs (Proikas-Cezanne et al., 2004). Indeed, the seven-bladed β-propeller structure was demonstrated by structural analyses of the WIPI homolog Hsv2 in *Kluyveromyces lactis* (Baskaran et al., 2012a; Baskaran et al., 2012b; Krick et al., 2012) and in *Kluyveromyces marxianus* (Watanabe et al., 2012). Conserved and invariant residues from yeast to human were identified throughout the seven WD repeats that form two clusters at opposite sides of the WIPI β-propeller (depicted in WIPI1; Fig. 2A,B) (Proikas-Cezanne et al., 2004). One of these clusters, harboring invariant residues within blades 5 and 6, the most conserved blades (Fig. 2A,B, magenta), was predicted to function as a phosphoinositide-binding site (Proikas-Cezanne et al., 2004). Within this cluster, two crucial arginine residues (R226 and R227 in human WIPI1) within an FRRG motif were identified as being essential for phosphoinositide binding in yeast homologs (Dove et al., 2004; Krick et al., 2006) and human WIPI1 (Gaugel et al., 2012; Jeffries et al., 2004; Proikas-Cezanne et al., 2007). The opposite site of the β-propeller, harboring a cluster of homologous residues (Fig. 2A,B, red), was proposed to associate with regulatory proteins (Proikas-Cezanne et al., 2004).

Indeed, based on the structural analysis of Hsv2, invariant residues were identified in the protein that confer specific binding to two individual phosphoinositides (Baskaran et al., 2012a; Baskaran et al., 2012b; Krick et al., 2012; Watanabe et al., 2012). Furthermore, alanine-scanning mutagenesis demonstrated that the cluster of invariant residues in human WIPI1 confers specific binding to PtdIns3*P* at phagophore membranes (Gaugel et al., 2012). It has been shown that identical amino acids in WIPI1 confer the ability to bind to either PtdIns3*P* or PtdIns(3,5)P_2 (Gaugel et al., 2012), indicating that one pool of WIPI members might bind to PtdIns3*P* and another pool to PtdIns(3,5)P_2. Of note, it was suggested that PtdIns3*P*-bound yeast Atg18 functions in autophagy, whereas PtdIns(3,5)P_2-bound Atg18 fulfills additional functions in alternative vesicle pathways (Box 2) (Efe et al., 2007).

A crucial arginine, R110, within the cluster of conserved residues in WIPI1 (Fig. 2A,B, red) was found to bind to an as-yet-unidentified regulator that should inhibit the binding of WIPI1 to PtdIns3*P* at phagophore membranes (Gaugel et al., 2012). Recently, autophagy regulation through arginine residues (R108, R125) within the cluster of conserved residues in human WIPI2B was reported, with these residues being found to confer specific binding to ATG16L1, an event preceding LC3 lipidation (Dooley et al., 2014). Interestingly, although these crucial arginines are conserved in all WIPI members, the binding of ATG16L1 is specific to WIPI2B (Dooley et al., 2014), supporting the idea of non-redundant and distinct functional contributions of WIPI members and splice variants to the process of autophagy.

WIPI proteins belong to an ancient seven-bladed β-propeller protein family referred to as PROPPINs

Phylogenetic analysis has demonstrated that human WIPI proteins belong to an ancient seven-bladed β-propeller protein family, with two paralogous groups, one containing human WIPI1 and WIPI2 and the ancestral yeast Atg18, and the other containing human WIPI3 and WIPI4 (Behrends et al., 2010; Polson et al., 2010; Proikas-Cezanne et al., 2004). This ancient β-propeller protein family was proposed to be named PROPPIN, for 'β-propellers that bind phosphoinositides' (Michell et al., 2006). Both paralogous groups of the PROPPIN family include representatives from plants, fungi and animals, and the WIPI3 and WIPI4 group is also present in some protozoans (Behrends

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et al., 2010; Polson et al., 2010; Proikas-Cezanne et al., 2004). Phylogenetic analysis further demonstrated that vertebrates have undergone an additional duplication in each of the two paralogous groups, indicating that the four human WIPI proteins should fulfill distinct functional roles in autophagy (Behrends et al., 2010; Polson et al., 2010; Proikas-Cezanne et al., 2004). Because the prototypical β-propeller was differentiated into its seven blades at the time of evolutionary divergence, phosphoinositide-binding likely represents an ancestral function of the PROPPIN family (Gaugel et al., 2012; Proikas-Cezanne et al., 2004).

WIPI1

WIPI1 was the first WIPI family member to be identified as having a role in autophagy. Prototypical cell treatments that induce autophagy above the basal level, such as starvation and treatment with rapamycin, promote a striking relocalization of cytoplasmic WIPI1 to ATG12-, ATG16L- and LC3-positive autophagosomal membranes (Gaugel et al., 2012; Itakura and Mizushima, 2010; Proikas-Cezanne et al., 2007; Proikas-Cezanne et al., 2004; Vergne et al., 2009). Furthermore, prototypical cell treatments that inhibit autophagy, such as blocking the generation of PtdIns3*P*, prevent the formation of autophagosomal membranes, causing WIPI1 to remain in the cytoplasm (Gaugel et al., 2012; Proikas-Cezanne et al., 2004; Vergne et al., 2009). In the cytoplasm, WIPI1 moves bidirectionally on microtubules, and the relocalization of cytoplasmic WIPI1 to autophagosomal membranes upon autophagy induction was found to be assisted by labile microtubules (Geeraert et al., 2010). Autophagosomal membranes that become decorated with WIPI1 upon autophagy induction have been shown to resemble both phagophores and autophagosomes (Proikas-Cezanne and Robenek, 2011; Proikas-Cezanne et al., 2007) (Fig. 1B). Freeze-fracture replica immunolabelling identified endogenous WIPI1, as well as overexpressed GFP–WIPI1, in both the inner and the outer autophagosomal membrane (Proikas-Cezanne and Robenek, 2011). Moreover, upon autophagy induction, WIPI1 localizes prominently to the plasma membrane and the ER, particularly the nuclear envelope (Proikas-Cezanne and Robenek, 2011), which suggests that these structures act as sources of membrane for autophagosomes. The specific localization of WIPI1 at autophagosomal membranes can be observed as punctate structures by using fluorescence microscopy. Consequently, quantifying the number of WIPI1-puncta-positive cells and the number of WIPI1 puncta per cell was introduced as a new method to assess autophagy (Klionsky et al., 2012; Proikas-Cezanne et al., 2007). Subsequently, WIPI1 puncta formation assessments have been used as the basis for automated high-throughput high-content image acquisition and analysis (Mauthe et al., 2011; Pfisterer et al., 2011). Apart from reflecting autophagy induction, an increase in the number of WIPI1 puncta can also be due to an imposed block in autophagic flux, e.g. by treatment with Ca^{2+}-mobilizing compounds (Engedal and Mills, 2014), according to an increase of LC3 punctae upon autophagic flux inhibition (Ganley et al., 2011). Of note, not only does the monitoring of WIPI1 protein localization represent a robust tool to assess autophagy, but the abundance of WIPI1 mRNA also reliably reflects the level of autophagosome formation (Tsuyuki et al., 2014).

Evidence for an essential PtdIns3*P* effector function of WIPI1 has been provided by several approaches. The induction of autophagy by starvation or rapamycin treatment results in specific and rapid relocalization of cytoplasmic WIPI1 to phagophores and autophagosomes; this can be counteracted by co-treatment with compounds that inhibit PtdIns3*P* production, such as wortmannin or LY294002 (Proikas-Cezanne et al., 2007; Proikas-Cezanne et al., 2004). The localization of WIPI1 to the phagophore is also counteracted upon functional inhibition of PtdIns3*P* production, either by siRNA-mediated downregulation of PI3KC3 (Itakura and Mizushima, 2010) or in genetically deficient PI3KC3 (Devereaux et al., 2013) or Vps15 (Nemazanyy et al., 2013) backgrounds. In line with this, mutant WIPI1 variants that are unable to bind to PtdIns3*P* are also unable to localize to autophagosomal membranes (Gaugel et al., 2012; Proikas-Cezanne et al., 2007). Moreover, treatment with YM201636, a selective PIKFYVE inhibitor (Jefferies et al., 2008) that prevents the phosphorylation of PtdIns3*P* to PtdIns(3,5)P_2 – thereby leading to PtdIns3*P* accumulation – results in an enhanced localization of WIPI1 to the phagophore and autophagosome (Gaugel et al., 2012). It is not only the pool of PtdIns3*P* and PtdIns(3,5)P_2 that regulates autophagy, but also the balance between PtdIns3*P* production and hydrolysis, with the latter being controlled by myotubularin phosphatases (MTMR) that remove D3-positioned phosphate from PtdIns3*P* or PtdIns(3,5)P_2 (Fig. 2C). Downregulation of Jumpy (also known as MTMR14) or MTMR3 was found to significantly increase the amount of WIPI1-decorated autophagosomal membranes and, thus, autophagosome formation (Taguchi-Atarashi et al., 2010; Vergne et al., 2009).

The positive regulation of the PtdIns3*P* effector activity of human WIPI1 follows the canonical route to autophagosome formation, i.e. the inhibition of mTORC1, activation of ULK1 and PI3KC3, and production of PtdIns3*P* (Codogno et al., 2012). It has been demonstrated that mTORC1 inhibition, either by rapamycin administration (Proikas-Cezanne et al., 2007), siRNA-mediated mTOR downregulation (Gaugel et al., 2012) or FOXO-mediated glutamine production (van der Vos et al., 2012), promotes the localization of WIPI1 at the nascent autophagosome. This specific localization of WIPI1 is counteracted by the downregulation of ULK1 or PI3KC3 (Itakura and Mizushima, 2010; McAlpine et al., 2013). Hence, the activation of ULK1 and PI3KC3, followed by localized PtdIns3*P* production, rapidly attracts WIPI1 through its PtdIns3*P*-binding specificity. Subsequently, PtdIns3*P*-bound WIPI1 at the nascent autophagosome is required for the conjugation of LC3 to PtdEtn (Fig. 1), as siRNA-mediated downregulation of WIPI1 counteracts LC3 lipidation (Mauthe et al., 2011). The role of WIPI1 as a crucial PtdIns3*P* effector in autophagy has been further underlined by the finding that WIPI1 also functions in selective autophagy, including mitophagy (Itakura et al., 2012) and xenophagy (Kageyama et al., 2011; Mauthe et al., 2012). Accordingly, WIPI1 prominently colocalizes with the autophagy adaptor p62 (also known as SQSTM1) (van der Vos et al., 2012).

The PtdIns3*P*-effector function of WIPI1 not only depends on ULK-mediated activation of PI3K3C and PtdIns3*P* production during the initiation step of autophagy, but also on Ca^{2+} signaling, as Ca^{2+} chelation counteracts starvation-induced accumulation of WIPI1 at autophagosomal membranes (Engedal and Mills, 2014; Pfisterer et al., 2011). AMPK, one of the conserved regulators of autophagy, can be activated by Ca^{2+} signaling through calmodulin-dependent kinase kinase α/β (CAMKKα and -β; also known as CAMKK1 and -2) (Means, 2008). Accordingly, cytoplasmic Ca^{2+} levels have been found to positively regulate autophagy through CaMKKα/β-mediated activation of AMPK followed by the inhibition of mTORC1 (Høyer-Hansen et al., 2007). However, Ca^{2+}-mediated induction of autophagy has been suggested to also occur in a non-canonical way, bypassing AMPK (Grotemeier et al.,

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2010). Accordingly, although WIPI1 localization at autophagosomal membranes is ablated upon Ca^{2+} chelation, WIPI1-positive autophagosomes are formed in AMPK-deficient mouse embryonic fibroblasts, probably involving an AMPK-independent route through CAMKI (Pfisterer et al., 2011). It is noteworthy that the PtdIns3*P* effector function of WIPI1 does not respond to glucose starvation (McAlpine et al., 2013; Pfisterer et al., 2011), which generally activates AMPK. Glucose starvation has been suggested to signal independently of PtdIns3*P* to trigger autophagy (McAlpine et al., 2013).

WIPI2

Initial expression analysis showed that WIPI2 is ubiquitously expressed in normal human tissues, with high levels in heart and skeletal muscle, and it appears to be aberrantly expressed in matched human pancreatic, kidney and uterine tumor samples (Proikas-Cezanne et al., 2004) (Box 1). Interestingly, non-silent mutations in WIPI2 have been identified in large-scale analyses (Box 1, Table 1).

In the course of the cloning of the human WIPI gene and protein family, WIPI2 splice variants have been identified that also encode seven-bladed β-propeller WIPI2 proteins (Proikas-Cezanne et al., 2004). So far, only the splice variants WIPI2B and WIPI2D (Proikas-Cezanne et al., 2004) follow the pattern of WIPI1, whereby punctate structures prominently increase upon autophagy induction. By contrast, full-length WIPI2A and WIPI2C show no increase in punctate staining upon autophagy induction (Mauthe et al., 2011). This indicates that different WIPI2 splice variants fulfill distinct functional roles, one of which is essential in autophagy (Lamb et al., 2013). Like WIPI1 (Itakura and Mizushima, 2010), WIPI2 functions downstream of ULK1 (McAlpine et al., 2013) and specifically binds to PtdIns3*P* at early autophagosomal membranes that are decorated with ULK1, DFCP1, ATG16L1 and LC3 during starvation-induced autophagy (Polson et al., 2010). Therefore, WIPI2 localizes to both ER-associated omegasomes and phagophore membranes (Polson et al., 2010). Immunofreeze fracture electron microscopy provided evidence that WIPI2B (Fig. 1C) and WIPI2D (Fig. 1D) further localize at the inner and outer membrane of autophagosomes (Proikas-Cezanne and Robenek, 2011). As is the case for the distribution of WIPI1, WIPI2 also appears to be enriched in the inner membrane of the autophagosome (Fig. 1B–D). This asymmetry seems to be distinct from the localization of PtdIns3*P* in yeast, which is enriched in the luminal monolayers of the autophagosome, thereby suggesting different initiating processes of autophagosome formation in yeast and mammals (Cheng et al., 2014). Immunofreeze fracture electron microscopy provides further evidence that WIPI2B and WIPI2D also localize at the plasma membrane and to the Golgi and ER area (Proikas-Cezanne and Robenek, 2011).

Functionally, siRNA-mediated downregulation of WIPI2 showed that WIPI2 is essential for LC3 lipidation (Mauthe et al., 2011; Polson et al., 2010). Interestingly, WIPI2 downregulation results in an accumulation of ATG9 (also known as ATG9A) at DFCP1-positive omegasomes (Orsi et al., 2012), suggesting that WIPI2 might have a role in the retrieval of ATG9 from early autophagosomal membranes, a functional role that has been assigned to the yeast Atg18 (Reggiori et al., 2004). However, WIPI2 was not found to be necessary for the recruitment of ATG9 to the omegasomes (Orsi et al., 2012).

Importantly, a recent study demonstrates that WIPI2 connects PtdIns3*P* production with LC3 lipidation at the onset of autophagy, because WIPI2 has been found to specifically bind to ATG16L1, thereby recruiting the ATG12–ATG5–ATG16L1 complex that is required for LC3 lipidation (Dooley et al., 2014). This indicates a scaffold function for WIPI2, in that it bridges PI3KC3-mediated PtdIns3*P* production and LC3 lipidation during phagophore formation and expansion. WIPI2 is also though to play a functional role in bridging the PtdIns3*P* signal with LC3 lipidation in xenophagy, as shown for *Salmonella* clearance (Dooley et al., 2014). In this context it has been reported previously that during xenophagy of *Shigella*, WIPI2 interacts with TECPR1, which also interacts with ATG5 (Ogawa et al., 2011). Moreover, a functional requirement for TECPR1 in regulating xenophagy of *Shigella* was extended to a general role of TECPR1 in selective autophagy, as TECPR1-deficient mouse embryonic fibroblasts were found to be defective in degrading depolarized mitochondria and protein aggregates (Ogawa et al., 2011).

The PtdIns3*P*-effector function of WIPI2 has also been shown to be crucial in non-canonical autophagy that is triggered by LRRK2 protein kinase activity (Manzoni et al., 2013) or rVP1, a recombinant capsid protein of the foot and mouth disease virus (Liao et al., 2014). By contrast, autophagy triggered by glucose starvation appears to be independent of WIPI2, as is the case for WIPI1 (McAlpine et al., 2013).

WIPI3

WIPI3 (also known as WDR45L) was found to be ubiquitously expressed in normal human tissue and aberrantly expressed in

Table 1. WIPI mutations identified in human tumors

WIPI	Cancer	Mutation	Reference
WIPI1	Breast	N154I	Stephens et al., 2012
WIPI1	Colon	V385I	Seshagiri et al., 2012
WIPI1	Lung	G313R	Rudin et al., 2012
WIPI1	Lung	G12R	Seo et al., 2012
WIPI1	Lung	L309V	Imielinski et al., 2012
WIPI1	Skin (melanoma)	Q300P	Nikolaev et al., 2012a
WIPI1	Skin (melanoma)	D243N	Berger et al., 2012
WIPI1	Skin (melanoma)	P291S	Krauthammer et al., 2012
WIPI1	Bone	Q47*	Joseph et al., 2014
WIPI1	Pancreas	A249T	Biankin et al., 2012
WIPI1	Skin (SCC)	S446L	Stransky et al., 2011
WIPI2	Colon	V410A	Muzny et al., 2012
WIPI2	Colon	S114I	Seshagiri et al., 2012
WIPI2	Esophagus	E26*	Dulak et al., 2013
WIPI2	Esophagus	K371T	Dulak et al., 2013
WIPI2	Lung	E445*	Peifer et al., 2012
WIPI2	Skin (melanoma)	A402V	Berger et al., 2012
WIPI2	Ovary	E65Q	Stransky et al., 2011
WIPI2	Pancreas	A206V	Jiao et al., 2014
WIPI3	Blood (ALL)	D341N	Roberts et al., 2012
WIPI3	Breast	Q229E	Stephens et al., 2012
WIPI3	Brain (glioma)	T161M	Bettegowda et al., 2011
WIPI3	Kidney (RCC)	N306D	Sato et al., 2013
WIPI4	Colon	R112H	Nikolaev et al., 2012b
WIPI4	Endometrial	R274H	Le Gallo et al., 2012
WIPI4	Lung	V230L	Seo et al., 2012
WIPI4	Lung	R7Q	Seo et al., 2012
WIPI4	Lung	K115*	Imielinski et al., 2012
WIPI4	Brain (medulloblastoma)	S50R	Pugh et al., 2012
WIPI4	Blood (myeloma)	I196fs*26	Walker et al., 2012
WIPI4	Pancreas	R112C	Jiao et al., 2014

*nonsense mutations; fs, frame shift; SCC, squamous cell carcinoma; ALL, acute lymphoblastic leukemia; RCC, renal cell carcinoma.

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ovarian, uterus and kidney cancers in an initial expression analysis (Proikas-Cezanne et al., 2004). WIPI3 should also fold into a seven-bladed β-propeller that specifically binds to phosphoinositides (Proikas-Cezanne et al., 2004); however, this has not been demonstrated as yet, and the functional role of WIPI3 is unknown.

WIPI4

WIPI4 (also known as WDR45) encodes a seven-bladed β-propeller protein that is expected to specifically bind to phosphoinositides; it is ubiquitously expressed at high levels in skeletal muscle and heart, and appears to be overexpressed in pancreatic and kidney cancer samples in an initial expression analysis (Proikas-Cezanne et al., 2004). The localization of WIPI4 has been analyzed in normal rat kidney (NRK) cells, and the protein shows a diffuse pattern in dividing cells (Lu et al., 2011). Specifically upon starvation, WIPI4 relocates to autophagosomal membranes and colocalizes with LC3 (Lu et al., 2011), but unlike WIPI1 and WIPI2, WIPI4 was not found on autophagosomes as analyzed by immunofreeze fracture electron microscopy (T.P.-C. and Horst Robenek, unpublished observations). Functionally, WIPI4 was found to be essential for proper omegasome maturation and autophagosome formation, as siRNA-mediated downregulation of WIPI4 impaired the formation of autophagosomes and increased the number of ATG5-positive phagophores (Lu et al., 2011). Thus WIPI4 was shown to be essential for autophagy in mammalian cells. The WIPI4 homolog EPG-6 in *C. elegans* is thought to function downstream of ATG-18, in complex with ATG-2 (Lu et al., 2011). From this, it is possible that mammalian WIPI4 binds to ATG2 and acts downstream of LC3 in autophagosome formation (Fig. 2C). However, the role of the WIPI members in recruiting ATG2 in human cells warrants further detailed studies, as it was also found that ATG2 functions hierarchically at the same position as WIPI1 (Itakura and Mizushima, 2010; Velikkakath et al., 2012), and if WIPI1 is not bound to PtdIns3*P* at omegasomes, ATG2 is not recruited to the initiation site of autophagosome formation (Pfisterer et al., 2014).

Strikingly, WIPI4 was found to be mutated *de novo* and causative for SENDA (static encephalopathy of childhood with neurodegeneration in adulthood), a sporadic NBIA (neurodegeneration with brain iron accumulation) subtype (Haack et al., 2012; Hayflick et al., 2013; Saitsu et al., 2013). Interestingly, although the *WIPI4* gene is localized on the X chromosome, the phenotype in female and male patients has been found to be indistinguishable (Hayflick et al., 2013; Saitsu et al., 2013). Functionally, it has been shown that the *de novo* mutations in the *WIPI4* gene, which probably result in the production of unstable WIPI4 protein variants, indeed confer a drastic reduction in autophagic function in lymphoblastoid cell lines derived from SENDA patients (Saitsu et al., 2013). Furthermore, this study showed that mutant WIPI4 proteins in SENDA patients were less abundant when compared with wild-type WIPI4 in unaffected individuals and that lipidated LC3 and LC3-positive autophagosomal membranes accumulated in these patients without being completely utilized in the process of autophagy (Saitsu et al., 2013). Furthermore, accumulated LC3-positive autophagosomal membranes were also positive for ATG9 and were classified as abnormal autophagic structures, and autophagy occurred at only very low levels (Saitsu et al., 2013). Thus, WIPI4 mutations in SENDA patients lead to the accumulation of early autophagosomal membranes and improper autophagic degradation. These findings are of utmost importance for both future diagnosis and classification of SENDA. Moreover, identifying WIPI4 mutations in SENDA patients provides the first direct evidence that autophagy malfunction is causative of neurodegeneration in humans (Saitsu et al., 2013).

Conclusions

Evidence suggests that WIPI1, WIPI2 and WIPI4 function as essential and non-redundant PtdIns3*P* effectors downstream of PI3KC3-mediated PtdIns3*P* production at the onset of autophagy. By this means, WIPI2 recruits the ATG12–ATG5–ATG16L1 complex, which mediates LC3 lipidation, for which WIPI1 is also essential. In addition, WIPI4 interacts with ATG2 and is anticipated to function downstream of LC3. It is of urgent interest to decipher the individual contributions of the WIPI members at the nascent autophagosome, as their concerted action and regulation should hold clues for the further understanding of autophagy initiation and for the understanding of autophagosomal membrane formation. On the basis of their important role in autophagy, the function of WIPI family members is likely to be compromised in a great variety of human pathologies that exhibit defective or misregulated autophagy, as indicated by the identification of WIPI mutations in cancer and neurodegeneration. Hence, a better understanding of WIPI proteins could help to elucidate the principles of autophagosome formation in health and disease.

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Competing interests

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