mTOR-Controlled Autophagy Requires Intracellular Ca$^{2+}$ Signaling

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Abstract

Autophagy is a lysosomal degradation pathway important for cellular homeostasis and survival. Inhibition of the mammalian target of rapamycin (mTOR) is the best known trigger for autophagy stimulation. In addition, intracellular Ca$^{2+}$ regulates autophagy, but its exact role remains ambiguous. Here, we report that the mTOR inhibitor rapamycin, while enhancing autophagy, also remodeled the intracellular Ca$^{2+}$-signaling machinery. These alterations include a) an increase in the endoplasmic-reticulum (ER) Ca$^{2+}$-store content, b) a decrease in the ER Ca$^{2+}$-leak rate, and c) an increased Ca$^{2+}$ release through the inositol 1,4,5-trisphosphate receptors (IP$_3$R$_s$), the main ER-resident Ca$^{2+}$-release channels. Importantly, buffering cytosolic Ca$^{2+}$ with BAPTA impeded rapamycin-induced autophagy. These results reveal intracellular Ca$^{2+}$ signaling as a crucial component in the canonical mTOR-dependent autophagy pathway.

Introduction

Macroautophagy (further referred to as “autophagy”) is a cellular degradation process characterized by the transfer of cellular material in double-membranous vesicles, termed autophagosomes, to the lysosomes. After fusion with lysosomes, the autophagosomal cargo becomes degraded. This intracellular cargo can consist of proteins, lipids or even entire organelles [1]. Basal levels of autophagy contribute to the maintenance of cellular homeostasis by removing damaged or toxic intrinsic components (e.g. damaged organelles, protein aggregates) [2]. Additionally, autophagy becomes stimulated during conditions of cellular stress. In these conditions, the recycling of their own material provides the cells with cellular building blocks that can be incorporated in newly synthesized macromolecules required for cellular anti-stress responses and energy production, so ensuring survival. Because of its role in these vital cellular functions, autophagy is implicated in various pathologies (reviewed in [3]).

The canonical signaling protein in autophagy regulation is the mammalian target of rapamycin (mTOR), a ubiquitously present protein kinase that is also involved in the regulation of cell growth, proliferation, motility, protein translation and transcription [4]. Depending on its binding partners, mTOR forms two different protein complexes (mTORC1 and mTORC2), but only mTORC1 is directly involved in autophagy regulation. In growth-promoting conditions, active mTORC1 inhibits autophagy through phosphorylation of the unc-51-like kinase (ULK1/2) complex members. Upon certain stress conditions, mTORC1 becomes inhibited, alleviating these phosphorylations, and allowing the activation of the autphagic ULK1/2 complex [5]. In this way, inhibition of mTORC1 will activate autophagy in response to amino-acid depletion, growth-factor depletion, low energy production or chemical mTORC1 inhibitors, like rapamycin. Additionally, the activity of mTORC1 is regulated by its association/dissociation from the lysosomal membranes, mediated by Rag GTPase heterodimers [6].

Intracellular Ca$^{2+}$ signaling was recently recognized as an important player in the regulation of autophagy, although its exact role still remains a matter of debate [7,8]. On the one hand, Ca$^{2+}$ signals mediated by the inositol 1,4,5-trisphosphate (IP$_3$) receptor (IP$_3$R), a ubiquitous endoplasmic-reticulum (ER) Ca$^{2+}$-release channel, were reported to inhibit autophagy [9,10,11]. On the other hand, an increase in the cytosolic [Ca$^{2+}$] enhanced autophagy [12,13,14,15]. The exact role of Ca$^{2+}$ and/or IP$_3$Rs probably depends on the cellular state; in growth-promoting conditions constitutive IP$_3$R-mediated Ca$^{2+}$ signals from the ER to the mitochondria promote cellular bioenergetics and so inhibit basal autophagy, while during stress different, possibly cytosolic, Ca$^{2+}$ signals stimulate autophagy [7].

The view that Ca$^{2+}$ stimulates autophagy is based on several reports using different Ca$^{2+}$-mobilizing compounds that stimulate autophagy [12,13,16,17]. Recently, we observed that also starvation-induced autophagy was dependent on IP$_3$R-mediated Ca$^{2+}$ signaling [18]. Interestingly, starvation led to a sensitization of the intracellular Ca$^{2+}$ machinery in different cell types, enhancing their Ca$^{2+}$-signaling capacity. Moreover, the results suggested that this sensitization was operative in promoting autophagy-stimulating Ca$^{2+}$ signals.
Moreover, intracellular Ca\(^{2+}\) signals were monitored on a FlexStation-3 microplate reader (Molecular Devices, LLC, Sunnyvale, CA) by alternately exciting the Ca\(^{2+}\) indicator at 340 and 380 nm and measuring fluorescence emission at 510 nm. Fluorescent [Ca\(^{2+}\)] measurements in permeabilized cells were performed at 25°C as previously described [21].

**Materials and Methods**

**Cell culture**

Doxycycline-inducible Atg5-knockout mouse embryonic fibroblasts (MEF cells), a kind gift from Prof. N. Mizushima (Tokyo Medical and Dental University, Japan), and wild-type human cervix carcinoma HeLa cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS) and 10 mM HEPES buffer. The cells were grown at 37°C and 5% CO\(_2\) in the presence of 85 IU ml\(^{-1}\) penicillin and 85 \(\mu\)g ml\(^{-1}\) streptomycin. Knockdown of Atg5 in MEF was achieved by addition of 10 ng ml\(^{-1}\) doxycycline (Sigma-Aldrich NV, Diegem, Belgium) 2 days before the experiment [20]. Medium was changed regularly to avoid nutritional stress. All materials were purchased from Gibco, Life Technologies (Ghent, Belgium).

**Antibodies and reagents**

The following antibodies were used for Western-blotting experiments: anti-GAPDH (G8795, Sigma-Aldrich NV), anti-BiP (G8918, Sigma-Aldrich NV), anti-LC3 (0231-100, NanoTools Antikörpertechnik GmbH & Co., Teningen, Germany), anti-SERC2 (9580, Cell Signaling Technologies, Danvers, MA), anti-St6R and anti-phospho-St6R (8207, Cell Signaling Technologies), anti-Atg12 (2011, Cell Signaling Technologies) and anti-calretilcin (anti-CRT) (PA1-903, Thermo Fisher Scientific, Erembodegem, Belgium). The chemicals used were: A23187 and IP\(_3\) (Sigma-Aldrich NV), EGTA (Acros Organics BVBA, Geel, Belgium), thapsigargin (Enzo Life Sciences BVBA, Antwerp, Belgium), ionomycin, rapamycin and bafilomycin A1 (LC laboratories, Woburn, MA), ATP (Roche Diagnostics), sodium phosphate buffer (pH 7.5), 150 mM NaCl, 1.5 mM MgCl\(_2\), 0.5 mM EDTA, 1% Triton X-100, 2.5% glycerol and Complete EDTA-free Protease Inhibitor Tablets (Roche Diagnostics). After 30 min of incubation on ice, the lysates were cleared via centrifugation. Protein concentrations were determined by the Bradford procedure. For sample separation we used commercial Tris-Glycine or Bis-Tris SDS-PAGE gels (Invitrogen, Life Technologies). After transfer to a PVDF membrane (Immobilon-P, Merck Millipore, Billerica, MA) the membranes were blocked with Tris-buffered saline containing 0.1% (v/v) Tween-20 and 5% (w/v) non-fat dry milk powder. Subsequently the membranes were incubated with primary antibody and horseradish peroxidase-conjugated secondary antibody. The immunoreactive bands were visualized with ECL substrate and exposed to CLXposure\textsuperscript{TM} film (Thermo Fisher Scientific). The film was developed using a Kodak X-Omat 1000. Alternatively, alkaline phosphatase-conjugated secondary antibodies were used and visualized with a Storm 840 imager (GE Healthcare GmbH, Diegem, Belgium). Quantification was done with ImageJ software (rsbweb.nih.gov/ij/).

**Calibration of the resting [Ca\(^{2+}\)]**

After trypsinization, suspensions of 5\(\times\)10\(^5\) cells ml\(^{-1}\) of intact HeLa cells were loaded for 30 min with 5 \(\mu\)M Fura2-AM at 25°C in modified Krebs solution. The cells were then incubated for another 30 min in the absence of Fura2-AM. Fluorescence was monitored in the cell suspensions at 25°C in an AMINCO-Bowman Series 2 spectrophuorometer (Thermo Electron Corporation, Rochester, NY) by alternately exciting the Ca\(^{2+}\) indicator at 340 and 380 nm and recording emission fluorescence at 510 nm. After 50 s, 0.06 mg ml\(^{-1}\) digitonin was added to permeabilize the plasma membrane and to record fluorescence at a maximal [Ca\(^{2+}\)]. Minimal fluorescence was measured 100 s later by adding 33 mM EGTA. The cytosolic [Ca\(^{2+}\)] was derived using the following equation:

\[K_d \times Q_{\text{cell}} \times (R_{\text{max}} - R)/R_{\text{min}}\]

where \(K_d\) is the dissociation constant of Fura2 for Ca\(^{2+}\), \(Q_{\text{cell}}\) is the cytosolic Ca\(^{2+}\) concentration, and \(R\) is the fluorescence ratio of the emission intensity excited by 380 nm in the absence of Ca\(^{2+}\) to that in the presence of saturating Ca\(^{2+}\). \(R_{\text{min}}\) and \(R_{\text{max}}\) are the minimal and maximal fluorescence ratios, respectively.

**ImmunobLOTS**

HeLa or MEF cells were scraped into ice-cold phosphate-buffered saline and lysed in a modified RIPA buffer containing 10 mM sodium phosphate (pH 7.5), 150 mM NaCl, 1.5 mM MgCl\(_2\), 0.5 mM DTT, 1% Triton X-100, 10% glycerol and Complete EDTA-free Protease Inhibitor Tablets (Roche Diagnostics). After 30 min of incubation on ice, the lysates were cleared via centrifugation. Protein concentrations were determined by the Bradford procedure. For sample separation we used commercial Tris-Glycine or Bis-Tris SDS-PAGE gels (Invitrogen, Life Technologies). After transfer to a PVDF membrane (Immobilon-P, Merck Millipore, Billerica, MA) the membranes were blocked with Tris-buffered saline containing 0.1% (v/v) Tween-20 and 5% (w/v) non-fat dry milk powder. Subsequently the membranes were incubated with primary antibody and horseradish peroxidase-conjugated secondary antibody. The immunoreactive bands were visualized with ECL substrate and exposed to CLXposure\textsuperscript{TM} film (Thermo Fisher Scientific). The film was developed using a Kodak X-Omat 1000. Alternatively, alkaline phosphatase-conjugated secondary antibodies were used and visualized with a Storm 840 imager (GE Healthcare GmbH, Diegem, Belgium). Quantification was done with ImageJ software (rsbweb.nih.gov/ij/).

**GFP-LC3 measurements**

HeLa cells were transfected with pcDNA3.1(-)-GFP-LC3 [18] with jetPRIME\textsuperscript{TM} from Polyplus Transfection (Illkirch, France). 48 h later, the cells were fixed in 4% parformaldehyde. Cells were then analyzed on a Zeiss LSM510 confocal microscope using a 63\(\times\) lens with resolution near Nyquist rate (xy dimensions: \(-0.09\) \(\mu\)m, z dimension: 0.14 \(\mu\)m). The number of punctae per
cell was determined using an adapted version of the WatershedCounting3D plug-in for ImageJ [23], using a threshold for punctae volumes corresponding to autophagosome diameters of 0.5 μm. Only cells displaying a modest overexpression level were included in the analysis.

Statistical analysis

Results are expressed as means±SEM, and n refers to the number of independent experiments. For statistical analyses, normal distribution (Shapiro-Wilk test) and equal variance (Levene’s test) were first tested. Accordingly, significance was determined using the appropriate tests, as mentioned in the figure legends. Differences were considered significant at p<0.05.

Results

Rapamycin induces autophagy in a time- and concentration-dependent manner

We treated HeLa cells with 1 μM of rapamycin for different time periods (2, 5 and 7 h) or for 5 h with different concentrations of rapamycin (0.1, 1 and 5 μM). First, the inhibition of mTORC1 by rapamycin was verified by assessing the phosphorylation of one of the downstream targets of mTORC1, S6 ribosomal protein (S6Rp), using a phospho-specific S6Rp antibody [24]. In all treatment conditions using rapamycin, the phosphorylation of S6Rp was inhibited (Fig. S1). Subsequently, autophagy was assessed by immunoblotting for detection of the essential autophagy protein LC3. In the autophagic pathway, this protein is conjugated to phosphatidylethanolamine and thereby recruited to the autophagosomal membrane. This lipidated form of LC3 can be detected as a band with an apparently lower molecular weight (LC3-II, 16 kDa) than the non-lipidated, non-autophagic form (LC3-I, 18 kDa). The level of LC3-II is therefore an indication for the extent of autophagy [25]. However, since LC3-II remains associated with the autophagosomes, it eventually becomes degraded in the lysosomes. Therefore, increased LC3-II levels can also be explained by defective autophagic flux and hence accumulation of LC3-II-positive autophagosomes. The addition of lysosomal inhibitors (e.g. bafilomycin A1) is therefore recommended as a proper control condition to verify ‘truly’ increased autophagy induction [26,27]. Therefore, bafilomycin A1 (100 nM) was added during the last hour of our treatment and the formation of LC3-II was monitored in this last hour (quantified as the LC3-II/GAPDH ratio, as recommended [27]). Our results show that LC3-II levels were increased consequently to both increasing time periods and concentrations of rapamycin treatment (Fig. 1A–B).

We also tested the effect of different concentrations of rapamycin on the localization of transiently expressed GFP-LC3 in HeLa cells. Autophagic GFP-LC3-II will concentrate at the autophagosomes, which can be detected as intracellular GFP-LC3 punctae. The amount of these punctae per cell correlates with the level of autophagy [27]. The number of GFP-LC3 punctae per cell was significantly increased upon rapamycin treatment (Fig. 1C). In agreement with the results obtained by LC3 Western blotting, the lowest concentration of rapamycin (0.1 μM) did not significantly increase the number of punctae.

Rapamycin treatment increases the intracellular Ca2+-store content and IP3-induced Ca2+ release

We loaded HeLa cells, treated with or without rapamycin, with the fluorescent cytosolic Ca2+ dye Fura2 and measured the response upon addition of the Ca2+-ionophore ionomycin, thapsigargin or ATP. Ionomycin can be used to determine the size of all Ca2+ stores. Thapsigargin is an inhibitor of the SERCA pumps and can be used to determine the ER Ca2+ content. ATP binds to its receptor at the plasma membrane, resulting in the production of IP3 and consequently inducing IP3-R-mediated Ca2+ release. Before treatment with the Ca2+-mobilizing agents, extracellular Ca2+ was chelated using 3 mM EGTA. As shown in Fig. 2A–B, cells treated with rapamycin concentrations triggering autophagy (1 and 5 μM) displayed an increased Ca2+ release in response to the different Ca2+-mobilizing agents tested. Interestingly, the lowest concentration (0.1 μM) of rapamycin did not result in a significantly increased Ca2+ release (Fig. 2B), correlating with its inability to significantly stimulate autophagy.

The traces from Fig. 2A before EGTA addition also suggest an increase in the resting cytosolic [Ca2+] upon rapamycin treatment. To verify this behavior, the Fura2-ratio signal was calibrated, revealing a significant increase in the cytosolic [Ca2+] in cells treated with rapamycin (Fig. 2C). As a control, it was verified that rapamycin addition by itself did not induce a shift in the spectral characteristics of the Fura2 signal (Fig. S2).

The results obtained with Fura2-loaded cells point to an increase in IP3-R-mediated Ca2+ release after rapamycin treatment. To verify this hypothesis, we performed Ca2+-flux experiments in plasma membrane-permeabilized cells. The benefit of using plasma membrane-permeabilized cells is the direct access to the cytosol and the possibility to directly activate the IP3-R via the addition of IP3. In this way, the extent of the IP3-R-mediated Ca2+ release can be assessed in a quantitative way without interference of plasma-membrane Ca2+ fluxes. The non-mitochondrial Ca2+ stores were loaded with 45Ca2+ to steady state and the release of 45Ca2+ from the cell layer was then measured every 2 min. We added IP3 at a submaximal concentration (0.7 μM) and measured IP3-induced Ca2+ release (Fig. 2D). In these conditions, IP3-induced Ca2+ release was enhanced in cells treated with 1 μM rapamycin for 5 h (Fig. 2E).

Thus, these independent Ca2+-assays indicate that an optimization of the Ca2+ signaling occurs upon rapamycin treatment by increasing the ER Ca2+-store content and the IP3-R-mediated Ca2+ release.

Rapamycin treatment reduces the ER Ca2+-leak rate

To evaluate the underlying cause of the increased ER Ca2+-store content upon rapamycin treatment, we analyzed several parameters that control the ER Ca2+ content. First, we analyzed the main Ca2+-buffering proteins of the ER: calreticulin and BIP/Grp78. Rapamycin treatment, however, did not significantly affect the levels of these proteins (Fig. 3A–B). We also assessed the levels of SERCA2, the major Ca2+-pump isoform in the ER of HeLa cells, but rapamycin treatment did not alter SERCA2 levels (Fig. 3C).

Finally, we also measured the Ca2+-leak rate using 45Ca2+-flux experiments in permeabilized cells, as previously described [28]. Cells were loaded with 45Ca2+ in the absence or in the presence of the Ca2+-ionophore A23187, the latter to determine the passively bound Ca2+. The value for the passively bound Ca2+ is then subtracted to calculate exclusively the amount of releasable Ca2+ in the internal stores. This experiment also revealed a significantly increased Ca2+-store content (Fig. 3D and Fig. 3E), similarly to the findings in the intact Fura2-loaded cells (Fig. 2A–B). The ER Ca2+-leak rate can be appreciated by the slope of the curve plotting the Ca2+ content that remains in the cell layer as (logarithmic scale) a function of time. As shown in Fig. 3D and the quantification in Fig. 3F, rapamycin treatment slightly but significantly reduced the slope of the curve and hence the Ca2+-leak rate.
In conclusion, rapamycin treatment reduced the ER Ca²⁺-leak rate, which may account for the increased Ca²⁺-store content observed.

Rapamycin-induced changes in Ca²⁺ signaling are independent of functional autophagy and occur upstream of the Atg12-Atg5 complex

To analyze whether the observed changes in Ca²⁺ signaling during rapamycin treatment are upstream or downstream of autophagy stimulation, we performed [Ca²⁺] measurements in doxycycline-inducible Atg5-knockout MEF cells. The addition of doxycycline to the medium results in the complete knockdown of Atg5, the absence of the autophagic Atg12-Atg5 complex and the inability to stimulate autophagy by rapamycin (Fig. 4A) [20]. [Ca²⁺] measurements in MEF cells showed a similar increase in the ATP- and ionomycin-induced Ca²⁺ release upon rapamycin treatment as in HeLa cells (Fig. 4B–D), indicating that these effects do not depend on the cell type. Even more interestingly, in the absence of Atg5, similar changes in Ca²⁺ signaling were observed, indicating that the rapamycin-induced increase in Ca²⁺ signaling is independent of functional autophagy.

**Intracellular Ca²⁺ is required for rapamycin-induced autophagy**

Since we observed changes in the Ca²⁺ machinery by rapamycin treatment that correlated with the induction of autophagy, we investigated whether intracellular Ca²⁺ signals played a role in rapamycin-induced autophagy. Therefore, we incubated HeLa cells during the rapamycin treatment (1 µM, 5 h) with the intracellular Ca²⁺ chelator BAPTA-AM (10 µM). Although incubation with BAPTA-AM had no significant effect on the basal levels of autophagy, rapamycin-induced autophagy was abolished by loading the cells with BAPTA-AM (Fig. 5). These results indicate that cytosolic Ca²⁺ was required for rapamycin-induced autophagy.
The major finding of this study is the occurrence of changes in the intracellular Ca\(^{2+}\) homeostasis during rapamycin treatment that correlated with the stimulation of autophagy. These changes include an increase in the intracellular Ca\(^{2+}\)-store content, a decrease in the ER Ca\(^{2+}\)-leak rate and more IP\(_3\)-induced Ca\(^{2+}\) release. This study also reveals that cytosolic Ca\(^{2+}\) is required for rapamycin-induced autophagy. These findings therefore identify intracellular Ca\(^{2+}\) as a novel and essential secondary messenger in the canonical mTOR-dependent autophagy pathway.

Recently, we have identified enhanced IP\(_3\)-mediated Ca\(^{2+}\) signaling as an essential player in starvation-induced autophagy [18]. We observed a sensitization of the cellular Ca\(^{2+}\)-release machinery during starvation, leading to increased IP\(_3\)-mediated Ca\(^{2+}\) signaling from the ER Ca\(^{2+}\) stores. However, it was not clear whether the observed starvation-induced alterations in Ca\(^{2+}\) homeostasis were caused by mTORC1 inhibition, or by another pathway affected by starvation. In the present study, we therefore used rapamycin as a specific tool to chemically and irreversibly inhibit mTORC1. Our results now provide unequivocal evidence that mTORC1-dependent autophagy stimulation causes sensitization of Ca\(^{2+}\)-signaling events and that these Ca\(^{2+}\) signals are essential to drive autophagy induced by mTORC1 inhibition. This is an important finding, since mTORC1 is the canonical upstream regulator of the autophagy pathway.

Similar to the effects of starvation, we found an increase in the ER Ca\(^{2+}\)-store content during rapamycin treatment, leading to increased IP\(_3\)-induced Ca\(^{2+}\) release. During starvation, the increase in the Ca\(^{2+}\)-store content was associated with an increase in the levels of intraluminal Ca\(^{2+}\)-buffering proteins and with a reduction in the ER Ca\(^{2+}\)-leak rate [18]. During rapamycin treatment, the levels of the intraluminal Ca\(^{2+}\)-buffering proteins remained unaltered, while the Ca\(^{2+}\)-leak rate was clearly reduced. The unaltered levels of the Ca\(^{2+}\)-buffering proteins suggest that they take no part in the regulation of the Ca\(^{2+}\)-leak rate during rapamycin-induced autophagy, in contrast to the situation upon starvation [18,29]. How the ER Ca\(^{2+}\) leak is regulated and which proteins are involved are however still a matter of debate [30].
In addition to the increased Ca\(^{2+}\)-store content, we also observed increased IP3-mediated Ca\(^{2+}\) release after rapamycin treatment. However, in contrast to our findings, other reports revealed a decrease in the IP3R-mediated Ca\(^{2+}\) release after rapamycin treatment, which was due to decreased interactions of mTORC1-protein members with the IP3R, and subsequent less mTORC1-dependent IP3R phosphorylation [31,32]. The reason for this discrepancy probably reflects experimental differences, including the time of rapamycin treatment (5–15 min in [32] versus 2–7 h in present study). Fifteen minutes of rapamycin treatment is probably not sufficient to cause autophagy stimulation and these short time periods were therefore not investigated in our study. In any case, the relevance of the mTORC1-dependent phosphorylation of the IP3R and its potential effect on IP3R activity after prolonged exposure to rapamycin requires further investigation.

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In HeLa cells, we also detected an increase in the resting cytosolic Ca\(^{2+}\) upon rapamycin treatment. The reason for this observation is unclear, and could possibly involve an enhanced Ca\(^{2+}\) influx across the plasma membrane. In contrast, MEF cells rather showed a reduced cytosolic Ca\(^{2+}\) upon rapamycin treatment (Fig. 4), suggesting that the increase in the cytosolic Ca\(^{2+}\) may be cell-type dependent, in contrast to the increase of the ER Ca\(^{2+}\)-store content and agonist-induced Ca\(^{2+}\) release, which occurs in both cell types.

Finally, we also found that mTORC1-controlled autophagy was dependent on proper intracellular Ca\(^{2+}\) signaling, since chelating cytosolic Ca\(^{2+}\) by BAPTA-AM treatment completely abolished rapamycin-induced autophagy. In contrast, inhibiting autophagy
by Atg5 knockout in MEF cells did not alter the observed rapamycin-induced changes in Ca\textsuperscript{2+} signaling. Taken together, these results suggest that the changes in Ca\textsuperscript{2+} signaling during rapamycin-induced autophagy are upstream of the Atg12-Atg5 complex and therefore identify intracellular Ca\textsuperscript{2+} as a novel critical player in the canonical mTORC1-dependent autophagy pathway.

**Figure 4. Changes in Ca\textsuperscript{2+} signaling are independent of autophagy stimulation and occur upstream of the Atg12-Atg5 complex.**

A) Representative Western-blot analysis for Atg12 (showing the autophagic Atg12-Atg5 complex), GAPDH and LC3 of protein lysates obtained from MEF cells pretreated with (+Dox) or without (-Dox) doxycycline and treated with DMSO or 0.1, 1 or 5 μM rapamycin (Rapa) for 5 h (n = 3). B–C) Representative measurements of cytosolic Ca\textsuperscript{2+} signals, displayed as Fura2 ratio (F340/F380), showing the effect of 1 mM ATP (B) or 10 μM ionomycin (Iono) in intact MEF cells pretreated with or without doxycycline and treated with different concentrations of rapamycin for 5 h. Prior to the addition of ATP or Iono, EGTA (3 mM) was added to chelate the extracellular Ca\textsuperscript{2+} as indicated. D) Quantification of the average amplitude of the response (F\textsubscript{2}−F\textsubscript{0}) (n = 3, 4, 5 and 6 for ATP-Dox, ATP+Dox, Iono-Dox and Iono+Dox, resp.) * p<0.05; ** p<0.01; *** p<0.001, repeated measurements ANOVA.

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**Figure 5. Rapamycin-induced autophagy is Ca\textsuperscript{2+}-dependent.**

Western-blot analysis for GAPDH and LC3 of protein lysates obtained from HeLa cells treated for 5 h with DMSO, 1 μM rapamycin (Rapa), 10 μM BAPTA-AM or both. One hour before harvesting, 100 nM bafilomycin A1 was added. Left: representative Western blots; right: quantification of the LC3-II/GAPDH ratio (n = 6). * p<0.05, repeated measurements ANOVA.

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The finding that intracellular Ca\(^{2+}\) is required for autophagy induction is in line with a series of reports showing that an increase in cytosolic Ca\(^{2+}\) can stimulate autophagy [12,13,14,15,16,17,54]. Other reports however have assigned an inhibitory role for Ca\(^{2+}\) in autophagy regulation [10,11,35,36]. We believe that this discrepancy may be explained by the specific role of different Ca\(^{2+}\) signals: a Ca\(^{2+}\) signal in normal growth-promoting conditions (probably targeted towards mitochondria) that inhibits basal autophagy and a different Ca\(^{2+}\) signal in conditions of cellular stress that stimulates autophagy (reviewed in [7]). We speculate that in order to generate these autophagy-stimulating Ca\(^{2+}\) signals, a sensitization of the Ca\(^{2+}\) machinery is required, as observed during starvation or during rapamycin treatment.

The target of this autophagy-stimulating Ca\(^{2+}\) signal remains elusive. CaMKK\(\beta\) [12,34], CaMKI [37], but also ERK [13] and PKC\(\alpha\) [16] have been proposed as potential targets for these cytosolic Ca\(^{2+}\) signals. As the exact target might depend on the stimulus or the cell type used, it is also likely that different downstream targets or pathways may be involved in the Ca\(^{2+}\)-dependent regulation of autophagy.

In conclusion, intracellular Ca\(^{2+}\) signaling should be considered as an essential component of the canonical mTORC1-regulated autophagy pathway. The further characterization of this Ca\(^{2+}\)-dependent pathway may reveal novel important players and targets in autophagy. Finally, affecting these intracellular Ca\(^{2+}\) signals by chemical compounds or genetic interventions may provide a unique way to modulate the canonical mTORC1-controlled autophagy pathway.

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Supporting Information

Figure S1 Rapamycin inhibits S6Rp phosphorylation. Western-blot analysis for total and phosphorylated S6Rp in HeLa cells treated with the indicated concentrations of rapamycin (Rapa) for 5 h or with 1 \(\mu\)M rapamycin for the indicated times. A representative blot is shown for 2 independent experiments. (TIFF)

Figure S2 Rapamycin addition does not induce a shift in the spectral characteristics of Fura2. Representative measurements (n = 2) of cytosolic Ca\(^{2+}\) signals, displayed as Fura2 ratio (F340/F380), showing the effect of the acute addition of DMSO or different concentrations of rapamycin in intact HeLa cells; control denotes no addition. The arrow indicates the time of addition. (TIFF)

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Author Contributions

Conceived and designed the experiments: JPD JBP GB. Performed the experiments: JPD TL KW. Analyzed the data: JPD DK LM HDS JBP GB. Wrote the paper: JPD DK LM HDS JBP GB.
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