Ca\(^{2+}\)/Calmodulin-Dependent Kinase (CaMK) Signaling via CaMKI and AMP-Activated Protein Kinase Contributes to the Regulation of WIPI-1 at the Onset of Autophagy

Simon G. Pfisterer, Mario Mauthe, Patrice Codogno, and Tassula Proikas-Cezanne

Autophagy Laboratory, Interfaculty Institute for Cell Biology, Eberhard Karls University Tuebingen, Tuebingen, Germany (S.G.P., M.M., T.P.-C.); and Institut National de la Santé et de la Recherche Médicale U984, Faculté de Pharmacie, University Paris-Sud 11, Châtenay-Malabry, France (P.C.)

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ABSTRACT

Autophagy is initiated by multimembrane vesicle (autophagosome) formation upon mammalian target of rapamycin inhibition and phosphorylation of phosphatidylinositol 3-phosphate [PtdIns(3)P] generation. Upstream of microtubule-associated protein 1 light chain 3 (LC3), WD-repeat proteins interacting with phosphoinositides (WIPI proteins) specifically bind PtdIns(3)P at forming autophagosomal membranes and become membrane-bound proteins of generated autophagosomes. Here, we applied automated high-throughput WIPI-1 puncta analysis, paralleled with LC3 lipidation assays, to investigate Ca\(^{2+}\)-mediated autophagy modulation. We imposed cellular stress by starvation or administration of etoposide (0.5–50 μM), sorafenib (1–40 μM), staurosporine (20–500 nM), or thapsigargin (20–500 nM) (1, 2, or 3 h) and measured the formation of WIPI-1 positive autophagosomal membranes. Automated analysis of up to 5000 individual cells/treatment demonstrated that Ca\(^{2+}\) chelation by BAPTA-AM (10 and 30 μM) counteracted starvation or pharmacological compound-induced WIPI-1 puncta formation and LC3 lipidation. Application of selective Ca\(^{2+}\)/calmodulin-dependent kinase (CaMKK) α/β and calmodulin-dependent kinase (CaMK) VII/VII inhibitors 7-oxo-7H-[1,2]-benzimidazole[1,1-benz[deisoxoquinoline-3-carboxylic acid acetate (STO-609; 10–30 μg/ml) and 2-[(2-hydroxyethyl)-N-(4-methoxybenzenesulfonyl)amino-N-(4-chlorocinnamyl)-N-methylamino-(K; N3; 1–10 μM), respectively, significantly reduced starvation-induced autophagosomal membrane formation, suggesting that Ca\(^{2+}\) mobilization upon autophagy induction involves CaMKII/IV. By small interfering RNA (siRNA)-mediated down-regulation of CaMKI or CaMKIV, we demonstrate that CaMKI contributes to stimulation of WIPI-1. In line, WIPI-1 positive autophagosomal membranes were formed in AMP-activated protein kinase (AMPK) α1/2-deficient mouse embryonic fibroblasts upon nutrient starvation, whereas basal autophagy was prominently reduced. However, transient down-regulation of AMPK by siRNA resulted in an increased basal level of both WIPI-1 puncta and LC3 lipidation, and nutrient-starvation induced autophagy was sensitive to STO-609/KN-93. Our data provide evidence that pharmacological compound-modulated and starvation-induced autophagy involves Ca\(^{2+}\)-dependent signaling, including CaMKI independent of AMPKα1/α2. Our data also suggest that AMPKα1/α2 might differentially contribute to the regulation of WIPI-1 at the onset of autophagy.

Introduction

Autophagy is a lysosomal bulk degradation system for cytoplasmic constituents, including long-lived proteins and organelles. This process of self-digestion is constitutively active (basal autophagy) and promotes the recycling of the cytoplasm, thereby supplying macromolecules and energy for metabolic reactions (e.g., Klionsky et al., 2007). Upon cellular...
stress such as nutrient starvation, autophagy is induced above basal level and critically secures cellular survival. This cytoprotective function is compromised in a variety of age-related human diseases, hence modulating autophagy as a new therapeutic strategy has attracted attention over the last few years (Kondo et al., 2005; Mizushima et al., 2008; Fleming et al., 2011). For this aim, vital molecular details need to be addressed, such as the molecular understanding of contributing signaling pathways that regulate autophagy (Codogno et al., 1997; Chen and Klionsky, 2011).

During the process of autophagy, multimeric vesicles, autophagosomes, are formed from initial membrane templates (phagophore) that begin to sequester the cytoplasmic cargo; formed autophagosomes fuse with lysosomes to autolysosomes where cargo degradation takes place (e.g., Klionsky and Emr, 2000). Autophagosome formation is regulated by the activity of TORC1, PtdIns3KC3 complex I, and PtdIns(3)P effectors; two ubiquitin-like-conjugation systems (Atg12-Atg5 and LC3); and the Atg9 pathway, which contributes to the delivery of membrane sources (e.g., Noda et al., 2010). Ubiquitin-like conjugation systems contribute to the elongation of early autophagosomal membranes, where LC3 is conjugated to phosphatidylethanolamine (LC3 lipidation), promoting membrane tethering and hemifusion (Ohsumi 2001; Nakatogawa et al., 2007). Monitoring LC3 lipidation is used to score for autophagosome formation, and the addition of lysosomal inhibitors provides further information regarding the autophagic flux (Rubinsztein et al., 2009). The initiation of autophagosome formation is critically governed by TORC1, which inhibits autophagy; consequently, TORC1 inhibitors (e.g., rapamycin) induce autophagy (Blommaart et al., 1995). Crucial for the formation of autophagosomes is the activation of the PtdIns3KC3 complex I, including Beclin 1, Vps15, and Atg14L, which generates PtdIns(3)P, an essential phospholipid for autophagosomal membrane formation (Petiot et al., 2000; Matsunaga et al., 2009). Consequently, compounds that inhibit PtdIns(3)P generation (e.g., wortmannin) abolish autophagosome formation (Blommaart et al., 1997).

We previously identified the human WIPI family, including WIPI-1 (Atg18 in yeast), which functions as a PtdIns(3)P effector at early autophagosomal membranes (Proikas-Cezanne et al., 2004, 2007). The specific autophagosomal membrane localization of PtdIns(3)P-bound WIPI-1 is inhibited by wortmannin (Proikas-Cezanne et al., 2004) and PtdIns3KC3 down-regulation (Itakura and Mizushima, 2010), suggesting that WIPI-1 binds PtdIns(3)P generated by the PtdIns3KC3 complex I (Nobukuni et al., 2007). Rapamycin-mediated TORC1 inhibition also stimulates WIPI-1 to localize at early autophagosomal membranes, suggesting that WIPI-1 also acts downstream of mTOR activity (Proikas-Cezanne et al., 2007). To use WIPI-1 for monitoring autophagy in human cells, we employ quantitative, fluorescence-based WIPI-1 puncta-formation analyses in which basal autophagy is reflected by the number of cells that display WIPI-1 at autophagosomal membranes (WIPI-1 puncta), and induced or inhibited autophagy is reflected by the elevated or decreased number of cells displaying WIPI-1 puncta.

In this study, we used WIPI-1 puncta-formation and quantitative LC3 lipidation analyses to provide molecular details on cytosolic Ca\(^{2+}\) increase-mediated modulation of autophagy. Several studies have analyzed the role of Ca\(^{2+}\) with regard to the regulation of autophagy; however, whether Ca\(^{2+}\) contributes to the activation or inhibition of autophagy is still under investigation (Demarchi et al., 2006; Brady et al., 2007; Hayer-Hansen et al., 2007; Williams et al., 2008; Grottemeier et al., 2010; Khan and Joseph, 2010; Fleming et al., 2011). Cyttoplasmic Ca\(^{2+}\) is bound by calmodulin, which associates to and activates calmodulin-dependent kinase \(\alpha/\beta\) (CaMKK\(\alpha/\beta\)), leading to AMPK stimulation and subsequent TORC1 inhibition (Hardie, 2008; Means, 2008). This notion implies that autophagy might be activated by Ca\(^{2+}\)/calmodulin–CaMKK\(\alpha/\beta\)–AMPK–TORC1 signaling. In fact, evidence that calcium signaling contributes to the induction of autophagy was provided by using compounds that elevate cytosolic Ca\(^{2+}\), such as thapsigargin (Hayer-Hansen et al., 2007). We previously found that Ca\(^{2+}\)-mediated induction of autophagy can also bypass AMPK (Grottemeier et al., 2010), suggesting that additional routes downstream of Ca\(^{2+}\)/calmodulin could activate autophagy. Because it is known that the Ca\(^{2+}\)/calmodulin signal also activates CaMKI/II/IV apart from AMPK (Means, 2008), we asked in this study whether or not this route might contribute to the regulation of autophagy. Herein, we provide evidence that nutrient starvation and pharmacological compound-modulated autophagy mobilizes cellular Ca\(^{2+}\), in part via CaMKI independent of AMPK, to regulate the PtdIns(3)P effector WIPI-1, and LC3.

### Materials and Methods

**Reagents.** Earle’s balanced salt solution, etoside (C\(_{39}\)H\(_{42}\)O\(_{13}\), CAS 39419-42-0), 3-[N-(2-hydroxyethyl)-N-methylamino]-N'-4-chloroquinamnamylyl-N-methylamine (KN-93; C\(_{29}\)H\(_{25}\)ClN\(_{2}\)O\(_{3}\)S\(_{2}\)H\(_{2}\)PO\(_{4}\), CAS 139298-40-1), staurosporine (C\(_{29}\)H\(_{25}\)NO\(_{5}\), CAS 62996-74-1), 7-amino-4-3H-benzimidazol2[1,2-b]benz[d]isoquinoline-3-carboxylic acid acetate (STO-609; C\(_{23}\)H\(_{14}\)N\(_{2}\)O\(_{4}\), CAS 52029-86-4), thapsigargin (C\(_{29}\)H\(_{25}\)NO\(_{5}\), CAS 67526-95-8), and wortmannin (C\(_{29}\)H\(_{25}\)NO\(_{5}\), CAS 19545-26-7) were obtained from Sigma-Aldrich (St. Louis, MO); sorafenib (C\(_{29}\)H\(_{25}\)NO\(_{5}\), CAS 67526-95-8) from Selleck Chemicals (Houston, TX); Baf A1 (bafilomycin A1; C\(_{29}\)H\(_{25}\)NO\(_{5}\), CAS 88899-55-2) and DAPI (C\(_{29}\)H\(_{25}\)NO\(_{5}\), CAS 28718-90-3) from Appligene (Carlsbad, CA). Anti-AMPK-\(\alpha\) and anti-CaMKI antibodies were purchased from Cell Signaling Technology (Danvers, MA); and anti-CaMKI, anti-LC3, and anti-\(\alpha\)-tubulin antibodies from Abcam Inc. (Cambridge, MA), nanoTools (Teningen, Germany), and Sigma-Aldrich, respectively. Anti-WIPI-1 antisemur was described previously (Proikas-Cezanne et al., 2004). Anti-rabbit IgG-Alexa Fluor 546 and TO-PRO-3 was obtained from Invitrogen.

**DNA.** GFP-WIPI-1 was described previously (Proikas-Cezanne et al., 2007). GFP-ULK2 was generated by cloning human ULK2 cDNA (imaGenes clone IRTp9709B0931D) into pEGFP.C1 (XhoI). Construct integrity was confirmed by PCR, automated DNA sequencing, and protein expression analysis.

**siRNA.** Control siRNA and human AMPK\(\alpha_1/\alpha_2\) siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Control human CaMKI or CaMKIV endoribonuclease-prepared siRNAs were obtained from Sigma-Aldrich.

**Cell Culture.** Human U2OS osteosarcoma cell line (American Type Culture Collection, Manassas, VA) and human G361 malignant melanoma cell line (American Type Culture Collection) were cultured in Dulbecco’s modified Eagle’s medium (DMEM), 10% FCS, 100 U/ml penicillin/100 \(\mu\)g/ml streptomycin, and 5 \(\mu\)g/ml Plasmocin (InvivoGen, San Diego, CA) at 37\(^\circ\)C, 5% CO\(_{2}\). Stable U2OS clones expressing GFP-WIPI-1 were cultured in DMEM, 10% FCS, 100 U/ml penicillin/100 \(\mu\)g/ml streptomycin, and 5 \(\mu\)g/ml Plasmocin (InvivoGen) supplemented with 0.6 mg/ml G418 (Invitrogen) (Grottemeier et al., 2010). AMPK\(\alpha_1/\alpha_2\)-deficient and wild-type mouse embryonic fibroblasts (MEF) were cultured as described previously.
(Laderoute et al., 2006) in DMEM/10% FCS supplemented with 25 mM HEPES, pH 7.2 to 7.5, 50 μM β-mercaptoethanol, and 100 μM nonessential amino acids (all from Invitrogen).

**Transfections.** Transient transfections were conducted by using Promofectin (PromoCell, Heidelberg, Germany) for DNA plasmids, or by using RNAiMax (Invitrogen) for siRNAs according to the manufacturer’s protocols. Three hours after transfection, the transfection medium of AMPKα1/2-deficient and wild-type MEFs was replaced by normal culture medium (see Cell Culture) with either 4.5, 1.0, or 0.1 g/l glucose.

**Treatments.** Cells were treated for 1 to 3 h with thapsigargin (0.02–0.5 μM), staurosporine (0.02–0.5 μM), sorafenib tosylate (1–40 μM), etoposide (0.5–50 μM), or wortmannin (233 nM) or by amino acid and serum deprivation (Earle’s balanced salt solution, nutrient-free medium). To inhibit the autophagic flux, cell culture medium was supplemented with bafilomycin A1 (200 nM). To chelate, cytoplasmic Ca2+-cells were pretreated with BAPTA-AM (10–30 μM). For CaMK and CaMK inhibition with STO-609 and KN-93, cells were pretreated for 30 min and autophagy assays were performed for 1 h.

**Quantitative Confocal Microscopy.** Quantitative confocal microscopy of GFP-WIPI-1 puncta-positive cells using a laser-scanning microscope (LSM 510; Carl Zeiss GmbH, Jena, Germany) and a 63×, 1.4 numerical aperture, differential interference contrast, Plan-Apochromat, oil-immersion objective was performed as described previously (Proikas-Cezanne et al., 2007).

**Automated High-Throughput Image Acquisition and Analyses.** Stable U2OS GFP-WIPI-1 cells were cultured in 96-well plates. Cells were treated as indicated, fixed in 3.7% paraformaldehyde, and stained with DAPI (5 μg/ml in phosphate-buffered saline). An automated imaging platform equipped with a Nikon Plan Fluor ELWD 40 × 0.6 objective was used for automated image acquisition (In Cell Analyzer 1000; GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) and analyses (In Cell Analyzer Workstation 3.4). Twenty images per well (approximately 20 cells per image) were routinely acquired from each experimental treatment, and the dual area object assay used to classify cells as WIPI-1 puncta-positive or -negative (see Fig. 1). Applying a decision tree to automatically analyze the acquired images (Grotemeier et al., 2010), cells were detected by using DAPI (cell nuclei) and corresponding GFP images. Using the parameters inclusion size and intensity versus cell intensity, cells were further classified to measure the resulting GFP-WIPI-1 puncta-positive or puncta-negative cells. High-intensity GFP inclusions (terminology from Workstation 3.4), counted per puncta-positive cell, provided the measure of the average of GFP-WIPI-1 puncta per puncta-positive cell.

**Quantitative LC3 Immunoblotting.** Cells were washed twice with phosphate-buffered saline and lysed with Tris-buffered saline/1% Triton X-100. Cell lysates were centrifuged at 15,000 g for 10 min at 4°C to remove nuclei and cell debris. Supernatants were mixed with 4× Laemmli loading buffer (200 mM Tris, pH 6.8, 5 mM EDTA, pH 8.0, 50% glycerol, 8% SDS, 200 mM dithiothreitol, 10% β-mercaptoethanol, and 0.1% bromphenol blue). Alternatively, cells were lysed by using hot Laemmli buffer, and the chromatin was disintegrated by using a 23-gauge needle. Protein extracts were subjected to SDS-polyacrylamide gel electrophoresis and Western blot analysis using anti-LC3 and anti-α-tubulin antibodies. Quantifications of Western blot results were conducted using ImageQuant 5.1 (GE Healthcare), and LC3-II signal intensities were normalized over tubulin.

**Statistical Analyses.** p Values were calculated by using two-tailed heteroscedastic t-testing.

**Results**

**Automated GFP-WIPI-1 Puncta-Formation Analysis Monitors PtdIns(3)P-Dependent Autophagy Modulated by Pharmacological Compound Administration.** WIPI-1 puncta-formation analysis is used to assess PtdIns(3)P-
dependent mammalian autophagy established for visualizing endogenous WIPI-1 or overexpressed variants of tagged WIPI-1 proteins followed by quantitative fluorescence microscopy (Prokask-Cezanne et al., 2007). In this study, we employed our recently established procedure for automated GFP-WIPI-1 puncta image acquisition and analysis (Grotemeier et al., 2010). In brief, stable GFP-WIPI-1 U2OS cells were assayed in 96-well plates and fixed, and cell nuclei stained with DAPI. GFP and DAPI images were automatically acquired in each field (20 cells/field; 20 fields/well) (Fig. 1A). Automated quantitative analyses included the recognition of 1) individual cells (Fig. 1B) by using both DAPI and GFP images (~400 cells/well), and 2) GFP-WIPI-1 puncta by applying a decision tree based on puncta intensity and size thresholds (Fig. 1C). During image analyses, a dynamic heat map presents the range of GFP-WIPI-1 puncta-positive cells detected per well (Fig. 1D). Here, we analyzed up to 5100 individual cells per treatment from two to six independent experiments (Figs. 2, 3A, 3B, 3C, 4, 5B, 5C, 6B, 6C). Using this system, we assessed the effects of the anticancer drugs sorafenib (SF) and etoposide (EP), the pan-kinase inhibitor staurosporine (SP), and the sarco(endo)plasmic reticulum Ca\(^{2+}\) ATPase inhibitor thapsigargin (TG) on WIPI-1 puncta formation (Fig. 2). Because it has been demonstrated that serum influences compound effects on autophagy (Yang et al., 2010), we conducted the experiments in the presence (Fig. 2, A and B) or absence (Fig. 2C) of 10% serum during the different treatments. Stable GFP-WIPI-1 U2OS cells were treated for 1, 2, or 3 h using five different concentrations for each drug. The concentrations for both TG and SP ranged between 0.02 and 0.5 \(\mu\)M, for SF, between 1 and 40 \(\mu\)M, and for EP, between 0.5 and 50 \(\mu\)M. As a positive control for the induction of autophagy, the cells were starved by using nutrient-free medium lacking amino acids and serum (NF); as a negative control for the inhibition of overall basal level of autophagy is already increased, thereby diminishing the compound-mediated effect. In contrast, SF or EP treatments, especially those using lower concentra-

![Fig. 2. Compound-mediated modulation of WIPI-1 puncta formation. The effect of thapsigargin, staurosporine, sorafenib, or etoposide treatment on WIPI-1 puncta formation was assessed in the presence (A and B) or absence of serum (C) over time. Stable GFP-WIPI-1 cells were treated with TG (0.02, 0.04, 0.1, 0.25, 0.5 \(\mu\)M), SF (0.02, 0.04, 0.1, 0.25, 0.5 \(\mu\)M), SP (0.02, 0.04, 0.1, 0.25, 0.5 \(\mu\)M), SF (1, 5, 10, 20, 40 \(\mu\)M), and EP (0.5, 1, 5, 25, 50 \(\mu\)M) for 1, 2, or 3 h. Nutrient starvation (NF) was used as positive control and WM treatment as negative control. Representative images from automated WIPI-1 puncta-formation analysis are shown. A, 40 nM TG, 100 nM SF, 40 \(\mu\)M EP, and 50 \(\mu\)M Etoposide for 3 h. Up to 3000 individual cells were quantified for every single treatment, \(n=2\) to 3. B and C, \(p<0.001\). Scale bar, 20 \(\mu\)m.](https://molpharm.aspetjournals.org/doi/10.1124/mol.117.1069)
tions, clearly resulted in a more prominent increase of WIPI-1 puncta-positive cells under serum-free conditions.

**Ca²⁺ Chelation Inhibits Nutrient Starvation-Mediated Autophagy and the Effects of Pharmacological Compounds on WIPI-1 and LC3.** We reported previously that thapsigargin-mediated cytosolic Ca²⁺ increase stimulated the localization of both WIPI-1 and LC3 at autophagosomal membranes (Grotemeier et al., 2010). Here, we sought to determine whether Ca²⁺ availability is generally required for autophagosome formation. U2OS cells stably expressing GFP-WIPI-1 were incubated for 1 h with control medium (CM), NF, TG (40 nM), SP (100 nM), SF (40 µM) or wortmannin (WM; 233 nM) with or without 10 or 30 µM BAPTA-AM (Fig. 3, A–C). Automatically acquired fluorescence microscopy images are presented (Fig. 3A) as well as the heat map from automated WIPI-1 puncta-formation analyses (Fig. 3B) and the statistical analyses of up to 2600 individual cells per treatment (Fig. 3C). This quantification demonstrates that BAPTA-AM-mediated Ca²⁺ chelation significantly prevents the increase of WIPI-1 puncta-positive cells upon NF, TG, SP, or SF treatments (Fig. 3C). In parallel, human U2OS osteosarcoma cells were treated with CM or NF for 1 h with or without BAPTA-AM; in addition, the autophagic flux was analyzed by introducing the lysosomal inhibitor Baf A₁ (Fig. 3D). We found that BAPTA-AM treatment drastically reduced LC3 lipidation under these conditions (Fig. 3D; Supplemental Fig. S1). These results imply that, in general, autophagy is prevented by Ca²⁺ chelation, indicating that Ca²⁺ availability is necessary for the induction of autophagy via WIPI-1 and LC3.

**Inhibition of CaMKI Signaling Reduces the Formation of WIPI-1 Positive Autophagosomal Membranes.** We found previously that the thapsigargin effect on WIPI-1 and LC3 is inhibited by using the selective CaMKKα/β inhibitor STO-609 (Grotemeier et al., 2010). Here, we used STO-609 during nutrient starvation of GFP-WIPI-1–expressing U2OS osteosarcoma cells (Fig. 4A). By automated analyses of up to 5100 individual cells, we found that STO-609 addition to NF, significantly reduced the number of GFP-WIPI-1 puncta-positive cells compared with NF alone (Fig. 4A, hatched symbols). However, even in the presence of STO-609 (10–30 µg/ml) nutrient starvation still significantly induced WIPI-1 puncta formation (Fig. 4A, star symbols).

This shows that CaMKKα/β inhibition partially reduces the nutrient-starvation-mediated formation of WIPI-1-positive autophagosomal membranes. Because CaMKKα/β acts upstream of AMPK, this result indicates that the Ca²⁺/CaMKKα/β AMPK signaling cascade contributes to starvation-induced WIPI-1-positive autophagosomal membrane formation. Because the inhibition by STO-609 on WIPI-1 was partial, we investigated whether the AMPK-independent CaMK signaling route might also contribute to the regulation of WIPI-1. Administration of the selective CaMKI/II/IV inhibitor KN-93 (1, 5, or 10 µM) showed that 5 or 10 µM KN-93 significantly reduced the number of WIPI-1 puncta-positive cells induced by NF (Fig. 4B, hatched symbols). Again, this reduction was partial, as found using STO-609. However, because either selective inhibitor, STO-609 or KN-93, affected WIPI-1 puncta formation induced by nutrient starvation, our results indicate that CaMKI/IV (Means, 2008) also contributes to the regulation of WIPI-1. In support, STO-609 and KN-93 cotreatments (10, 20, or 30 µg/ml STO-609 in combination with 1, 5, or 10 µM KN-93) for 1 h in nutrient-free medium reduced WIPI-1 puncta-positive cells to basal control levels (Fig. 4B). Furthermore, autophagic flux assays on LC3 lipidation further confirmed this finding, in that LC3 lipidation decreased upon coadministration of STO-609 and KN-93 (Fig. 4C; Supplemental Fig. S2).

These results warranted address of the functional involvement of CaMKI and CaMKIV in regulating WIPI-1 by siRNA-mediated down-regulation. By introducing human siCaMKI or siCaMKIV in the GFP-WIPI-1 cell line, both proteins were prominently down-regulated; by combining siCaMKI and siCaMKIV, simultaneous down-regulation of CamKI/IV was achieved (Fig. 5A). Furthermore, upon 48 h of silencing, we treated the cells with CM or NF and coanalyzed the number of WIPI-1 puncta per puncta-positive cell (Fig.
Although nutrient starvation resulted in a significant increase in the number of WIPI-1 puncta-positive cells (Fig. 5C), the number of WIPI-1 puncta per puncta-positive cell was significantly reduced in both nutrient-rich (CM) and NF conditions when CaMKI was down-regulated (Fig. 5B). Furthermore, although we observed a significant increase in WIPI-1 puncta per puncta-positive cell upon nutrient-starvation induced autophagy in siRNA-transfected cells targeting either CaMKI (siCaMKI), CaMKI/IV (siCaMKI/IV), or CaMKIV (siCaMKIV), this elevation was much less prominent compared with the siControl cells (Fig. 5B). This result, a partial inhibition of WIPI-1 puncta formation upon down-modulated CaMKI/IV protein levels, correlates with the partial inhibition of starvation-induced WIPI-1 puncta formation upon KN-93 treatment alone (Fig. 4B). The reduction of WIPI-1 puncta was not further lowered when CaMKIV was simultaneously down-regulated along with CaMKI (Fig. 5B), suggesting that the results achieved by using STO-609 and KN-93.
Knockout mouse embryonic fibroblasts display reduced WIPI-1 puncta formation under basal conditions. AMPKα2 deficiency did not alter LC3 lipidation and WIPI-1 puncta formation in WIPI-1 cells transfected with control siRNA (siControl) and STO-609/KN-93 (Fig. 6B). However, transient down-regulation of AMPKα2 significantly decreased LC3 lipidation and WIPI-1 puncta formation. Stable U2OS GFP-WIPI-1 cells were transiently transfected with 50 nM unique siRNAs (siControl, AMPKα1/α2) for 48 h. Cells were pretreated with CM or NF with or without STO-609 (30 μM) plus KN-93 (10 μM) for 1 h. Protein extracts were subjected to 8% (top) or 15% (bottom) SDS-polyacrylamide gel electrophoresis and Western blot analysis using anti-AMPK, anti-LC3, or anti-tubulin antibodies. Representative Western blot result (n = 2) is shown (A), and supplemental information is available (Supplemental Fig. S3). In parallel, automated WIPI-1 puncta-formation analysis was expressed as the number of GFP-WIPI-1 puncta-positive cells (C). Up to 2600 cells were analyzed for each treatment. Image fields from four independent experiments were used for both quantifications (B and C) and to calculate p values: *p < 0.05; **p < 0.01; ***p < 0.001.

Fig. 6. Transient siRNA-mediated down-regulation of AMPKα1/α2 increased LC3 lipidation and WIPI-1 puncta formation. Stable U2OS GFP-WIPI-1 cells were transiently transfected with 50 nM unique siRNAs (siControl, AMPKα1/α2) for 48 h. Cells were pretreated with CM or without 30 μg/ml STO-609 plus 10 μM KN-93 for 30 min. Subsequently, cells were treated with CM or NF with or without STO-609 (30 μg/ml) plus KN-93 (10 μM) for 1 h. Protein extracts were subjected to 8% (top) or 15% (bottom) SDS-polyacrylamide gel electrophoresis and Western blot analysis using anti-AMPK, anti-LC3, or anti-tubulin antibodies. Representative Western blot result (n = 2) is shown (A), and supplemental information is available (Supplemental Fig. S3). In parallel, automated WIPI-1 puncta-formation analysis was expressed as the number of GFP-WIPI-1 puncta-positive cells (B) or the number of GFP-WIPI-1 puncta-positive cells (C). Up to 2600 cells were analyzed for each treatment. Image fields from four independent experiments were used for both quantifications (B and C) and to calculate p values: *p < 0.05; **p < 0.01; ***p < 0.001.

regulation of WIPI-1. In support, by confocal microscopy studies on the colocalization of WIPI-1 and Atg1 (Ulk2), a target of both AMPK and TORC1, we found a partial colocalization of WIPI-1 and Ulk2 puncta (Fig. 8).
Discussions

Providing for macromolecules and energy, the process of cellular autophagy is constitutively active on a basal level. In response to a variety of imposed cellular stress, including nutrient and energy shortage, autophagic bulk degradation is induced above basal level to secure cellular survival. Alterations in the process of autophagy are associated with human diseases, such as cancer, neurodegeneration, and diseases of the heart, liver, and muscle (Mizushima et al., 2008). Alterations in autophagy genes have also been associated with human diseases, monoallelic deletions in \textit{BECN1} with breast and ovarian cancer (Liang et al., 1999), and \textit{ATG16L1} single-nucleotide polymorphism with Crohn’s disease (Barrett et al., 2008; Cadwell et al., 2008). Although mutations in both genes contribute to different diseases, the encoded autophagosomal proteins Beclin 1 and Atg16L1 both function in the assembly of the autophagic machinery. The autophagosomal PtdIns(3)P effector protein WIPI-1 is thought to function downstream of the Beclin 1/PtdIns3KC3 complex I and upstream of Atg16L (Nobukuni et al., 2007; Itakura and Mizushima, 2010) and might also be disease-associated because of its aberrant expression in analyzed matched normal/tumor patient tissues (Proikas-Cezanne et al., 2004). Activating the Beclin 1/PtdIns3KC3 complex I to generate PtdIns(3)P that is subsequently bound by PtdIns(3)P effectors, such as WIPI-1, is prerequisite for initiating canonical autophagosome formation (Proikas-Cezanne and Codogno, 2011). WIPI-1 is inhibited by counteracting PtdIns(3)P availability [wortmannin, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002)] or by mutating PtdIns(3)P-binding motifs in WIPI-1, and WIPI-1 is stimulated by rapamycin-mediated TORC1 inhibition (Proikas-Cezanne et al., 2007). TORC1 is the target of the energy sensor AMPK, both of which regulate autophagy via differential Atg1 (Ulk1) phosphorylation (Egan et al., 2011; Kim et al., 2011). This suggests that rapamycin-mediated stimulation of WIPI-1 should at least be partially guarded by this pathway, explaining that both amino acid and serum starvation lead to WIPI-1 stimulation (Fig. 2) and partial colocalization of WIPI-1 and Atg1 (Ulk2) (Fig. 8).

CaMKs Contribute to WIPI-1 Regulation at Autophagy Onset

\begin{figure}[h]
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\caption{AMPKα1/α2-deficient MEFs display reduced WIPI-1 puncta under nutrient-rich conditions. AMPK WT and AMPKα1/α2-deficient MEFs were subjected to anti-AMPK Western blotting confirming the absence of AMPK in knockout cells (A). AMPK WT and AMPKα1/α2-deficient MEFs were transiently transfected with GFP-WIPI-1 and treated with CM, NF, TG (100 nM), or WM. Representative images were acquired by confocal microscopy, n = 3 (B). Scale bars, 20 μm. Upon transient GFP-WIPI-1 transfection, AMPK WT and AMPKα1/α2-deficient MEFs were incubated for 24 h in CM with either 4.5, 1.0, or 0.1 g/l glucose medium and further treated with NF for 3 h or not. Quantitative fluorescence microscopy of 400 cells is presented, n = 4 (C). p Values: *, p < 0.05; **, p < 0.01; ***, p < 0.001. Scale bars, 20 μm.}
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\centering
\includegraphics[width=\textwidth]{fig8}
\caption{Colocalization of endogenous WIPI-1/Alexa Fluor 546 (red) and overexpressed GFP-Ulk2 (green) upon nutrient starvation (NF) and TG treatment in human G361 cells. Nuclei were stained with TO-PRO-3 (blue). Arrows mark prominent colocalization of endogenous WIPI-1/Alexa Fluor 546 (red) and GFP-Ulk2 (green) (zoom). Scale bars, 20 μm.}
\end{figure}
membranes upon nutrient starvation (Fig. 4). Although WIPI-1 puncta formation was not completely abolished upon either STO-609 or KN-93 alone, coapplication of STO-609 and KN-93 inhibited the induction of autophagy, measured by WIPI-1 puncta formation and LC3-II protein abundance (Fig. 4). It was suggested that an effect of KN-93 argues for an involvement of CaMKII/IIV and rules out CaMKII if a similar effect is found with STO-609 (Means, 2008). However, CaMKII has long been suggested to be involved in autophagy (Holen et al., 1992). By using siRNA-mediated down-regulation of CaMKII and CaMKIV, we found that the function of CaMKII indeed contributes to the formation of WIPI-1 positive autophagosomal membranes (Fig. 5). It is noteworthy that although the number of WIPI-1 puncta per cell significantly increased upon nutrient starvation in siRNA-transfected cells targeting CaMKII, this elevation was predominantly reduced compared with the siControl setting. In support, KN-93 treatment also resulted in a partial inhibition of WIPI-1 puncta-formation. This shows that CaMKII signaling is involved in the regulation of WIPI-1 mediated autophagy, but complete inhibition is not achieved by siRNA-mediated CaMKII down-regulation or by the use of selective inhibitors alone. Full inhibition is achieved only upon BAPTA-AM-mediated chelation of intracellular Ca$^{2+}$ or upon PtdIns(3)P depletion (Fig. 3).

However, our results demonstrate that CaMKs contribute in part to the regulation of WIPI-1-mediated autophagy. In line with Ca$^{2+}$ signaling opportunities via CaMKs in the absence of AMPK, we found that nutrient starvation-mediated autophagy can take place in AMPKα1/α2 mouse embryonic fibroblasts from knockout mice compared with the wild-type control (Fig. 7). However, basal autophagy was significantly reduced in the AMPKα1/α2-deficient background (Fig. 7). This result indicates that starvation-induced autophagy can bypass AMPKα1/α2 either because catalytic subunits of AMPK-related protein kinase family members (Dale et al., 1995) substitute for AMPK-null background as suggested (Laderoute et al., 2006). However, because the localization of WIPI-1 at autophagosomal membranes under nutrient-rich conditions is significantly reduced without AMPKα1/α2, AMPK should be a crucial regulatory factor for basal autophagy. Furthermore, by siRNA-mediated transient down-regulation of AMPKα1/α2, we found an increase of both autophagosomal markers LC3-II protein and WIPI-1 positive membranes, arguing that low AMPKα1/α2 protein levels might induce autophagy, prominently under nutrient-free conditions (Fig. 6). It is tempting to speculate that evolutionary highly conserved pathways, such as generation of PtdIns(3)P and AMPK-mediated mTOR inhibition, regulate basal autophagy and consequently promote adaptation to nutrition/energy supply; additional and distinct signal cascades such as Ca$^{2+}$ mobilization via CaMKIV that we found here to contribute to the regulation of WIPI-1 and LC3, independent of AMPK, might present further signaling opportunities to modulate autophagy. In fact, noncanonical pathways that modulate autophagy but bypass canonical Atg proteins have been identified (e.g., Nishida et al., 2009). It is noteworthy that the study (Nishida et al., 2009) that provides evidence for an Atg5/Atg7-independent entry into autophagic sequestration upon etoposide treatment depends on the activity of PtdIns3K/C3 to generate PtdIns(3)P. In line with this, we found in the current study that etoposide treatment increases the number of cells in which WIPI-1 localizes at autophagosomal membranes, suggesting that WIPI-1 marks also noncanonical autophagy pathways as long as PtdIns(3)P is generated. Forced cellular stress by further compounds (etoposide, sorafenib, staurosporine, thapsigargin) or nutrient starvation (amino acids, serum) always increased the number of WIPI-1 puncta-positive cells, and Ca$^{2+}$ chelation nullified the localization of WIPI-1 at autophagosomal membranes; in line with this, LC3-II protein abundance was also decreased upon Ca$^{2+}$ chelation (current study).

The most compelling interpretation from this might be that all of the treatments employed promote Ca$^{2+}$ mobilization from intracellular stores and modulate autophagy. Work from Park et al. (2008) regarding the molecular characterization of sorafenib-treated tumor cells demonstrated that sorafenib promotes both autophagy and cell death by acting synergistically with vorinostat. Staurosporine treatment has long been correlated with both autophagy and cell death, reporting the appearance of autophagosomes upon staurosporine treatment (30 nM) for 22 to 28 h in Tetrahymena thermophila and apoptotic cell blebbing between 12 and 44 h (Christensen et al., 1998). Our current study demonstrates that both starvation-induced and pharmacological compound-modulated autophagy depends on the availability of cytosolic Ca$^{2+}$ to permit the localization of WIPI-1 (Proikas-Cezanne and Robenek, 2011) and LC3 at autophagosomal membranes. WIPI-1 should be regulated by 1) PtdIns(3)P generation, 2) Ca$^{2+}$ mobilization, and 3) mTOR inhibition, indicating that WIPI-1 functions as a PtdIns(3)P effector that receives additional required signals during regulated autophagosome formation (see our proposed model in Supplemental Fig. S4). The current study provides evidence for this hypothesis and for an involvement of CaMKI independent of AMPK. In light of future putative employment of therapeutic compounds to modulate autophagy, stimulating Ca$^{2+}$ signaling might represent a further opportunity to influence this cellular process.

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Authorship Contributions

Participated in research design: Codogno and Proikas-Cezanne.

Conducted experiments: Pfisterer and Mauthe.

Performed data analysis: Pfisterer, Mauthe, and Proikas-Cezanne.

Wrote or contributed to the writing of the manuscript: Pfisterer and Proikas-Cezanne.

References


Address correspondence to: Tassula Proikas-Cezanne, Autophagy Laboratory, Interfaculty Institute for Cell Biology, Eberhard Karls University Tuebingen, Auf der Morgenstelle 15, 72076 Tuebingen, Germany. E-mail: tassula.proikas-cezanne@uni-tuebingen.de
**Supplementary Figure S1: Ca**²⁺**/Calmodulin-dependent kinase signaling via CaMKI and AMPK contribute to the regulation of WIPI-1 at the onset of autophagy**

According to Fig. 3D, additional experiments are shown here (A, B). Each of the 3 independent experiments (Fig. 3D, Suppl. Fig. S1A, Suppl. Fig. S1B) provides evidence that BAPTA-AM treatment prominently reduced LC3-lipidation.
Supplementary Figure S2: Both selective inhibitors STO-609 and KN-93 reduce LC3 lipidation. According to Fig. 4C, additional experiments are shown here (A, B). Each of the 3 independent experiments (Fig. 4C, Suppl. Fig. S2A, Suppl. Fig. S2B) provides evidence that STO-609/KN-93 treatment prominently reduced nutrient-starvation induced LC3-lipidation.
Supplementary Figure S3: Transient siRNA-mediated downregulation of AMPK increased LC3 lipidation. The quantification of the western blot result shown in Fig. 6A is provided (A) and an additional independent experiment is shown here (B). Both experiments (Fig. 6A/Suppl. Fig. S3A, Suppl. Fig. S3B) provide evidence that STO-609/KN-93 treatment reduced nutrient-starvation induced LC3-lipidation in siAMPK transfected cells (boxed in red).
Ca$^{2+}$/Calmodulin-dependent kinase signaling via CaMKI and AMPK contribute to the regulation of WIPI-1 at the onset of autophagy

Simon G. Pfisterer, Mario Mauthe, Patrice Codogno, and Tassula Proikas-Cezanne*
*Correspondence: tassula.proikas-cezanne@uni-tuebingen.de

Supplementary Figure S4: Model for the regulation of WIPI-1 in human U2OS osteosarcoma cells.