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BRIEF REPORT

Stress-response transcription factors Msn2 and Msn4 couple TORC2-Ypk1 signaling and mitochondrial respiration to *ATG8* gene expression and autophagy

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ABSTRACT

Macroautophagy/autophagy is a starvation and stress-induced catabolic process critical for cellular homeostasis and adaptation. Several Atg proteins are involved in the formation of the autophagosome and subsequent degradation of cytoplasmic components, a process termed autophagy flux. Additionally, the expression of several Atg proteins, in particular Atg8, is modulated transcriptionally, yet the regulatory mechanisms involved remain poorly understood. Here we demonstrate that the AGC kinase Ypk1, target of the rapamycin-insensitive TORC2 signaling pathway, controls *ATG8* expression by repressing the heterodimeric Zinc-finger transcription factors Msn2 and Msn4. We find that Msn2 and Msn4 promote *ATG8* expression downstream of the histone deacetylase complex (HDAC) subunit Ume6, a previously identified negative regulator of *ATG8* expression. Moreover, we demonstrate that TORC2-Ypk1 signaling is functionally linked to distinct mitochondrial respiratory complexes. Surprisingly, we find that autophagy flux during amino acid starvation is also dependent upon Msn2-Msn4 activity, revealing a broad role for these transcription factors in the autophagy response.

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Introduction

Autophagy is a catabolic cellular process that functions as a quality control mechanism that promotes homeostasis and adaptation. It does so through the formation of a double-membrane compartment, the phagophore, that captures cytoplasmic cargo, including damaged proteins and organelles, ultimately maturing into an autophagosome, which fuses with the cellular lytic compartment (vacuole or lysosome) for degradation and recycling.[1-4] In several different cell types, autophagy often occurs selectively and at a basal rate, but can increase markedly upon nutrient starvation or other forms of metabolic stress, an event conventionally referred to as macroautophagy.[1,5,6] Autophagy is involved in a myriad of cellular processes and its dysregulation can lead to metabolic and degenerative diseases including, but not limited to, cancer, osteoarthritis, Alzheimer and Parkinson diseases, as well as several cardiomyopathies.[7-9]

Many studies over the years have focused on defining the molecular mechanisms and machinery involved in autophagosomal biogenesis and subsequent degradation of cargo, a process commonly referred to as autophagy flux.[2,10-13] Indeed, pioneering studies in the yeast *S. cerevisiae* led to the discovery of more than 40 autophagy genes (*ATG* genes) essential for this process, many of which are conserved in higher eukaryotes.[11,12,14-17] Recently, it has also become clear that the transcription of a select number of autophagy-related genes is coupled to autophagy induction and that regulated expression of these genes is important for the proper control of autophagy.[1,18-20] By comparison to autophagy flux, the molecular mechanism(s) regulating the transcriptional induction of these *ATG* genes remain(s) relatively poorly uncharacterized.

Of these transcriptionally regulated *ATG* genes, *ATG8* is unique in that it is one of the few genes encoding an Atg protein that is physically incorporated into the phagophore membrane.[10,13,21] Accordingly, Atg8 is suggested to regulate both autophagosome size as well as the binding of selective cargo receptor proteins.[4,10,22-24] Our current understanding of *ATG8* transcriptional regulation is defined primarily by recent studies showing that it is negatively regulated by Ume6, a subunit of the Rpd3 histone deacetylase complex (HDAC) (Fig. 1A).[25,26] Significantly, the mechanism by which *ATG8* expression is positively controlled downstream of Ume6, including the transcription factor(s) subject to Ume6 regulation, remains unknown.

Our understanding of the regulation of autophagy has also expanded in recent years with the discovery that different environmental stresses initiate autophagy through distinct signaling pathways.[5,20,27] For example, we have demonstrated that the TORC2 signaling pathway, via its target kinase Ypk1, functions as a positive regulator of autophagy flux during amino acid-limited growth conditions.[27,28] In this context, TORC2-Ypk1 signaling promotes autophagy flux by negatively regulating the calcium-regulated phosphatase calcineurin and promoting the general amino acid control (GAAC) response. More recently, we have demonstrated that aberrant mitochondrial respiration, as a result of decreased TORC2-Ypk1 signaling, activates calcineurin and inhibits autophagy.[27-29]

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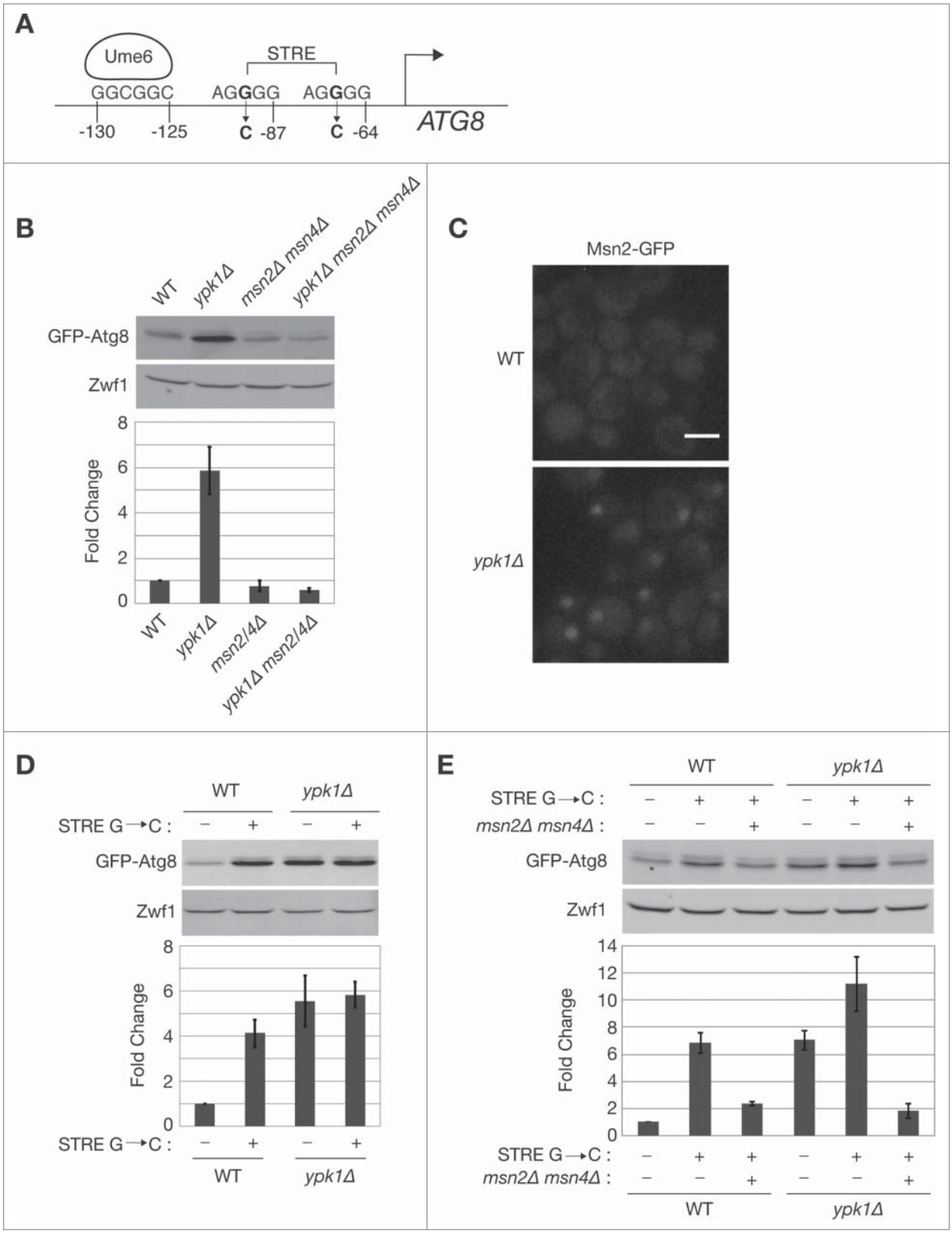

Figure 1. TORC2-Ypk1 signaling regulates *ATG8* expression via the heterodimeric zinc-finger transcription factor Msn2-Msn4. (A) Schematic representation of the *ATG8* promoter region, indicating the identified recognition site for the HDAC subunit Ume6[25] as well as potential stress response elements (STRE) as determined by YEASTRACT.[30] Also indicated are positions of G to C mutations introduced into the STRE sites. (B) Indicated strains carrying pRS416 *prATG8-GFP-ATG8* were grown to log phase in SCD medium without uracil. GFP-Atg8 protein was visualized via western blot analysis as described in Materials and Methods. GFP-Atg8 protein expression was normalized to the Zwf1 loading control and quantification represents Atg8 protein levels relative to WT, presented as the mean ± standard deviation (SD) of at least 3 independent experiments. (C) Indicated strains expressing endogenously tagged *MSN2-GFP* were visualized by fluorescence microscopy as described in Materials and Methods. Scale bar: 5 μm. ((D)and E) Indicated strains carrying pRS416 *prATG8-GFP-ATG8* or pRS416 *prATG8-GFP-ATG8* possessing STRE mutations depicted in (A) were grown to log phase in SCD medium without uracil. GFP-Atg8 protein levels were detected by western blot and analyzed as described in (B).

In addition to its role as a positive regulator of autophagy flux, TORC2-Ypk1 also negatively regulates *ATG8* expression.[29] In particular, we have demonstrated that in both nutrient-rich and starvation conditions, loss of Ypk1 activity (in *ypk1*Δ cells) results in increased levels of Atg8 protein. This increase is not due to a block in autophagy flux as the expression of a GFP reporter gene fused to the *ATG8* promoter is also elevated in Ypk1-deficient cells.[27] Moreover, this regulation is independent from calcineurin, because increased *ATG8* expression is still observed in *ypk1*Δ cells following deletion of *CNB1*, encoding the calcineurin B regulatory subunit.[27,28] Similarly, no change in *ATG8* expression is observed in *ypk1*Δ cells following deletion of *CRZ1*, encoding a stress-responsive transcription factor that functions downstream of calcineurin. Together, these findings point to a unique regulation for *ATG8* induction downstream of TORC2-Ypk1 signaling that is independent from calcineurin.[27]

In this study, we demonstrate that the stress-responsive transcription factor complex composed of proteins Msn2 and Msn4 is a positive regulator of *ATG8* expression. We describe functional interactions between TORC2-Ypk1 signaling and mitochondrial respiration that contribute to *ATG8* transcriptional regulation by Msn2-Msn4. Finally, we demonstrate that Msn2 and Msn4 are also involved in regulating mitochondrial respiration and autophagy flux in collaboration with TORC2-Ypk1 signaling following amino acid starvation, highlighting broad roles for TORC2 signaling and Msn2-Msn4 within this regulatory pathway.

Results

TORC2-Ypk1 signaling regulates ATG8 expression via the heterodimeric zinc-finger transcription factor Msn2-Msn4

Inhibition of TORC2 or Ypk1 results in increased basal expression of *ATG8* that is distinct from defects observed in autophagy flux during amino acid starvation.[27] Inspection of the promoter region of the *ATG8* gene revealed the presence of 2 stress response elements (STREs) (Fig. 1A), predicted to be targets of the Msn2-Msn4 heterodimeric zinc-finger transcription factor complex.[30,31] Significantly, several genes that are negatively regulated by TORC2-Ypk1 signaling have identifiable STRE motifs within their promoters.[32] Therefore, we tested whether *ATG8* induction in *ypk1Δ* cells requires Msn2-Msn4 activity by deleting *MSN2* and *MSN4* within these cells. We observed that elevated GFP-Atg8 expression in *ypk1Δ* cells was indeed absent in *ypk1Δ msn2Δ msn4*Δ cells (Fig. 1B). We confirmed that Msn2-Msn4 activity was induced in *ypk1Δ* cells by examining the intracellular location of GFP-tagged Msn2, which adopted a punctate nuclear localization pattern specifically in *ypk1Δ* but not in WT cells (Fig. 1C).

We next tested whether Msn2-Msn4 utilize the 2 STRE elements within the *ATG8* promoter, by introducing a single G to C base change within each element (Fig. 1A), based upon prior studies demonstrating this single mutation is sufficient to disrupt recognition by the Msn2-Msn4 complex.[31] Surprisingly, we observed that a reporter construct harboring these base changes maintained elevated expression of *ATG8* in *ypk1Δ* cells (Fig. 1D, compare lanes 3 and 4). Moreover, these mutations resulted in increased *ATG8* expression even when introduced into WT cells alone (Fig. 1D, compare lanes 1 and 2). Importantly, however, increased expression of *ATG8* in either WT or *ypk1Δ* cells required the presence of Msn2-Msn4 (Fig. 1E). From these results we conclude that Msn2-Msn4 is unlikely to bind directly or solely to the STRE elements and control expression of *ATG8*, at least compared with mechanisms described for other Msn2-Msn4 target genes. Nevertheless, these findings confirm that *ATG8* expression is regulated by TORC2-Ypk1 signaling in an Msn2-Msn4-dependent manner.

Msn2-Msn4 link the HDAC subunit Ume6 to ATG8 transcription

Recently, Klionsky and colleagues demonstrated that inhibition of the HDAC subunit Ume6 results in a dramatic increase in *ATG8* induction in nutrient-rich conditions.[25,26] They demonstrated further that Ume6 is inactivated following nutrient deprivation, in part via phosphorylation by the nutrient-responsive kinase Rim15, which results in increased expression of *ATG8*.[25] However, the transcription factor(s) involved in promoting *ATG8* expression under these conditions was not identified.

Given our present results, we tested whether functional interactions exist between Ume6 and Msn2-Msn4, with respect to *ATG8* expression. In agreement with prior findings, we observed that deletion of *UME6* resulted in significant (~20-fold) levels of *ATG8* induction (Fig. 2A). Remarkably, this expression was completely dependent upon Msn2-Msn4, as

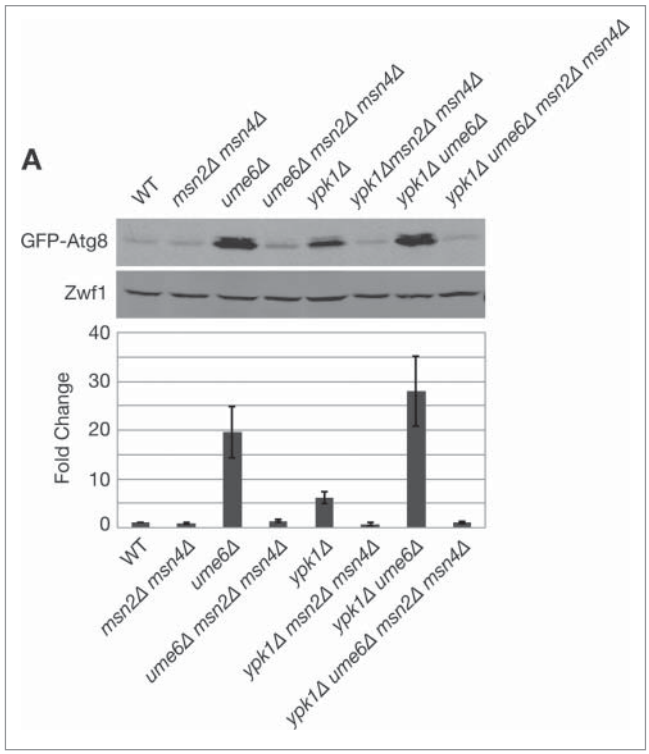

Figure 2. Msn2-Msn4 link the HDAC subunit Ume6 to *ATG8* transcription. Indicated strains expressing pRS416 *prATG8-GFP-ATG8* were grown to log phase in SCD medium without uracil and western blot analysis was performed as described in Materials and Methods. GFP-Atg8 protein expression was normalized to the Zwf1 loading control and quantification represents Atg8 protein levels relative to WT, presented as the mean ± SD of at least 3 independent experiments.

ATG8 expression returned to basal levels in *ume6Δ msn2Δ msn4Δ* cells (Fig. 2A). Moreover, the additive effects of deleting both *YPK1* and *UME6*, with respect to increased *ATG8* expression, were also reversed completely when combined with deletions of *MSN2* and *MSN4* (Fig. 2A). These findings demonstrate an important role for Msn2-Msn4 in the transcriptional control of *ATG8* in response to both TORC2-Ypk1 signaling as well as HDAC-regulated expression. Based upon our above analyses of the STRE site mutations, one possibility is that these base changes within the *ATG8* promoter alleviate Ume6-dependent repression of *ATG8* via an Msn2-Msn4-dependent mechanism.

Mitochondrial respiration links TORC2-Ypk1 and Msn2-Msn4 to ATG8 expression

We have demonstrated recently that TORC2-Ypk1 signaling regulates autophagy flux in part by controlling mitochondrial respiration. In particular, Ypk1 represses the accumulation of mitochondria-derived reactive oxygen species (ROS), which play a key role in activating calcineurin.[29] Accordingly, we explored the relationship between TORC2-Ypk1 signaling and mitochondrial respiration with respect to *ATG8* induction. We systematically tested the role of individual electron transport chain (ETC) complexes in regulating *ATG8* expression in Ypk1-deficient cells by deleting specific nuclear-encoded genes important for the assembly of ETC complex III (*cbs1Δ*), complex IV (*mss51Δ*), or complex V (*atp10Δ*). We observed that deletion of *MSS51* or *ATP10* significantly reduced *ATG8* expression in *ypk1Δ* cells but had no significant effect on their own (Fig. 3A). By contrast, we found that deletion of *CBS1* alone resulted in increased *ATG8* expression that was exacerbated in *ypk1Δ cbs1Δ* cells, demonstrating a unique behavior for complex III in *ATG8* expression (Fig. 3A).

To explore these differences further, we performed epistasis analyses by examining *ATG8* expression in strains deleted for both *CBS1* as well as one of the other genes encoding ETC components, *MSS51* or *ATP10*. We observed that increased expression of *ATG8* was abolished in both double mutants, demonstrating that induction of *ATG8* in the absence of complex III activity requires functional complexes IV and V (Fig. 3B). Importantly, we observed that *ATG8* induction in *cbs1Δ* cells also required Msn2-Msn4 activity, confirming that perturbation of mitochondrial respiration affects *ATG8* via a common regulatory pathway involving Msn2-Msn4 (Fig. 3C). Thus, the status of mitochondrial respiration serves as a branch-point that mediates the impact of TORC2 and Ypk1 on both arms of the autophagy response: transcriptional induction of *ATG8* as well as autophagy flux.

Msn2-Msn4 function upstream of mitochondria to regulate ROS and autophagy flux

As *ATG8* expression is responsive to both Msn2-Msn4 activity as well as mitochondrial respiration, we next examined the relationship between Msn2-Msn4 and mitochondria in the context of the regulatory scheme we have described recently, wherein autophagy flux is controlled by TORC2-Ypk1 signaling, mitochondrial oxidative stress, and calcineurin.[27-29] We have

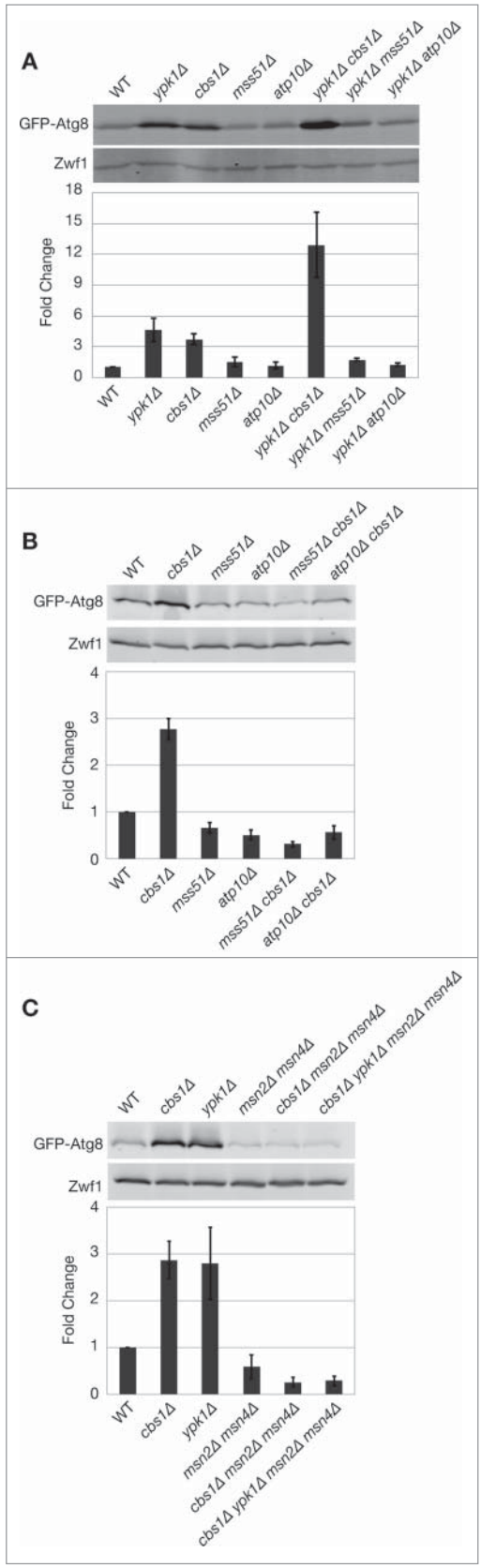

Figure 3. Specific mitochondrial respiratory complexes link TORC2-Ypk1 and Msn2-Msn4 to *ATG8* expression. (**A-C**) Indicated strains expressing pRS416 *prATG8-GFP-ATG8* were grown to log phase in SCD medium without uracil. Western blot analysis was performed as described in Materials and Methods. GFP-Atg8 protein expression was normalized to Zwf1 loading control and quantification represents Atg8 protein levels relative to WT, presented as the mean ± SD of at least 3 independent experiments.

demonstrated previously that ROS accumulation in *ypk1*Δ cells occurs strictly through mitochondrial-derived sources.[29] Accordingly, we tested whether Msn2-Msn4 regulates accumulation of mitochondrial ROS, using the ROS-reactive fluorescent dye H2DCF-DA (DCF).[33,34] Here we observed that ROS was absent in *ypk1*Δ *msn2*Δ *msn4*Δ cells (Fig. 4A). These results indicate that in addition to being activated by aberrant mitochondrial respiration, Msn2 and Msn4 also contribute to the accumulation of ROS when TORC2-Ypk1 signaling is impaired.

We have shown previously that accumulation of mitochondria-derived ROS is essential for activation of calcineurin.[29] Thus, based on our above findings we predicted that increased calcineurin activity in *ypk1*Δ cells might also require Msn2 and Msn4. To test this, we examined calcineurin activity using a calcineurin-dependent response element (CDRE)-driven *lacZ* reporter in both *ypk1*Δ as well as *ypk1*Δ *msn2*Δ *msn4*Δ cells, as described previously.[35] Indeed, we observed that calcineurin activity was elevated in *ypk1*Δ cells, as reported previously, but that it returned to basal levels in the triple mutant (Fig. 4B).

As an independent approach for testing the role of Msn2-Msn4 in regulating both ROS and calcineurin, we used the anti-athrymic and antifungal drug amiodarone, which we have shown inhibits growth of *ypk1*Δ cells in a manner that correlates with activation of calcineurin in the presence of

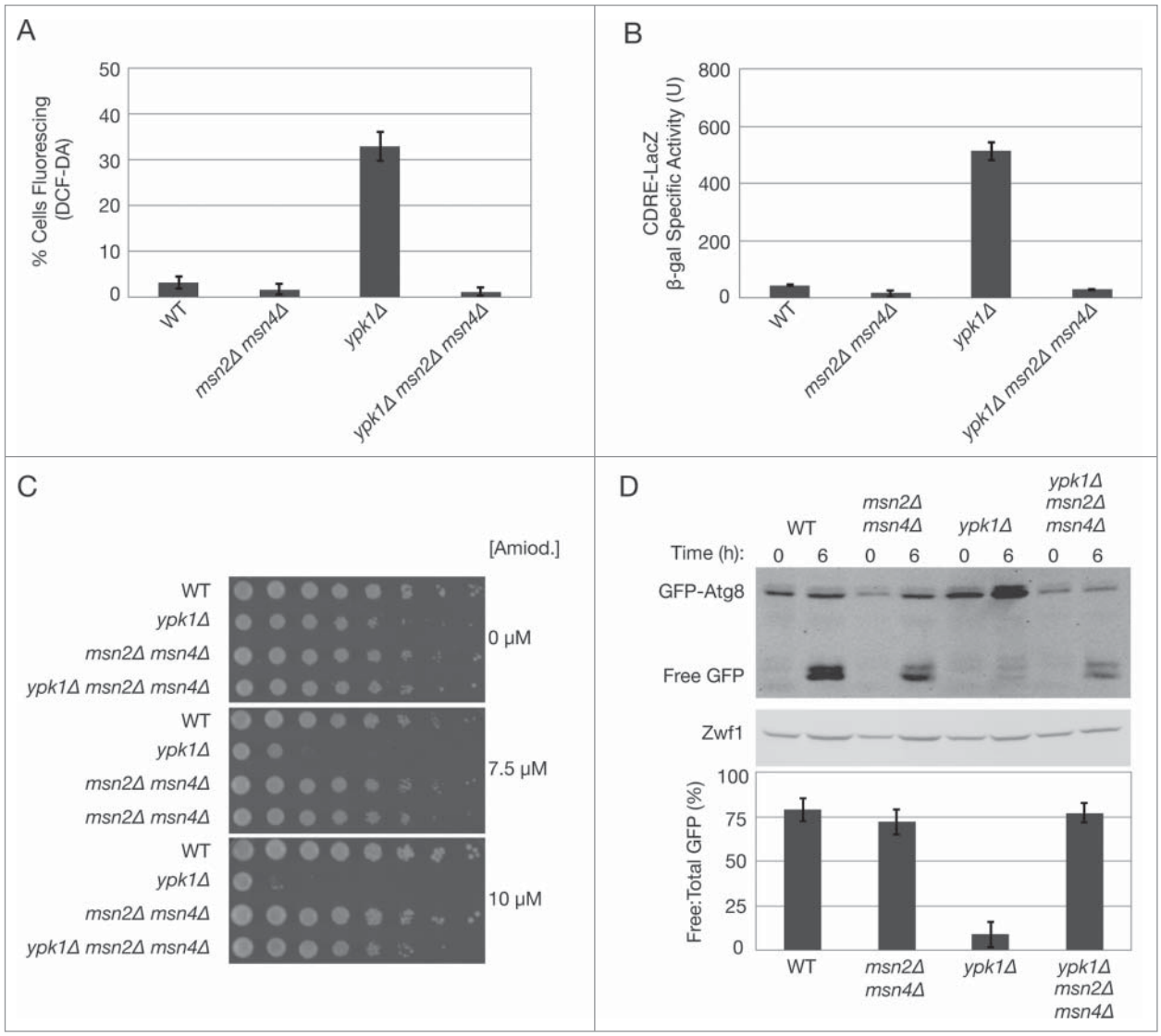

Figure 4. Msn2-Msn4 function upstream of mitochondria to regulate ROS and autophagy flux. (**A**) Indicated strains were grown to log phase and treated with 10 μM DCF for 30 min at 30°C before imaging by fluorescence microscopy, as described in Materials and Methods. Quantification represents the percentage of DCF-positive fluorescing cells, where a minimum of 250 cells for each strain was counted. (**B**) Indicated strains carrying plasmid pAMS363 that expresses 2xCDRE:LacZ were grown to log phase in SCD medium without uracil, and β-galactosidase activity was measured as described in Materials and Methods. β-galactosidase activity is represented as units of ONPG (nmol) converted/min/mg of protein. Data are presented as means ± SD of 3 independent experiments. (**C**) Indicated strains were grown to log phase and serial dilutions were plated onto agar plates containing YPD and the indicated concentrations of amiodarone. Cells were grown for ~2–3 d at 30°C. (**D**) Indicated strains carrying pRS416 *prATG8-GFP-ATG8* were grown to log phase in SCD medium without uracil medium and transferred to amino acid starvation medium (0.05% yeast extract, 2% dextrose) for 6 h. 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 3 independent experiments.

mitochondrial ROS.[29,36] Indeed, we used this drug to characterize the importance of the calcium channel regulator Mid1 as a key intermediate for calcineurin activation in the presence of ROS-derived signals, as increased Mid1 activity results in amiodarone hypersensitivity.[29,36] Because loss of Msn2-Msn4 activity prevented both accumulation of ROS as well as induction of calcineurin in *ypk1*Δ cells, we predicted that *ypk1*Δ *msn2*Δ *msn4*Δ cells may possess decreased Mid1 activity, which would be manifest as amiodarone-resistant growth. Indeed, we observed that while *ypk1*Δ cells were hypersensitive to sublethal concentrations of the drug, the triple mutant displayed significant resistance under these conditions (Fig. 4C). We conclude therefore that Msn2-Msn4 promotes Mid1 and calcineurin activity by facilitating dysfunctional mitochondrial respiration and ROS production in *ypk1*Δ cells.

Because inhibition of Msn2-Msn4 activity in *ypk1*Δ cells reduced calcineurin activity, we next tested whether they function in autophagy flux following amino acid starvation. Accordingly, we used western blotting to monitor processing of GFP-Atg8 during autophagy, where free GFP accumulates within the vacuole and autophagy flux is determined as the percentage of free GFP relative to total GFP signal.[37] Indeed, we observed that while autophagy flux was severely compromised in *ypk1*Δ cells, it was restored in *ypk1*Δ *msn2*Δ *msn4*Δ cells to levels comparable to WT (Fig. 4D). Importantly, we observed that overall levels of GFP-Atg8, and hence levels of free GFP, were significantly reduced in the triple mutant, consistent with *ATG8* induction also being impaired in the absence of Msn2-Msn4 activity. From these results we conclude that Msn2-Msn4 activity plays a dual role in both the induction of *ATG8* expression as well as in autophagy flux following amino acid starvation in *ypk1*Δ cells.

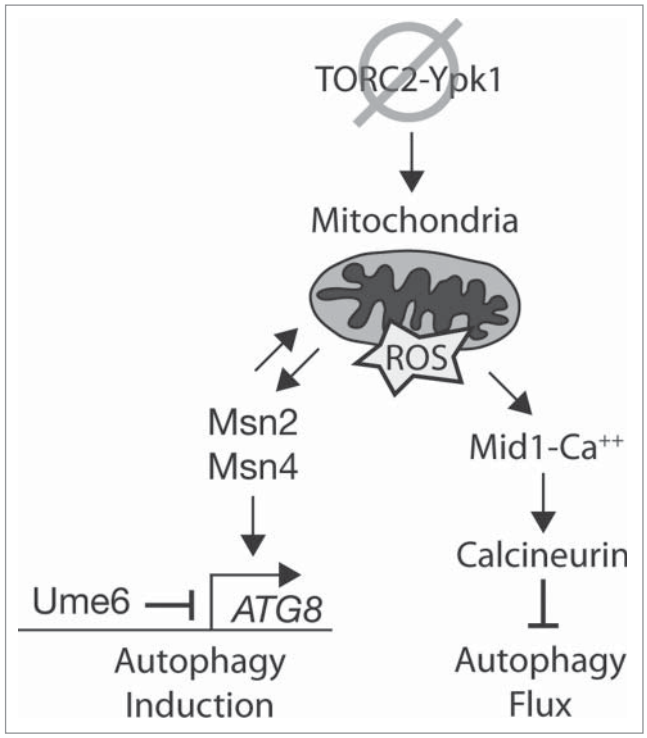

Figure 5. Model for regulation of *ATG8* expression and autophagy flux by TORC2-Ypk1, mitochondrial respiration, and Msn2-Msn4 during amino acid starvation. Model depicts the consequences of decreased TORC2-Ypk1 signaling and impaired mitochondrial function with respect to Msn2-Msn4 activity during both autophagy induction as well as autophagy flux. See text for details.

Discussion

Our key findings described here are summarized in a model presented in Fig. 5. First, we have extended our understanding of the transcriptional regulation of the key autophagy gene *ATG8*. In particular, we have demonstrated that the stress-inducible transcription factor heterodimer Msn2-Msn4 functions as a positive regulator of *ATG8* expression. Importantly, we find that this transcription factor complex interacts functionally with the HDAC subunit Ume6, where increased expression of *ATG8* observed in *ume6*Δ cells requires Msn2-Msn4. In addition, we find that Msn2-Msn4 activity is also linked to TORC2-Ypk1 signaling, where increased *ATG8* expression in *ypk1*Δ cells occurs in an Msn2-Msn4-dependent manner. By promoting autophagosome turnover while in turn limiting autophagy induction, TORC2-Ypk1 signaling plays opposing roles in autophagy that may provide a balanced response to cellular stress. Interestingly, both Msn2-Msn4 as well as Ume6 are controlled by direct phosphorylation by the nutrient-responsive kinase Rim15, suggesting TORC2-Ypk1 signaling may interface with Rim15 regulation (Fig. 4).[25,26,38] While Ume6 has been shown to interact directly with the *ATG8* promoter, our findings suggest that Msn2-Msn4 may regulate *ATG8* indirectly, as mutations in 2 predicted STRE sites within the *ATG8* promoter did not abolish Msn2-Msn4-dependent expression of *ATG8*. The possibility remains, however, that Msn2-Msn4 may interact with Ume6 or bind alternatively to the *ATG8* promoter by a previously uncharacterized mechanism.

Our findings also demonstrate that, in addition to TORC2-Ypk1 signaling, activation of *ATG8* expression by Msn2-Msn4 is responsive to the respiratory state of mitochondria. We recently determined that activation of calcineurin and inhibition of the GAAC response and autophagy flux in Ypk1-deficient cells requires functional mitochondrial respiratory complexes and is associated with accumulation of mitochondrial-derived ROS.[29] Similarly, here we demonstrate that Msn2-Msn4-dependent induction of *ATG8* in Ypk1-deficient cells is also linked to mitochondrial respiration, in that increased *ATG8* expression is abolished when genes encoding components of ETC complexes IV or V are deleted from *ypk1*Δ cells. Interestingly, we observe that deletion of *CBS1*, a component of ETC complex III, results in increased *ATG8* expression, either alone or in combination with deletion of *YPK1*. However, this expression is both dependent upon functional ETC complex IV and complex V as well as Msn2-Msn4, indicating these complexes converge on a common mechanism to regulate *ATG8* expression.

This complexity in the behavior of different ETC components suggests that each ETC complex is capable of possessing distinct signaling properties that affect autophagy induction. This notion is consistent with recent reports in human cells where diverse neurodegenerative diseases are linked to mutations in different ETC complex subunits and/or their specific

assembly factors.[39] Relevant to this conclusion, a study in MELAS patient cell lines demonstrates that autophagy degrades ETC complex I, linking defects in specific ETC complexes to the induction of autophagy in this neuronal disease.[40] Thus, further investigation into the roles of each ETC complex in mediating autophagy may uncover novel aspects of neuronal pathogenesis and lead to unique avenues for treatment of autophagy-related diseases.

In addition to a positive role in *ATG8* induction, we have also found that Msn2-Msn4 activity inhibits autophagy flux during amino acid starvation in Ypk1-deficient cells (Fig. 5). Our analyses indicate that this regulation is additionally connected to mitochondrial respiration, where accumulation of ROS in *ypk1*Δ cells requires both the activity of mitochondrial ETC complexes as well as Msn2-Msn4. Thus, in the absence of Msn2-Msn4, ROS fail to accumulate in *ypk1*Δ cells and calcineurin activity is not stimulated by a Mid1-dependent mechanism. Under these conditions, the GAAC response senses amino acid starvation and autophagy is induced. These findings highlight the complexity that exists between Msn2-Msn4 and mitochondria, where reciprocal regulation governs their behavior to control *ATG8* expression as well as autophagy flux.

This complexity is reminiscent of the relationship between protein kinase A (PKA) activity and mitochondrial respiration during autophagy, where PKA both regulates the accumulation of ROS as well as being controlled by the respiratory state of mitochondria, according to the precise nutritional state of the cell.[41,42] Consistently, a prior study revealed that in addition to the TORC1 downstream substrate Sch9, PKA negatively regulates autophagy flux via a pathway that requires Msn2 and Msn4.[18] Given that PKA is an established negative regulator of Msn2-Msn4,[18,43,44] it will be essential to understand the precise relationship between PKA and TORC2-Ypk1 signaling in regulating Msn2-Msn4 activity under different autophagy-inducing conditions.

Materials and methods

Yeast strains, media, and plasmids

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 open reading frames with *kanMX6, HIS3MX6, NAT* or *TRP1* cassettes where indicated.[45] Strains expressing endogenously tagged Msn2-GFP were constructed using a *GFP:HIS3MX6* PCR product generated from the yeast GFP collection and targeted homologous recombination.[46] Yeast transformations were performed using lithium acetate as previously reported.[47] All strains were grown to log phase (OD_{600} = approximately 1) in synthetic complete dextrose (SCD) yeast medium (0.8% yeast nitrogen base without amino acids [Becton Dickinson, 291940], 2% [wt:vol] dextrose [Fisher Scientific, D16-3], pH 5.5) supplemented with amino acids as described previously.[48] Where indicated, yeast strains contained the described previously plasmid *prATG8-GFP-ATG8*.[37] Mutations in the predicted STRE sites within the promoter of *ATG8* were introduced by site-directed mutagenesis of *prATG8-GFP-ATG8* using "QuikChange" (Agilent technologies, 200523), according to the manufacturer's instructions.

Whole-cell extraction, antibodies, and western blot analysis

Whole cell protein extracts were prepared from yeast using the NaOH cell lysis method, loaded onto SDS-PAGE gels and transferred to nitrocellulose membrane for western blot analysis.[49] Membranes were probed with anti-GFP N86/8 mouse monoclonal antibody (1 μg/mL; UC Davis/NeuroMab Facility, 73–131:RRID AB_10671444) and rabbit anti-Zwf1/G6PDH (1:1,000; Sigma-Aldrich, HPA000247), and visualized using the appropriate secondary antibodies conjugated to IR Dye (1:5,000; LI-COR Biosciences, 926–32210 and 926–68071). All western blot images were quantified using Fiji software.[50] Averages are presented with means ± standard deviation (SD) of at least 3 independent experiments.

Fluorescence microscopy

Fluorescence microscopy was performed using a Nikon E600 fluorescence microscope and an Orca ER charge-coupled device camera (Hamamatsu) controlled by Micro Manager 1.2 ImageJ software. Strains were grown to log phase (OD_{600} = approximately 1) in SCD medium supplemented with amino acids as described above. Image capture and processing were done using Fiji and Photoshop (Adobe). For Msn2-GFP microscopy, precautions were taken to prevent Msn2 from becoming activated by aberrant exposure to blue light, specifically, by using proper UV filters and fast exposure times and proper controls, as outlined previously.[51] Visualization of cellular ROS via fluorescence microscopy was performed using log phase growing cells in SCD complete medium that had been treated with 10 μM 2′,7′-dichlorofluorescin diacetate (DCFDA; Cayman Chemical, 20656) for 30 min at 30°C.

β-Galactosidase activity assay

Exponentially growing cells were incubated at 30°C in SCD medium supplemented with amino acids as required. β-Galactosidase activity was measured at 30°C using the substrate *O*-nitrophenyl-β-D-galactopyranoside (ONPG; Sigma-Aldrich, 73600) as described previously.[52] In brief, 10 OD_{600} units of a yeast cell pellet was 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 8.0, [Trizma base; Sigma-Aldrich, T1503], 1 mM dithiothreitol [Fisher Scientific, BP 172–25], 20% glycerol [Sigma-Aldrich, G5516]) on ice, and 12.5 μl of 100 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich, P7620; diluted in ethanol) was added. Cells were broken and homogenized using glass beads and a mini–bead beater (Biospec Products, Bartlesville, OK, USA) 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 centrifugation at 14,000 × g 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 $MgSO_4$–$7H_2O$, 50 mM β-mercaptoethanol [Sigma-Aldrich, M-3148], 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 the 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, B6916). β-Galactosidase activity is given in units of nanomoles of ONPG converted per min per mg of protein. Data are presented as means ± SD of 3 independent experiments.

Amiodarone growth assay

Amiodarone (Cayman Chemical, 15213) was prepared as a 50 mM stock in DMSO and then diluted into sterile water as appropriate and overlaid onto solid agar plates containing YPD medium. Exponentially growing yeast cells were diluted and plated onto these agar plates and allowed to grow at 30°C for 2–3 d.

Statistical analyes

To calculate autophagy flux, the ratio of free versus total GFP was determined for 3 independent biologic samples and Microsoft Excel was used to calculate and plot the mean and standard deviation. For CDRE-LacZ reporter assays, the mean of 3 independent biologic samples was similarly calculated and plotted using Excel.

Abbreviations

ATG	autophagy related
CDRE	calcineurin-dependent response element
ETC	electron transport chain
GFP	green fluorescent protein
HDAC	histone deacetylase complex
PKA	protein kinase A
ROS	reactive oxygen species
STRE	stress response element
TORC2	target of rapamycin complex 2

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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