

Evidence for an evolutionary relationship between Vmp1 and bacterial DedA proteins

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ABSTRACT VMP1 and DedA proteins are conserved families of transmembrane proteins in eukaryotes and prokaryotes respectively. Despite numerous reports involving these proteins in multiple cellular processes, their molecular function is still unknown. They share the domain of unknown function PF09335, suggesting a possible functional relationship between these protein families. Here we show that VMP1 from different species contain two short motifs conserved in the bacterial DedA proteins and the yeast protein Tvp38. The hallmark of one of these motifs is a glycine residue previously shown to be strictly conserved in all the DedA proteins. Substitution of this residue to leucine, glutamate or arginine in *Dictyostelium* Vmp1 inactivates the protein, as shown by the inability of the mutants to rescue the phenotypes associated with the lack of Vmp1 including development and lipid homeostasis. This is the first experimental approach that supports an evolutionary relationship between Vmp1 and DedA proteins and highlights the importance of the conserved glycine residue in the PF09335 domain.

KEY WORDS: *autophagy, DedA, Dictyostelium, lipid homeostasis, Vmp1, PF09335, SNARE-associated Golgi protein*


A proteomic analysis in *S. cerevisiae* identified several proteins present in a Golgi sub-compartment defined by the SNARE protein Tlg2. Among these proteins, Tvp38 (Tlg2-compartment vesicle protein of 38 kDa) is a predicted transmembrane protein of unknown function (Inadome *et al.*, 2005). A region of this protein was annotated as a pfam conserved domain (PF09335) and named “SNARE-associated Golgi protein”. Despite the lack of an overall similarity, this domain is also detected in other eukaryotic proteins: VMP1, TMEM41A/B and TMEM64. Interestingly, the same domain is also found in a family of prokaryotic proteins, the so-called DedA proteins.

Prokaryotic DedA proteins are a family of conserved membrane proteins of unknown function initially identified in *Escherichia coli*. Phylogenetic analysis based on sequence homologies identified the presence of distantly related proteins of this family in bacteria, archaea and cyanobacteria (Boughner and Doerrler, 2012, Doerrler *et al.*, 2013). They have been often denoted as the DedA/Tvp38 protein family to highlight the presence of the conserved PF09335 domains in these proteins (Keller *et al.*, 2015, Keller and Schneider, 2013). DedA proteins are essential for cell viability and have been involved in a wide variety of functions, including cell division, membrane lipid composition, envelope

stress responses and drug resistance (Boughner and Doerrler, 2012, Doerrler *et al.*, 2013, Kumar and Doerrler, 2014, Ledgham *et al.*, 2005, Sikdar *et al.*, 2013).

The eukaryotic vacuole membrane protein 1 (VMP1) is now attracting much attention due to its important role in autophagy and ER homeostasis. VMP1 is a predicted multispansing membrane protein of unknown molecular function located in the endoplasmic reticulum (ER). VMP1 is highly conserved in most eukaryotes but is absent in the fungi lineage (Calvo-Garrido *et al.*, 2008a). The effects of VMP1 deficiency have been studied in a number of model organisms and in cultured mammalian cells. Cells deficient in this protein display pleiotropic defects in cell growth, osmoregulation, protein secretion, Golgi and ER structure, and autophagy in *Dictyostelium discoideum* (Calvo-Garrido *et al.*, 2008a, Calvo-Garrido *et al.*, 2008c, Calvo-Garrido and Escalante, 2010, Calvo-Garrido *et al.*, 2014); cytokinesis, cell shape, structural defects of different organelles and alterations in the lipidic profile in *Chlamydomonas reinhardtii* (Tenenboim *et al.*, 2014); autophagy and protein se-

Abbreviations used in this paper: ER, endoplasmic reticulum; TVP, Tlg2-compartment vesicle protein; VMP, vacuole membrane protein.

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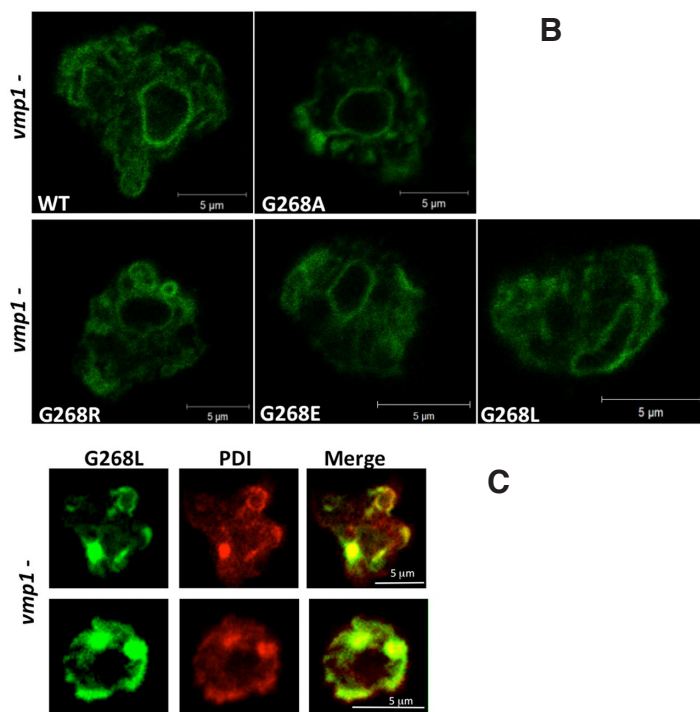
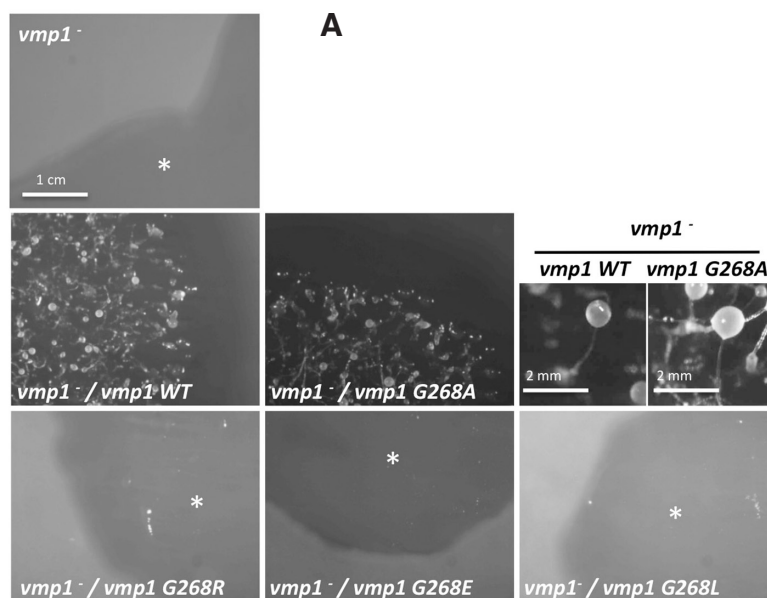
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| | [FY]XXX[RK] | | GXXX[VILM]XXXX[FY] |
|------------------|--|-----|--------------------|
| YqjA (Ecol) 77 | VAASLCWVSYIQGRNLGN-----TRTVQNWLSHLPAPHYQRAHHLFKHGLSALLIGRFTAF-VRTLPTIAGLSGNNARFOFNWMSGLL | 163 | |
| DedA (Bbur) 66 | WGAYLGDIIISFYIGKLMGN-----KLFKNNKDNLLDKINYYGQYGVLTGFRFIPFGRVNAIFMSACGMGNKSNLITVSDFFATLL | 149 | |
| Tvp38 (Scer) 178 | LGSVTGSIASFVVFVKTIHL-----SRAEKLVLNRRFEALASIQENNSYWTLLALLRLCFFPYSLTNGAIAAGVYCHSVRNFSIANITTPK | 263 | |
| KMS1 (Atha) 198 | IGTALGELPPYFISRAASISGSTVDGMEELD-----SSTEDSGFMATHLNVRKRWLLTHSOHLNFFVVLVVASVPNPLFDLAGIMCQFCHFPWFELATLIGKAI | 299 | |
| VMP1 (Ptet) 153 | IGTAIGELPPYFVARAAALANKKLEEEVLE-----KKDENTFMHRMKLLIYNKLOKNAFLVYMLCASIPNPLFDLAGITCGHFLISFWTFPGATVVLGKI | 249 | |
| VMP1 (Ddis) 182 | AGTAIGELPPYFVARTARLKLKLEQEKLLK-----QQEKPIDEKDQPKKGLLERLSEKVPALIGNLGFFGILAFASIPNPLFDLAGITCGHFLVPPFWKFFGATFIGKAV | 287 | |
| EPG3 (Cele) 206 | AGTALGELPPYFMARAARISGQEPDDEYREFLELMNADKESDADQKLSIVERAKSWVEHNIHRLGFPFCILLFASIPNPLFDLAGITCGHFLVPPFWSFFGATLIGKAL | 313 | |
| VMP1 (Hsap) 196 | IGTAIGELPPYFMARAARISGAEPDDEEYQEF-----EEMLEHAESAQDFASRAKLVQKLVQKVGFFGILACASIPNPLFDLAGITCGHFLVPPFWSFFGATLIGKAI | 298 | |
| PFAM | LGALLCASLAVLLGRYLGR-----KALRRKLLSEKRVKALERLVERYGFLALLLRLRLIVPRDLVNYVAGLTRVSEKRFELATLIGSIP | | * |

Fig. 1. Conservation of the PF09335 domain in DedA, Tvp38 and VMP1 proteins. The alignment includes amino acid sequences from YqjA (*E. coli*), DedA (*B. burgdorferi*), Tvp38 (*Scer. S. cerevisiae*), VMP1 from different organisms: Kms1 (*Atha*, *Arabidopsis Thaliana*), VMP1 (*Ptet*, *P. tetraurelia*); Ddis, *D. discoideum*; Hsap, *H. sapiens*), EPG3 (*Cele*, *C. elegans*), and the consensus for the PF09335 PFAM domain. Conserved (BLOSUM62) residues were shaded according to degree of conservation: black, >90%; gray, 50-90%. Numbers indicate amino acid positions and dashes indicate gaps. Shown above are consensus sequences of the two conserved motifs previously identified in the Tvp38/DedA family. Asterisk indicates the position of the glycine residue conserved in all DedA proteins.



cretion defects in *Caenorhabditis elegans* and *Drosophila melanogaster* respectively (Bard et al., 2006, Tian et al., 2010); ER and Golgi structure defects in plants (Wang et al., 2011). Recent studies in mammalian cells provide an explanation for the complexity of these phenotypes as VMP1 has a role in the distribution and dynamics of ER subdomains enriched in lipid-synthesizing enzymes that are involved in a variety of membrane contact sites between the ER and other organelles (Tabara et al., 2018). VMP1 has been proposed to regulate lipid signalling at these contact sites and to play an essential role in cellular lipid and Ca²⁺ homeostasis (Tabara and Escalante, 2016, Tabara et al., 2018, Zhao et al., 2017). How VMP1 performs this function at the molecular level is still unknown.

The possibility of a functional relationship between eukaryotic VMP1 and prokaryotic DedA proteins is intriguing. Since the molecular function of VMP1 and DedA proteins is unknown, it is of great interest to investigate the functional conservation of the PF09335 domain in these proteins.

Results and Discussion

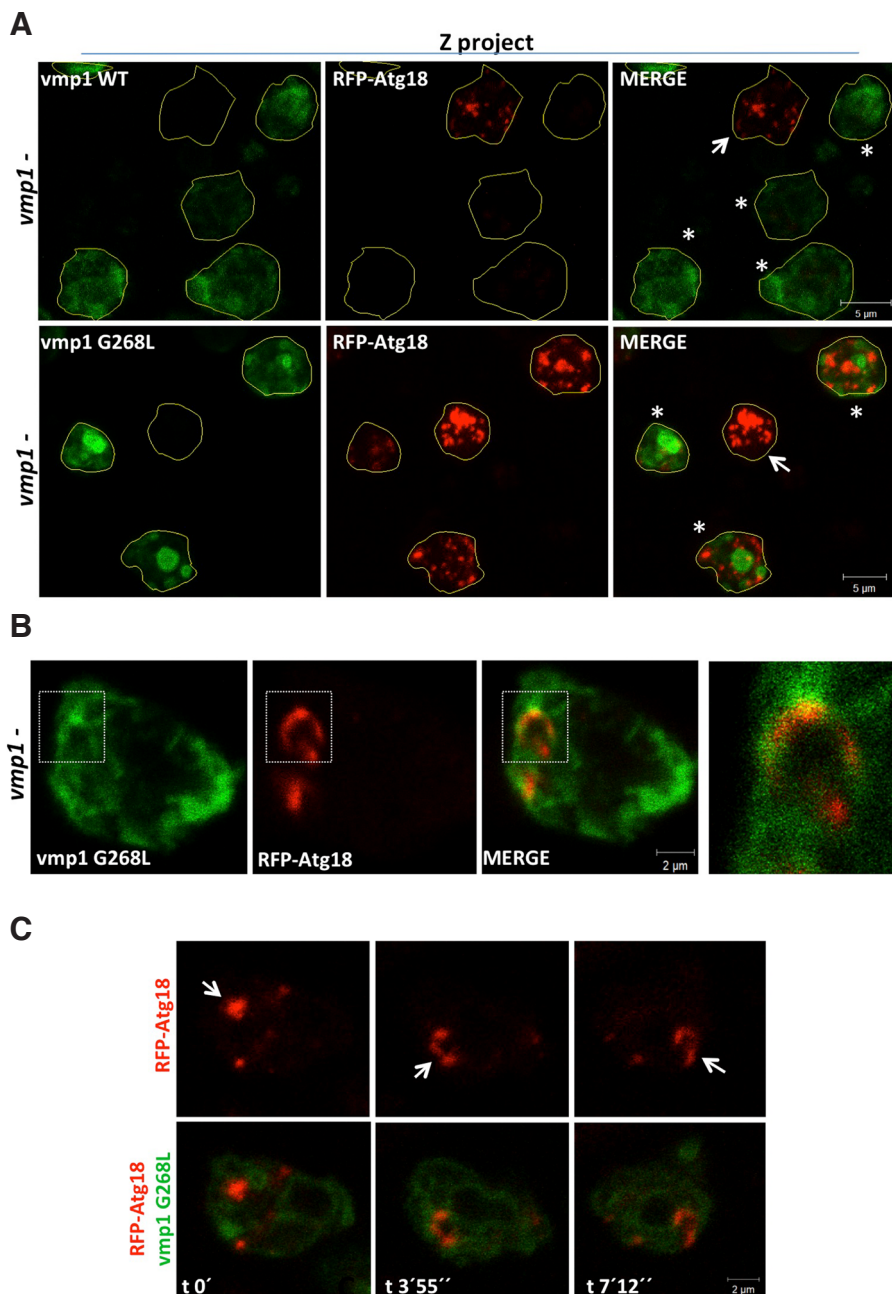
Previous sequence alignments of the PF09335 domain of DedA proteins and yeast Tvp38 revealed the presence of two short conserved motifs separated by variable length spacer sequences of approximately 50 amino acids. These motifs have the following consensus sequences: F/YxxxR/K and GxxxM/V/L/lxxxxF/Y (Keller and Schneider, 2013). The Gly in the second motif is the most conserved residue in all

Fig. 2. The conserved Gly residue of the PF09335 domain of Vmp1 is essential for Dictyostelium development. (A) *vmp1*⁻ strain was transfected with expression plasmids carrying WT and mutated forms of Vmp1. After selection, transformants were analyzed in association with bacteria and representative pictures taken after 5 days. It should be noted that *vmp1*⁻ cells are able to grow in association with bacteria forming a growth plaque (the dark zones labeled by asterisks) but are unable to develop under these conditions. Normal aggregation and fruiting body formation were only accomplished by the expression of WT and G268A Vmp1 protein. Details of the fruiting bodies are displayed at the right. (B) Representative confocal images of cells expressing the different Vmp1 proteins. All proteins showed similar levels and localization. (C) Optical sections of two *Dictyostelium vmp1*⁻ cells expressing the mutated Vmp1 G268L (green) and the colocalization with the ER membrane marker PDI (red). Some cells present accumulations of ER membrane.

DedA proteins analyzed (Keller and Schneider, 2013). We have now performed sequence alignments of the predicted PF09335 domain of VMP1 and DedA proteins in different organisms, including the yeast protein Tvp38 (Fig. 1). Although the overall similarity between VMP1 and DedA proteins is very low, the two motifs of the PF09335 domain are very well conserved.

All tested VMP1 sequences from different species contain the conserved Gly residue, the only amino acid fully conserved in all DedA proteins (Keller and Schneider, 2013). It should be noted that the members of the TMEM41 and 64 families lack this Gly residue, which may indicate a higher divergence of these proteins with DedA proteins. In order to determine the functional importance of this residue, we used *Dictyostelium discoideum* since the inactivation of Vmp1 in this organism impairs its developmental stage and thus,

the functionality of the protein is relatively easy to assess. Besides developmental defects, *Dictyostelium* cells lacking Vmp1 are unable to grow in liquid media and have altered lipid homeostasis, as evidenced by the accumulation of the phospholipid PtdIns3P in the ER (Calvo-Garrido *et al.*, 2014). We generated point mutations in the *Dictyostelium vmp1* gene to convert the conserved Gly (G) residue at position 268 to the following amino acids: Ala (A), Leu (L), Arg (A) and Glu (E). The protein was fused to GFP at the C-terminus to determine its subcellular localization. The mutated constructs were transfected into *Dictyostelium* cells lacking *vmp1* (Calvo-garrido *et al.*, 2008b), and phenotypic rescue was evaluated. Both wild-type and G268A mutant were able to complement the growth in liquid media (data not shown) and develop into normal-looking fruiting bodies (Fig. 2A). In contrast, the other mutants containing the G268L, G268E and G268R substitutions were unable to complement the Vmp1-associated phenotypes, suggesting that the functionality of these mutant proteins is impaired (Fig. 2A).



As shown in Fig. 2B, WT and mutant proteins showed similar levels of expression and similar localization at the ER, thus indicating that these mutations do not affect the stability or the correct subcellular localization of this protein. Interestingly, around 50% of non-rescued Vmp1 mutant cells showed accumulation of the Vmp1 signal in abnormal ER structures (confirmed by the colocalization with the ER marker PDI) (Fig. 2C). This is in agreement with the defect in ER structure previously described in *Dictyostelium* and *Arabidopsis* Vmp1-null cells (Calvo-Garrido *et al.*, 2008b, Wang *et al.*, 2011).

It should be noted that the *E. coli* protein YqjA is, like *Dictyostelium* Vmp1, still functional when the conserved Gly residue is converted to Ala (Keller *et al.*, 2015). Due to their small size and chemical properties, Gly and Ala seem to be interchangeable at least for the phenotypes we have examined. In contrast, a substitution to Leu, another non polar amino acid, or a conversion to a charged residue disrupt the functionality of Vmp1, suggesting

Fig. 3. Mutated Vmp1 is not able to rescue the homeostasis of PtdIns3P. *vmp1*⁻ cells expressing WT and G268L Vmp1 proteins fused to GFP were transfected with the PtdIns3P marker RFP-Atg18 and visualized by confocal microscopy. **(A)** Maximum intensity projections of x-optical sections are shown. In the upper panel cells expressing WT Vmp1 (green cells marked by asterisks) did not show the abnormal accumulation of RFP-Atg18 while the cell not expressing Vmp1 (marked by an arrow) show the accumulation of the marker. In the lower panel, cells show abnormal accumulation irrespective of the expression of the mutated form of Vmp1. **(B)** Colocalization analysis of Vmp1 G268L fused to GFP with Atg18 marker. A detail is shown on the right. **(C)** Different images at the times indicated from a confocal time lapse showing that the aberrant expression of the Atg18 marker is persistent and colocalizes with the mutated form of Vmp1-GFP.

that this conserved Gly residue and the corresponding short motif is important for Vmp1 function.

To determine whether the function of Vmp1 in lipid signalling is also impaired in the mutants, we analyzed the distribution of the autophagic protein Atg18, which interacts with the signaling lipid PtdIns3P during autophagosome formation. It has been previously reported that PtdIns3P is transiently generated in *Dictyostelium* WT cells but strongly accumulates in Vmp1 mutant cells (Calvo-Garrido *et al.*, 2014). The *vmp1* null strain expressing either wild-type Vmp1 or the G268L mutant derivative were transformed with an expression marker encoding RFP-Atg18 and the fluorescence pattern was analyzed. As expected, expression of the WT form of Vmp1 results in a low RFP-Atg18 signal due to the rapid turnover of PtdIns3P (Calvo-Garrido *et al.*, 2014) and thus rescues the described aberrant accumulation of this marker (Fig. 3A). In contrast, expression of the protein containing the G268L substitution was unable to rescue the abnormal and characteristic accumulation of the Atg18 marker (Fig. 3A). Furthermore, this aberrant PtdIns3P signaling event is associated with ER membranes containing the Vmp1 mutant form (Fig. 3B) and persists during long periods of times (Fig. 3C). All together, these results indicate that the G268L substitution leads to a loss of function of *Dictyostelium* Vmp1.

Although the molecular function of DedA proteins in bacteria remain elusive, it has been suggested that their function may be related to the stability of cellular membranes (Keller and Schneider, 2013; Sikdar *et al.*, 2013). Interestingly, DedA mutants display defects in protein and solute transport across membranes and alterations in the proton motive force (Kumar and Doerrler, 2014, Kumar and Doerrler, 2015, Sikdar and Doerrler, 2010). It has been proposed that these defects could be primarily caused by alterations in the phospholipid composition of the cellular membrane (Doerrler *et al.*, 2013, Sikdar *et al.*, 2013, Thompkins *et al.*, 2008). In fact, deletion of the unique DedA gene in *Borrelia* leads to decreased levels of phosphatidylethanolamine and increased levels of both phosphatidylglycerol and cardiolipin (Liang *et al.*, 2010). Moreover, the major envelope stress response pathways that control the expression of genes involved in membrane integrity are strongly activated in DedA mutants (Sikdar *et al.*, 2013).

VMP1 has been also involved in a wide variety of membrane-dependent processes (Calvo-garrido *et al.*, 2008b, Calvo-Garrido *et al.*, 2014, Tenenboim *et al.*, 2014, Wang *et al.*, 2011). In mammalian cells, VMP1 regulates the length of multiple membrane contact sites (Tabara and Escalante, 2016) and the dynamics of ER subdomains enriched in different phospholipid-synthesizing enzymes (Tabara *et al.*, 2018). Moreover, metabolomics studies in *Chlamydomonas* demonstrated that the amount of phospholipid involved in membrane biogenesis decreases in VMP1-KO cells (Tenenboim *et al.*, 2014). As in prokaryotes, a primary defect in membrane organization (the ER membrane in eukaryotes) may affect cellular lipid homeostasis, which would explain the pleiotropic defects in organelle biogenesis. In line with this hypothesis, the aberrant recruitment of the PtdIns3P binding protein Atg18 that colocalize with the Vmp1 mutant isoforms strongly suggest that Vmp1 function could be related to the maintenance of a proper lipid signalling. Remarkably, it has been recently reported that the absence in mammalian cells of the VMP1-related protein TMEM41B (which also contains the PFAM09335 domain) leads to similar defects in both autophagosome and LD biogenesis to those observed after VMP1 depletion (Moretti *et al.*, 2018, Morita

et al., 2018). Furthermore it has been proposed that TMEM41B is also required for cellular lipid mobilization and distribution (Moretti *et al.*, 2018).

In conclusion, our study provides experimental evidence for the functional conservation of the PF09335 domain and the essential role of a conserved Gly residue in Vmp1 and DedA proteins. Remarkably, the defective phenotypes associated with the absence or mutation of this domain in both bacteria and mammalian cells are linked to altered membrane homeostasis. The study of the function of this protein domain will be essential to decipher the molecular function of eukaryotic VMP1 and the prokaryotic DedA proteins.

Materials and Methods

Dictyostelium growth and transformation

All strains used in this work were derived from *Dictyostelium discoideum* AX4 strain. *Dictyostelium* cells were grown at 22°C axenically in HL5 liquid medium (Foremedium) supplemented with glucose 1% and penicillin–streptomycin (10 000 U/ml penicillin and 10 000 mg/ml streptomycin; Gibco). For growth in solid medium, *Dictyostelium* was seeded in association with a non pathogenic *Klebsiella pneumoniae* strain (Lima *et al.*, 2018). Transformation were carried out by electroporation as previously described (Pang *et al.*, 1999) and selected with the appropriate antibiotic. The mutant strain in Vmp1 was described previously (Calvo-Garrido *et al.*, 2008c).

The plasmid RFP-Atg18 was kindly provided by Jason King (University of Sheffield).

Site-directed mutagenesis

Vmp1 coding sequence was cloned in a modified pBluescript SK between SpeI and BglII. 10 ng of the plasmid was used as a template in a PCR reaction with a HiFi polymerase (KAPA biosystems) using overlapping oligonucleotides containing the desired mutations as described in the Stratagene QuickChange Site-Directed Mutagenesis protocol (<https://www.genomics.agilent.com/files/Manual/200523.pdf>). The mutation was verified by sequencing of the entire fragment. The mutated forms were cloned into pDM323 (Veltman *et al.*, 2009).

Confocal microscopy

Cells were observed *in vivo* using Ibidi 15 µ-Slide 8-well. For immunofluorescence cells were fixed with 2% paraformaldehyde in PBS 2X (1X PBS: 133 mM NaCl, 8 mM Na₂HPO₄, 2 mM KH₂PO₄; pH 7.4) for 15 min at room temperature, washed 3 times for 5 min each with PBS 1X and then incubated with blocking buffer (2% BSA; 0.5% NP40 in PBS 1X) for 1 hour. Cells were incubated overnight at 4°C with mouse monoclonal antibody against PDI (kindly provided by Dr. Pierre Cosson) at 1:1000 in blocking buffer. After three washes with PBS 1X cells were incubated for 1 h with the secondary antibody (1:500 in blocking buffer) conjugated to Alexa 546. After three washes with PBS 1X cells were mounted with Prolong for confocal visualization. Confocal images were acquired using an inverted Zeiss LSM 710 laser-scanning microscope.

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