Calcium channel regulator Mid1 links TORC2-mediated changes in mitochondrial respiration to autophagy

Ariadne Vlahakis, Nerea Lopez Muniozguren, and Ted Powers

Department of Molecular and Cellular Biology, College of Biological Sciences, University of California, Davis, Davis, CA 95616

Autophagy is a catabolic process that recycles cytoplasmic contents and is crucial for cell survival during stress. The target of rapamycin (TOR) kinase regulates autophagy as part of two distinct protein complexes, TORC1 and TORC2. TORC1 negatively regulates autophagy according to nitrogen availability. In contrast, TORC2 functions as a positive regulator of autophagy during amino acid starvation, via its target kinase Ypk1, by repressing the activity of the calcium-dependent phosphatase calcineurin and promoting the general amino acid control (GAAC) response. Precisely how TORC2-Ypk1 signaling regulates calcineurin within this pathway remains unknown. Here we demonstrate that activation of calcineurin requires Mid1, an endoplasmic reticulum–localized calcium channel regulatory protein implicated in the oxidative stress response. We find that normal mitochondrial respiration is perturbed in TORC2-Ypk1–deficient cells, which results in the accumulation of mitochondrial-derived reactive oxygen species that signal to Mid1 to activate calcineurin, thereby inhibiting the GAAC response and autophagy. These findings describe a novel pathway involving TORC2, mitochondrial oxidative stress, and calcium homeostasis for autophagy regulation.

Introduction

Autophagy is a catabolic cellular stress response that recycles cytoplasmic components to facilitate cellular homeostasis and adaptation (Onodera and Ohsumi, 2005; Ryter et al., 2013). Impaired regulation of autophagy leads to the progression of metabolic and neurodegenerative diseases including, but not limited to, cancer, Alzheimer's disease, and Parkinson's disease (Choi et al., 2013). Upon nutrient stress, autophagy proteins coordinate the formation of cargo-containing double-membrane vesicles, termed autophagosomes, which nucleate at ER exit sites and fuse to the vacuole or lysosome, where cargo is degraded (Takeshige et al., 1992; Suzuki et al., 2001; Wang and Klionsky, 2003; Graef et al., 2013).

Autophagy is tightly regulated by several nutrient-sensing cellular pathways, including the target of rapamycin (TOR) signaling network (Noda and Ohsumi, 1998; Stephan et al., 2009). The central component of this network is the TOR kinase, which assembles with different proteins to form two structurally and functionally distinct complexes termed TOR complex 1 (TORC1) and TORC2 (Loewith et al., 2002). TORC1 functions as a negative regulator of autophagy in response to the availability of nitrogen (Noda and Ohsumi, 1998; Stephan et al., 2009). In contrast, recent studies have demonstrated that TORC2 functions as a positive regulator of autophagy (Renna et al., 2013; Vlahakis et al., 2014; Vlahakis and Powers, 2014). During amino acid starvation, TORC2 promotes autophagy via its downstream target kinase, Ypk1, which inhibits the calcium-regulated phosphatase calcineurin, a negative regulator of the autophagy response (Niles et al., 2012; Vlahakis et al., 2014). Calcineurin inhibits activation of the eIF2α kinase, Gcn2, and subsequent translational de-repression of the transcription factor Gcn4, events required for both initiation of the general amino acid control (GAAC) response and autophagy during amino acid–limited growth conditions (Ecker et al., 2010; Vlahakis et al., 2014; Vlahakis and Powers, 2014). Thus, TORC1 and TORC2 collaborate to tune the autophagy response according to the metabolic state of the cell.

Mitochondria also play an important role in the regulation of autophagy during amino acid starvation (Graef and Nunnari, 2011a,b). When mitochondrial function is compromised, autophagy-mediated protein turnover, or flux, is impaired under amino acid, but not nitrogen, starvation conditions (Graef and Nunnari, 2011a,b). Importantly, the relationship between TORC2-Ypk1 and mitochondria with respect to the regulation of autophagy has not yet been examined. In this study, we demonstrate that TORC2-Ypk1 signaling regulates mitochondrial respiration to promote the general amino acid control response and autophagy. In particular, we find that TORC2-

Correspondence to Ted Powers: tpowers@ucdavis.edu

Abbreviations used: DCF, 2,7-dichlorofluorescin diacetate; ETC, electron transport chain; GAAC, general amino acid control; HACS, high-affinity calcium uptake system; NAC, *N*-acetyl-L-cysteine; ONPG, *O*-nitrophenyl-β-D-galactopyranoside; PM, plasma membrane; ROS, reactive oxygen species; SCD, synthetic complete dextrose; TOR, target of rapamycin; WT, wild type.

The Rockefeller University Press $30.00
J. Cell Biol. Vol. 215 No. 6 779–788
https://doi.org/10.1083/jcb.201605030

Ypk1–mediated changes in mitochondrial respiration signal through the calcium channel regulatory protein Mid1, which we demonstrate is required for activation of calcineurin and modulation of autophagy flux.

Results and discussion

Mitochondria regulate autophagy flux downstream of TORC2-Ypk1

To test whether TORC2 signaling and mitochondria converge to regulate autophagy, we examined amino acid starvation–induced autophagy in wild-type (WT) and *ypk1Δ* cells and respiratory-incompetent rho^0 and *ypk1Δ* rho^0 cells. Because Ypk1 is the relevant target of TORC2 involved in autophagy regulation (Vlahakis et al., 2014), for simplicity we used exclusively *ypk1Δ* cells as a surrogate for disrupting TORC2 activity. Autophagy was monitored via Western blot analysis using GFP-Atg8, which associates with autophagosomes and becomes degraded in the vacuole to release free GFP, and autophagosome turnover, or flux, is represented as the percentage of free GFP relative to total GFP signal (Kirisako et al., 1999; Abeliovich et al., 2000, 2003). Consistent with our previous findings (Vlahakis et al., 2014), we observed a severe defect in GFP-Atg8 turnover in *ypk1Δ* cells during amino acid starvation (Fig. 1 A). Remarkably, we observed that inhibition of mitochondrial respiration in *ypk1Δ* cells restored autophagy, as both rho^0 and *ypk1Δ* rho^0 cells exhibited WT-like levels of flux under these conditions (Fig. 1 A).

In that experiment, glucose was used as a carbon source, and no defect in autophagy was observed in *rho*^{0} cells alone (Fig. 1 A). In contrast, when galactose was used as a carbon source, we observed a defect in autophagic flux in rho^0 cells that persisted in *ypk1Δ* rho^0 cells (Fig. 1 B). These differences are consistent with prior observations (Graef and Nunnari, 2011a) and may be related to the effects of glucose repression on mitochondrial biogenesis and function (Böker-Schmitt et al., 1982; Herrero et al., 1985; Ulery et al., 1994).

We next tested whether rescue of autophagy flux in *ypk1Δ* rho^0 cells was caused specifically by changes in mitochondrial respiration by deleting specific nuclear-encoded genes important for the assembly of mitochondrial electron transport chain (ETC) complex III (*cbs1*Δ), complex IV (*mss51*Δ), or complex V (CV; *atp10*Δ). Inhibition of mitochondrial respiration by these gene deletions restored autophagy flux in *ypk1Δ* cells in response to amino acid starvation (Fig. 1 C). Accordingly, we conclude that mitochondrial respiration acts downstream of TORC2-Ypk1 signaling to regulate autophagy under amino acid–limiting conditions. As an extension of these findings, we determined that TORC2-Ypk1 signaling also promotes mitophagy, the autophagy-dependent selective degradation of mitochondria (Kissová et al., 2007; Kanki and Klionsky, 2008; Okamoto et al., 2009), during amino acid starvation (Fig. S1).

Ypk1-deficient cells have several mitochondria-associated defects, including reduced mitochondria membrane potential and a concomitant increase in mitochondria-associated reactive oxygen species (ROS; Niles et al., 2014; Niles and Powers, 2014). These defects in mitochondrial function are all linked to aberrant activation of Tpk3, one of three catalytic subunits of protein kinase A, and are reversed when *TPK3* is deleted from Ypk1-deficient cells (Niles et al., 2014). We tested whether protein kinase A also links TORC2-Ypk1 signaling and mitochondria with respect to autophagy by examining autophagy flux in WT, *ypk1Δ*, *tpk3Δ*, and *ypk1Δ tpk3Δ* mutant cells. We observed that deletion of *TPK3* resulted in significant restoration of autophagy flux in *ypk1Δ* cells after amino acid starvation (Fig. 1 D). Thus, TORC2-Ypk1 signaling promotes autophagy in part by preventing Tpk3-dependent changes in mitochondrial respiration.

TORC1 is also a negative regulator of autophagy and inhibits Atg13 activity by direct phosphorylation (Kamada et al., 2010). However, upon examination of TORC1-dependent phosphorylation of native Atg13 in WT versus *ypk1Δ* cells, we observed no changes in phosphorylation after amino acid starvation (Fig. 1 E). These findings are consistent with prior conclusions that autophagy flux under amino acid–limited conditions is largely independent of TORC1 (Graef and Nunnari, 2011a; Vlahakis et al., 2014).

Mitochondria function upstream of calcineurin during autophagy

TORC2-Ypk1 signaling regulates autophagic flux by repressing the activity of calcineurin (Vlahakis et al., 2014). Given our results, we next wanted to determine whether mitochondria act upstream or downstream of calcineurin. Toward this end, we examined calcineurin activity in respiratory-competent WT and *ypk1*Δ cells, as well as in cells made respiratory deficient by introduction of ETC gene deletions. Calcineurin activity was measured using a CDRE-driven *lacZ* reporter, as described previously (Stathopoulos and Cyert, 1997; Vlahakis et al., 2014). In agreement with our prior findings, calcineurin activity was elevated in *ypk1*Δ cells compared with basal levels observed in WT cells (Fig. 2 A). Importantly, calcineurin activity was reduced in each of the respiratory-deficient *ypk1*Δ strains, suggesting that elevated calcineurin activity in *ypk1*Δ cells requires aberrant mitochondrial respiratory function (Fig. 2 A).

We previously reported that *ypk1*Δ cells possess an increase in mitochondria-associated ROS (Niles et al., 2014; Niles and Powers, 2014). Therefore, to address the mechanism by which mitochondria regulate calcineurin, we examined levels of ROS in *ypk1*Δ and *ypk1*Δ *cnb1*Δ cells, using the ROS-reactive fluorescent dye (H2DCF-DA; Lee et al., 2011; Niles et al., 2014; Niles and Powers, 2014). We observed that ROS was elevated in *ypk1*Δ compared with WT cells (WT: 3% ± 0.8 vs. *ypk1Δ*: 43% ± 12 2,7-dichlorofluorescin diacetate [DCF]–positive cells; Fig. 2 B). Importantly, ROS remained elevated in *ypk1*Δ *cnb1*Δ cells (45% ± 10 DCF-positive cells), a condition in which autophagic flux is restored after amino acid starvation (Vlahakis et al., 2014). This indicates that changes in calcineurin activity do not influence mitochondrial respiratory function, as measured by this assay (Fig. 2 B). Control experiments demonstrated that the ROS observed in *ypk1*Δ cells could be accounted for exclusively by mitochondria-derived sources, as elevated levels of ROS were not detected in any of the respiratory deficient *ypk1*Δ strains (Fig. 2 B).

Given these results, we next tested whether mitochondrial-derived ROS activates calcineurin. Specifically, we measured calcineurin activity in WT and *ypk1Δ* cells grown in the absence or presence of the ROS scavenger *N*-acetyl-L-cysteine (NAC), which ameliorates defects associated with deficient Ypk1 signaling (Niles et al., 2014). Indeed, we observed that calcineurin activity was reduced in *ypk1Δ* cells treated with

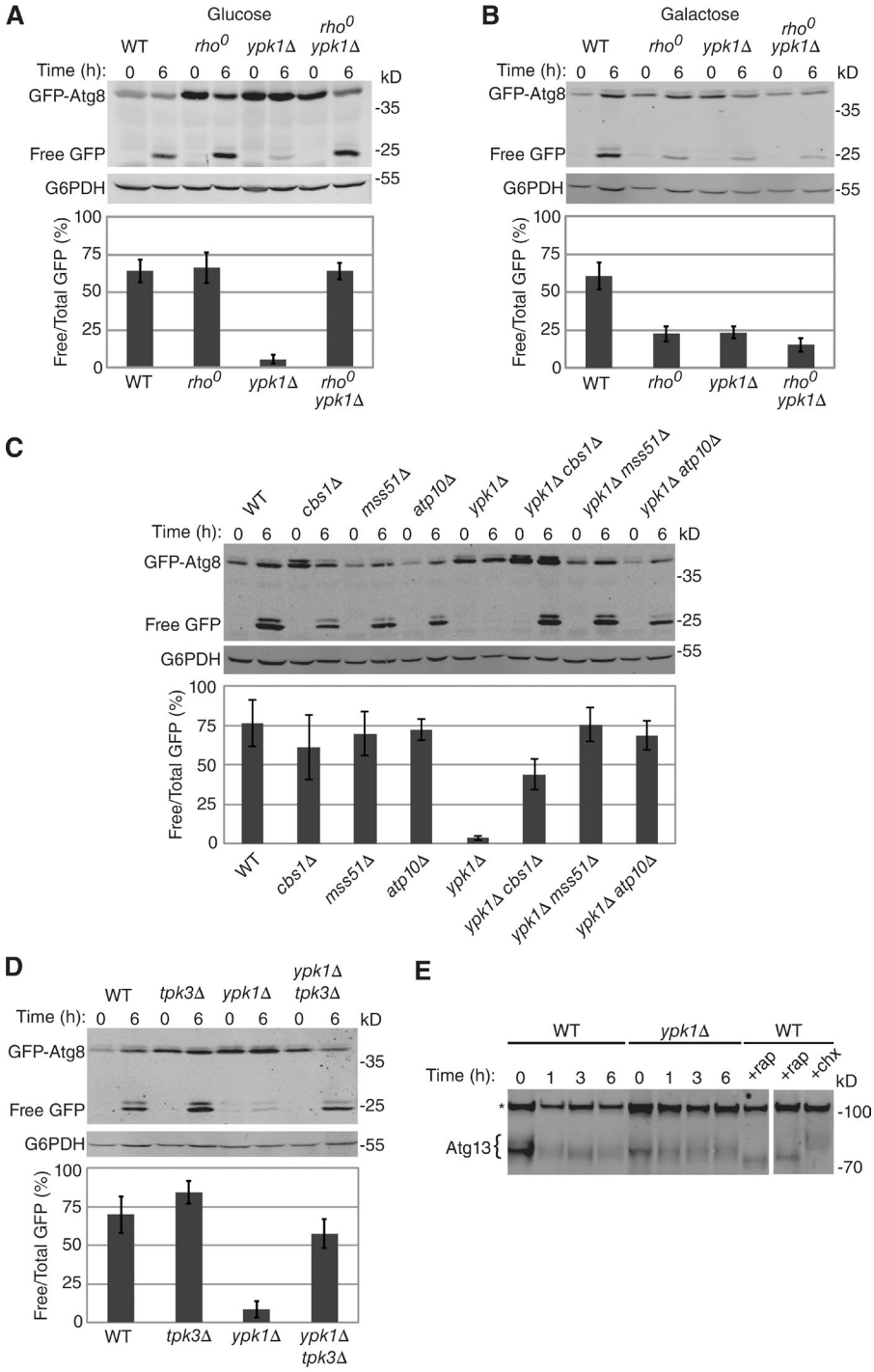

Figure 1. **TORC2-Ypk1 signaling and mitochondria regulate autophagy flux.** (A–D) Cells carrying pRS416 *pr^{ATG8}GFP-ATG8* were grown to log phase in SCD medium (A, C, and D) or synthetic complete galactose medium (B) without uracil and transferred to amino acid starvation medium with 2% glucose (A, C, and D) or galactose (B) as the carbon source. GFP and GFP-Atg8 protein levels were visualized by Western blot analysis as described in Materials and methods. Quantification of autophagy flux at 6 h of starvation is represented as a percentage of the ratio of free GFP to total GFP (GFP + GFP-Atg8) signal. Data are presented as means ± SD of three independent experiments for A, B, and D and four independent experiments for C. (E) WT and *ypk1Δ* cells were grown and starved as in A, and cells were harvested at the indicated time points. WT cells were also treated with 200 nM rapamycin in SCD medium for 1 h or 25 µg/ml cycloheximide (CHX) for 2 h. Protein extracts were run on 3–8% NuPage Tris-acetate gels, and Western blot analysis was performed using α-Atg13 and α-G6PDH antibodies. Asterisk denotes nonspecific protein band for loading reference.

NAC (Fig. 2 C). These findings support a model wherein aberrant mitochondrial activity leads to elevated ROS, which stimulates calcineurin activity in *ypk1Δ* cells. These findings are also consistent with findings in mammalian cells that chronic mitochondrial respiratory stress leads to activation of calcineurin (Guha et al., 2010). Moreover, exposure to ROS has been reported to activate calcineurin in cardiomyocytes and lead both to an attenuation of autophagy as well as an increase in apoptosis (He et al., 2014). Thus, a coupling between mitochondrial respiration and calcineurin activation is likely to be a conserved feature in the regulation of autophagy in eukaryotic cells.

Mitochondria function upstream of the GAAC response during autophagy

We next examined the relationship between mitochondrial respiration and the GAAC response. We determined previously that Gcn2-mediated eIF2α (Sui2) phosphorylation as well as subsequent *GCN4* mRNA translation was impaired in *ypk1Δ* cells after amino acid starvation because of increased calcineurin activity (Wek et al., 1995; Zhu et al., 1996; Vlahakis et al., 2014). We therefore reasoned that respiratory-deficient *ypk1Δ* cells should display a normal GAAC response after amino acid starvation because calcineurin activity is reduced in these cells. Indeed, we observed that whereas Gcn2-dependent

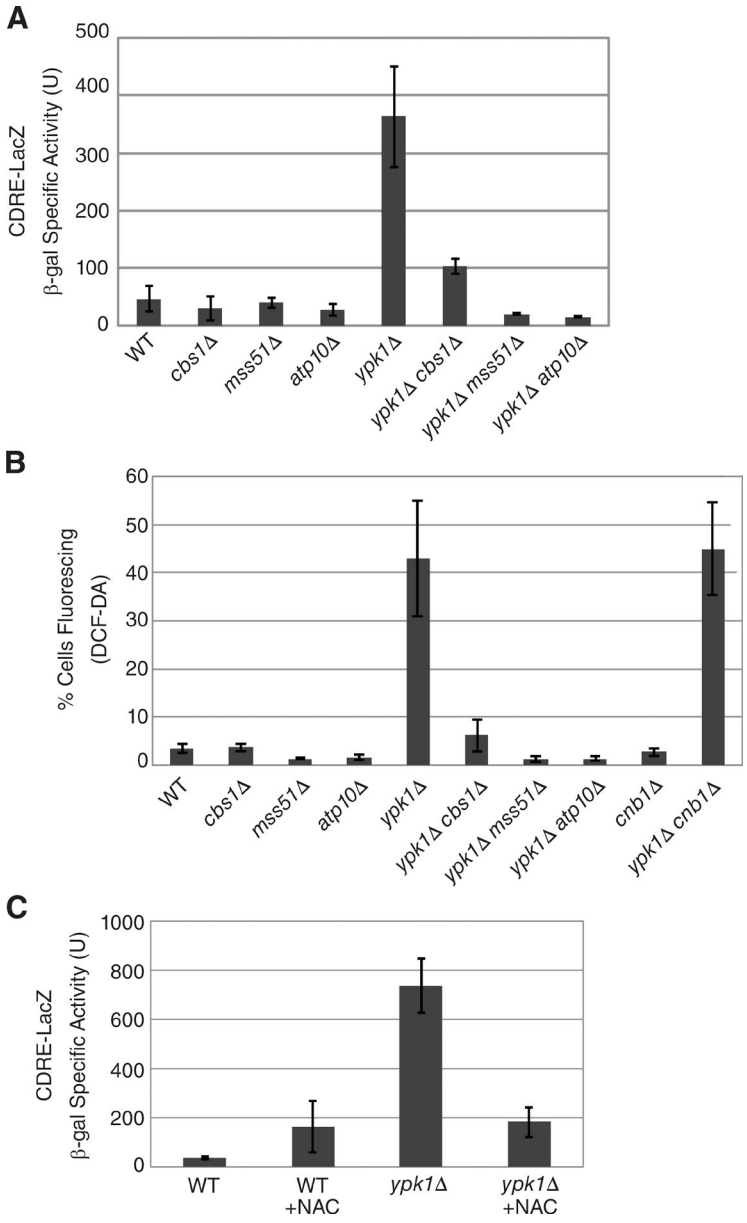

Figure 2. **Mitochondria regulate calcineurin activity in response to TORC2-Ypk1.** (A) Cells carrying pAMS363 that expressed *2xCDRE:lacZ* were grown to log phase in SCD medium without uracil. β-Galactosidase (β-gal) activity was measured and is displayed as units of nmol ONPG converted/min/mg of protein (see Materials and methods). Data are presented as means ± SD of three independent experiments. (B) Strains were grown to log phase as in A and incubated with 10 µM DCF for 30 min before fluorescence microscopy imaging. Quantification represents the percentage of DCF-positive fluorescing cells where $n \geq 300$ total cells per strain. Data are presented as means ± SD of three independent experiments. (C) WT and *ypk1Δ* cells carrying pAMS363 were grown as in A in the presence and absence of 20 mM NAC, and β-galactosidase activity was measured. Data are presented as means ± SD of three independent experiments.

eIF2α phosphorylation at Ser-51 and *GCN4* expression were impaired in *ypk1*Δ cells, both readouts were restored in respiratory-deficient *ypk1*Δ cells (Fig. 3 A). These findings demonstrate that the GAAC response functions downstream of normal mitochondrial function to promote autophagic flux.

Mid1 links mitochondrial respiration to calcineurin

We sought to explore the mechanism of calcium signaling within the TORC2-Ypk1–mediated autophagy response. The source of calcium that regulates calcineurin downstream of TORC2-Ypk1 is unknown, as TORC2 signaling has been reported to not affect cytoplasmic calcium (Mulet et al., 2006). Because the vacuole represents a major intracellular reservoir of calcium, we first examined a role for vacuolar calcium regulators in promoting calcineurin activity in Ypk1-deficient cells. However, we observed that calcineurin activity remained elevated in *ypk1Δ* cells lacking the major vacuolar calcium homeostasis regulators; Vcx1, the vacuolar membrane Ca^{2+}/H^{+} anti-porter; or Yvc1, the channel that mediates vacuolar calcium release during a variety of cellular stress conditions (Ton and Rao, 2004; Fig. 4 A).

We next examined a role for the conserved high-affinity calcium uptake system (HACS) responsible for importing extracellular calcium into the cell (Ton and Rao, 2004; Martin et al., 2011). The HACS regulates calcium import through a plasma membrane (PM)-localized calcium channel formed by the proteins Cch1 and Mid1 (Iida et al., 1994; Ozeki-Miyawaki et al., 2005; Martin et al., 2011; Vu et al., 2015). Interestingly, we observed that deletion of Mid1, but not Cch1, was sufficient to return calcineurin activity to basal WT levels in *ypk1Δ* cells (Fig. 4 A). These results demonstrate that Mid1 functions as a positive regulator of calcineurin downstream of Ypk1 via a mechanism that is distinct from the traditional HACS involving Cch1. This conclusion is consistent with prior evidence that Mid1 possesses a Cch1-independent function in calcium homeostasis (Courchesne and Ozturk, 2003). Because inhibition of Mid1 in *ypk1Δ* cells reduced calcineurin activity, we also

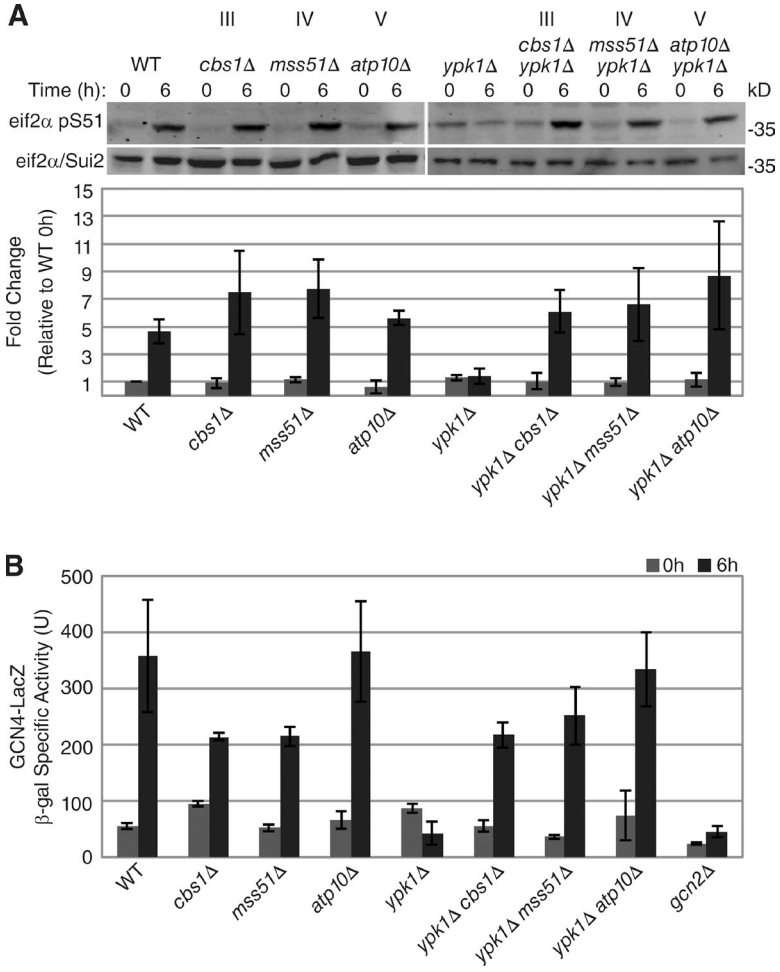

Figure 3. **Mitochondria function upstream of the GAAC response during autophagy.** Cells were grown to log phase in SCD medium without uracil, then transferred to amino acid starvation medium with 2% glucose for 6 h. (A) Gcn2-dependent phosphorylation of eIF2α was determined by Western blot analysis using α-phospho Ser-51 eIF2α and α-Sui2 (eIF2α) antibodies. Quantification represents the ratio of phosphorylated eIF2α Ser-51 and total eIF2α (Sui2) signal with fold change relative to WT at t = 0 h. Data are presented as means ± SD of three independent experiments. (B) *GCN4* de-repression was measured using the p180 *GCN4 URE-1* to *-4:lacZ* reporter plasmid expressed in all strains. β-Galactosidase (β-gal) activity was determined as described in the legend to Fig. 2. Data are presented as means ± SD of three independent experiments.

examined a role for Mid1 in autophagy. Indeed, we observed that autophagic flux was restored to WT levels in *ypk1Δ mid1Δ* cells (Fig. 4 B). Thus, we conclude that Mid1 negatively regulates autophagy by activating calcineurin in Ypk1-deficient cells in a HACS-independent manner.

We developed an independent approach to assay Mid1 activity by using the anti-arrhythmic and antifungal drug amiodarone, which induces a rapid increase in intracellular calcium by a mechanism that specifically requires Mid1, but not Cch1 (Courchesne and Ozturk, 2003). Because cells possessing high levels of Mid1 activity are hypersensitive to amiodarone, we reasoned we could use this sensitivity as an assay for Mid1 activity. Indeed, we observed that *ypk1Δ* cells were hypersensitive to sublethal concentrations of amiodarone, consistent with a model wherein Mid1 activity is elevated in Ypk1-deficient cells (Fig. 4 C, top). We note that this finding is consistent with a prior study that identified *ypk1Δ* cells as hypersensitive to amiodarone (Gupta et al., 2003). In further support for this model, the hypersensitivity of *ypk1Δ* cells to amiodarone was abolished in *ypk1Δ mid1Δ* cells (Fig. 4 C, top). Importantly, we observed that *ypk1Δ cnb1Δ* cells remained hypersensitive to the drug, confirming that this assay likely applies to Mid1 function specifically, rather than calcium signaling in general (Fig. 4 C, top).

We next examined the sensitivity of a respiratory-deficient *ypk1Δ* strain to amiodarone and observed that *ypk1Δ atp10Δ* cells lacking ETC CV were as resistant to the drug as WT cells (Fig. 4 C, top). Similarly, we determined that treating *ypk1Δ* cells with the ROS scavenger NAC significantly reduced the hypersensitivity of these cells to amiodarone, confirming a role for ROS in promoting Mid1 activity (Fig. 4 C, bottom). We also determined that Mid1 activity does not itself influence ROS, as ROS levels remained elevated in both *ypk1Δ* and *ypk1Δ mid1Δ* cells (Fig. 4 D), indicating that Mid1 functions downstream of TORC2-Ypk1 and mitochondria to activate calcineurin.

Mid1 is an integral membrane protein and has been localized previously to both the PM and the ER (Iida et al., 1994; Yoshimura et al., 2004; Ozeki-Miyawaki et al., 2005). We determined that an mCherry-tagged version of Mid1 (Mid1-mCherry) colocalized in a pattern essentially indistinguishable from the ER marker HDEL-GFP in both WT and *ypk1Δ* cells (Fig. 4 E and Fig. S2 A). Because expression of Mid1-mCherry in *ypk1Δ mid1Δ* cells restored amiodarone sensitivity, we conclude that this construct expresses a functional fusion protein (Fig. 4 F). To test whether a strong cortical ER–localized signal might mask a subpopulation of Mid1 localized at the PM, we examined Mid1-mCherry in a strain (termed Δ-tether) that possesses mutations in numerous ER–PM tethering proteins and results in partial separation of cortical ER from the PM (Manford et al., 2012). Using this approach, we indeed observed two distinct populations of PM- and ER-localized Mid1 (Fig. S2 B), consistent with previous studies on the cellular localization of Mid1 (Iida et al., 1994; Yoshimura et al., 2004; Ozeki-Miyawaki et al., 2005). Nevertheless, because Mid1 localization does not change in *ypk1Δ* cells, we conclude that its

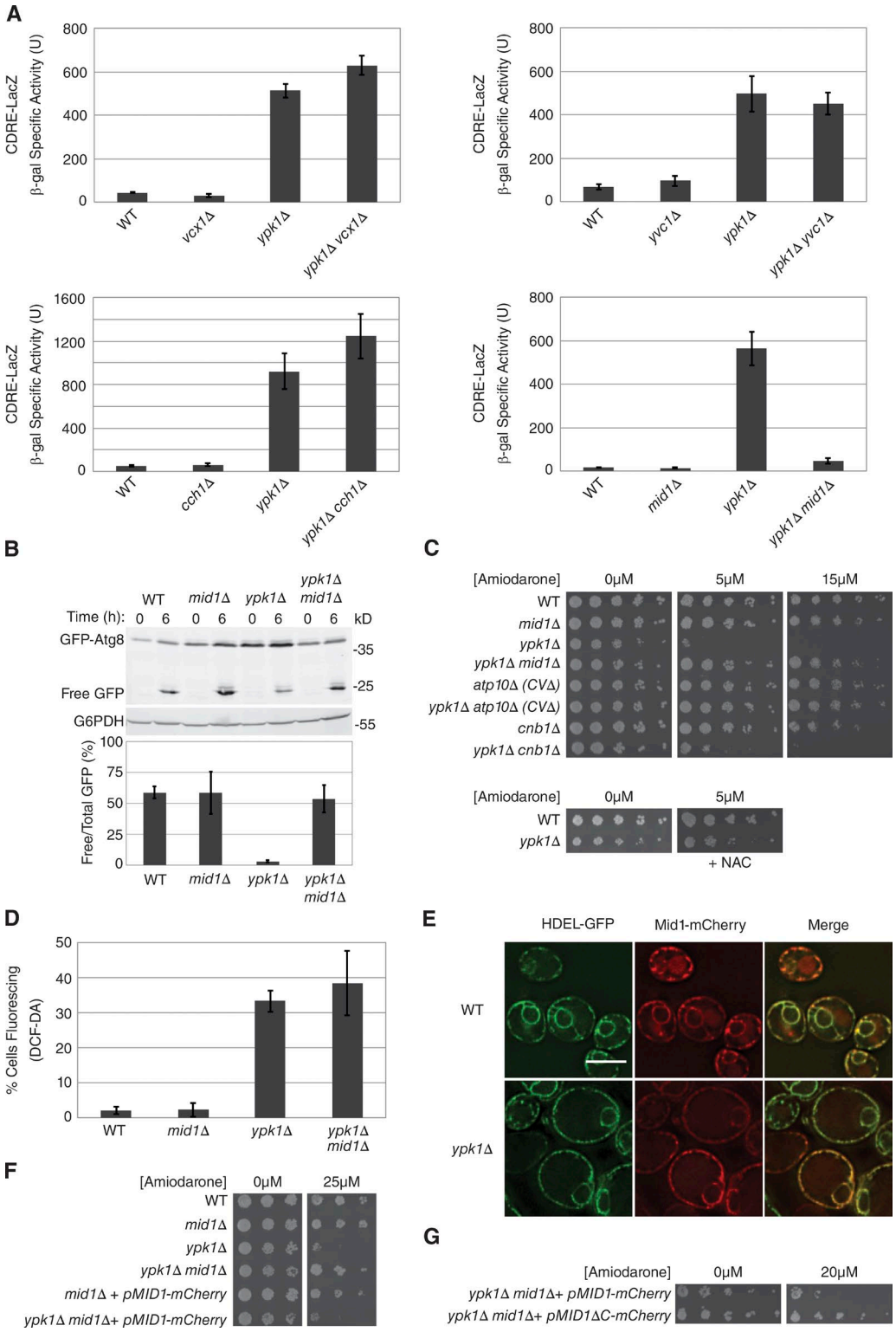

Figure 4. **Mid1 links mitochondrial respiration to calcineurin during autophagy.** (A) Cells carrying pAMS363 that expressed *2xCDRE:lacZ* were grown, and β-galactosidase (β-gal) activity was determined as described in the legend to Fig. 2. Data are presented as means ± SD of three independent experiments. (B) Strains were grown and subjected to amino acid starvation with 2% glucose, and autophagy flux was quantified as described in the legend to Fig. 1. Data are presented as means ± SD of three independent experiments. (C) Cells were grown to log phase, and serial dilutions were plated on agar plates containing SCD medium and the indicated concentrations of amiodarone. Where indicated, plates also contained 20 mM NAC. (D) Cells were grown to log phase, and DCF-DA fluorescence was determined as described in the legend to Fig. 2. Quantification represents the percentage of DCF-positive fluorescing cells where $n \geq 200$ total cells per strain. Data are presented as means ± SD of three independent experiments. (E) Cells carrying pRS416 MID1-mCherry and the ER marker HDEL-GFP at the endogenous LEU2 locus were grown to log phase and visualized using deconvolution fluorescence microscopy with the Applied Precision Delta Vision Real-Time microscope as detailed in Materials and methods. Deconvolved images are shown. Bar, 5 µm. Cells containing either empty vector prs416-MET25 (PPL187) or prs416-MET25 MID1-mCherry (PPL610; F) or pRS416 MET25-MID1-ΔC-mCherry (PPL611; G) were grown to log phase and plated on SCD plates without uracil and containing amiodarone as in C.

molecular function in the regulation of calcineurin activity is likely to be performed, at least in part, within the ER or ER-associated regions of the PM.

The C-terminal domain of Mid1 contains several phylogenetically conserved cysteine residues that have the potential to be oxidized during conditions of oxidative stress (Finkel, 2011; Funato and Miki, 2013; Vu et al., 2015). To begin to explore the mechanism by which Mid1 is regulated by mitochondrial ROS, we asked whether this cysteine-rich domain is important for Mid1 activity in Ypk1-deficient cells. Indeed, we observed that whereas expression of WT Mid1-mCherry in *ypk1Δ mid1Δ* cells exhibited hypersensitivity to amiodarone as expected, expression of a truncated version of Mid1 lacking its cysteine-rich C-terminal domain (Mid1ΔC-mCherry) was sufficient to reverse this hypersensitivity (Fig. 4 G). Importantly, deletions within the C-terminal region of Mid1 do not prevent localization within the ER/PM (Ozeki-Miyawaki et al., 2005). Together, these findings are consistent with a model wherein Mid1 activity responds to oxidative stress in Ypk1-deficient cells to positively regulate calcineurin activity.

In mammalian cells, mitochondria are an important reservoir of calcium; however, in yeast this is not the case, raising the question of how mitochondrial function is linked to calcineurin activation in TORC2-Ypk1 mutants (Rizzuto et al., 1992; Csordás and Hajnóczky, 2009; Kovács-Bogdán et al., 2014; Suzuki et al., 2014). The ER is known to be involved in direct physical interactions with mitochondria, via a multiprotein complex termed ERMES, suggesting that intra-organelle communication may interface with TORC2-Ypk1 signaling to regulate calcium availability (Rapizzi et al., 2002; Kornmann et al., 2009, 2011; Murley et al., 2015). Our demonstration that Mid1 both localizes to the ER and responds to aberrant mitochondrial respiration and increased ROS underscores the appeal of this model. Indeed, our preliminary findings indicate that, like loss of Mid1 activity, mutant alleles of the ERMES component Gem1 abolished calcineurin activity and rescued the amiodarone hypersensitivity of Ypk1-deficient cells (Fig. S3, A and B). Interestingly, these Gem1 alleles also suppressed ROS in *ypk1Δ* cells (Fig. S3 C), raising the intriguing possibility that mitochondrial–ER contacts are directly involved in ROS accumulation. Understanding the precise function and regulation of Mid1, as well as the potential role of ERMES within this pathway, represents a high-priority goal.

Our understanding of autophagy has deepened with the discovery that distinct signaling pathways and upstream nutritional cues converge to regulate this important cellular process. Our present findings demonstrate that mitochondria are a key component of the pathway that links TORC2-Ypk1 signaling to autophagy under amino acid starvation conditions. In particular, we find that impaired Ypk1 signaling leads to accumulation of mitochondrial ROS, which activates calcineurin and negatively regulates the GAAC response (Fig. 5). Importantly, we have identified the ER/PM-localized calcium channel regulator Mid1 as a key mediator of the effects of mitochondrial ROS that promotes calcineurin activity (Fig. 5). Because dysregulation of TORC2, mitochondria, calcium signaling, and autophagy are all implicated in metabolic disorders, our findings are significant, as they link these components and suggest a common mechanism that may contribute to pathogenesis of related diseases.

Materials and methods

Yeast strains and media

Strains of yeast used in this study are derivatives of W303α (*leu2-3,112*; *ura3-1*; *his3-11,15*;*trp1-1*; *ade2-1*; *can1-100*) and are listed in Table S1. PCR-based targeted homologous recombination was used to replace complete ORFs with kanMX6, HIS3MX6, NAT, or TRP1 cassettes where indicated (Longtine et al., 1998). Strains expressing HDEL-GFP at the endogenous *LEU2* locus were created by transforming strains with pRS305 YIPLac204 HDEL-GFP linearized with Cla1 (provided by J. Nunnari, University of California, Davis, Davis, CA; Rossanese et al., 2001). Strains expressing endogenous Idh1-GFP:HISMX6 were created by PCR-based targeted homologous recombination after PCR amplifying GFP:HISMX6 flanked with regions of homology using genomic DNA obtained from the yeast GFP collection (Huh et al., 2003). Yeast transformations were performed using lithium acetate as previously reported (Gietz and Woods, 2002). In brief, logarithmically growing yeast (OD_{600} 0.5–1) were washed in 100 µl of 100 mM lithium acetate then resuspended in a mixture containing 240 µl PEG 3350 (50% wt/vol), 36 µl of 1 M lithium acetate, 10 µl salmon sperm DNA (10 mg/ml), and 70 µl DNA/sterile water. Once resuspended, cells were incubated at 30°C for 1 h. Cells were then heat-shocked at 42°C for 15 min and centrifuged for 30 s at 8,000 rpm. Supernatant was removed, and cells were resuspended in 100 µl of sterile water, plated on agar plates with required nutrients, and incubated at 30°C. All strains were grown to log phase (OD_{600} = ∼1.0) in synthetic complete dextrose (SCD) or synthetic complete galactose yeast medium (0.8% yeast nitrogen base without amino acids and 2% [wt/vol] dextrose or galactose, pH 5.5) supplemented with amino acids as described previously (Sherman, 1991). Where indicated, strains were then exposed to amino acid starvation medium (0.05% yeast extract and 2% [wt/vol] dextrose or galactose) previously shown to be specifically limiting in auxotrophic amino acids (Graef and Nunnari, 2011a). To determine cellular ROS, log-phase cells were incubated with 10 µM DCF (Cayman Chemical) for 30 min before imaging by fluorescence microscopy. For the amiodarone sensitivity assay, amiodarone (Cayman Chemical) was diluted in sterile water and overlaid onto agar plates containing appropriate yeast media to the indicated final concentrations. Logarithmically growing yeast cells were diluted and plated on solid agar plates and allowed to grow at 30°C for 2–3 d. To induce mitophagy, cells expressing endogenous IDH1-GFP were grown overnight in YPL medium (yeast extract, peptone, and 2% lactate, pH 5.5) to an OD_{600} of 1.0 and subjected to amino acid starvation medium, pH 5.5, with 2% lactate for 24 h, followed by the workup described in the Whole-cell extraction, antibodies, and Western blot analysis section.

Plasmids

For this study, pRS416 MET25-MID1-mCherry (PPL610) was generated by inserting MID1 ORF (1,647 base pairs) flanked by XbaI and SalI and mCherry flanked by SalI and XhoI restriction sites into pRS416-MET25 plasmid (pPL187). The plasmid pRS416 MET25-MID1-ΔC-mCherry (pPL611) expressing the C-terminal truncation mutant of Mid1 (Mid1-ΔC) was generated by replacing the WT MID1 ORF in pPL610 with a truncated MID1 ORF containing base pairs 1–1,491 using XbaI and SalI. Where indicated, yeast strains contained the following previously described plasmids: pRS416 *prATG8-GFP-ATG8* (Abeliovich et al., 2003), pAMS363 *2xCDRE:lacZ* (Stathopoulos and Cyert, 1997), p180 *GCN4 URE-1* to *-4:lacZ* (Hinnebusch, 1985), pRS315 Gem1E225K, Gem1E354K, and Gem1E225K/E354K (Kornmann et al., 2011).

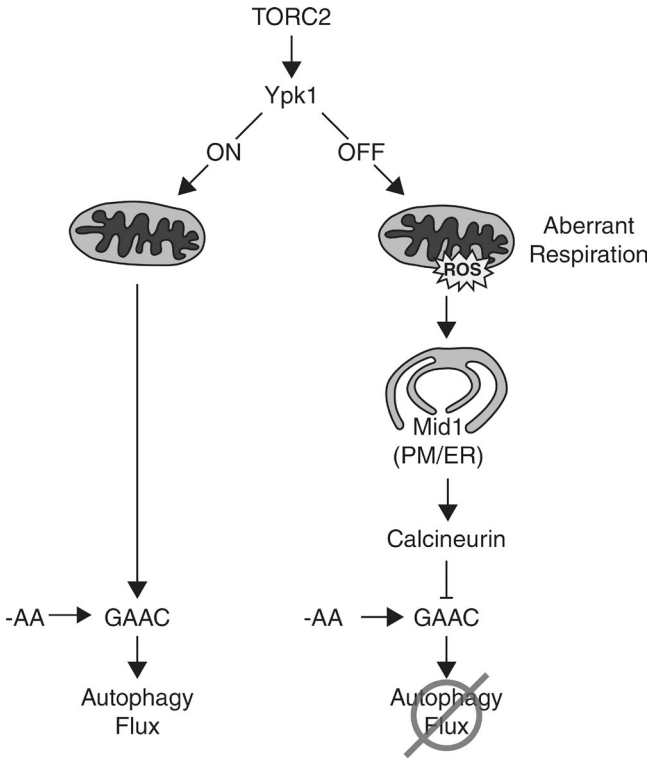

Figure 5. **Model for role of TORC2-Ypk1 and mitochondria in regulating autophagy after amino acid starvation.** When active, TORC2 and Ypk1 promote healthy mitochondrial function, which is required for the GAAC response and subsequent induction of autophagy after amino acid starvation. Decreased TORC2-Ypk1 signaling leads to aberrant mitochondrial respiration and increased mitochondrial ROS, which activates calcineurin via the ER/PM-localized calcium-channel component, Mid1. Hyperactive calcineurin in turn negatively regulates the GAAC response and blocks autophagy under conditions of amino acid starvation.

Whole-cell extraction, antibodies, and Western blot analysis

Whole-cell protein extracts were prepared from yeast using the NaOH cell lysis method. In brief, per sample, 1.5 OD_{600} units of yeast pellet was resuspended in 580 µl of 0.255 M NaOH/1% β-mercaptoethanol and incubated on ice for 10 min. 80 µl of 50% TCA was added and incubated for an additional 10 min on ice. Protein precipitates were centrifuged at 12,000 rpm for 15 min at 4°C, and supernatant was discarded. Protein pellet was washed in 0.5 ml of 1 M Tris-HCl, pH 6.8, and spun, and the pellet was resuspended in 100 µl of protein sample buffer. 10–25 µl of extracted proteins in sample buffer were loaded onto 10% SDS/PAGE gels and transferred to nitrocellulose membranes for Western blot analysis using LI-COR secondary antibodies (Sekito et al., 2000). The Atg13 phospho shift experiment was performed using Invitrogen NuPage Tris-acetate 3–8% gradient gels run at 4°C. Membranes were probed with α-GFP N86/8 monoclonal antibody (1 µg/ml; NeuroMab), α-G6PDH (Zwf1; 1:1,000; Sigma-Aldrich), α-Atg13 (1:1,000; polyclonal, gift of D. Klionsky, University of Michigan, Ann Arbor, MI), α-phospho eIF2α Ser-51 antibody (1:1,000; Cell Signaling Technology), and α-Sui2 (eIF2α; 1:1,000; gift of T. Dever, National Institutes of Health, Bethesda, MD) primary antibodies and visualized using the appropriate secondary antibodies conjugated to IR dye (1:5,000; LI-COR Biosciences). All Western blot images were quantified using ImageJ software. Data are presented as means ± SD of at least three independent experiments.

β-Galactosidase activity assay

Exponentially growing cells (OD_{600} = ∼1) were incubated at 30°C in SCD medium supplemented with amino acids, and where indicated, were transferred to amino acid starvation medium for 6 h. β-Galactosidase activity was measured at 30°C using the substrate *O*-nitrophenyl-β-D-galactopyranoside (ONPG; Sigma-Aldrich) as described previously (Rose and Botstein, 1983). In brief, 10 OD_{600} units of yeast cell pellet were acquired per sample and washed with 1 ml of 4°C sterile water. Cell pellets were resuspended in 250 µl of 4°C breaking buffer (100 mM Tris-HCl, pH 8, 1 mM DTT, and 20% glycerol) on ice, and 12.5 µl of 100 mM PMSF (diluted in ethanol) was added. Cells were broken and homogenized using glass beads and a mini–bead beater (Biospec Products) for 1 min in the cold. After homogenization, an additional 250 µl of breaking buffer was added, and extract was removed from glass beads and clarified by centrifuging at 12,000 rpm for 15 min at 4°C. Supernatant (cell lysate) was removed from the pellet for subsequent β-galactosidase measurement. To measure β-galactosidase activity, the following were added per sample in a 96-well plate: 14.2 µl of clarified cell lysate, 128.57 µl of z-buffer with fresh β-mercaptoethanol (60 mM Na_2HPO_4-$7H_2O$, 40 mM NaH_2PO_4-H_2O, 10 mM KCl, 1 mM $MgSO_4$-$7H_2O$, and 50 mM β-mercaptoethanol, pH 7), and 28.57 µl ONPG stock solution (4 mg/ml ONPG in z-buffer). Before adding ONPG, samples were incubated at 30°C to ensure that reaction occurred at this temperature. The reaction was incubated at 30°C, and time was recorded upon addition of ONPG. When the reaction turned a faint yellow color, the OD of samples was measured at 420 nm using a 96-well plate reader (SpectraMax M5; Molecular Devices). The protein concentration of cell lysate was quantified using a BSA assay with Bradford reagent (Sigma-Aldrich). β-Galactosidase activity is given in units of nanomoles of ONPG converted per minute per milligram of protein. Data are presented as means ± SD of three independent experiments.

Fluorescence microscopy

Cells expressing pRS416-MET25-MID1-mCherry and ER marker HDEL-GFP at the endogenous LEU locus were grown to log phase and visualized using a DeltaVision real-time microscope (Applied Precision Ltd.) fitted with a 60×, 1.4-NA objective and CoolSnap HQ camera (Photometrics). Capture and postcapture image processing was done using softWoRx software (Applied Precision Ltd.) and Fiji (Schindelin et al., 2012). Where indicated, cells were visualized using a Nikon E600 fluorescent microscope fitted with a 100×, 1.4-NA objective and an Orca ER charge coupled device camera (Hamamatsu Photonics) controlled by Micro Manager 1.2 ImageJ software. Image capture and processing were done using Fiji (Schindelin et al., 2012) and Photoshop (Adobe Systems).

Online supplemental material

Fig. S1 shows that TORC2-Ypk1 signaling is required for mitophagy. Fig. S2 shows that Mid1 localizes to both the ER and PM. Fig. S3 shows that the ERMES component Gem1 is involved in TORC2-Ypk1 signaling to Mid1 and calcineurin.

Acknowledgments

We thank Dr. Thomas Dever for antibodies and Dr. Martin Graef for strains. We thank Drs. Martin Graef and Jodi Nunnari and members of the Powers laboratory for helpful discussions. We thank Samya Faiq, Nicole Dudaney, Elizabeth Iliuta, and Allison Gabbert for technical help during this study.

This work was supported by National Institutes of Health grant GM086387 (to T. Powers) and T-32 Training Grant in Molecular and Cellular Biology (to A. Vlahakis).

The authors declare no competing financial interests.

Submitted: 10 May 2016
Revised: 2 September 2016
Accepted: 8 November 2016

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