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Autophagy dysfunction and ubiquitin-positive protein aggregates in Dictyostelium cells lacking Vmp1

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Abbreviations: ER, endoplasmic reticulum; GFP, green fluorescence protein; RFP, red fluorescence protein; PDI, protein disulfide isomerase; VMP1, vacuole membrane protein 1; cAMP, cyclic adenosine monophosphate; DIF-1, differentiation inducing factor-1; ACD, autophagic cell death

Ubiquitin-positive protein aggregates are a hallmark of many degenerative diseases. Their presence can be induced by dysfunction in protein degradation pathways such as proteasome and autophagy. We now report several lines of evidence suggesting a defect in autophagy in Dictyostelium cells lacking Vmp1 (vacuole membrane protein 1), an endoplasmic reticulum (ER)-resident protein involved in pathological processes such as cancer and pancreatitis. *vmp1*$^-$ null cells are unable to survive starvation or undergo autophagic cell death under the appropriate inductive signals. Moreover, confocal studies using the autophagy marker Atg8 and previous transmission electron microscopy analysis showed defects in autophagosome formation. Although Vmp1 is localized in the ER, we found colocalization with Atg8 suggesting a contribution of both Vmp1 and ER in autophagosome biogenesis or maturation. Interestingly, *vmp1*$^-$ *mutant* cells showed accumulation of huge ubiquitin-positive protein aggregates containing the autophagy marker GFP-Atg8 and the putative Dictyostelium p62 homologue as described in many degenerative human diseases. The analysis of other Dictyostelium autophagic mutants (*atg1*$^-$, *atg5*$^-$, *atg6*$^-$, *atg7*$^-$ and *atg8*$^-$) showed a correlation in the severity of their corresponding phenotypes and the presence of ubiquitin-positive protein aggregates suggesting that the deleterious effects associated with development of these aggregates might contribute to the complex phenotypes observed in autophagy deficient mutants. Our results suggest that Vmp1 is required for the clearance of these ubiquitinated protein aggregates through autophagy and highlight a potential role for Vmp1 in protein-aggregation diseases.

Introduction

Many neurodegenerative diseases are associated with the presence of intracellular or extracellular protein aggregates. These disorders include Parkinson disease (PD), Alzheimer disease (AD), polyglutamine expansion diseases (PQD) and prion-related diseases among others. Collectively, these pathologies can be considered proteinopathies or protein conformation disorders, but the precise role of the protein aggregates in the pathological outcome is a matter of intense debate.[1] It is therefore important to identify key factors involved in the formation, degradation and toxicity of these aggregates at the cellular level. These protein aggregates are composed of primary constituents such as the Aβ (β-amyloid), the protein Tau or α-synuclein. However, the analysis of the protein composition of these brain inclusions have shown a complex and varied composition that might contribute to the pathology.[2] Ubiquitin is frequently present in protein aggregates indicating the activation of protein degradation pathways aimed to prevent the accumulation of misfolded and aggregated proteins. The ubiquitin-proteasome system is devoted mainly to degradation of soluble and short-lived proteins.[3] As insoluble aggregates are poor substrates for the proteasome, autophagy, a lysosomal degradation pathway, is believed to play a major role in their clearance.

Macroautophagy (autophagy hereafter) is a degradative process of cellular components that has been conserved in eukaryotic evolution. In certain circumstances, like starvation or cellular stress, parts of the cytoplasm are included in double membrane vesicles called autophagosomes that fuse later to lysosomes where they are degraded. At the molecular level, several proteins involved in the initial formation and maturation of the autophagosomes have been characterized. These proteins have been identified initially in yeast (named Atg for "Autophagosome proteins"). They are grouped in functional complexes that are necessary for the origin, elongation and completion of the autophagosomes, although the precise mechanism of action of many of these proteins and the way in which they are regulated temporarily are not yet completely understood (recent reviews of the topic include[4-7]). There are at least three signaling complexes required for autophagy:

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Tor 1 kinase, Atg1 kinase complex and the complex of class III PI3Kinase. Tor 1 controls the nutritional state of the cell regulating protein synthesis and it is also involved in autophagy through the regulation of Atg1. This signaling level is required for the induction of autophagy. Tor 1 is in turn being regulated, among other pathways, by the key kinase AMPK, a master regulator of the cellular energetic metabolism.[8,9] The PI3K complex is composed of a ser/thr kinase (Vps34), Vps15 and a coiled-coil containing protein named Vps30/Atg6 (Beclin 1). The activation of this complex is necessary for the recruitment of additional proteins to the autophagosome membrane.

Autophagy is also induced in other circumstances like the elimination of protein aggregations, organelles or bacteria and it is therefore of immense importance in diverse pathological processes as well as in aging.[7,10,11] Loss of autophagy caused the intracellular accumulation of polyubiquitinated protein aggregates.[12,13] Formation of these aggregates and their clearance by autophagy are dependent on p62/SQSTM1, an ubiquitin-binding scaffold protein that links polyubiquitinated proteins to the autophagic machinery through the direct interaction with atg8 and polyubiquitin.[14-17]

The social amoeba *Dictyostelium discoideum* is increasingly being used as a simple model to address biological problems that are relevant to human health.[18] Its genetic tractability and the presence of many conserved genes that are absent in *Saccharomyces cerevisiae* make Dictyostelium a valuable simple model for studying the function of new proteins.[19,20] Regarding the study of protein aggregates, Dictyostelium has been used to address the formation of Hirano bodies, actin-rich inclusions frequently associated with neurodegenerative diseases.[21-23] Interestingly, Hirano bodies can be cleared by autophagy in Dictyostelium.[24]

Dictyostelium cells feed on bacteria by phagocytosis and remain in the form of individual cells while bacteria are present. However, when bacteria are exhausted, starvation triggers a process of cellular aggregation and development leading to the formation of fruiting bodies containing spores that allow Dictyostelium to survive.[25] Autophagy is essential for Dictyostelium to get through starvation and complete its developmental program.[26] Multiple origins of nascent autophagosomes are formed in mammals and Dictyostelium, in contrast with *S. cerevisiae* where they are concentrated in a single location of the cytoplasm (called PAS, "Pre-autophagosomal structure or phagosome assembly site"). Autophagosomes appear as a punctate pattern in the cytoplasm when they are analyzed using specific autophagosome markers like Atg8.[27,28] Autophagy mechanisms are highly conserved and the genes involved are present in Dictyostelium and humans. The function of many of these genes has been studied in Dictyostelium.[26-30] Since autophagy in this model system is necessary for several aspects of its life cycle (such as survival under starvation and the completion of its developmental program), the lack of this process leads to phenotypes that are easily recognized. Intriguingly, Dictyostelium autophagy mutants vary in the severity of their phenotypes and the precise cause of these differences are not known.

Fruiting body formation in Dictyostelium requires the differentiation of cells into stalk and spores. The spores will maintain the life cycle by germinating when environmental conditions are adequate. In contrast, stalk cells sacrifice themselves to allow a better dispersal of their siblings. Stalk cells die by a specific programmed cell death that has the characteristics of autophagic cell death.[31-34] In vitro induction of this cell death in Dictyostelium requires at least three components including starvation and the presence of the extracellular signaling molecules cAMP and DIF-1.[34-37]

Vmp1 is a Dictyostelium protein identified in a functional genomic study[19,20,38] and highly similar proteins can be recognized in other organisms including human but it is absent in fungi.[39] The social amoeba is consequently the simplest experimental model to study this protein. Moreover, the expression of a Vmp1 mammalian homologue in the Dictyostelium mutant is able to complement the phenotype suggesting a functional conservation across species and validates Dictyostelium as experimental model to analyze Vmp1 function.[38] Besides the presence of several putative transmembrane domains, Vmp1 does not show any recognizable functional motif and this has complicated its study. As a matter of fact, the function of mammalian Vmp1 is controversial since it has been involved in unrelated processes such as autophagy and cell adhesion.[40,41] In Dictyostelium Vmp1 is localized in the endoplasmic reticulum and has been involved in diverse membrane-traffic-dependent processes.[38,39]

In this study we show that Vmp1 is required for autophagy in Dictyostelium and its absence causes defects in autophagic cell death and the accumulation of ubiquitin-positive protein aggregates. These aggregates contain, among other proteins, p62 and Atg8, revealing a remarkable similarity to other ubiquitin-positive aggregates found in neurodegenerative diseases. We hope that this simple cellular model will shed some light on the basic principles governing autophagy, protein aggregation and cell survival. We have extended our studies to other well-characterized autophagy Dictyostelium mutants to show that protein aggregates are formed in those mutants with a stronger developmental phenotype. Remarkably, *vmp1*$^{-}$ mutant cells show the most severe phenotype among the autophagy mutants, stressing its potential role in human disease.

Results

Vmp1 localization and its role in starvation and autophagic cell death. Our previous studies in Dictyostelium *vmp1*$^{-}$ mutant suggested its possible role in autophagy. In order to further validate this hypothesis, we first wanted to determine if Vmp1 is necessary for autophagy-dependent processes that are essential for Dictyostelium such as cell survival under starvation and the induction of autophagic cell death (ACD). ACD takes place during the terminal differentiation of stalk cells in Dictyostelium development and is necessary for the correct formation of the stalk, a structure required for fruiting body formation and spore dispersal. ACD can be induced in vitro under the appropriate extracellular signals.[32]

Wild-type (WT) and mutant cells were washed free of nutrients and resuspended in PDF buffer for starvation survival assay. Cell viability at different times was measured by scoring the

colony-forming units after plating the cells in association with bacteria. A dramatic reduction in cell viability for the mutant cells was observed within the first three days while the WT strain retained high viability for the same period of time (**Fig. 1A**).

When starvation is combined with cAMP and DIF-1 stalk cells differentiate in vitro and die by a process dependent on autophagy.[36] As shown in **Figure 1B**, control wild-type cells under starvation formed small aggregates of cells surrounded by extracellular sheath (marked by an asterisk). However, when wild-type cells were starved and treated with cAMP and DIF-1 they differentiated and became highly vacuolized, a specific feature of ACD and a previous step before membrane disruption. Conversely, mutant cells were unable to aggregate and remained as single cells under control conditions. They were also insensitive to the induction of ACD by the treatment with cAMP and DIF-1 as evident by the absence of vacuolization. In accordance with these results Dictyostelium development is completely blocked at the aggregative stage either in filter conditions or in association with bacteria.[38]

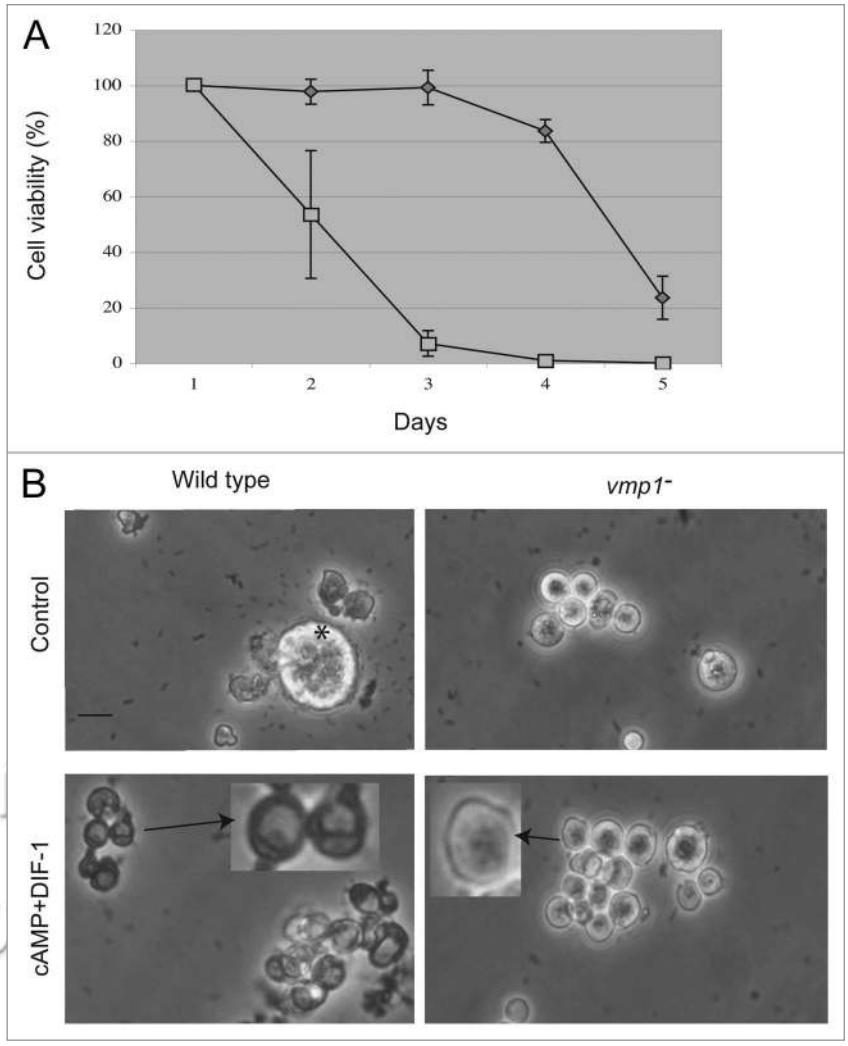

Figure 1. Vmp1 is required both for survival upon starvation and autophagic cell death in Dictyostelium. (A) Dictyostelium WT and mutant cells were washed free of nutrients and maintained in starvation for 5 days. At the indicated times viability of the cells were measured. Viability of the mutant cells dropped very rapidly. The data correspond to three independent experiments. Bars represent the standard deviation of the mean. (B) Autophagic cell death was induced in WT and *vmp1- mutant* cells. Control conditions correspond to cells kept in starvation for the same period of time without treatment. Under these control conditions (starvation in buffer), WT cells remained as single cells except for some aggregation centers (marked by an asterisk) composed of cells embedded in extracellular sheath. Mutant cells did not show any sign of aggregative behavior remaining as single round cells. WT cells treated with cAMP and DIF-1 formed a huge vacuole as they differentiated to stalk cells. The mutant cells did not show vacuolization. Bar: 10 μm.

It has been previously described that Vmp1 colocalized with Atg8, a marker of autophagosomes and induced autophagosome formation in pancreatic acinar cells.[42] Therefore, we next asked whether or not Vmp1 is localized in autophagosomes in Dictyostelium cells. Our own previous studies using a strain expressing Vmp1 fused to GFP showed colocalization with the ER marker PDI.[38] The same strain expressing Vmp1-GFP was now transformed with the autophagosome marker RFP-Atg8. The resulting strain showed a characteristic autophagosome punctate pattern as described previously.[26] Cells from this strain were prepared for immunofluorescence to detect PDI. Confocal analysis showed colocalization of Vmp1 with the ER-marker PDI as described previously (**Fig. 2**). Interestingly, a proportion of the RFP-Atg8-positive structures showed a clear colocalization with Vmp1-GFP and PDI as shown in **Figure 2**. After counting the signals of at least 50 cells we found 50% colocalization between Vmp1 and the markers both during growth (HL5) and starvation conditions (PDF). These observations indicate that Vmp1 in Dictyostelium is present in the ER and autophagosomes suggesting a possible role in autophagosome biogenesis or maturation.

Aberrant autophagosome formation in cells lacking Vmp1. The localization of Vmp1 in autophagosomes and its role in cell survival under starvation, autophagic cell death and development suggested a possible role in autophagosome formation. To test this hypothesis *vmp1* gene was disrupted in a strain expressing the autophagic marker GFP-Atg8. The resulting strain showed the expected phenotype as previously described (data not shown). Wild-type and mutant strains expressing GFP-Atg8 were analyzed in vivo by confocal microscopy. It should be noted that in vivo analysis provided a better resolution of autophagosome visualization than that obtained in fixed cells. This allowed us to detect slight differences in the fluorescent pattern in wild-type cells between growth conditions (HL5) and starvation (PDF). During growth there was a very dynamic pattern, mostly formed by puncta (**Fig. 3A**). However, during starvation the autophagosomes became larger and most of them showed a clear vesicle appearance (**Fig. 3B**). A count of the fluorescence signal in 35 cells

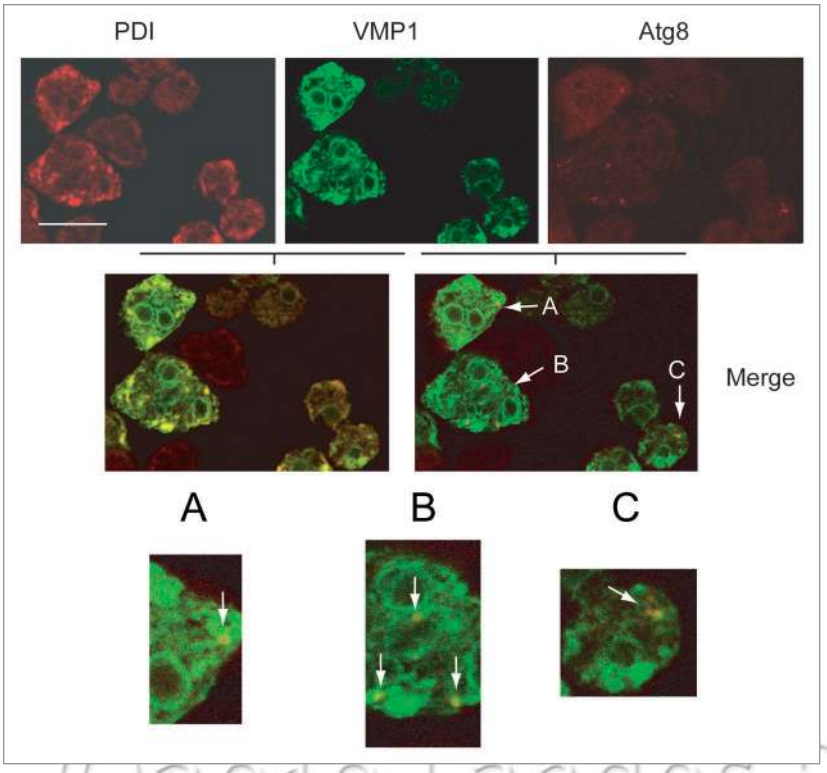

Figure 2. Vmp1 colocalizes with autophagosome marker RFP-Atg8. Cells expressing Vmp1-GFP and RFP-Atg8 were prepared for immunolocalization of PDI, an ER specific marker. A secondary antibody conjugated with far-red Alexa-647 was used to discern the three fluorophores simultaneously by confocal microscopy. To facilitate the visualization of the experiment the fluorescence corresponding to PDI and Atg8 (Alexa and RFP) was merged to that of Vmp1 (GFP) in a sequential two-step fashion. High magnification of the cells from the merged Vmp1-Atg8 (A–C) are displayed below to show the colocalization. Bar: 10 μm.

taken randomly in each condition showed that while only 29% of the signal were vesicle-like in HL-5, this proportion increased to 61% in starvation (PDF) conditions. However, the fluorescence pattern in mutant cells was clearly different showing a huge accumulation of GFP-Atg8, irrespective of the growth or starvation conditions. Video-lapse of mutant cells clearly showed the static nature of the GFP-Atg8 signal (**Suppl. Movie 1**) with no formation of vesicle-like autophagosomes. In contrast, the WT cells in a similar temporal framework showed a very dynamic pattern of multiple foci moving rapidly and forming vesicle-like structures (**Suppl. Movie 2**).

Ubiquitin-positive protein aggregates in *vmp1*⁻ mutant cells. There is a growing body of evidence that autophagy dysfunction might contribute to the accumulation of protein aggregates in aging and disease. These aggregates are often associated with ubiquitin suggesting that degradative pathways are being activated to avoid the deleterious effect of their accumulation. We have now addressed this issue in Dictyostelium *vmp1*⁻ mutant and other autophagy-deficient strains. Our aim was to determine the interplay between autophagy and protein accumulation and gain insight into a potential role of Vmp1 in proteinopathies.

Interestingly, as shown in **Figure 4A**, Dictyostelium *vmp1*⁻ mutant cells showed huge ubiquitin-positive aggregates as determined by immunofluorescence confocal microscopy using a specific antibody (**Fig. 4A**). The large size of the aggregate might facilitate their fractionation into an insoluble detergent-resistant fraction. To address this, cells were lysed and incubated with a buffer containing 0.1% NP40 and 1% TritonX-100 as described in materials and methods. After a low-speed centrifugation the supernatant and the pellet were subjected to western blot analysis to detect ubiquitinated proteins. As shown in **Figure 4B**, a typical signal marking a wide region of high molecular size proteins was accumulated in the pellet fraction of the mutant cells. This simple method confirmed the insolubility of these structures and allowed us to identify some of the proteins present in the aggregates (as explained below).

We next wanted to determine if these ubiquitinated protein aggregates colocalized with the aberrant accumulation of the autophagosome marker GFP-Atg8 described in the mutant cells. WT and *vmp1*⁻ mutant strains expressing GFP-Atg8 were used to detect ubiquitinated proteins by immunofluorescence (**Fig. 5A**). WT staining using the anti-ubiquitin antibody gave a fairly uniform signal that did not colocalize with autophagosomes. However, the mutant strain showed a clear colocalization of these markers suggesting that GFP-Atg8 is being recruited to these ubiquitin-positive aggregates. We next asked whether or not these aggregates colocalize with lysosomes as this would indicate a functional autophagy flux. As expected colocalization was not found between the aggregates and lysosomes as detected by lysotracker staining (**Fig. 5B**).

Autophagy is required for the clearance of ubiquitin-positive protein aggregates. Dictyostelium is a suitable model for the study of autophagy and several mutants affecting key autophagy genes have been generated.[26,27] The multicellular development of this organism is affected by autophagy dysfunction but the severity of the phenotype was found to vary among the mutants. It is not clear how differences in the level of autophagy can affect specific aspects of development. The presence of protein aggregates is believed to alter cell function in many different ways. The cause of these effects is not well understood but several lines of evidence suggest that aggregates might sequester proteins required in other cellular functions, in addition to the possible steric effects that might affect cell movement or intracellular traffic. We wanted to determine whether ubiquitin-positive aggregates are also present in other well-defined autophagy mutants and whether or not the severity of their phenotype correlate with the presence of protein aggregates.

We have used Dictyostelium *atg1*⁻ mutant cells and *atg1*⁻ mutant cells expressing GFP-Atg8 to study the presence of ubiquitinated-protein aggregates by immunofluoresce and western blot. As previously described for *vmp1*⁻ mutant, we found the presence of large ubiquitin-positive aggregates in *atg1*⁻ cells and

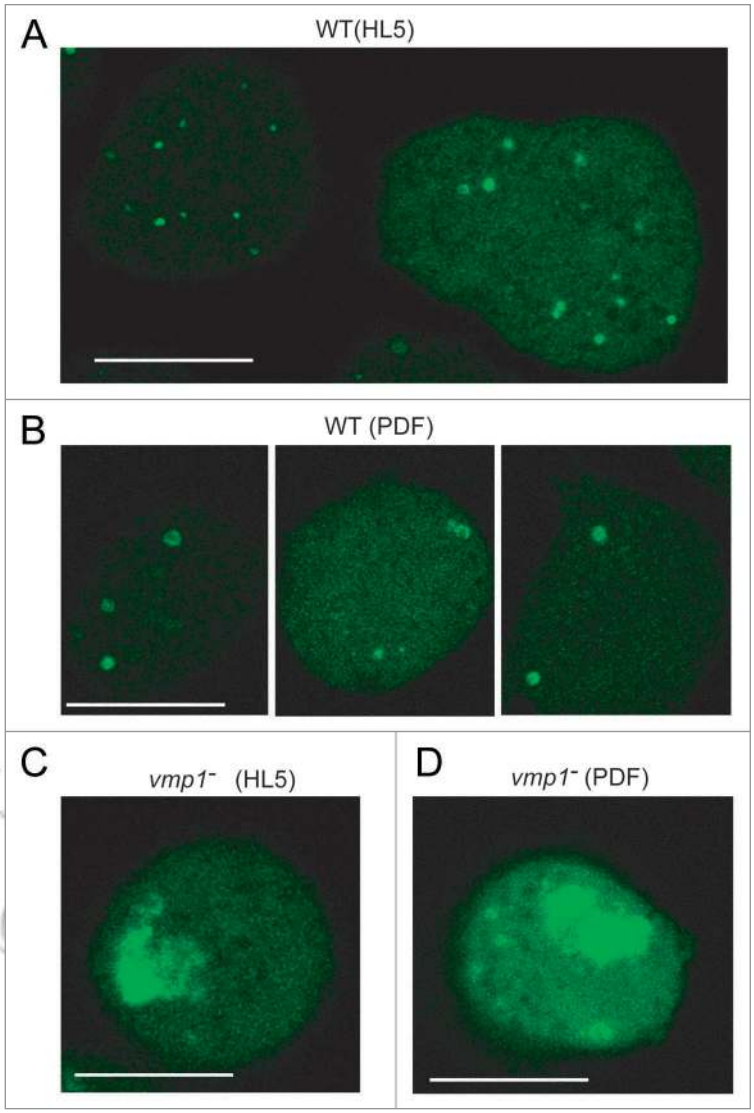

Figure 3. Aberrant autophagosome formation in *vmp1*- Dictyostelium cells. In vivo confocal microscopy of GFP-Atg8 autophagosome marker in WT and *vmp1*- cells. (A and B) Wild-type cells show a different pattern under growth (HL5) or starvation (PDF) conditions. During starvation cells had fewer structures but they were larger and most of them showed cup-like or vesicle-like appearance. However, during growth conditions most of the signals displayed a punctate pattern. (C and D) Mutant cells showed a strong aberrant GFP-Atg8 fluorescence under growth or starvation conditions. Bar: 10 μM.

colocalization of these aggregates with GFP-Atg8 (**Fig. 6A**). The size of the aggregates and the proportion of cells containing them were not as prominent as in *vmp1*- cells. The presence of these ubiquitin-protein aggregates was also tested by immunofluorescence in Dictyostelium *atg5*-, *atg6*-, *atg7*- and *atg8*- mutants. As shown in **Figure 6B**, *atg5*- and *atg7*- mutants had ubiquitin-positive accumulations. However these aggregates were not as noticeable as in *vmp1*- or *atg1*- mutants. Ubiquitin-positive aggregates were not detected in *atg6*- and *atg8*- mutant cells (the later not shown). Detection by western blot of ubiquitinated proteins in the pellet fraction after detergent cell lysis only showed clear accumulation in *atg1*- mutant. Interestingly, there is a good correlation between the severity of the phenotype of the different mutants and the presence of these protein aggregates as shown in **Table 2**.

Protein aggregates contain ubiquitin and other ubiquitin-related proteins. As we have shown above, the ubiquitinated protein aggregates present in *vmp1*- *mutant* cells can be separated by a low-speed centrifugation after detergent lysis. We took advantage of this property to identify proteins present in the aggregates. Wild-type and mutant cells were lysed under those conditions, centrifuged and the pellet solubilized in SDS-loading buffer. High molecular proteins were separated by SDS-PAGE and the gel stained with commassie. We found a strong enrichment in protein bands in the mutant pellet that were barely visible in wild type (data not shown). Bands were excised and processed for MALDI-TOF analysis. **Table 1** shows a list of the identified proteins. As expected, we found the presence of ubiquitin, presumably bound to other proteins of high molecular size. Interestingly, the analysis detected the presence of a putative Dictyostelium homologue of p62

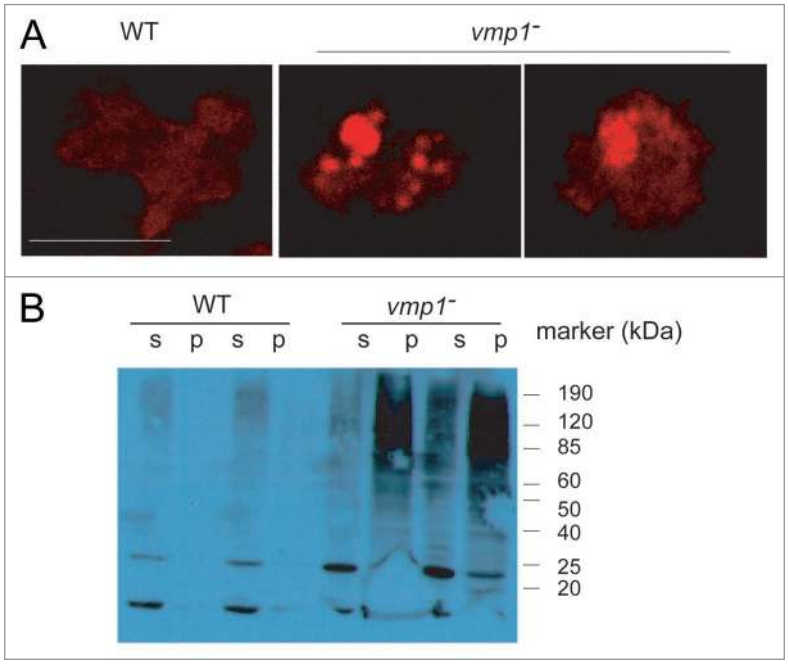

Figure 4. Ubiquitin-positive protein aggregates in *vmp1*- mutant. (A) Ubiquitin-positive aggregates were analyzed by immunofluorescence using anti-ubiquitin antibody. WT showed a uniform staining while most of the mutant cells showed huge accumulations. (B) WT and mutant cells were lysed in buffer containing 1% Triton X-100 and 0.1% NP-40. After two centrifugation steps the pellets and supernatants were separated by SDS-PAGE and transferred to a filter for western blot to detect ubiquitinated proteins. A representative experiment of two independent samples is shown. The lanes corresponding to the protein pellets of the mutant strain showed a strong signal at high molecular size. Bar: 10 μm.

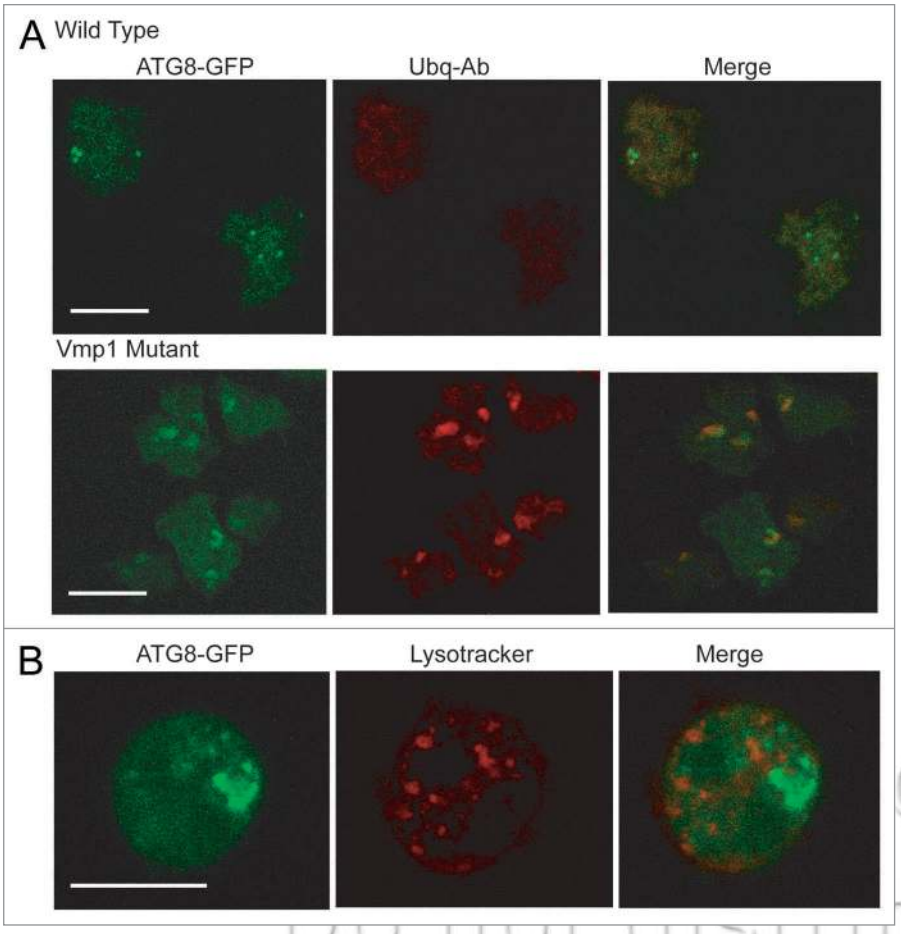

Figure 5. GFP-Atg8 marker colocalizes with ubiquitin-positive aggregates. (A) WT and *vmp1*⁻ mutant strains expressing the autophagosome marker GFP-Atg8 were prepared for immunodetection of ubiquitin-positive protein aggregates. Colocalization of both markers can be observed in the mutant aggregates. (B) Lysosomes were labeled with lysotracker in the *vmp1*⁻ mutant strain expressing the autophagosome marker GFP-Atg8. The protein aggregates were not found in close proximity to the lysosomes. Bar: 10 μm.

(DDB_G0270098), a protein involved in directing ubiquitinated protein aggregates to autophagosomes and, like ubiquitin, is often found accumulated in protein aggregation diseases. A comparative analysis of DDB_G0270098 primary sequence with the human p62 is shown in **Supplementary Figure 1**. The Dictyostelium protein contains the characteristic functional motifs present in the human p62 protein such as the PB1 domain required for oligomerization, the ubiquitin-associated domain (UBA) necessary for the interaction with ubiquitin and a ZZ-type zinc finger domain.[43] Other ubiquitin-related proteins have been detected such as DDB_G60269462, an ubiquitin-domain containing protein and DDB_G0284757, an ovarian tumor (OTU)-domain containing protein. The OTU-domain family comprises a group of putative proteases involved in de-ubiquitination processes.[44,45] Our analysis also detected the presence of other proteins potentially involved in other cellular functions such as DDB_G0291127, a major vault protein; DDB_G0289467, an AAA ATPase domain-containing protein and other proteins of unknown function.

Discussion

Vmp1 is a multispanning membrane protein of unknown molecular function. Its subcellular localization suggests multiple cellular roles not yet fully elucidated. Our results indicate that Vmp1 is an ER-resident protein in Dictyostelium as also described in Drosophila and plants. In Drosophila, Vmp1 (known as TANGO-5) was identified in a functional genomic RNAi screening as a protein required for protein secretion and Golgi organization.[46] Our previous results in Dictyostelium also indicated that Vmp1 is necessary for protein secretion and other membrane-traffic processes.[38] We postulate a possible traffic defect originating from the ER and affecting multiple membrane-traffic-dependent processes.[38] Intriguingly a recent report showed that Vmp1 was located in the plasma membrane playing a role in cell-cell contact in kidney cancer cell line Caki-2.[47] However, localization studies in other mammalian cell lines (HELA and NIH3T3) showed that Vmp1 was located in autophagosomes.[40]

Our results now seem to reconcile in part some of these data. At least in Dictyostelium Vmp1 can be located simultaneously in the ER and autophagosomes suggesting a functional interplay between the ER and autophagy. However, we have not found the protein in the plasma membrane. According to our results in Dictyostelium, a recent report showed evidence that cup-shaped protrusions from the ER, the omegasomes, might serve as platforms for autophagosome formation in mammalian cells.[48,49] Moreover, it has been described that ER-phagy, a form of autophagy that selectively include ER membranes, might use ER as a membrane source for autophagosome biogenesis.[50] The interplay between the ER and autophagy has also been revealed in yeast where membrane traffic from the ER is somehow required in autophagosome formation.[51] The physical presence of Vmp1 in a proportion of autophagosomes leave open the possibility of a direct role, perhaps by supplying or regulating membrane constituents. In agreement with this hypothesis a protein-protein interaction of Vmp1 with Beclin 1, a mammalian autophagy initiator, has been reported in mammalian cells.[40] More work will be required to test these hypotheses and determine the precise molecular function of Vmp1 in autophagy.

Autophagy is required for several aspects of Dictyostelium life cycle including survival of starvation, morphogenesis and differentiation into stalk cells, a process that takes place by autophagic cell death.[26,27,34,35,52] We have now shown that all these aspects are dependent on Vmp1. Confocal analyses with the autophagic

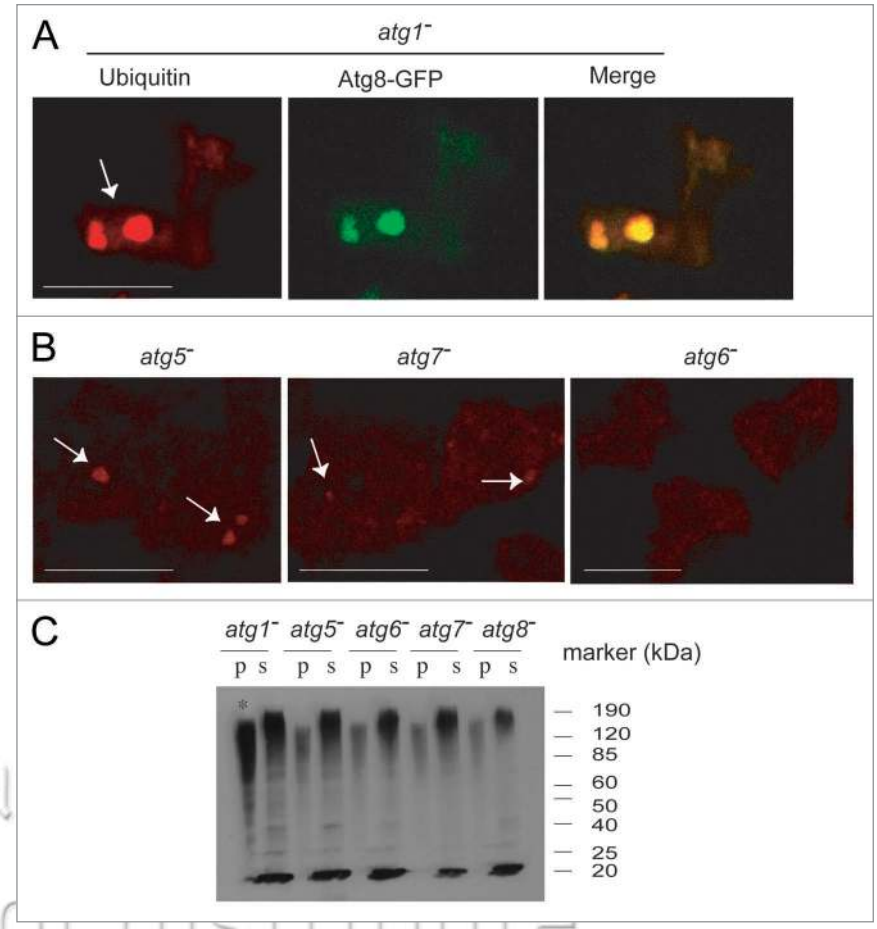

Figure 6. Presence of ubiquitin-positive protein aggregates in Dictyostelium autophagy mutants. (A) Ubiquitin-positive protein aggregates were detected by confocal immunofluorescence in *atg1*- cells expressing the autophagosome marker GFP-Atg8. (B) Presence of ubiquitinated-protein aggregates in *atg5*-, *atg7*- and *atg6*- mutants. While *atg5*- and *atg7*- mutants showed smaller protein aggregates, *atg6*- mutant cells (and *atg8*- mutant, not shown) did not show any. Bar: 10 μm.

marker GFP-Atg8 showed an aberrant pattern suggesting a defect in autophagosome formation. In accordance with this observation, electronic microscopy studies in *vmp1*- cells failed to detect normal autophagosomes.[38]

Dictyostelium cells, in contrast with the yeast model and similarly to mammalian cells, have multiple autophagosome origins displaying the typical punctate pattern when studied using the marker Atg8. Unexpectedly, this punctate pattern is also present under growth conditions while classical TEM studies have shown that the formation of autophagosome vesicles in Dictyostelium was only triggered by starvation and few autophagosomes were detected during growth.[53] Our in vivo confocal studies in wild type have shown a change in the punctate pattern between growth and starvation that might explain this discrepancy. During growth we found that most of the GFP-Atg8 signals displayed a punctate appearance and only few vesicle-like autophagosomes could be clearly observed. However, during starvation, the number of puncta was reduced and the vesicle-like appearance of the autophagosomes became more evident. The punctate pattern observed under growing conditions might reflect initial stages of autophagosome formation. The completion of these foci into vesicle-like autophagosomes would probably require additional signaling events triggered by starvation. Additional work would be required to test this hypothesis. In any case, this normal pattern observed in wild type was severely altered by the lack of Vmp1 as the autophagosome marker appeared aggregated both under growth and starvation conditions.

Protein aggregation has an enormous interest as it is involved in many human diseases. The presence of mutated aggregate-prone proteins or dysfunction in protein degradation pathways, including autophagy, can contribute to this phenotype. We now report that the lack of Vmp1 in Dictyostelium leads to the accumulation of detergent-resistant protein aggregates containing ubiquitinated proteins. We have partially characterized these aggregates to determine the presence of other proteins. Interestingly, among the identified proteins we found DDB_G0270098, a putative Dictyostelium homologue of p62. This hypothesis is supported by sequence comparison since p62 in Dictyostelium has not yet been characterized functionally. DDB_G0270098 is the most similar protein to mammalian p62 at the amino acid level and more importantly, it contains the expected functional motifs required for p62 multimerization and interaction with Atg8 and ubiquitin. Interestingly GFP-Atg8 was also found to be present in the protein aggregates by confocal analysis. In mammalian cells p62 plays a key role in the formation of ubiquitinated aggregates and provides a link with the autophagic machinery by its interaction with Atg8. Our data suggest the possibility of a similar mechanism in Dictyostelium. The inability of *vmp1*- cells to clear these aggregates by autophagy would explain their accumulation as described in mouse models where autophagy has been disturbed by mutations in *atg5* and *atg7*.[13,54]

Table 1. Proteins identified by MALDI-TOF in the protein aggregates

Dicty-base entry	Domains
DDB_G0289467	AAA ATPase domain-containing protein
DDB_G0276361	Hypothetical protein of unknown function
DDB_G0291127	Major vault protein
DDB_G0282295	Ubiquitin
DDB_G0288947	Hypothetical protein of unknown function
DDB_G0284757	OTU domain-containing protein
DDB_G0269462	Ubiquitin domain-containing protein
DDB_G0270098	Ubiquitin-associated (UBA) domain-containing protein
DDB_G0292188	Hypothetical protein of unknown function
DDB_G0269482	Hypothetical protein of unknown function

Table 2. Autophagy mutants in Dictyostelium and their phenotype

Mutant	Ubiq. + aggregates	Growth	Starvation survival	Development	References
vmp1$^-$	+++	+++	+++	+++	Calvo-Garrido et al. 2008[38]
atg1$^-$	+++	++	+++	+++	Otto et al. 2004[27]
atg5$^-$	++	-	++	++	Otto et al. 2003[26]
atg7$^-$	+	-	++	++	Otto et al. 2003[26]
atg6$^-$	-	-	++	+	Otto et al. 2004[27]
atg8$^-$	-	-	++	+	Otto et al. 2004[27]

+++, severely affected; ++, affected; +, slightly affected; -, similar to wild type.

It should be noted that Atg8, that we have observed to be present in the aggregates by means of confocal microscopy was not detected by MALDI-TOF of partially purified aggregates. It is possible that our simple experimental conditions might solubilize some of their constituents. Further analysis using different conditions and more sophisticated protein purification protocols would be necessary for a comprehensive analysis of the molecular composition of these aggregates.

The similarity of these aggregates with those found in neurodegenerative diseases highlights the interest of Dictyostelium as a cellular model system in proteinopathies and their possible interplay with autophagy. Therefore, we extended our studies to other well-characterized Dictyostelium autophagic mutants. The presence of ubiquitin-positive protein aggregates in *atg1*$^-$ and to a lesser extent in *atg5*$^-$ and *atg7*$^-$ mutants confirmed the importance of autophagy in the clearance of ubiquitinated protein in Dictyostelium. However, their absence in *atg6*$^-$ and *atg8*$^-$ mutants revealed a second layer of complexity as we found a fairly good correlation between the severity of the phenotype and the presence of these protein aggregates. We have summarized these observations in **Table 2** to facilitate the comparison. *vmp1*$^-$ and *atg1*$^-$ mutants show a striking phenotypic similarity as they have reduced growth rates both in axenic media and in association with bacteria, development is blocked in the aggregative stage and the response under starvation is severely compromised. We have now found the presence of huge ubiquitin-positives aggregates in both strains and similar defects in the abnormal morphology of the autophagosome marker GFP-Atg8. However, Dictyostelium *atg5*$^-$ and *atg7*$^-$ mutants show a less severe phenotype. These proteins are part of an ubiquitin-like conjugation reaction where Atg5 is covalently conjugated with Atg12. Both mutant strains showed normal growth in contrast to *vmp1*$^-$ and *atg1*$^-$ mutant strains. Regarding development, although they have defects on aggregation when developed in bacterial lawns, they are able to form multitipped aggregates in filter development. These structures culminate to form small fruiting bodies with no spores. We have found small ubiquitin-positive protein aggregates in both strains.

Atg6, the yeast homologue of mammalian Beclin 1, and Atg8, also known as LC3 (microtubule associated protein light chain 3), show the mildest phenotype among the autophagy mutants. Development is almost normal and although they form spores, their viability is reduced. No ubiquitin-positive aggregates were detected in these strains. Therefore, the complexity of Dictyostelium autophagy phenotypes and the different degree of developmental affectations might be related to the presence of these aggregates.

It has been postulated that the presence of protein aggregates might affect cell function not only by means of its steric effects but also by sequestering other proteins that might be attracted to the aggregate by specific interaction with other existing proteins. Recently the formation of actin inclusions in Dictyostelium cells by mistargeting VASP, an actin-binding protein, to endosomes[23] has been described. These actin aggregates sequester other actin-binding proteins and endosomal proteins promoting their disappearance from the cytosol.[23] In neurodegenerative diseases, besides the major molecular constituents of protein aggregates (usually a mutated aggregate-prone protein), there have been identified other proteins that might contribute to the pathology.[2] The aggregates generated in *vmp1*$^-$ cells contained proteins that might not be related to autophagy or ubiquitin such as DDB_G0291127, a major vault protein, DDB_G0289467, an AAA ATPase domain-containing protein, and other proteins of unknown function. The presence of these proteins in the aggregates might alter their correct localization and concentration in the cell and contribute to the phenotype in intricate ways that remain to be explored.

Materials and Methods

Dictyostelium cell culture, transformation and generation of *vmp1* null mutant. Cells were grown axenically in HL5 medium or in association with *Klebsiella aerogenes* in SM plates.[55] The original *vmp1*$^-$ strain was generated in AX4 as described previously.[38] DH1 was the parental strains for the autophagy mutants *atg1*$^-$, *atg5*$^-$, *atg6*$^-$, *atg7*$^-$ and *atg8*$^-$ as well as the GFP-Atg8 expressing strains. They were kindly provided by Dr. Kessin's laboratory.[26,27] Disruption of Vmp1 in DH1 strains showed the same phenotype as previously described in AX4.[38] Transformations were carried out by electroporation as described previously.[56] For starvation assays, cells were resuspended in PDF buffer (20 mM KCl, 9 mM K_2HPO_4, 13 mM KH_2PO_4, 1 mM $CaCl_2$, 1 mM $MgSO_4$, pH 6.4) for the indicated times and cell viability was determined by counting the number of colony-forming units after plating on SM plates in association with bacteria. Disruption of Vmp1 gene in the Dictyostelium strain expressing GFP-Atg8 was performed as previously described.[38]

Immunocytochemistry and confocal microscopy. For immunocytochemistry, cells were allowed to adhere to coverslips and fixed in 2.5% paraformaldehyde (Polysciences, Inc., 00380) in

PBS for 30 minutes. After two washes with PBS, cells were permeabilized with chilled methanol during 2 minutes and incubated during 20 minutes in blocking buffer (0.2% BSA in PBS). The samples were then incubated with the first antibody in blocking buffer for 1–3 hours. After five washes with blocking buffer the appropriate secondary antibody was added at a dilution of 1/1,000 in blocking buffer for 30 minutes. After three washes with blocking buffer cells were mounted for microscopic observation. Confocal analysis was performed in a Leica TCS SP5 using a PL APO 63X/1.4-0.6 objective and a LAS-AF (Leica Application Suite) software. For excitation of GFP a 488-nm Argon laser was used. PDI antibody (221-64-1 ascitis, mouse monoclonal) was kindly provided by Pierre Cosson from the University of Geneva (Switzerland) and used at a dilution 1:1,000. Ubiquitin monoclonal antibody was from Cell Signalling (3936).

Autophagic cell death assay. Cells were washed free of nutrients in phosphate buffer and deposited in multiwell plates at 5 x 10^5 cells/ml in spore buffer (KCl 20 mM; NaCl 20 mM; $CaCl_2$ 1 mM; $MgCl_2$ 1 mM; MES pH 6.2 10 mM) containing 5 mM cAMP. After 18 hours the media was washed twice with spore medium and incubated with 100 nM DIF-1 (Biomol International, GR324) in spore buffer for 24 hours. Photographs were taken directly in the plates with an inverted Leica microscope.

Detection of ubiquitinated proteins by western blot. 5 x 10^6 cells were resuspended in 100 µl of PBS supplemented with 0.1% NP40 (Sigma, I-3021) and 1% TritonX-100 (Merck, 1.08603.1000) and incubated for 10 minutes on ice. Cells were then passed trough a syringe four times for a complete cell disruption. The extracts were then centrifuge at 4,000 rpm for 5 minutes. Supernatants were kept for further analysis and the pellets resuspended again in the same buffer and subjected to the same process. After the last centrifugation the supernatants were now discarded and the pellets were solubilized directly in 25 µl of SDS loading buffer. Pellets and the first supernatant fractions were separated by SDS-PAGE, transferred to a filter and analyzed by western blot with an anti-ubiquitin monoclonal antibody from cell-signalling (3936).

Enrichment of protein aggregates, in-gel digestion of proteins, MALDI-MS/MS and database searching. Cells were treated as described in the above section and the pellets were separated by SDS-PAGE using a 6% acrylamide concentration to allow the separation of high molecular size proteins. After coomassie blue staining the protein bands were excised manually from the gel and then digested automatically using a Proteineer DP protein digestion station (Bruker-Daltonics). The digestion protocol used was previously described,[57] with minor variations: gel plugs were submitted to reduction with 10 mM dithiothreitol (Plusone, 17-1318-02) in 50 mM ammonium bicarbonate (99.5% purity; Fluka, 09830) and alkylation with 55 mM iodoacetamide (Sigma, I-6125) in 50 mM ammonium bicarbonate. The gel pieces were then rinsed with 50 mM ammonium bicarbonate and acetonitrile (gradient grade; Sigma, 34967) and dried under a stream of nitrogen. Modified porcine trypsin (sequencing grade; Promega, V5111) at a final concentration of 8 ng/µl in 50 mM ammonium bicarbonate was added to the dry gel pieces and the digestion proceeded at 37°C for 8 h. Finally, 0.5% trifluoroacetic acid (99.5% purity; Fluka, 91699) was added for peptide extraction. An aliquot of the above digestion solution was mixed with an aliquot of cyano-4-hydroxycinnamic acid (Bruker-Daltonics, 205931) in 33% aqueous acetonitrile and 0.25% trifluoroacetic acid. This mixture was deposited onto a 600 µm AnchorChip prestructured MALDI probe (Bruker-Daltonics, 209518) and allowed to dry at room temperature. MALDI-MS/MS data were obtained in an automated analysis loop using an Ultraflex time-of-flight mass spectrometer (Bruker-Daltonics) equipped with a LIFT MS/MS device.[58] Spectra were acquired in the positive-ion mode at 50 Hz laser frequency, and 100 to 1,000 individual spectra were averaged. For fragment ion analysis in the tandem time-of-flight (TOF/TOF) mode, precursors were accelerated to 8 kV and selected in a timed ion gate. Fragment ions generated by laser-induced decomposition of the precursor were further accelerated by 19 kV in the LIFT cell and their masses were analyzed after passing the ion reflector. Automated analysis of mass data was performed using the flexAnalysis software (Bruker-Daltonics). Internal calibration of MALDI TOF mass spectra was performed using two trypsin autolysis ions with m/z = 842.510 and m/z = 2211.105; for MALDI-MS/MS, calibrations were performed with fragment ion spectra obtained for the proton adducts of a peptide mixture covering the 800–3,200 m/z region. MALDI-MS and MS/MS data were combined through the BioTools program (Bruker-Daltonics) to search a nonredundant protein databases (NCBI: National Center for Biotechnology Information; and SwissProt: Swiss Institute for Bioinformatics) using the Mascot software (Matrix Science).[59]

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Note

Supplementary materials can be found at:
www.landesbioscience.com/supplement/CalvoGarridoAUTO6-1-Sup.pdf
www.landesbioscience.com/supplement/CalvoGarridoAUTO6-1-mov1.mov
www.landesbioscience.com/supplement/CalvoGarridoAUTO6-1-mov2.mov

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