

A conserved mechanism of TOR-dependent RCK-mediated mRNA degradation regulates autophagy

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Autophagy is an essential eukaryotic pathway requiring tight regulation to maintain homeostasis and preclude disease. Using yeast and mammalian cells, we report a conserved mechanism of autophagy regulation by RNA helicase RCK family members in association with the decapping enzyme Dcp2. Under nutrient-replete conditions, Dcp2 undergoes TOR-dependent phosphorylation and associates with RCK members to form a complex with autophagy-related (ATG) mRNA transcripts, leading to decapping, degradation and autophagy suppression. Simultaneous with the induction of ATG mRNA synthesis, starvation reverses the process, facilitating ATG mRNA accumulation and autophagy induction. This conserved post-transcriptional mechanism modulates fungal virulence and the mammalian inflammasome, the latter providing mechanistic insight into autoimmunity reported in a patient with a PIK3CD/p110 δ gain-of-function mutation. We propose a dynamic model wherein RCK family members, in conjunction with Dcp2, function in controlling ATG mRNA stability to govern autophagy, which in turn modulates vital cellular processes affecting inflammation and microbial pathogenesis.

Autophagy is a conserved essential eukaryotic catabolic process¹. Cells undergo rapid initiation of autophagy in response to cellular stress. Induction is followed by nucleation of a sequestering compartment, the phagophore, which expands into a double-membrane autophagosome. Although autophagy is cytoprotective, excessive autophagy is deleterious, suggesting a necessity for tightly governed regulation, exemplified by target of rapamycin (TOR)-mediated post-translational phosphorylation of autophagy-related (Atg) proteins² that maintains autophagy at a basal level under growing conditions. Transcription-dependent regulation of autophagy-related genes occurs in both *Saccharomyces cerevisiae*³ and mammalian cells⁴. However, less is known about post-transcriptional regulatory mechanisms modulating messenger RNA steady-state levels during autophagic induction and maintenance.

Recent work has shown that some TOR-dependent processes are regulated through control of mRNA stability. Although the mechanisms of its regulation remain poorly understood, steps leading

to deadenylation-dependent mRNA degradation in eukaryotes are well known⁵. mRNA degradation begins with a reversible deadenylation step⁵, followed by the irreversible removal of the 5' cap by the decapping enzyme, Dcp2, that allows the mRNA to be degraded in a 5'-3' direction by the Xrn1 exoribonuclease⁶. Additional decapping accessory proteins include a DExD/H-box RNA helicase compromising the RCK family, known as Dhh1 in *S. cerevisiae*, Vad1 in the fungal pathogen *Cryptococcus neoformans* and DDX6 in mammals⁷⁻⁹. These RCK family members function at the interface of translation and mRNA degradation by recruiting transcripts to the Dcp2 decapping complex¹⁰. Nevertheless, how mRNA post-transcriptional regulation is linked to signal-transduction machinery and autophagy remains the subject of intense investigation.

The post-transcriptional regulation of autophagy is still not fully understood even though the core components have been identified. A recent study pointed out dynamic changes in protein-RNA interactions under conditions of nutrient limitation¹¹, suggesting that

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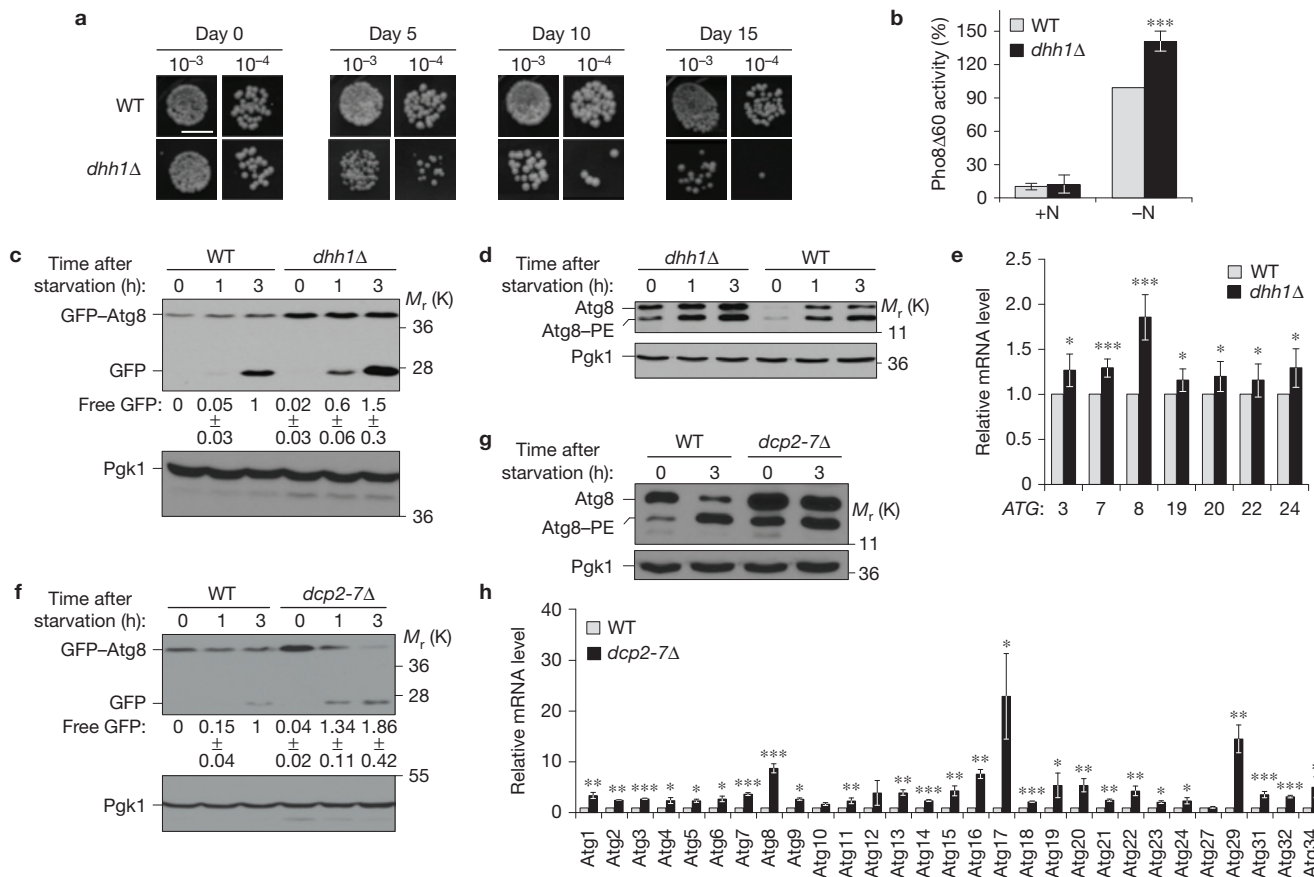


Figure 1 The RCK member Dhh1 is a post-transcriptional repressor of autophagy in yeast. **(a)** The *dhh1Δ* mutation reduces cell survival. Wild-type (WT) and *dhh1Δ* cells (BY4741) were grown in SMD, starved and dilutions grown on YPD plates for 2 days. Scale bar, 0.5 cm. **(b)** Dhh1 negatively regulates autophagy. WT (WLY176) and *dhh1Δ* (YAB269) cells were grown in YPD (+N) and then starved (-N). Pho8 Δ 60 activity was normalized to WT cells (set to 100%). Error bars: standard deviation (s.d.). $n=5$ independent experiments for (+N); $n=3$ independent experiments for (-N). Student's t -test, *** $P < 0.001$. **(c)** Autophagy is increased in *dhh1Δ* cells. WT (WLY176) and *dhh1Δ* cells expressing GFP-Atg8 were grown in rich medium, and then starved. Free-GFP intensity was normalized to the loading control. Free GFP was then normalized to the amount in WT cells after 3 h of starvation (set to 1). Average values \pm s.d. of $n=3$ independent experiments. **(d)** Dhh1 represses the expression of Atg8. WT (WLY176) and *dhh1Δ* cells were grown in YPD, and then starved. **(e)** Dhh1 represses *ATG* gene expression in nutrient-replete conditions. WT (WLY176) and *dhh1Δ* cells were grown in YPD. Total RNA

was extracted and the mRNA levels were quantified by qRT-PCR. The mRNA levels of individual *ATG* genes were normalized to the corresponding gene in WT cells (set to 1). Error bars: s.d. $n=5$ independent experiments for *ATG3*, *19*, *20*, *22*, *24*; $n=6$ independent experiments for *ATG7* and *8*. Student's t -test, * $P < 0.05$, *** $P < 0.001$. **(f)** Dcp2 negatively regulates autophagy. WT (BY4742) and *dcp2-7Δ* cells expressing GFP-Atg8 were grown in rich medium until early log phase. Cells were shifted to 38.5°C for 1 h, and then starved. Quantification of free GFP was carried out as in **c**. Average values \pm s.d., $n=3$ independent experiments. **(g)** Dcp2 represses the expression of Atg8. WT and *dcp2-7Δ* cells were grown as in **f**. **(h)** Dcp2 represses the expression of *ATG* genes in nutrient-replete conditions. WT (BY4742) and *dcp2-7Δ* cells were grown in rich medium until early log phase. Cells were shifted to 38.5°C for 1 h. Total RNA was extracted and analysed as in **e**. Error bars indicate s.d., $n=3$ independent experiments. Student's t -test, * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. Unprocessed original scans of blots are shown in Supplementary Fig. 8.

RNA-binding proteins (RBPs) could regulate autophagy. Also, defects in autophagy activity are associated with increased cell death during nitrogen starvation¹². Therefore, to identify regulators of autophagy among RBPs, a comprehensive library of RBP mutants was screened for a cell survival phenotype, which identified the yeast *S. cerevisiae* RCK member Dhh1 as a potential autophagy modulator. In addition, an RNA immunoprecipitation screen demonstrated that *ATG8* mRNA bound to the decapping complex containing the RCK yeast cryptococcal member Vad1. Further studies identified a conserved role for RCK members and binding partners in the recruitment of key transcriptionally controlled autophagy gene mRNAs to the Dcp2 decapping complex in yeast and mammals. Mammalian TOR (MTOR)-dependent phosphorylation of DCP2 was identified by

targeted ion mass spectrometry and found to play a role in the function of the decapping complex. Genetic manipulation, either by transcriptional modulation of RCK mRNA levels or by DCP2 phosphomimetic or phosphodeficient mutations, recapitulated TOR-dependent effects on decapping, resulting in alterations of autophagy. These changes in autophagy were sufficient to modulate the function of fungal virulence and the mammalian inflammasome by human differentiated THP-1 macrophages. This regulatory pathway was then used to characterize an autoimmune phenotype in a patient with a PIK3CD/p110 δ gain-of-function mutation with elevated MTOR activity¹³, linking pathological increases in MTOR-dependent DCP2 phosphorylation to reduced autophagy and increased IL1B production.

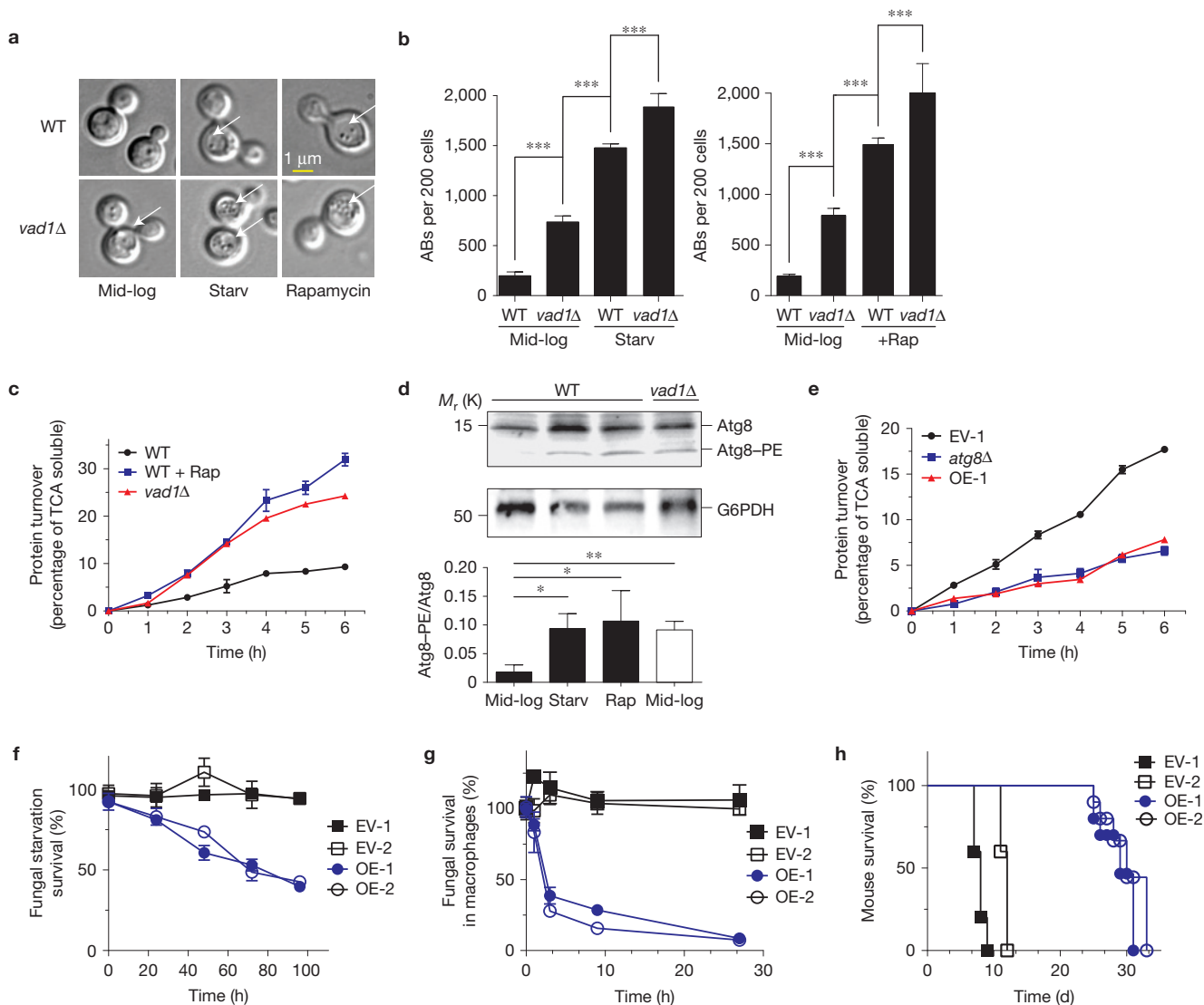


Figure 2 A Vad1 RCK/p54 homologue plays a role in repression of autophagy and virulence in *C. neoformans*. **(a,b)** Indicated strains were observed in mid-log phase, starvation in phosphate-buffered saline (PBS), pH 7.4 (Starv) or in the presence of rapamycin by differential interference contrast microscopy (DIC) for the presence of autophagic bodies (AB, white arrows) and quantified in 200 cells per condition by DIC in $n=3$ independent experiments. Student's *t*-test, error bars indicate s.d., $***P < 0.001$. **(c)** Autophagic flux determined by [35 S] pulse-labelling of cells followed by precipitation in 10% trichloroacetic acid (TCA) at the indicated times followed by measurement of TCA-soluble peptides by liquid scintillation. Autophagic flux was determined for the indicated strains in mid-log phase (wild type (WT), *vad1Δ*) or in mid-log cells in the presence of rapamycin (+Rap) for the indicated times. Time points $n=3 \pm$ s.e.m. **(d)** Lysate of mid-log phase cells either treated (+) or untreated (–) with rapamycin or after starvation in PBS, pH 7.4 (Starv) were subjected to urea electrophoresis and detected on western blot using an anti-Atg8 rabbit antibody. Lower arrow indicates lipidated Atg8 bands. Lower panel

represents results of $n=3$ independent experiments \pm s.d. $*P < 0.05$; $**P < 0.01$. **(e)** Autophagic flux in starved cells was determined as in **a**. *C. neoformans* expressing empty vector strain no. 1 (EV-1) and *VAD1*-overexpressing vector, strain no. 1 (OE-1). Time points $n=3 \pm$ s.e.m. **(f)** Fungal cell survival under starvation conditions. Strains were starved by incubation in PBS, pH 7.4, for the indicated times, followed by live-cell determination using an assay of colony-forming units (CFUs). *C. neoformans* expressing empty vector strain no. 1 and 2 (EV-1 and 2) and *VAD1*-overexpressing vector (under the *ACT1* promoter), strain no. 1 and 2 (OE-1 and 2). Time points $n=3 \pm$ s.e.m. **(g)** Fungal survival in macrophages. Indicated strains were opsonized with 40% mouse serum, phagocytosed using a J774.16 cell line induced by IFNG/IFN- γ , and fungal viability measured by CFUs. Time points $n=3 \pm$ s.e.m. **(h)** Mouse mortality after cryptococcal infection. Indicated strains (1×10^6 CFUs) were inoculated intravenously into 10 female Swiss albino mice for each strain and followed until moribund. $n=10$ mice per group. Unprocessed original scans of blots are shown in Supplementary Fig. 8.

RESULTS

Dhh1 and the mRNA decay pathway coordinately repress the autophagy transcriptome in *S. cerevisiae*

To identify potential regulators of autophagy among RBPs, a comprehensive library of RBP mutants was screened for a cell

survival phenotype during nitrogen starvation. The RBP mutants exhibited a range of phenotypes, and *dhh1Δ* cells in particular showed reduced survival compared with wild type, noticeable after 5 days of treatment, which was further aggravated with prolonged starvation (Fig. 1a), suggesting that Dhh1 might regulate autophagy. On nitrogen

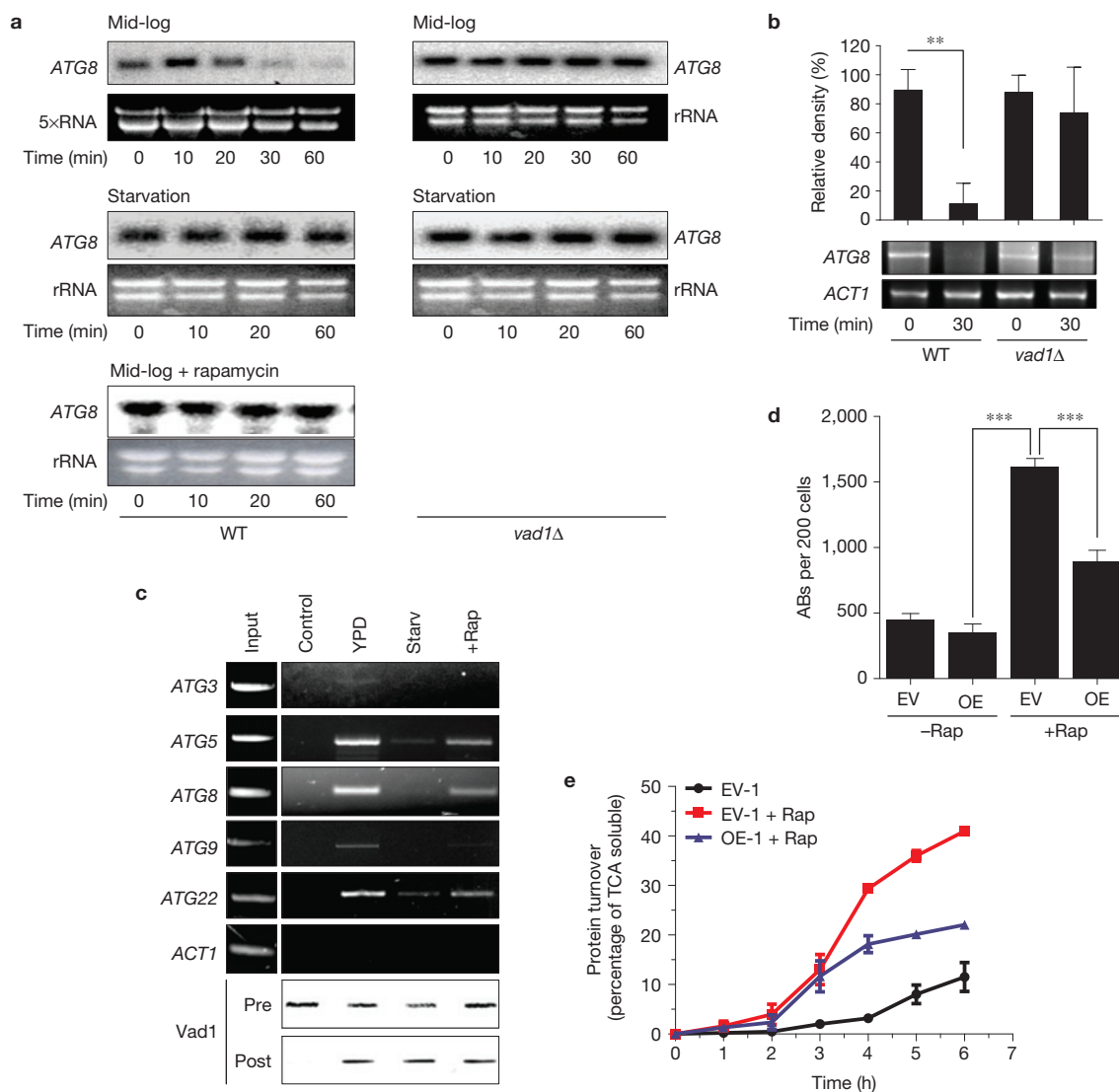


Figure 3 Vad1 plays a role in decapping and degradation of *ATG8* in *C. neoformans*. **(a)** Degradation assays. Indicated cells were grown to mid-log phase in 2% glucose, 2% bacto-peptone, 1% yeast extract (YPD), and incubated under starvation conditions (PBS, pH 7.4) for 1 h or treated with rapamycin followed by transcription inhibition with 1,10-phenanthroline as described in the Methods for the indicated times. Northern blots were used to monitor *ATG8* transcripts. The designate, '5×RNA' was used to show that 5× the amount of RNA was required to be loaded on the gel to identify the repressed *ATG8* transcript under mid-log conditions. **(b)** The presence of capped *ATG8* transcripts was assayed over 30 min using the indicated strains by PCR amplification of a cDNA product obtained after sequential ligation of a 5' RNA adapter following treatment with tobacco acid pyrophosphatase as described in the Supplementary Methods. Error bars indicate s.e.m. of $n=3$ independent experiments. Student's *t*-test, $**P < 0.01$. **(c)** Lysate from cells incubated

under the indicated conditions, expressing a *c-myc*-tagged Vad1 protein, or untagged Vad1 (control) was immunoprecipitated followed by RT-PCR using primers for the indicated gene transcripts. To normalize RT-PCR reactions, a cross-reactive RCK antibody was used to quantify Vad1 in the lysate before (pre) and in the pellet after (post) immunoprecipitation. Input RT-PCR on the indicated transcripts was performed to show detectability of the indicated mRNA from cells grown in YPD. **(d)** Autophagic bodies (ABs) assayed as in Fig. 2a. Results from $n=3$ independent experiments, 200 cells each, Student's *t*-test, error bars represent s.d., $***P < 0.001$. **(e)** Autophagic flux in mid-log cells in the presence or absence of rapamycin was determined by [35 S] pulse-labelling of cells followed by precipitation in 10% TCA at the indicated times followed by measurement of TCA-soluble peptides by liquid scintilligraphy. Time points $n=3 \pm$ s.e.m. Unprocessed original scans of blots are shown in Supplementary Fig. 8.

starvation, autophagy was induced to a higher level in *dhh1* Δ cells compared with wild type (Fig. 1b) as measured by the Pho8 Δ 60 assay. This assay measures autophagy-dependent alkaline phosphatase activity of a modified vacuolar alkaline phosphatase precursor that can be delivered to the vacuole for proteolytic activation only through autophagy. Although insufficient autophagy can result in a loss of cell viability, excessive autophagy activity could cause a similar phenotype.

The Pho8 Δ 60 data suggested that the latter may explain the decreased survival in the *dhh1* Δ strain, and that Dhh1 may be a negative regulator of autophagy. Therefore, we extended our analysis using a GFP-Atg8 processing assay. When transported into the vacuole during autophagy, GFP-tagged Atg8 is hydrolysed to yield free GFP, reflecting the level of autophagy¹⁴. In *dhh1* Δ cells, autophagy was induced more rapidly and to a higher extent, as indicated by the level of free GFP

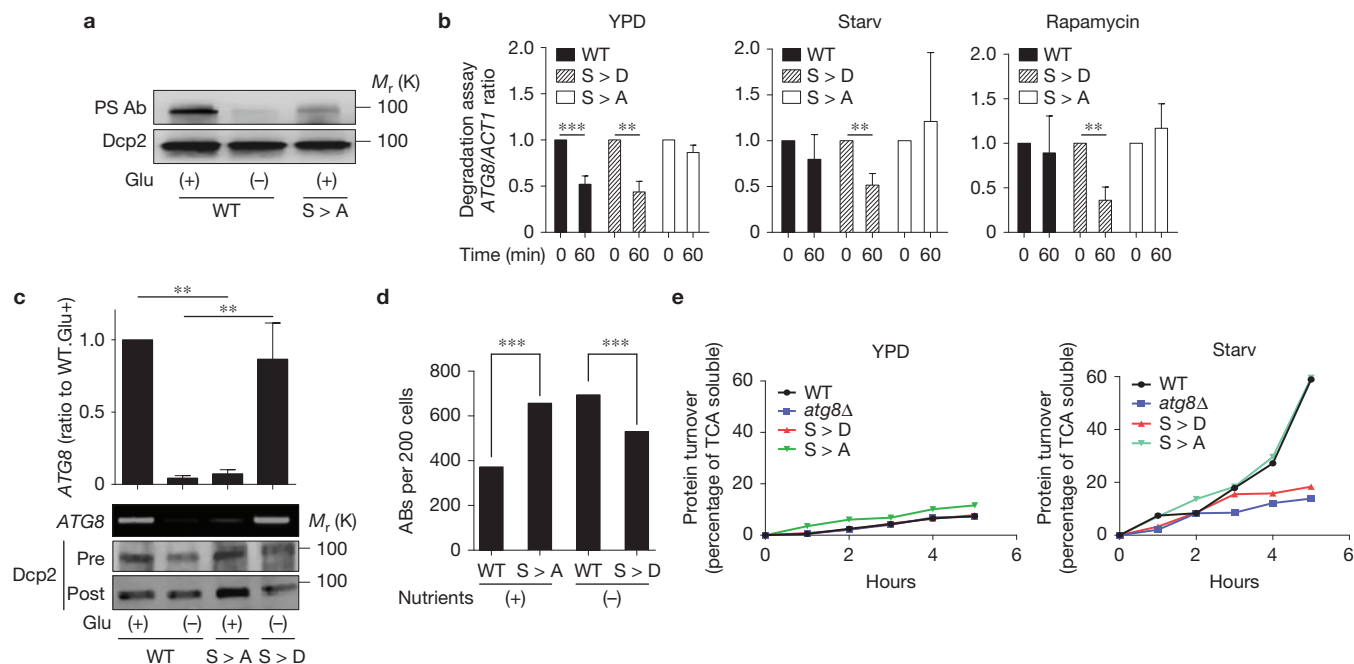


Figure 4 TOR-dependent phosphorylation of Dcp2 modulates recruitment of *ATG8* transcripts to the decapping complex and autophagic body formation in *C. neoformans*. (a) *C. neoformans* cells expressing a WT 3xFLAG–Dcp2 fusion protein or an equivalent fusion protein containing S>A mutations at positions 614 and 617 were grown in nutrient-rich medium (WT, S>A) or the strain expressing WT Dcp2 was subjected to starvation in PBS, pH 7.4 (–). Cells were collected and the Dcp2 fusion protein was immunoprecipitated and assayed using a phosphoserine or 3xFLAG antibody, as loading control, respectively. (b) Degradation assay. *C. neoformans* cells expressing either WT Dcp2 or an equivalent protein containing either S>D mutations or S>A mutations at positions 614 and 617 were assayed for *ATG8* and *ACT1* transcripts by qRT-PCR at the indicated times after 1,10-phenanthroline addition in the indicated conditions as in Fig. 3a. Results of $n=3$ independent experiments \pm s.d., Student's *t*-test, ** $P < 0.01$; *** $P < 0.001$. (c) Lysates from a Vad1–myc-expressing strain were treated as in Fig. 3d to assess *ATG8* target transcript binding to Vad1. Cells contained plasmid expressing either WT 3xFLAG–Dcp2 or Dcp2 fusions containing S>A phosphodeficient or S>D phosphomimetic mutations at positions 614 and 617. Results from $n=3$ independent experiments \pm s.d. Student's *t*-test, ** $P < 0.01$. (d) Yeast cells expressing the indicated Dcp2 as in Fig. 4c were assessed for autophagic body formation by DIC microscopy and quantified in $n=200$ cells. Chi square: *** $P < 0.001$. (e) Autophagic flux in YPD or in starved cells was determined by [³⁵S] pulse-labelling of cells followed by precipitation in 10% TCA at the indicated times followed by measurement of TCA-soluble peptides by liquid scintillation. Data represent 1 of 2 experiments. Unprocessed original scans of blots are shown in Supplementary Fig. 8.

compared with wild type (Fig. 1c), further suggesting that Dhh1 acts as a repressor of autophagy.

We also noticed a higher level of the GFP–Atg8 fusion protein (Fig. 1c) as well as endogenous Atg8 (Fig. 1d) in *dhh1Δ* cells compared with wild type (Fig. 1c). In nutrient-rich conditions, Atg8, as well as its lipidated form, Atg8–PE, is expressed at a very low level in wild-type cells, but the level increases substantially when autophagy is induced. Atg8 is a key autophagy-related protein involved in formation of the phagophore, and previous studies showed that an elevated Atg8 level correlates with larger autophagosomes and increased autophagic flux¹⁵. Dhh1 is a DEXD/H-box helicase that has a role in the activity of the decapping machinery. Finding that Dhh1 affects the abundance of Atg8 led us to postulate that Dhh1 represses autophagy by regulating mRNA stability of one or more *ATG* genes. To test this idea, quantitative PCR with reverse transcription (RT–qPCR) was used to examine the expression of known *S. cerevisiae* *ATG* genes in *dhh1Δ* cells compared with wild type. In nutrient-replete conditions, the *DHH1* deletion caused an accumulation of *ATG3*, *ATG7*, *ATG8*, *ATG19*, *ATG20*, *ATG22* and *SNX4/ATG24* transcripts (Fig. 1e and Supplementary Fig. 1a). Conversely, there was no significant difference in mRNA levels of *ATG* genes between the *dhh1Δ* and wild-type cells

after starvation (Supplementary Fig. 1b). According to the literature, Dhh1 is a highly abundant protein in *S. cerevisiae* and is present in large excess over its mRNA substrates⁷. Consistent with this observation, mild overexpression of the protein resulted in a modest decrease in autophagy (Supplementary Fig. 1c–e). These results suggested a model in which Dhh1 blocks autophagy in nutrient-rich conditions by, at least partly, repressing the expression of a set of *ATG* genes. To further confirm the involvement of mRNA decapping in regulating autophagy, we assessed the role of the decapping enzyme Dcp2, using a *dcp2-7Δ* temperature-sensitive mutant. At the non-permissive temperature, autophagy activity was increased in the *dcp2-7Δ* cells compared with wild-type cells as measured by both the GFP–Atg8 processing and Pho8Δ60 assays (Fig. 1f and Supplementary Fig. 1f). As in the *dhh1Δ* cells, enhancement of autophagy in the *dcp2-7Δ* cells was associated with an accumulation of autophagy-related proteins (Fig. 1g and Supplementary Fig. 1g) and mRNA (Fig. 1h), compared with wild-type cells in nutrient-rich conditions. After nitrogen starvation, the difference in the level of *ATG* transcripts between the *dcp2-7Δ* and the wild-type cells was either abolished or strongly reduced for many of the genes (Supplementary Fig. 1h). The level of mRNA induction as well as the number of targets among

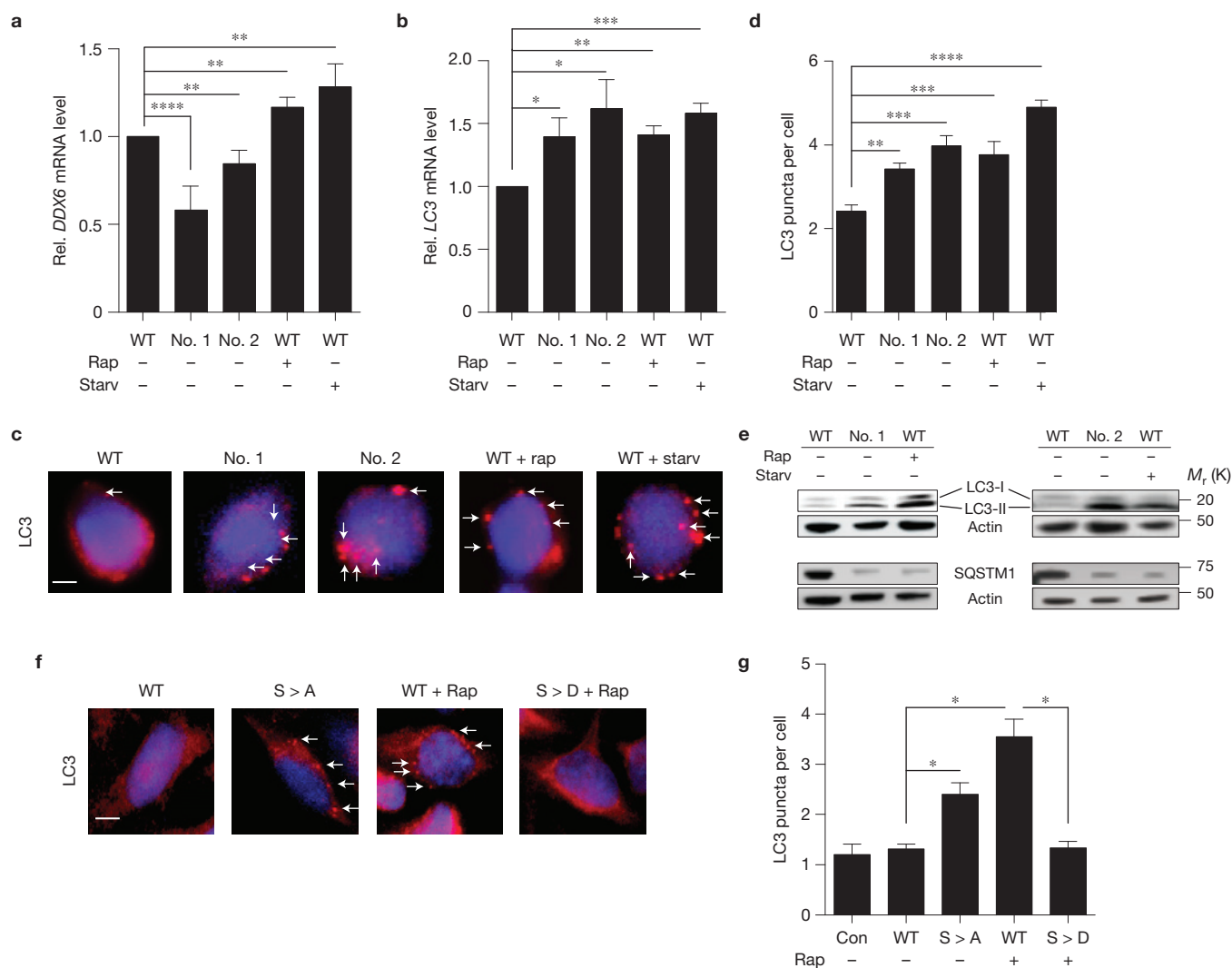


Figure 5 Mammalian DDX6 is a suppressor of autophagy in mouse primary ESCs and human HeLa cells. **(a,b)** *RCK* and *LC3* transcript levels of the indicated C57/BL6 mouse ESC strains in nutrient-rich media or after treatment with rapamycin (Rap +) or subjected to starvation (Starv +). $N=3$ independent experiments \pm s.d. Student's *t*-test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. **(c)** LC3 puncta formation in ESCs visualized by fluorescence microscopy using an anti-LC3 antibody (red fluorescence, white arrows) of the indicated strains under nutrient-rich conditions or after addition of rapamycin (Rap +) or incubation under starvation conditions (Starv +) with 4',6-diamidino-2-phenylindole used for nuclear localization (blue fluorescence). Scale bar, 2 μ m. **(d)** Quantification of **c**. Results

of $n=3$ independent experiments, 100 cells per experiment \pm s.d. Student's *t*-test, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. **(e)** Indicated ESC strains incubated under nutrient-rich conditions assayed for LC3-I/II and SQSTM1 by western blot. **(f)** LC3 puncta in HeLa cells assayed by fluorescence microscopy (red fluorescence, white arrows) in the indicated strains in nutrient-rich conditions or after addition of rapamycin (Rap +) with 4',6-diamidino-2-phenylindole used for nuclear localization (blue fluorescence). Scale bar, 2 μ m. **(g)** Quantification of LC3 puncta in HeLa cells 100 cells per experiment from **f**. $n=3$ independent experiments \pm s.d. Student's *t*-test; * $P < 0.05$. Unprocessed original scans of blots are shown in Supplementary Fig. 8.

the *ATG* genes was much higher in the *dcp2-7 Δ* cells compared with *dhh1 Δ* , a result consistent with the essential function of Dcp2 as a core component of the decapping complex in contrast with a regulatory role of Dhh1. Together, these data support the idea that Dhh1 and the decapping machinery repress autophagy in nutrient-replete conditions by promoting the degradation of *ATG* transcripts.

Vad1/Dhh1 repression of autophagy regulates the pathogenesis of the yeast *C. neoformans*

Autophagy is required for the virulence of pathogenic fungi¹⁶. Thus, we tested the significance of decapping and autophagy in the

fungal pathogen *C. neoformans*, a leading cause of infectious death globally¹⁷. Deletion of *VAD1*, the *DHH1* homologue in *C. neoformans*, recapitulated increases in autophagy activity observed in *S. cerevisiae* based on an accumulation of autophagic bodies, the single-membrane vesicle that is released into the vacuole lumen after fusion with the double-membrane autophagosome, in nutrient-replete conditions as well as after starvation of glucose and amino acids or rapamycin treatment (Fig. 2a,b). An increase in autophagic bodies can result from an increase in autophagic flux, or a block in a late step of autophagy, such as degradation within the vacuole¹⁸. To distinguish these possibilities, we carried out an analysis of long-lived protein

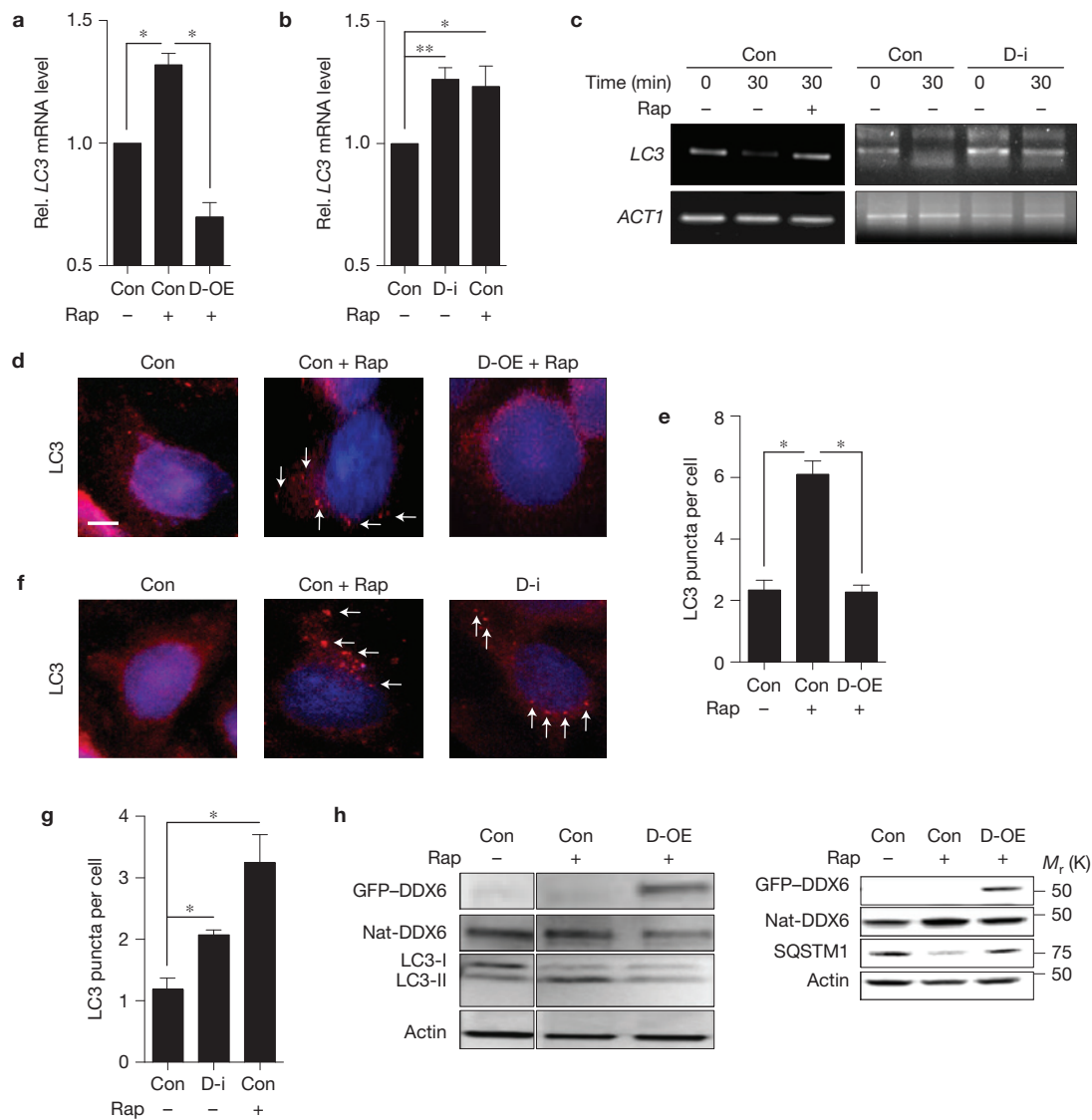


Figure 6 DDX6 is a suppressor of autophagy in HeLa cells. **(a,b)** LC3 transcript levels in HeLa cells in nutrient-rich media, transformed with empty vector (Con) or empty vector + rapamycin (Con + Rap) or with a plasmid expressing human DDX6 in the presence of rapamycin (D-OE + Rap) or treated with non-targeted siRNA (Con), or DDX6-directed siRNA (D-i), or non-targeted siRNA in the presence of rapamycin (Con + Rap). $N=3$ independent experiments \pm s.d., Student's t -test; $*P < 0.05$, $**P < 0.01$. **(c)** The presence of capped LC3 transcripts was assayed in HeLa cells subjected to DDX6-directed siRNA (DDX6-i) or non-targeted siRNA (Con) under the indicated conditions at the indicated times after transcriptional suppression by the specific PCR method detailed in the Methods. **(d,e)** HeLa cells subjected to conditions in Fig. 3a and examined by fluorescence microscopy for LC3 puncta formation using an LC3-II antibody (red fluorescence, white arrows); 4',6-diamidino-2-

phenylindole used for nuclear localization (blue fluorescence) and quantified in $n=3$ independent experiments of 100 cells each \pm s.d. Scale bar, $2\mu\text{m}$. Student's t -test, $*P < 0.05$. **(f,g)** HeLa cells subjected to conditions in Fig. 3b, and examined by fluorescence microscopy for LC3 puncta formation using an LC3-II antibody (red fluorescence, white arrows); 4',6-diamidino-2-phenylindole used for nuclear localization (blue fluorescence) and quantified in $n=3$ independent experiments of 100 cells each \pm s.d.; Student's t -test; $*P < 0.05$. **(h)** HeLa cells in conditions equivalent to Fig. 3a were assayed for the indicated proteins including cells containing plasmids expressing GFP-DDX6 and native DDX6 (Nat-DDX6) by western blot according to the Methods. Spliced Con sample run on the same gel as each of the other samples. Unprocessed original scans of blots are shown in Supplementary Fig. 8.

degradation; short-lived proteins are targeted primarily by the ubiquitin-proteasome system, whereas long-lived proteins are mostly degraded by autophagy. The *vad1* Δ cells showed an increase in protein turnover in nutrient-rich conditions, similar to that observed after autophagy induction by rapamycin treatment in wild-type cells (Fig. 2c), thus confirming an increase in autophagy flux in the *vad1* Δ mutant. This was accompanied by increased accumulation of ATG8 mRNA (Supplementary Fig. 2a) and protein, in particular its lipidated

form, in the mutant strain versus wild type during mid-log growth (Fig. 2d). *VAD1* deletion also led to an accumulation of many of the ATG gene transcripts in nutrient-rich conditions, similar to that induced after starvation (Supplementary Fig. 2a). These data show that, as in *S. cerevisiae*, *Vad1* acts as a repressor of autophagy in *C. neoformans*.

Conversely, higher gene dosing of *VAD1* in an overexpressing strain led to a reduction of ATG8 mRNA (Supplementary Fig. 2b),

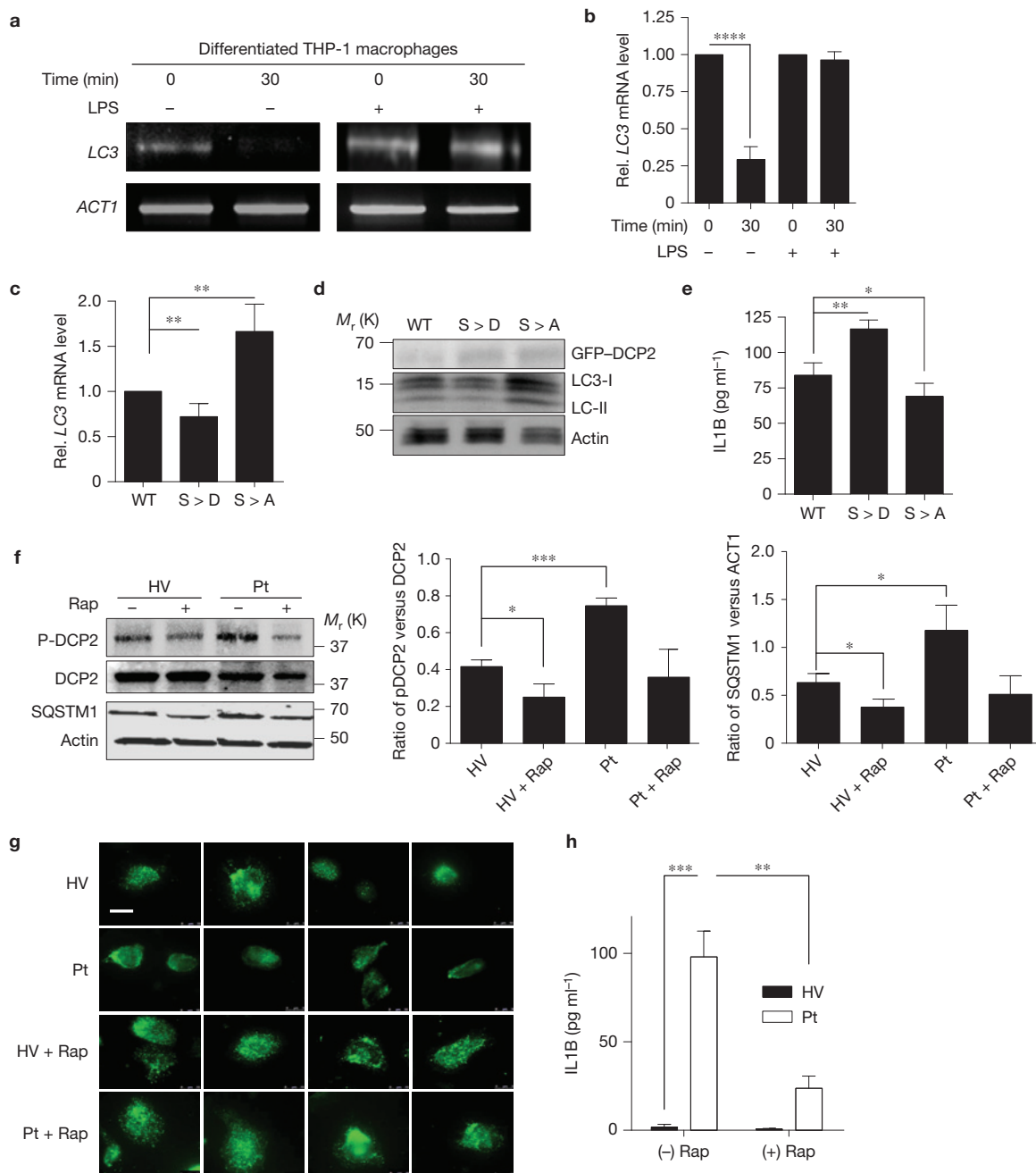


Figure 7 The DDX6 binding partner DCP2 regulates LC3 and IL1B levels during inflammasome stimulation in macrophages and in a patient with a PIK3CD/p110 δ gain-of-function mutation. (**a,b**) The presence of capped LC3 transcripts was assayed in THP-1-differentiated macrophage cells by PCR amplification. (**c–e**) THP-1-differentiated macrophages were transfected with either a plasmid containing DCP2 WT or DCP2 S249A, or DCP2 S249D, treated with LPS, and then analysed for LC3-I/II levels by qRT-PCR and western blotting as well as IL1B secretion by ELISA. Statistical analysis was determined from $n=3$ (**b,e**) or $n=6$ (**c**) independent experiments \pm s.d.

(**f–h**) PBMCs from a patient with a PIK3CD/p110 δ gain-of-function mutation (Pt) or an age-matched healthy volunteer (HV) with (+) or without (-) treatment with 200 nM rapamycin were assayed for phosphorylated DCP2 (P-DCP2) using an anti-pS249-DCP2 (**f**, top panel), SQSTM1 (**f**, bottom panel), LC3-containing autophagic vacuoles (**g**, white arrows) or IL1B by ELISA (**h**). Results of **b,c,e,f** (middle and right panel) and **h** the result of $n=3$ independent experiments \pm s.d. Student t -test: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Scale bar, 10 μm . Unprocessed original scans of blots are shown in Supplementary Fig. 8.

autophagic bodies (Supplementary Fig. 2c), and autophagic flux, the last of these to a level similar to that of an autophagy-incompetent *atg8 Δ* mutant (Fig. 2e). *VAD1* overexpression also resulted in defects in starvation tolerance (Fig. 2f) as well as a reduction in survival within macrophages (Fig. 2g), a key determinant of virulence for this

facultative intracellular pathogen¹⁹. Note that in this situation, we propose that the decrease in *C. neoformans* viability is due to the decrease in fungal autophagy resulting from *Vad1* overexpression, in contrast to the analysis in *S. cerevisiae* where we examined the null strain (Fig. 1a). However, starvation studies in *C. neoformans* were

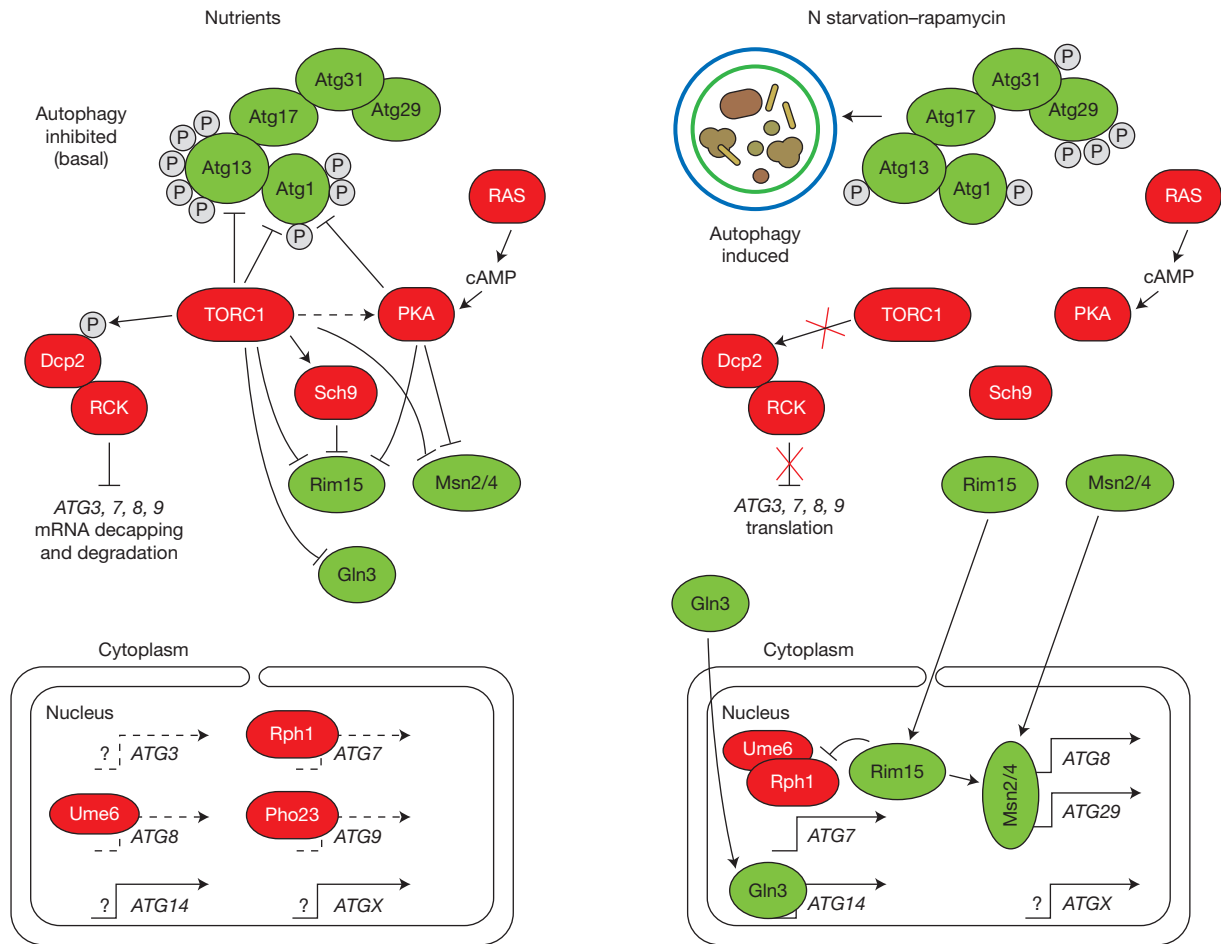


Figure 8 A TOR-mediated RCK-dependent pathway negatively regulates autophagy through mRNA decapping and degradation. Under normal growth conditions (that is, nutrient-replete), DEAD-box RNA helicases in the RCK family recruit *ATG* mRNA to the Dcp2 decapping complex, whose actions lead to cytoplasmic 5′–3′ mRNA degradation. TOR-associated phosphorylation of Dcp2 is required for transcript decapping and subsequent degradation. This process occurs simultaneously with transcript synthesis inhibition, facilitated

by inactivation of positive transcription factors, such as Rim15, Msn2/4 and Gln3. Environmental stress, such as nutrient deficiency or rapamycin treatment, inactivates TOR, thereby preventing Dcp2 phosphorylation and causing RCK dissociation from *ATG* mRNA in a coordinate fashion. This facilitates accumulation of *ATG* transcripts and autophagy induction and acts in concert with transcriptional synthetic induction by positive *ATG* transcription factors.

conducted using glucose and nitrogen starvation, to better simulate conditions encountered for this pathogen in the infected brain¹⁶. These gene dosing effects of *VAD1* were significant to pathogenesis in that independent *VAD1*-overexpressor strains demonstrated attenuated virulence in mice (Fig. 2h). The hypovirulent phenotype was similar to that of an *ATG8*-RNAi-suppressed strain described previously¹⁶. Together, these data demonstrate a significant role in pathogenesis for *VAD1*-mediated suppression of autophagy.

Vad1 controls the degradation of *ATG8* transcripts in nutrient-rich conditions

To gain insight into the molecular mechanism by which Vad1/Dhh1 represses autophagy, we examined its regulation of Atg8 in more detail. After inhibition of mRNA synthesis using the mRNA synthetic inhibitor 1,10-phenanthroline, northern blot and qRT-PCR analysis showed that during nutrient-replete conditions, wild-type cryptococcal cells exhibited time-dependent degradation of *ATG8* mRNA (Fig. 3a left, upper panel, Supplementary Fig. 2d) with

a half-life of 18 ± 4 min, whereas starvation stabilized the *ATG8* transcripts (Fig. 3a, middle left panel). As observed under starvation conditions, deletion of *VAD1* resulted in stable mRNA levels of *ATG8* for over 60 min (Fig. 3a, right panels; Supplementary Fig. 2d).

Decapping of mRNA represents a critical first irreversible step in mRNA turnover mediated by members of the DEAD-box family of RNA helicase proteins, which includes Vad1. We used a PCR-based method²⁰ and confirmed Vad1-dependent decapping of *ATG8* mRNA transcripts (Fig. 3b). Together these results support a model in which, during mid-log phase, Vad1 and the decapping machinery degrade *ATG8* mRNA, thus keeping autophagy activity at a low level. During nutrient limitation Vad1-mediated mRNA degradation is inhibited, thereby allowing for increased expression of *ATG* genes and induction of autophagy. Confirming this finding and extending it to other transcriptionally regulated autophagy genes, RNA immunoprecipitation of a *c-myc*-tagged Vad1 fusion protein demonstrated binding of autophagy-related transcripts, including *ATG3*, *ATG5*, *ATG8*, *ATG9* and *ATG22* under

mid-log nutrient-rich conditions (Fig. 3c), whereas under starvation conditions, Vad1 protein–transcript complexes were less prominent. This finding suggests a role for transcript binding to the Vad1 complex under nutrient-replete conditions and dissociation under starvation conditions. As RCK family members form complexes with decapping enzymes such as Dcp2 (ref. 21), dissociation of target mRNA from this complex would be expected to result in a loss of decapping and attenuated degradation, as seen in the *vad1Δ* mutant. Further studies demonstrated a role for the 3'UTR of *ATG8* in this process as expression levels of a *GFP-ATG8* fusion gene substituted with an unrelated 3'UTR resulted in GFP-Atg8 protein accumulation (Supplementary Fig. 2e). *ATG8* 3'UTR replacement alone resulted in a diffuse cytoplasmic accumulation of GFP-Atg8, whereas, under the same conditions, *VAD1* deletion resulted in both protein accumulation and autophagic body formation, suggesting regulation of multiple steps in the autophagic process by Vad1, in agreement with the observation that several *ATG* genes are regulated in a Vad1-dependent manner. Together, these data are consistent with Vad1 playing an important role in coordinate repression of autophagy-related mRNA transcripts under nutrient-replete conditions.

Vad1-mediated repression of autophagy is regulated by TOR-dependent phosphorylation of the decapping enzyme Dcp2

To identify a pathway(s) inhibiting Vad1-mediated autophagy repression on starvation, we evaluated the relationship between TOR and Vad1. We found that inactivation of TOR with rapamycin resulted in a similar reduction of transcript binding by Vad1 as seen with starvation, although to a lesser extent (Fig. 3c). In addition, degradation of *ATG8* transcripts was inhibited after rapamycin treatment (Fig. 3a, left bottom panel), similar to that exhibited during starvation. Furthermore, overexpression of *VAD1* resulted in inhibition of TOR-dependent induction of autophagic bodies and autophagic flux (Fig. 3d,e). These data thus suggest an epistatic relationship between induction of autophagy following TOR inactivation and Vad1-dependent transcript degradation.

As Dcp2 in *S. cerevisiae* was found to modulate autophagy (Fig. 1), we investigated whether this function was conserved in *C. neoformans* and if Dcp2 was a phosphorylation target of TOR. Work by us (data not shown) and others²² failed to show TOR-dependent phosphorylation of Vad1. However, previous whole proteome work in *S. cerevisiae* identified TOR-dependent phosphorylation of Dcp2, which had not reached false discovery rate statistical significance because of the large number of comparisons inherent in such studies²². Thus, to assess the role of this phosphorylation in autophagy, Dcp2–3XFLAG wild type, phosphomimetic (S > D) and phosphodeficient (S > A) double mutants were constructed in homologous sites at Ser 614 and Ser 617 and expressed in *C. neoformans*. In these studies, Dcp2 phosphoserine levels decreased after starvation as well as in the phosphodeficient mutant (Fig. 4a). Residual phosphoserine in the phosphodeficient mutant suggests the presence of additional starvation-related phosphorylation sites in Dcp2. A functional role for Dcp2 phosphorylation was demonstrated by measuring *ATG8* transcript degradation by qPCR. As shown in Fig. 4b, expression of the double-phosphodeficient Dcp2 resulted in competition with endogenous Dcp2 and retardation of degradation

in nutrient-rich conditions, but there was essentially no effect by the phosphomimetic mutant because endogenous Dcp2 is phosphorylated under these conditions as shown in Fig. 4a. However, expression of the phosphomimetic Dcp2 under starvation conditions or following rapamycin treatment (Fig. 4b middle and right panels) accelerated *ATG8* degradation as it competed with endogenous dephosphorylated Dcp2; in this case there was little effect of the phosphodeficient Dcp2 because the endogenous protein is also dephosphorylated under these conditions. This was associated with analogous effects on decapping (Supplementary Fig. 3b). Residual rates of degradation and decapping persisted in YPD after expression of the phosphodeficient Dcp2, likely owing to incomplete competition with endogenous Dcp2.

As *ATG* transcript binding to the decapping complex was dependent on TOR activity (Fig. 3c), we examined the effect of TOR Dcp2 phosphorylation on *ATG8* transcript binding under conditions that demonstrated alterations in mRNA-decapping activity (Fig. 4b and Supplementary Fig. 3b). As observed previously, in nutrient-replete conditions (nutrient +), wild-type cells exhibited binding of *ATG8* mRNA, whereas binding was lost on starvation (Fig. 4c). Cells expressing the Dcp2 phosphodeficient mutation showed a reduction of Vad1-dependent binding of the *ATG8* transcript in mid-log cells. Conversely, cells expressing the phosphomimetic Dcp2 maintained binding of *ATG8* mRNA even under starvation conditions. These effects correlated with autophagy activity based on changes in autophagic body accumulation (Fig. 4d) and autophagy flux (Fig. 4e). Flux was not as elevated in the phosphomimetic mutant in YPD compared with elevations due to rapamycin or after starvation, most likely owing to incomplete competition of the phosphomimetic mutant with endogenous Dcp2. Mutation of either the Ser 614 or the Ser 617 site produced detectable changes in *ATG8* degradation (Supplementary Fig. 3c) and decapping (Supplementary Fig. 3d) as well as *ATG5* decapping (Supplementary Fig. 4a,b), although the effects were predominant after mutation of the Ser 617 site. These data suggest that TOR-dependent phosphorylation of Dcp2 under nutrient-rich conditions is associated with increased recruitment of mRNA transcripts by the Dcp2–Vad1 decapping complex, resulting in mRNA degradation and autophagy inhibition. On starvation, TOR inhibition results in dephosphorylation of Dcp2, leading to a release of *ATG* mRNA from Vad1 and subsequent translation of *ATG* transcripts.

In addition to a role in mRNA decapping, *in vitro* studies have suggested that RCK homologues such as Dhh1 and Vad1 may undergo translational repression whereby mRNAs that have exited translation may accumulate within discrete cytoplasmic foci, referred to as processing (P)-bodies^{23,24}. Thus, to further assess for a role for translational suppression in autophagy, we monitored colocalization of *ATG5* and *ATG8* transcripts with P-bodies to estimate translational suppression during growth and starvation. As shown in Supplementary Fig. 5a, *ATG8* and *ATG5* transcripts were repressed under mid-log growth conditions and showed accumulation during starvation conditions. However, less than 5% of visible transcripts colocalized with P-bodies under either condition, suggesting very minor levels of translational suppression for these key autophagy transcripts. To determine the translational status of these transcripts, polysome analysis on sucrose gradients was performed. Consistent with earlier work in *S. cerevisiae*, starvation led to a reduction in polysomes in wild-type cells when overall synthetic needs are reduced²⁵.

However, unlike growth-induced transcripts that are transported from polysomes during mid-log growth to untranslated mRNP fractions on starvation²³, the autophagic *ATG5* and *ATG8* transcripts were predominantly localized to ribosomes and polysomes, consistent with a preponderance of active translation initiation and extension and consistent with their absence within P-bodies, demonstrated in Supplementary Fig. 5a.

Vad1/Dhh1-mediated repression of autophagy is conserved in mammalian cells

To extend the relationship between Vad1/Dhh1 and autophagy, we assessed the role of the mammalian homologue DDX6 in key autophagic processes. Two independent sets of mouse C57/BL6 embryonic stem cells (ESCs) were assessed that contained heterozygote gene-trap insertions within intron 2 (*DDX6*^{+/-} no. 1) and intron 1 (*DDX6*^{+/-} no. 2) (Supplementary Fig. 6a)²⁶. The insertion in *DDX6*^{+/-} no. 1 resulted in degradation and a 50% reduction in *DDX6* steady-state transcript levels (Fig. 5a). The *DDX6* transcript level was also decreased in *DDX6*^{+/-} no. 2 (18% reduction; Fig. 5a) but was found on RT-PCR followed by sequencing to contain a fragment of the gene-trap construct (data not shown). As a measure of residual DDX6 activity in the ESCs, decapping activity was assessed by PCR using specific *DDX6* primers, which demonstrated a 50% reduction in decapping in *DDX6*^{+/-} no. 1 and an almost complete absence in *DDX6*^{+/-} no. 2 (Supplementary Fig. 6b) accompanied by reductions in *LC3* (the mammalian *ATG8* homologue) mRNA degradation relative to the wild type (Supplementary Fig. 6c). The greater reductions observed with *DDX6*^{+/-} no. 2 are likely due to dominant-negative interactions of the native protein expressed from the wild-type gene copy and the aberrantly spliced copy of *DDX6* that included the gene-trap fragment. Defective decapping activity in the two *DDX6* ESC lines was associated with increased steady-state levels of *LC3* transcripts (Fig. 5b), formation of *LC3*-containing autophagosomes (Fig. 5c,d), increased *LC3*-II formation relative to both *LC3*-I and actin (Fig. 5e), and reduced levels of SQSTM1/p62 (Fig. 5e), an autophagy receptor whose degradation is a marker of autophagic flux in mammalian cells¹⁸. These data indicate that, analogous to its function in yeast, DDX6 suppresses autophagy in mammalian cells.

In contrast, overexpression of DDX6 in HeLa cells significantly suppressed rapamycin-induced *LC3* mRNA transcription, *LC3* lipidation and autophagic flux (Fig. 6a,d,e,h). As in the ESCs, DDX6 suppression resulted in accumulation of *LC3* transcripts, inhibition of *LC3* decapping, and increased *LC3*-labelled autophagosomes (Fig. 6b,c,f,g). DDX6 suppression also increased formation of *LC3*-II relative to actin but we were not able to induce SQSTM1 degradation after DDX6 suppression (data not shown), which may be due to species-related differences in autophagy induction or the transformed nature of the HeLa cell line.

In addition, we showed that the regulation of the mammalian pathway relies on the phosphorylation of the DCP2 protein by MTOR. As the smaller mammalian DCP2 protein does not contain a carboxy terminus homologous to the yeast phosphorylation site, high-density mass spectrometry (MS/MS; 97% coverage) was used to identify Ser 249 as a phosphorylated site under nutrient-rich conditions (Supplementary Fig. 7a) that was not present after rapamycin

treatment. Targeted ion mass spectroscopy was used to quantify a tenfold reduction in phosphorylation at Ser 249 after rapamycin treatment (Supplementary Fig. 7b). Although not homologous to yeast, the regions surrounding Ser 249 are highly conserved between human, mouse and rat (data not shown). DCP2 also seemed to be a direct target of MTOR phosphorylation as evidenced by *in vitro* phosphorylation data using a pSer 249 antigen-purified antibody (Supplementary Fig. 7c–e). Consensus sites are not highly conserved in MTOR targets²⁷, making assignment based on sequence less reliable. Again, similar to the result in yeast and fungus, expression of a DCP2^{S249A} phosphodeficient mutant resulted in accumulation of autophagosomes under normal growth conditions whereas the S249D phosphomimetic resulted in reduced autophagosome formation in HeLa cells under autophagy-inducing conditions (Fig. 5f,g). These data thus strongly suggest that DCP2 is a target of MTOR.

DCP2-dependent decapping modulates autophagy-dependent innate immune responses

Autophagy plays several roles in immunity^{28,29}, including modulation of the inflammatory response. For example, initial induction of autophagy occurs following stimuli eliciting inflammasome activation, resulting in activation of CASP1/caspase-1 and secretion of the proinflammatory cytokines IL1B and IL18, but maturation of the nascent autophagosome tempers the inflammatory response by engulfing and eventually destroying the inflammasome³⁰. We thus examined the role of mRNA decapping in the autophagic regulation of IL1B in differentiated THP-1 macrophages following lipopolysaccharide (LPS) stimulation. LPS treatment led to inhibition of *LC3* decapping (Fig. 7a), resulting in reduced *LC3* transcript degradation (Fig. 7b) and increased steady-state levels of *LC3* (data not shown). In addition, compared with wild-type DCP2, the S249D phosphomimetic mutant exhibited lower levels of *LC3* transcripts and protein, whereas the S249A mutant showed the opposite result (Fig. 7c,d). Furthermore, these changes in *LC3* transcript and protein correlated inversely with levels of IL1B, with higher levels of the cytokine seen when autophagy was inhibited (Fig. 7e).

To establish the significance of this regulatory pathway connecting MTOR to IL1B in human disease, we tested whether phospho-DCP2 levels were increased in PBMCs of a patient from a recently described cohort with gain-of-function mutations in PIK3CD encoding the PI(3)K delta subunit (p100δ) that conveys MTOR overactivity leading to lymphoproliferation and autoimmune cytopenias¹³. Using an immunopurified p249-DCP2 antibody, an age-matched healthy volunteer demonstrated steady-state P-DCP2 levels in PBMCs that were reduced after treatment with rapamycin. Interestingly, P-DCP2 levels were elevated in a patient with the PIK3CD/p110δ mutation (Fig. 7f) with resultant reductions in autophagic vacuoles (Fig. 7g) and autophagic flux, the latter demonstrated by accumulation of SQSTM1 (Fig. 7f). This alteration in autophagic flux was accompanied by high levels of IL1B in the patient, reduced after *ex vivo* treatment with rapamycin (Fig. 7h).

In sum, these results uncover the role of DDX6/Vad1/Dhh1 and the mRNA decay machinery in the repression of autophagy and strongly support a conserved function of this pathway in eukaryotic cells, playing a role in cell survival, infectivity and the immune response, applicable to human disease.

DISCUSSION

We propose a conserved pathway where a DEXD/H-box RNA helicase in the RCK family, in association with the decapping enzyme Dcp2, negatively regulates autophagy through modulation of *ATG* mRNA stability in a TOR-dependent manner (Fig. 8). In our model we predict that during nutrient-replete conditions RCK family members such as Dhh1, Vad1 and DDX6 recruit *ATG* mRNA to the Dcp2 decapping complex, leading to mRNA decapping followed by degradation and autophagy suppression. This is consistent with RCK members acting through an mRNA chaperone activity, rather than by induction of Dcp2 enzymatic activity. Along these lines, previous *in vitro* studies demonstrated a physical association of the RCK family member Dhh1 with Dcp2, but did not demonstrate Dcp2-enhancing activity³¹. The decapping activity thus acts as a coordinated, TOR-dependent process, synchronous with previously described mechanisms that inhibit transcriptional synthesis, dependent on such factors as Rim15, Gln3 and Msn2/4 (refs 3,15,32–34). Starvation-dependent dephosphorylation reverses the process, facilitating the induction of autophagy in concert with mRNA synthesis. The present data showing significant steady-state levels of *ATG*-related transcripts after RCK suppression in yeast also suggest that significant mRNA synthesis of these transcripts occurs even under ‘repressive’ conditions; this may allow a more rapid induction of *ATG* transcripts in response to cellular stress, by simultaneously inducing mRNA synthesis and inhibiting its degradation, much akin to previously described ‘futile cycles’ involving metabolic components³⁵. Combining a post-transcriptional inductive mechanism with increases in transcriptional synthesis in response to transcription factors such as Gln3 and TFEB (refs 3,4) thus facilitates a rapid autophagic induction. Environmental stresses, including glucose deprivation, can modulate mRNA stability^{24,36}. We identified RCK family members, which are recognized for facilitating mRNA decapping and decay¹⁰, as possessing a conserved functional role linking environmental conditions to autophagy induction. Analysis of protein–mRNA complexes identified *ATG8* as a prominent gene whose fate was likely determined by RNA binding and function, further supporting our model^{37,38}. Data in *C. neoformans* as well as in mammalian systems show a more highly penetrant phenotype under both vegetative conditions and following TOR suppression, suggesting that evolutionary pressures have placed a higher dependence on post-transcriptional regulation and support the need to conduct such cross-species studies when considering conserved regulatory mechanisms.

A reversible TOR-dependent Dcp2 serine phosphorylation was an important component linking RCK member recruitment and *ATG* target transcript decapping/degradation. TOR is a key global regulator that controls protein translation through its interactions with RPS6KB1/p70S6 kinase 1 and EIF4EBP1/4E-BP1 (ref. 39). In mammalian systems, the transition from rapid growth to growth arrest induced by MTOR inhibition results in translational arrest of synthetic machinery including ribosomal and translational initiation factors⁴⁰. TOR promotes the synthesis of most genes in vegetative conditions, but selectively represses a small subset by mechanisms including transcriptional suppression⁴¹. TOR has previously been linked to stress-responsive mRNA stability programs, but the mechanism of this regulation was unclear⁴². Along with mRNA stability regulation, it would seem that these post-transcriptional mechanisms may

be an important component of the stress response in eukaryotes. For the transcripts studied in the present paper, we did not find large amounts of translational suppression. However, Dhh1-mediated translational suppression has been predominantly studied using transcripts highly expressed under growth conditions that require translational suppression on transfer to starvation conditions to rapidly shut down unneeded translational synthesis from pre-formed mRNA. In this process, mRNA transcripts are rapidly shunted from the polysome to RNP particles including P-bodies²³. However, autophagy transcripts are repressed under growth conditions and are activated in yeast predominantly under nutrient stress, which may reduce the need for translational suppression under initial growth conditions because steady-state levels of transcripts are already quite low. Indeed, protein translational suppression of *ATG* genes did not feature predominantly in a recent whole genome ribosomal profiling study⁴⁰. In addition, we did not study the transition from starvation back to nutrient-rich growth where translational suppression could figure more predominantly. Sequence-specific factors that differentiate stress transcripts such as *ATG* genes versus those of the cellular synthetic machinery are the subject of active investigation.

The present data also demonstrated that RCK family members significantly affected cellular processes, including pathogen virulence and host immunity. In *C. neoformans*, Vad1 overexpression reduced autophagy sufficiently to affect the ability of the fungus to survive within macrophages, an important property for this facultative intracellular pathogen^{19,43}. A number of *ATG* transcripts are induced during macrophage residence⁴⁴ and suppression of autophagy transcripts such as *ATG8* results in severely attenuated fungal virulence¹⁶. Thus, the present data demonstrate a significant pathway of TOR signalling, connecting macrophage survival, autophagy and microbial virulence.

We also demonstrated that key aspects of MTOR-dependent DCP2–DDX6-dependent decapping of the autophagy-related transcript *LC3* modulated rates of LPS-induced IL1B levels produced by the inflammasome⁴⁵. Blocking autophagy potentiates inflammasome activity, measured by IL1B production, and stimulating autophagy limits it³⁰. This effect was mimicked in the present studies by mutations in *DCP2*, phenocopying by expression of an MTOR-associated phosphomimetic mutation (resulting in increased *LC3* degradation), and a phosphodeficient mutation (resulting in a reduction in *LC3* degradation), respectively. These data show that manipulation of autophagy by the MTOR-associated phosphorylation status of DCP2 can consequentially affect the human inflammatory response, and likely plays a role in MTOR-dependent modulation of inflammatory states exemplified by patients with dominant-activating mutations in PIK3CD/p110δ (ref. 13). In summary, this post-transcriptional mechanism of autophagy regulation is an important pathway for promoting cell survival as well as pathogen virulence and host inflammatory response. □

METHODS

Methods and any associated references are available in the [online version of the paper](#).

Note: Supplementary Information is available in the online version of the paper

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AUTHOR CONTRIBUTIONS

G.H.: experimental work, project planning, data analysis, writing; T.M.: experimental work, project planning, data analysis, writing; A.B.: experimental work, project planning, data analysis, writing; Y.-D.P.: experimental work, project planning, data analysis, writing; J.Q.: experimental work, data analysis, writing; A.V.: experimental work, data analysis, writing; N.Z.: experimental work, data analysis, writing; S.R.W.: experimental work, data analysis, writing; N.H.B.: experimental work, data analysis, writing; T.G.M.: experimental work, data analysis, writing; R.J.M.: data analysis, project planning, writing; J.H.K.: data analysis, project planning, writing, editing; G.U.: experimental work, data analysis, protocol preparation, writing, editing; D.J.K.: project planning, data analysis, writing, editing; P.R.W.: project planning, data analysis, writing, editing.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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METHODS

S. cerevisiae strains and media. Gene disruptions were performed using a standard method⁴⁶. Yeast cells were grown in YPD (1% yeast extract, 2% peptone, and 2% glucose (all wt/vol)), YPgal (1% yeast extract, 2% peptone, and 2% galactose (all wt/vol)) or synthetic minimal medium (SMD; 0.67% yeast nitrogen base, 2% glucose, supplemented with the appropriate amino acids and vitamins). Autophagy was induced in starvation medium (SD-N; 0.17% yeast nitrogen base without amino acids, containing 2% glucose or SGal-N containing 2% galactose). The yeast strains used in this study are listed in Supplementary Table 1. Protein extraction, immunoblot, GFP-Atg8 processing, and alkaline phosphatase (Pho8Δ60) assays were performed as described previously^{3,14}. Antisera against Atg8 (1:2,000), Atg1 (1:3,000), Atg9 (1:5,000), Pgl1 (1:50,000; a gift from J. Thorner, University of California, Berkeley, USA), monoclonal YFP (1:5,000; JL-8, Clontech) and a commercial antibody that reacts with PA (1:20,000; anti-PA, no longer available) were used for western blotting.

RNA and RT-qPCR. Total RNA was extracted using the RNeasy mini kit (Qiagen). To eliminate genomic DNA contamination, an additional DNase treatment was performed according to the RNeasy kit instructions with the RNase-free DNase set (Qiagen). One microgram of total RNA was reverse-transcribed into cDNA in a 20- μ l reaction mixture using the High-capacity cDNA Reverse Transcription kit (Applied Biosystems). The cDNA levels were then analysed using the Eppendorf Realplex⁴ with the gene-specific primers listed in Supplementary Table 2. Each sample was tested in a 96-well plate (Applied Biosystems). The reaction mix (15 μ l final volume) consisted of 7.5 μ l of Power SYBR Green master mix (Applied Biosystems), 0.5 μ l of each primer (333.3 nM final concentration), 1.5 μ l of H₂O, and 5 μ l of a 1:5 dilution of the cDNA preparation. The thermocycling program consisted of one hold at 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. After completion of these cycles, melting-curve data were then collected to verify PCR specificity, contamination and the absence of primer dimers. The transcript abundance in samples was determined using a comparative threshold cycle method. The relative abundance of reference mRNAs in each sample was determined as described previously⁴⁷ and used to normalize for differences of total RNA amount according to the method described in ref. 48. For the comparison between wild type and *dhh1Δ* the mRNAs of *ALG9*, *TAF10* and *TFC1* were used as references for the growing condition samples and the mRNAs of *TAF10* and *TFC1* were used as references for the starved samples. As the expression of *TAF10* and *TFC1* was affected by the *dcp2-7Δ* mutation, *ALG9* was used as the only internal reference in the comparison between wild type and *dcp2-7Δ* for both the growing and starvation condition samples.

RNA-binding-protein screen. Wild-type and RBP mutant cells were grown in YPD medium until mid-log phase. The cells were then washed twice in SD-N and resuspended in SD-N. The attenuation of each culture was adjusted to 0.8–0.9 and the cells were grown at 30 °C for the time indicated in Fig. 1a. Immediately after the shift to starvation (day 0) or after 5, 10 and 15 days the same volume of each culture was collected and serial dilutions were spotted on YPD plates; the cells were grown at 30 °C for 2 days before being imaged.

C. neoformans strains and media. All experiments were conducted in a genetic background of *C. neoformans* strain serotype A H99 (ATCC 208821), a gift from J. Perfect (Duke University, USA). A complete list used in this study is described in Supplementary Table 3. *Escherichia coli* DH10B (Invitrogen) was the host strain for recovery and amplification of plasmids. The fungal strains were grown in YPD medium (2% glucose, 1% yeast extract, 2% Bacto-peptone) or on YPD agar medium (YPD and 2% agar) or in YNB plus glucose (yeast nitrogen base without amino acids and ammonium sulphate + 2% glucose). Asparagine minimum selective media (ASN) for transformant selection was previously described⁴⁹. Starvation medium was sterile phosphate-buffered saline (PBS), pH 7.4, unless otherwise noted.

Generation of VAD1 overexpression strains. A *VAD1* gene fragment was generated by PCR amplification from an H99 cDNA mass-excised library with a pair of primers VAD1-S-Mun1 VAD1-A-Mun1, and then inserted into an EcoRI restriction site downstream from an *ACT1* promoter of a pORA-KUTAP cryptococcal shuttle vector described previously¹⁶. The construct was linearized with SclI, and then introduced into H99FOA, a recipient strain. Stable transformants were selected on ASN minimal medium. The overexpression of *VAD1* was confirmed by northern blots probed with isotope-tagged *VAD1* cDNA sequence, and transformants were selected for equivalent copy number to the empty vector controls as described previously⁵⁰.

Generation of an N-terminal green-fluorescent-tagged Atg8 expression strain. A fragment of the *ATG8* promoter region was PCR amplified using primers ATG8-PRO-Bgl II and ATG8PRO-BglIINedIPstr and cloned into a

pORA *C. neoformans* shuttle vector previously described⁵¹ to produce vector pORA-ATG8PRO. A fragment of a *C. neoformans* codon-optimized green fluorescent protein gene was then PCR amplified and cloned into pORA-ATG8PRO into a PstI compatible site, and then the *ATG8* codon region was amplified from *C. neoformans* genomic DNA and cloned into pORA-ATG8PRO:GFP through NdeI compatible sites, leaving the original *TEF1/EF1 α* 3'-UTR to yield pATG8-PCG-EF1. Finally, a fragment of the *ATG8* 3'-UTR was PCR amplified and cloned into pATG8-PCG-EF1, using compatible NdeI sites, replacing the *TEF1* 3'-UTR with the *ATG8* 3'-UTR to yield pATG8-PCG-Native. All inserted regions were confirmed by sequencing.

Construction of an atg8Δ mutant strain. To make the deletion construct, 2,500 base pair (bp) genomic fragments of the *ATG8* gene were PCR amplified using primers ATG8-62-S-Xba1/ATG8-572A-R1 and ATG8-1541-S-BglIII/ATG8-1866-A-XhoI as well as a 1.3-kb fragment of the *URA5* gene using primers URA5-S-R1/URA5-A-BglII and the 3 fragments ligated simultaneously after digestion using compatible sites (see Supplementary Table 4 for sequence information). The final disruption allele with a 1.3-kb *URA5* marker flanked on either side by a 500-bp DNA sequence homologous to genomic regions of the *ATG8* gene was PCR amplified and purified by spin column (Invitrogen) and introduced into H99FOA cells using a biolistic approach⁵¹. Transformants were screened for potential deletion mutants by a PCR approach using one set of primers, designed to detect disruption events, including a primer just outside the transforming vector and an opposing primer based on the *URA5* sequence. The specific 2.6-kb disruption of the *ATG8* gene in candidate mutants was verified by using a set of primers outside the sequences of the knockout construct and by Southern blot analysis.

Production of Dcp2 phosphomimetic- and phosphodeficient-expressing strains. Homologous sites in *C. neoformans* Dcp2 were determined from sites homologous to the rapamycin-sensitive sites described for Dcp2 from *S. cerevisiae*²². Two EMBOSS global alignment programs⁵², needle and stretcher, were used with default parameters to align the SAC sequence against the CRY sequence. The two programs aligned our site of interest ('SNKS') to two different regions; stretcher aligned 'SNKS' of SAC to 'SQSS' of CRY with the 'S' positions at 614 and 617, and needle aligned our site of interest ('SNKS') to 'ASFG' of CRY with the 'S' position at 84. The phosphorylation site predictions were done using six different web server tools (NetPhos 2.0 (ref. 53), DISPHOS (ref. 54), NetPhosK 1.0, MotifScan http://myhits.isb-sib.ch/cgi-bin/motif_scan, ScanProsite (ref. 55), KinasePhos 2.0 (ref. 56). Three (DISPHOS, NetPhosK, KinasePhos) of the six tools predicted significant phosphorylation sites at both positions 614 and 617. NetPhos predicted a significant phosphorylation site at 617 alone. None of the six tools predicted any phosphorylation site at position 847.

DNA, RNA preparation and blotting. Primers used in the *C. neoformans* study are described in Supplementary Table 4. DNA extraction was conducted according to a previously published protocol⁵⁷. RNA was isolated and northern blots performed as previously described⁸. DNA probes were labelled with [α -³²P]ATP for both Southern and northern blots using the RadPrime DNA Labelling System (Invitrogen) according to the manufacturer's protocol. Transcript levels of indicated genes were monitored by northern blot analysis hybridized with a PCR-amplified fragment of the indicated genes using primers listed in Supplementary Table 4.

RNA degradation assays. The method of ref. 8 was used. Briefly, cultures were grown to the log phase, at a final density of 10⁷ cells ml⁻¹ in yeast-peptone-dextrose medium at 30 °C with vigorous shaking. Starvation was induced by incubating mid-log phase cells in PBS, pH 7.4, for 1 h and cells were collected. Transcription was suppressed by addition of 1,10-phenanthroline (200 μ g ml⁻¹ in ethanol, Sigma). At the indicated time points, cells were collected by 5 min of centrifugation at 2,000g in a tabletop centrifuge at room temperature and immediately frozen in a -80 °C freezer for later use. Total RNA was extracted with the RNeasy Mini Kit (Qiagen). The degradation of *ATG8* mRNA was detected by a standard northern blot protocol⁸.

Capped RNA analysis. RNA ligase-mediated rapid amplification of cDNA ENDS (RLM-RACE) analysis was performed with total RNA isolated from the indicated strains at the indicated times after transcription suppression by 1,10-phenanthroline by using the FirstChoice RLM-RACE kit according to the manufacturer's (Applied Biosystems) protocol²⁰. PCR amplification was performed by using the gene-specific primers ATG8-A for *ATG8*, ATG5-A for *ATG5*, and ACT1-A for *ACT1* (Supplementary Table 4) and two universal primers 5' RACE Outer Primer and 5' RACE Inner Primer (supplied by the manufacturer). The actin gene, *ACT1*, served as loading control. This analysis was performed in 3 independent experiments, and *ATG8/ACT1* ratios were calculated for the indicated strains by densitometry (UN-SCAN-IT gel 6.1, Silk Scientific Corporation).

RNA and protein immunoprecipitation. RNA immunoprecipitation was performed by an adaptation of a previously described method⁵⁸ using strains expressing *c-myc*-Vad1 or 3XFLAG-Dcp2. Briefly, protein G-Sepharose (Sigma) was washed 3 times with 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% NP40, and either 30 µg of anti-*myc* monoclonal antibodies (Sigma) or anti-FLAG monoclonal (Sigma) was added and incubated for 1 h at 4 °C. The Sepharose-bound monoclonal antibody was washed 5 times in the same buffer at 4 °C. For immunoprecipitation, cells were grown to mid-log phase in YPD at 30 °C. Cells were collected by centrifugation and the cells were washed twice in 25 mM HEPES-KOH (pH 7.5), 150 mM KCl, 2 mM MgCl₂. Cells were resuspended in the above buffer containing 20 mM vanadyl ribonucleoside complexes (Sigma), 200 units ml⁻¹ RNaseOUT (Sigma), 0.1% Nonidet P-40, 1 mM dithiothreitol, with protease inhibitor cocktail. Glass beads were added and the samples vortexed for 1 min. The supernatant fractions were removed and centrifuged for 10 min at 3,000 × *g*_{max}. Extracts were incubated with either anti-*myc* antibodies, or 3XFLAG-Dcp2 coupled to protein G-Sepharose for 1 h at 4 °C. Beads were washed 7 times in 25 mM HEPES-KOH (pH 7.5), 150 mM KCl, 2 mM MgCl₂, and were eluted in 50 mM Tris · HCl (pH 8.0), 100 mM NaCl, 10 mM EDTA, 1% SDS for 10 min at 65 °C. A portion of each eluate was digested with proteinase K for 1 h at room temperature and then extracted with phenol/chloroform and ethanol precipitated to isolate RNA. RNA was analysed by RT-PCR using primers listed in Supplementary Table 4 and detected on ethidium bromide-labelled gels. Quantities of Vad1 in the lysate before immunoprecipitation as well as that in pellets after immunoprecipitation were estimated on western blots using a DDX6/Vad1 cross-reactive antibody (Bethyl).

Fluorescent *in situ* hybridization (FISH). FISH was adapted for *C. neoformans* on the basis of refs 59,60. Briefly, all strains were grown aerobically under continuous shaking in YPD broth (2% glucose, 1% yeast extract, 2% Bacto-peptone) at 30 °C and either collected during mid-log phase (A 0.2–0.5) or subjected to starvation by incubating mid-log phase cells in phosphate-buffered saline, pH 7.4 for 3 h. Cells were collected by centrifugation for 5 min at 7,800*g*. Cells were washed once with 1 × PBS and fixed for 4 h with 4% w/v paraformaldehyde in PBS at 4 °C. After fixation, cells were washed twice with PBS, resuspended in one volume of PBS and one volume of cold absolute ethanol and stored at –20 °C until use. All probes were labelled at the *ATG5* or *ATG8* ORF and terminator with the fluorochrome Cy3 (Supplementary Table 5; Affymetrix). Approximately 10⁶ fixed yeast cells were hybridized in 20 µl of hybridization buffer (0.9 M NaCl, 0.01% w/v SDS, 20 mM Tris-HCl, pH 7.2 and 20% formamide), with 5 ng of Cy3-labelled probe and incubated at 46 °C for 16 h. After incubation, cells were pelleted by centrifugation and resuspended in 1 ml of prewarmed washing buffer (20 mM Tris-HCl, pH 8.0, 0.01% w/v SDS, 5 mM EDTA, 225 mM NaCl) for 30 min at 46 °C. The suspension was then centrifuged and the pellet fraction resuspended in 50 µl of 1 × PBS, placed on ice and analysed within 3 h. The slides were then mounted in ProLong Gold antifade reagent (Invitrogen) and observed using a Leica DMI 6000B microscope with a Hamamatsu camera using LAS AF6000 ver. 2.1.2 software (Leica).

Polysome fractionation. The method of ref. 61 was used. Briefly, yeast cells were grown to mid-log phase (an *A*_{600nm} between 0.6–0.8) in YPD. Half of each culture (500 ml) was rapidly collected by centrifugation at 30 °C, the media poured off, then cells were washed with PBS and collected by centrifugation at 30 °C. The remaining 500 ml of culture was washed with PBS, and then incubated in PBS buffer 1 h for starvation conditions. Cell pellets were flash frozen by immersion in liquid nitrogen; frozen cell pellets were transferred to a mortar filled with liquid nitrogen and surrounded by dry ice. Yeast cells were lysed using a mortar and pestle under cryogenic conditions, and lysed yeast powder was transferred to 15-ml conical tubes and stored in dry ice until all samples were collected. Five hundred microlitres of ice-cold lysis buffer (20 mM HEPES, pH 7.3, 5 mM MgCl₂, 150 mM KCl, 40 U ml⁻¹ Rnasin Plus (Promega)) was added to each sample, and lysates were thawed on ice. Insoluble debris was pelleted at 4 °C for 10 min at 1,400*g*, and the supernatant fraction was transferred to ice-cold 1.5-ml tubes. A 1/200 dilution of each lysate was made and quantified using a DeNovix DS-11 spectrophotometer. On the basis of *A*₂₆₀ readings, 15 *A*₂₆₀ units of each lysate was layered on top of 7–47% w/v sucrose gradients, and polysomes separated for 2.5 h at 170,000*g* in a SW41 rotor at 4 °C. Gradients were then fractionated into 1 ml fractions using a Brandel tube piercer, Foxy Jr. fraction collector, and Teledyne Isco chart recorder. Sucrose fractions were stored at –80 °C until RNA was extracted. RNA was extracted from 500 µl of each fraction using the Maxwell 16 instrument (Promega), and SimplyRNA kit (Promega).

Autophagic flux measurements. The method of ref. 62 was used to measure protein turnover of radiolabelled proteins after serial trichloroacetic acid precipitation. Briefly, mid-log phase yeast cells grown in YNB without amino acids and ammonium sulphate containing 2% glucose were labelled with fresh [³⁵S]-labelled methionine for 1 h at 30 °C, then washed 3 × with cold YNB containing

20 mg l⁻¹-methionine and 20 mg l⁻¹-cysteine and incubated in either YNB without amino acids and ammonium sulphate containing 1 mg ml⁻¹ cysteine, 1 mg ml⁻¹ methionine (starvation) or YPD containing 1 mg ml⁻¹ each of methionine and cysteine (rich medium) at 30 °C. At the indicated times, cell aliquots were transferred to microcentrifuge tubes, the cells lysed and protein precipitated with 10% final volume trichloroacetic acid at 95 °C for 5 min followed by centrifugation at 12,000*g* and radiation measured in the supernatant and pellet fractions by liquid scintillation.

Mouse virulence studies. Virulence studies were conducted using a previously described intravenous mouse meningoencephalitis model with 10 Swiss albino female mice (6–8 weeks old) per group on cryptococcal strains expressing plasmids in equivalent copy number determined by uncut Southern blot⁶³. Episomal expression of plasmids in *C. neoformans* have statistically insignificant differences in virulence from wild-type parental strains¹⁶. Fungal viability was confirmed by growth of inocula on YPD agar. All experimental procedures involving animals were conducted under guidelines of the National Institutes of Health and protocols approved by the Institutional Animal Care Committees (IACUC) of the Intramural NIH/NIAID. Statistical comparison of survival times was made by a log-rank comparison using GraphPad Prism 5. No statistical method was used to determine sample size and investigators were not blinded to allocation during experiments and outcome assessment.

Identification of autophagy-related genes. Homologues of autophagy-related genes in *C. neoformans* were identified as described previously¹⁶ and additional ones identified by use of a bidirectional best hits (BBH) method⁶⁴. Briefly, the autophagy-related genes of *S. cerevisiae* were used to perform an all-versus-all BLASTP similarity search⁶⁵ against the *C. neoformans* genome. The resulting hits were analysed for the BBH using a custom PHP script.

Mammalian cells and cell culture. HeLa cells were freshly obtained from the American Type Culture Collection (ATCC) and were tested for mycoplasma contamination and cell identity as per the ATCC protocol before shipment. Cells were cultured were cultured in ATCC-formulated Eagle's minimum essential medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin solution. Embryonic stem cell (ESC) RCK gene-trap insertion was carried out as described previously²⁶ (Supplementary Fig. 2). Briefly, a retroviral gene-trap vector containing the 5' selectable/reporter gene βGeo (βGal and Neo fusion) downstream of a splice acceptor sequence was introduced into wild-type C57BL/6J embryonic stem cells. Insertion of the gene-trap cassette into an intron located at the 5' end of the target gene *DDX6* produces incorrect splicing such that downstream exons are not properly expressed, thereby resulting in gene inactivation²⁶. Inverse PCR was used to determine the insert location in the *DDX6* gene in the RCK gene-trap clone ESCs (Supplementary Fig. 2). ESC recombinant clones of the C57BL/6 background carrying the gene-trap cassette between exons 1 and 2 of the *DDX6* locus (Clone IST11384H1, Mouse Accession NM_00110826), referred to as *DDX6*^{+/–} no. 2 in this manuscript, or exons 2 and 3 of the RCK/*DDX6* locus (Clone IST12067C10, Mouse Accession NM_00110826), referred to as *DDX6*^{+/–} no. 1 in this manuscript, were generated by the Texas A&M Institute for Genomic Medicine (<http://www.tigm.org>). ESCs were cultured on mouse embryonic fibroblasts (PMEF-N; Millipore), which acted as feeder cells, for maintaining the ESC culture. In preparation for experiments, ESCs were separated from the feeder cells and plated onto gelatin-coated cell culture dishes. ESCs were then split an additional time and plated again onto gelatin-coated cell culture dishes to assure no feeder cells remained. ESCs were cultured in Knockout DMEM with glucose and sodium pyruvate (Life Technologies) containing 15% ES-grade FBS (Atlanta Biologicals), 1% penicillin–streptomycin solution, 2 mM glutamine, 0.1 mM non-essential amino acids, and 1,000 U ml⁻¹ leukaemia inhibitory factor of mouse origin (Santa Cruz Biotechnology). Wild-type C57BL/6J ESCs were a gift provided by K. Francis (NICHD, NIH, Bethesda, MD, USA) and were used as controls in all ESC experiments. THP-1 cells were obtained from American Type Culture Collection (Cat. No. TIBG 202) and tested for mycoplasma contamination before use using the Mycoalert Mycoplasma detection kit (Lonza). THP-1 cells were maintained in RPMI-1640 medium containing 10% (vol/vol) FBS, 1% penicillin–streptomycin–amphotericin B and 50 mM 2-mercaptoethanol. THP-1 cells were differentiated into macrophages through 50 nM PMA treatment for 3 h. Differentiated THP-1 cells were seeded into 6-well plates at 2.0 × 10⁶ cells per well and treated with 200 ng ml⁻¹ LPS overnight, when indicated.

Mammalian DNA and RNA preparation. DNA extraction and RNA extraction was accomplished using the DNeasy and RNeasy Mini Kits according to the manufacturer's (Qiagen) protocols. Primers used in this study are described in Supplementary Table 5.

Mammalian RNA interference. HeLa cells were transfected with small interfering RNA molecules specifically targeting RCK using the Amaxa Cell Line Nucleofector Kit R in conjunction with the Nucleofector System (Lonza). Cells were cultured for 48 h before all experiments to achieve optimal results. Following sample collection, knockdown efficiency was determined using PCR methods to examine RCK transcript levels and western blotting to establish protein levels.

Mammalian plasmids and transfection. Plasmids pT7-EGFP-C1-HsRCK (Plasmid 25033) and pT7-EGFP-C1-HsDCP2 (Plasmid 25031) were created by the laboratory of E. Izaurralde and obtained from Addgene (Addgene.org). pT7-EGFP-C1-HsDCP2 was used to express a site-directed mutagenesis HsDCP2 by a standard method. The plasmid as a template was amplified in a mutagenesis reaction with two overlapped primers containing the target mutations, DCP2-S249D-S 5'-A GATTGGCGATTCTCAGACGATGACAATGGATTTCTCAAC-3' and DCP2-S249D-A 5'-TCTAAACCGTAAGGAGTCTGCTACTGTTACCTAA AAGGAGTTG-3' for the phosphomimetic mutation (PT7-EGFP-C1-HsDCP2-S249D); DCP2-S249A-S 5'-GATTGGCGATTCTCAGACGCTGACAATG GATTTCTCAAC-3' and DCP2-S249A-A 5'-CTAAACCGCTAAGGAGTC TGCGACTGTTACCTAAAAGGAGTT-3' for the phosphodeficient mutation (pT7-EGFP-C1-HsDCP2-S249A). The PCR products were digested with DpnI (Invitrogen) to eliminate template plasmid and purified with a PCR Purification Kit (Qiagen), and then transformed into competent *E. coli* (Bioline). Individual transformants were picked and cultured in LB broth with ampicillin. The plasmids containing HsDCP2 with the target mutagenesis were collected with a Miniprep Kit (Qiagen) and verified by DNA sequencing. HeLa cells were transfected using X-tremeGene 9 DNA Transfection Reagent (Roche) using a 5:2 ratio of transfection reagent to plasmid DNA and cultured for 48 h before all experimentation. Following sample collection, transfection efficiency and protein levels were determined using fluorescence microscopy and western blotting.

HeLa and ESC autophagy induction. Cells were treated with control medium, medium containing 5 μ M rapamycin, or GIBCO Hanks' balanced salt solution (Life Technologies) for 2 h to examine autophagy unless otherwise stated. In experiments where LC3 conversion from cytosolic LC3-I to the lipidated LC3-II form was examined using western blotting, cells were also treated with 100 nM bafilomycin A₁ (Sigma) at the initiation of the experiment⁶⁶. In experiments where SQSTM1 degradation was examined for autophagosomal maturation, no lysosomal inhibitors were added to the cells. All samples were processed immediately for analysis or stored at -80 °C until a later date.

THP-1 cell autophagic induction and IL1B level measurements. pT7-EGFP-C1 (empty vector), pT7-EGFP-C1-HsDCP2 (wild-type), pT7-EGFP-C1-HsDCP2-S249D (phosphomimetic mutant), and pT7-EGFP-C1-HsDCP2-S249A (phosphodeficient mutant) were transformed using nucleofection into non-differentiated THP-1 monocytes (0.5 μ g plasmid per 2.5 \times 10⁶ cells) using Human Monocyte Nucleofection Kit (Lonza) as instructed in the 'Efficient non-viral transfection of THP-1 cells' paper. Six hours following nucleofection, THP-1 cells were differentiated into macrophages through 50 nM PMA treatment for 3 h. Differentiated THP-1 cells were seeded into 12-well plates and treated with 20 ng ml⁻¹ LPS overnight. The next morning, the supernatant fractions were collected for IL1B ELISA quantification and the cells were lysed for LC3, GFP and actin immunoblotting. IL1B measurement was performed as instructed in the eBioscience human IL1B ELISA kit.

Mammalian RNA degradation assays. To inhibit transcription, cells were treated with 5 μ g ml⁻¹ actinomycin D (Sigma) and 20 μ g ml⁻¹ 5,6-dichlorobenzimidazole 1- β -D-ribofuranoside (DBR) (Sigma) in control media or media containing 5 μ M rapamycin⁶⁷. Control cells were untreated and collected at initiation of treatment (time 0 min). After 30 min, samples were collected and RNA was extracted for analysis using PCR methods.

Capped RNA analysis in mammalian cells. Using the FirstChoice RLM-RACE Kit (Life Technologies), the presence of capped LC3 transcripts was assayed in mammalian cells by PCR amplification of a cDNA product obtained from sequential ligation of a 5' RNA adapter to full-length capped mRNA following treatment with tobacco acid pyrophosphatase. Samples were processed according to the manufacturer's protocol and analysed accordingly.

Western blotting and immunoprecipitation. Cells were lysed using cComplete Lysis-M (Roche) with the addition of PhosSTOP Phosphate Inhibitor Cocktail (Roche) and protein concentration was determined using a colorimetric assay. Fifty micrograms of each sample was run on 4–20% or 7.5% Mini-Protein TGX precast gels (Bio-Rad) and proteins were transferred onto 0.2- μ m PVDF or 0.45- μ m nitrocellulose membranes (Bio-Rad). Membranes were immunoblotted

with rabbit anti-DDX6 antibody (A300-461A, Bethyl), rabbit anti-DCP2 antibody (A302-597A, Bethyl), mouse anti-GFP antibody (clones 7.1 and 13.1, Roche), rabbit anti-SQSTM1/p62 antibody (A302-857A), mouse anti-LC3 antibody (NB600-1384, Novus) and mouse anti-actin (MAB1501, Millipore). Membranes were then probed with horseradish peroxidase-conjugated anti-rabbit secondary antibodies (Bethyl) or anti-mouse secondary antibodies (Jackson) and proteins were visualized using Clarity Western ECL Substrate (Bio-Rad). For immunoprecipitation, 1 mg of total protein from GFP-DCP2-expressing HeLa cells lysate was precipitated using mouse anti-GFP antibody (Roche) attached to Dynabeads Protein G (Invitrogen) crosslinked by bis(sulphosuccinimidyl) suberate (BS3) (Invitrogen) for 2 h at room temperature. Samples were then washed 3 times, prepared for gel electrophoresis and run on gels as described above. Lysate input was used as a control and immunoblotted accordingly to demonstrate equal loading.

Targeted ion tandem mass spectrometry. GFP-DCP2 was immunoprecipitated from samples as described, and gels were then fixed and stained with Coomassie brilliant blue. The band corresponding to the molecular weight of GFP-DCP2 was excised and sent to W. S. Lane at the FAS Center for Systems Biology of the Harvard Mass Spectrometry and Proteomics Resource Laboratory, USA (<http://labs.mcb.harvard.edu/microchem>) for peptide phosphorylation analysis. Samples were analysed in detail as previously described⁶⁸.

Analysis of MTOR-dependent DCP2 phosphorylation. Affinity-purified rabbit anti-DCP2-pSer249 antiserum was produced by a commercial vendor (GenScript) using standard methods. Briefly, a synthetic DCP2_pSer249 phosphopeptide (SSD(pSer)DNGFSSTGSTC) was used to immunize rabbits and produce polyclonal serum and purified by affinity chromatography using the synthetic phosphorylated peptide. Serum was positive at a greater than 1:128,000 dilution by ELISA against the phosphorylated peptide and negative at 1:1,000 against the corresponding non-phosphorylated peptide. Recombinant mammalian DCP2 was prepared from an *E. coli* vector pHI902 expression system as described previously⁶⁹. Briefly, primers pMAL-c-BamHI-Dcp2F: 5'-AGGCCTGGATCCATGGAGACCAACCGGTGGA GATTCC-3' and pMal-c-SalI-DCP2 R 5'-GCTTGGGATATCGTCTGACTCAAAG GTCCAAGATTTTCAT-3' were used to PCR amplify a 1,200-bp fragment, inserted into the pH902 vector, expressed in *E. coli* strain and purified over an amylose-agarose affinity matrix.

The MTOR kinase assay of ref. 70 was used to test for *in vitro* phosphorylation of DCP2 recombinant protein. Briefly, MTOR was immunoprecipitated from CHAPS-solubilized HeLa cells using an anti-MTOR antibody (Abcam), washed, then incubated with 100 ng of DCP2-MBP and 250 μ M ATP in kinase buffer containing 10 mM MgCl₂ at 30 °C for 20 min and phosphorylated product was detected by western blot using the anti-DCP2-pSer249 antibody described above and an anti-rabbit HRP antibody and developed using the manufacturer's directions.

Analysis of LC3 puncta in mammalian cells. Cells were grown on Lab-Tek II Chamber Slide System slides. After experimentation, cells were fixed using formalin, permeabilized using 0.5% BSA, 0.2% saponin in a 1 \times PBS solution, and blocked with 0.5% BSA, in a 1 \times PBS solution. Samples were probed with rabbit anti-LC3B antibody (LI0382, Invitrogen) and a fluorescence conjugated secondary anti-rabbit antibody was then used for visualization. DAPI was used for nuclear counterstaining. LC3 puncta in cells were observed using a Leica EL6000 microscope and analysed using Leica software. A minimum of 100 cells were examined for the presence of LC3 puncta in each condition of the individual experiments.

Analysis of inflammasome regulation in a patient with a PIK3CD/p110 δ mutation. For western blots of p-DCP2 and SQSTM1 2 \times 10⁶ PBMCs ml⁻¹ in 3 ml of RPMI from both the control and the patient were loaded into 6-well plates in the presence or absence of 200 nM rapamycin overnight at 37 °C with 5% CO₂. Cells were collected the following morning and lysed for western blot analysis. About 25 μ g of total protein was loaded into each well of a 4–20% SDS-PAGE gel. Following gel electrophoresis, proteins were transferred to a nitrocellulose membrane and probed with either primary antibodies or both primary and secondary antibodies and developed according to the manufacturer's directions (GenScript). For autophagy body visualization 5 \times 10⁴ PBMC cells in 800 μ l of RPMI from an age-matched healthy volunteer or the patient were loaded into each well in an 8-chamber glass slide. Cells were incubated at 37 °C with 5% CO₂ for 1 h for monocyte attachment followed by removal of the supernatant fraction and replacement with fresh RPMI in the presence or absence of 200 nM rapamycin. Cells were incubated at 37 °C with 5% CO₂ overnight. The following morning, cells were washed with PBS and fixed in 3% formalin for 15 min and permeabilized with permeabilization buffer (eBioscience, 00-5523-00) for 15 min at room temperature. After blocking with 10% anti-goat serum in 5% BSA for 2 h at room temperature, cells were probed with rabbit anti-human LC3 primary antibodies and Alexa Flour 488-labelled goat anti-rabbit secondary antibodies. Following staining, cells were mounted with ProLong antifade

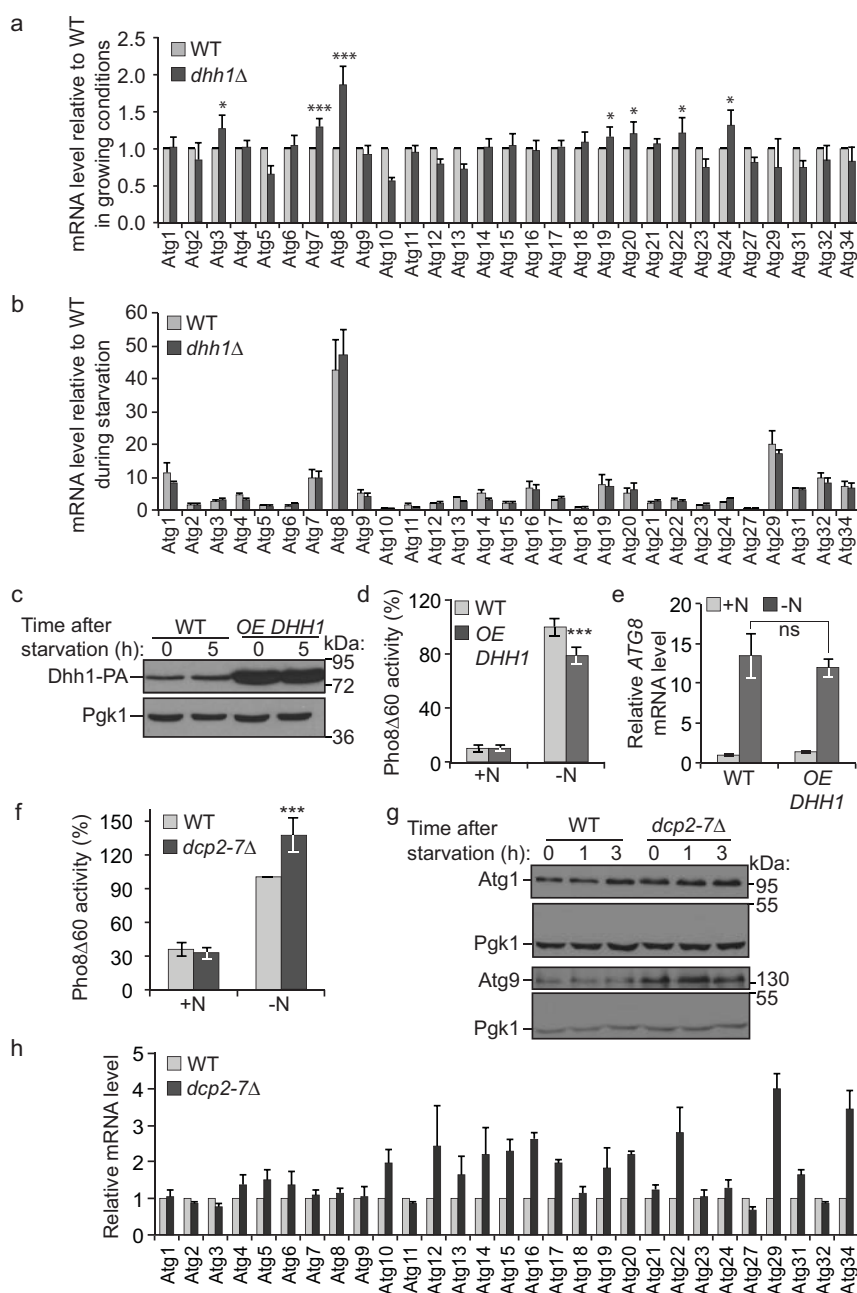
medium and observed under a Leica confocal microscope. For the IL1B assay, 70,000 cells in RPMI medium were loaded into each well of a 96-well plate. Cells were treated either with 200 ng ml⁻¹ rapamycin or PBS control for 16 h in a 37°C incubator with 5% CO₂. Triplicates were made for each condition. The next day, cells and media mixtures were transferred to microcentrifuge tubes and supernatant fractions collected by centrifugation at 5,000g for 5 min. The amount of IL1B cytokines in each supernatant fraction was measured according to the instructions of a human IL1B Elisa kit (eBioscience, 88-7010-88).

Patient informed consent and Institutional Review Board (IRB) approval. The National Institute of Allergy and Infectious Diseases (NIAID) IRB approved the study under NIAID Protocol no. 93-I-0119. All subjects provided written informed consent directly.

Statistics. In the fungal studies, differences in autophagic body formation were determined by χ^2 testing using GraphPad Prism 6. Transcriptional differences were compared in at least 3 independent experiments using unpaired Student's *t*-test using GraphPad Prism 6. Slopes of *ATG8* degradation were determined by differences in slopes by *t*-test using N-2 degrees of freedom and GraphPad Prism 5. In the mammalian studies statistical significance in transcript levels, band density of PCR products from capped RNA analysis assay, and LC3 puncta was determined by unpaired *t*-tests of at least 3 independent experiments using GraphPad Prism 6. Differences with a *P* value < 0.05 were considered significant.

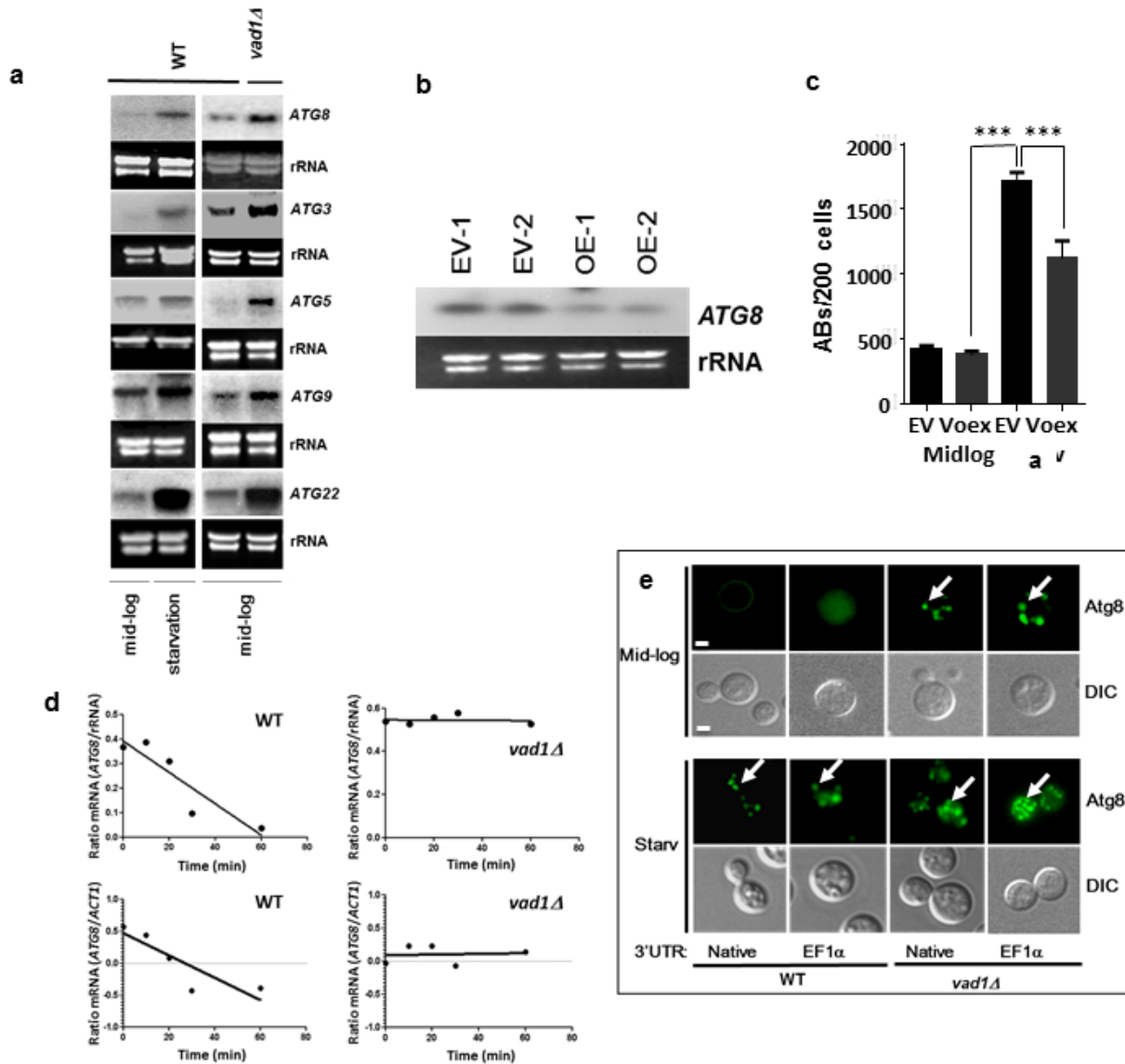
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DOI: 10.1038/ncb3189



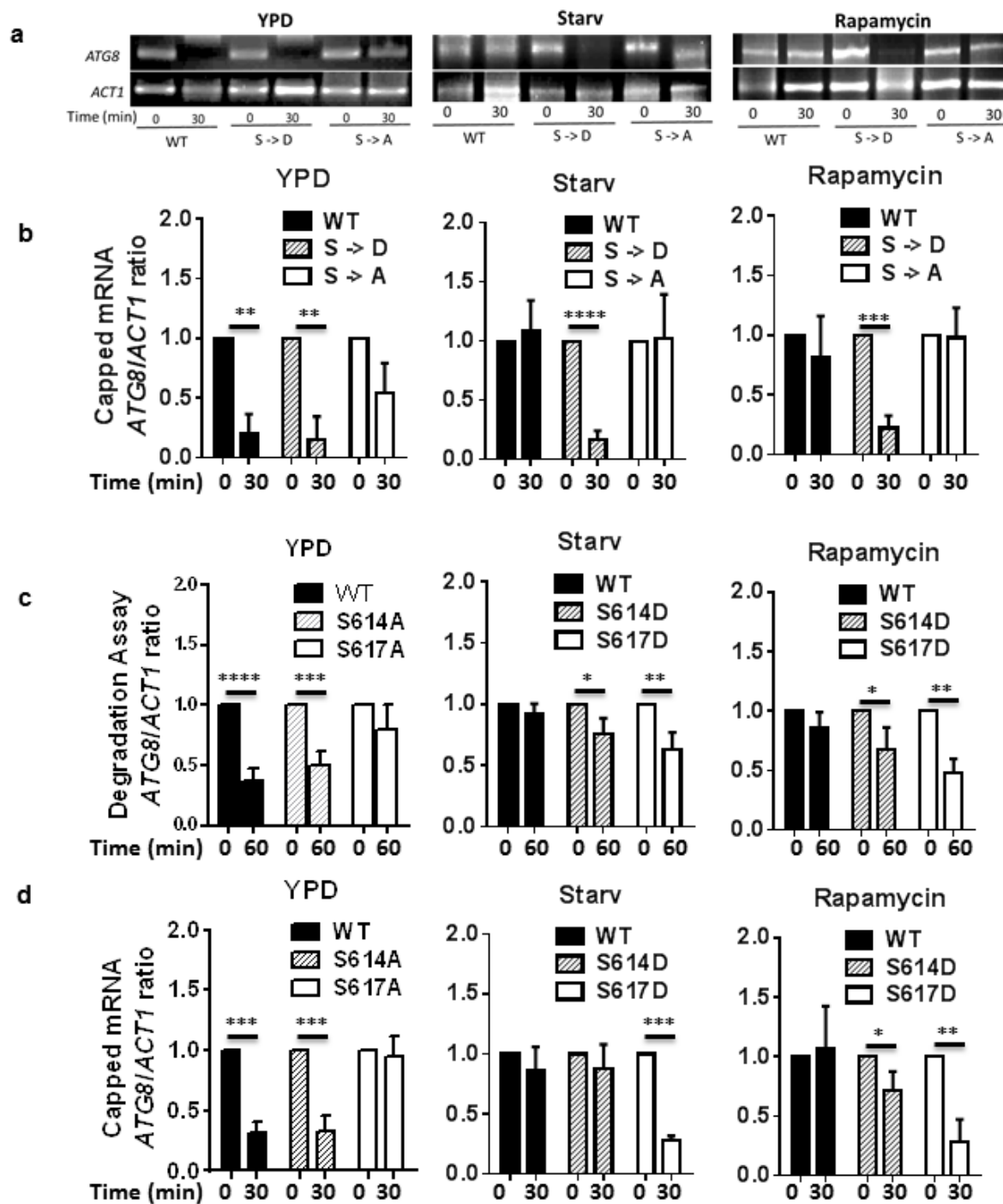
Supplementary Figure 1 RCK Member Dhh1 is a Post-transcriptional Repressor of Autophagy in Yeast, (a-b) Dhh1 represses the expression of *ATG* genes in nutrient-replete conditions. Wild-type (WT; WLY176) and *dhh1Δ* cells (YAB269) were grown in rich medium (a), then shifted to nitrogen starvation for 1 h (b). Total RNA was extracted and analyzed as in Figure 1a. Error bars: standard deviation (SD). (a) n=5 independent experiments except n=3 for *ATG9*, 32,34; n=4 for *ATG2* and n=6 for n=5 for *ATG1*, 7,8. (b) n=3 independent experiments except n=2 for *ATG4* in WT cells. Student's t-test, * p < 0.05, *** P < 0.001. (c-e) Mild overexpression of *DHH1* modestly represses autophagy. WT (YAB281) and OE *DHH1* cells (YAB282) were grown in YPGal medium (+N) until mid-log phase, then starved for nitrogen (-N) for 5 h (c,d) or 1 h (e). (c) Protein extracts were analyzed by western blot. (d) Pho8Δ60 activity was normalized to that of WT cells (set to 100%). Error bars: SD, n=4 independent experiments. Student's t-test, ** p < 0.01. (e) Total RNA was extracted and the

mRNA levels were quantified by RT-qPCR. The mRNA level of *ATG8* was normalized to that in WT cells in rich conditions (set to 1). Error bars: SD, n=4 independent experiments. Student's t-test, NS > 0.05. (f) Dcp2 represses autophagy. WT (YTS158) and *dcp2-7Δ* cells were grown in YPD at 24°C until early log-phase, then shifted to 38.5°C for 1 h and starved for 3 h at the same temperature. Pho8Δ60 activity was normalized to that of WT cells (set to 100%). Error bars: SD, n=5 independent experiments. Student's t-test, *** p < 0.001. (g) Dcp2 represses the expression of *Atg1* and *Atg9*. WT and *dcp2-7Δ* cells were grown in rich medium until early-log phase. Cells were shifted to 38.5°C for 1 h then starved. Protein extracts were analyzed by western blot. (h) WT (BY4742) and *dcp2-7Δ* cells were grown in rich medium until early log phase. Cells were then shifted to 38.5°C for 1 h then starved for 1 h. The mRNA level of individual *ATG* genes was normalized to the mRNA level of the corresponding gene in WT cells (set to 1). Error bars: SD, n=3 independent experiments.



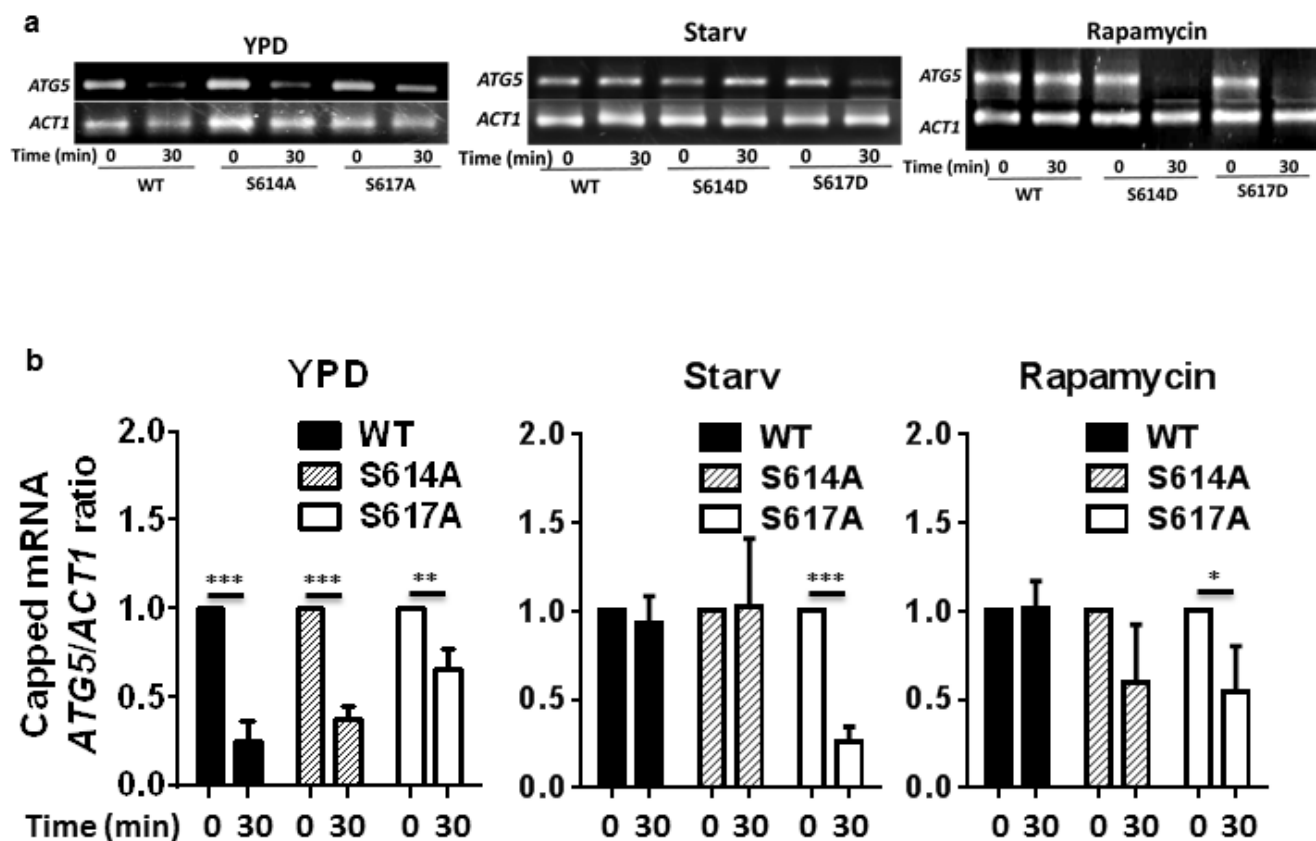
Supplementary Figure 2 The RCK Fungal Homolog Vad1 Plays a Role in Decapping and Degradation of Autophagy-related Transcripts (a) Northern blots of RNA from the indicated strains were hybridized with fragments of the indicated genes. (b) Wild-type fungal cells overexpressing *VAD1* from an *ACT1* promoter (OE-1, 2) or containing empty vector alone (EV-1, 2) were induced for autophagy by starvation for 1 h followed by northern blot analysis. (c) The indicated strains were observed by DIC for the presence of autophagic bodies (ABs) and quantified in n=3 independent experiments of 200 cells each by DIC +/- SD. Student's t-test, *** p < 0.001. (d) RNA from WT (left

panel) or *vad1Δ* mutant mid-log phase strains (right panel) was subjected to either northern blot (hybridized with a fragment of *ATG8*) and ratios of signal by densitometry to rRNA plotted over time (top panels) or *ATG8* was assayed by quantitative RT-PCR normalized to actin (bottom panels) slope: WT vs *vad1Δ* p < 0.001. (e) The indicated strains expressing a GFP-Atg8 fusion protein containing either the native or heterologous 3'UTR (EF1a) under the indicated conditions were incubated with phenylmethanesulfonyl fluoride for 30 min and observed by DIC microscopy for the presence of autophagic bodies (arrows). Scale bar = 2 μm. Representative image from n=50 cells.



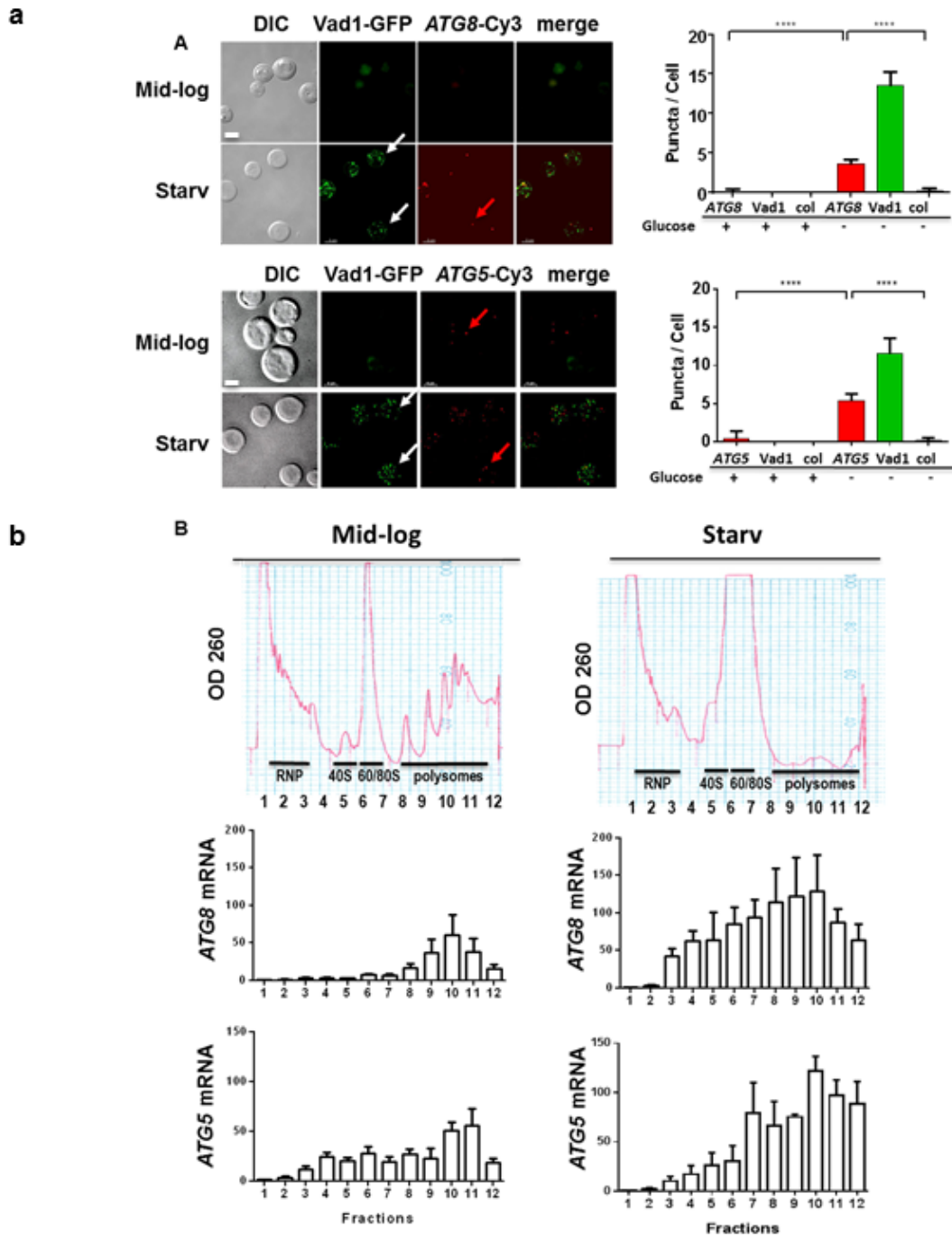
Supplementary Figure 3 Phosphorylation of TOR-Dependent Sites S614 and S617 Mediates Decapping and Degradation of *ATG8* Transcripts, Related to Figure 4. (a) Yeast cells under the indicated conditions expressing either WT Dcp2 or an equivalent protein containing either an S->D mutation or S->A mutation at positions S614 and S617 were assayed for decapping of *ATG8* transcripts over 30 min by a PCR assay and visualized by ethidium bromide as in Figure 3b. (b) Decapping assay: Densitometry of n=3

independent assays Dcp2 containing an S->D mutation or S->A mutation at a single position S614 or S617 were assayed for degradation as in Figure 4b. Degradation (c) and Decapping (d) assays conducted on yeast cells expressing WT or Dcp2 with single mutations; decapping assay conducted as in Figure S3a and B. All experiments the results of n = 3 independent assays +/- SD. Student's t-test, * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.



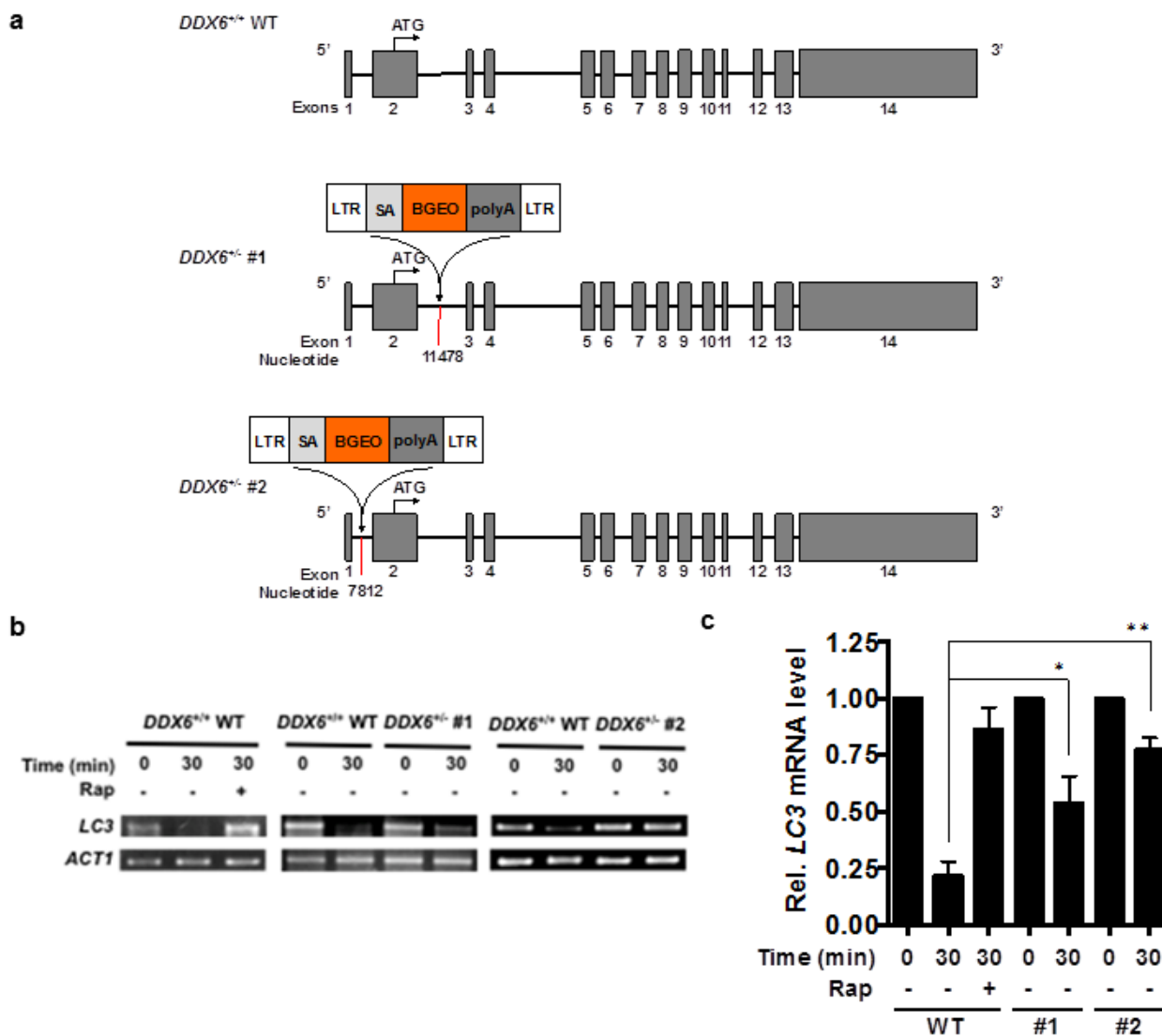
Supplementary Figure 4 Phosphorylation of TOR-Dependent Sites S614 and S617 Mediate Decapping of *ATG5* Transcripts, Related to Figure 4. (a) Yeast cells under the indicated conditions expressing either WT Dcp2 or an equivalent protein containing either the S → D mutation or S → A mutation at

positions S614 and S617 were assayed for decapping of *ATG5* transcripts over 30 min by a PCR assay and visualized by ethidium bromide as in Figure 3B. (b) Densitometry of n=3 independent decapping assays performed as in Figure S3a. Bars +/- SD; Student's t-test, * p < 0.05, ** p < 0.01, *** p < 0.001.



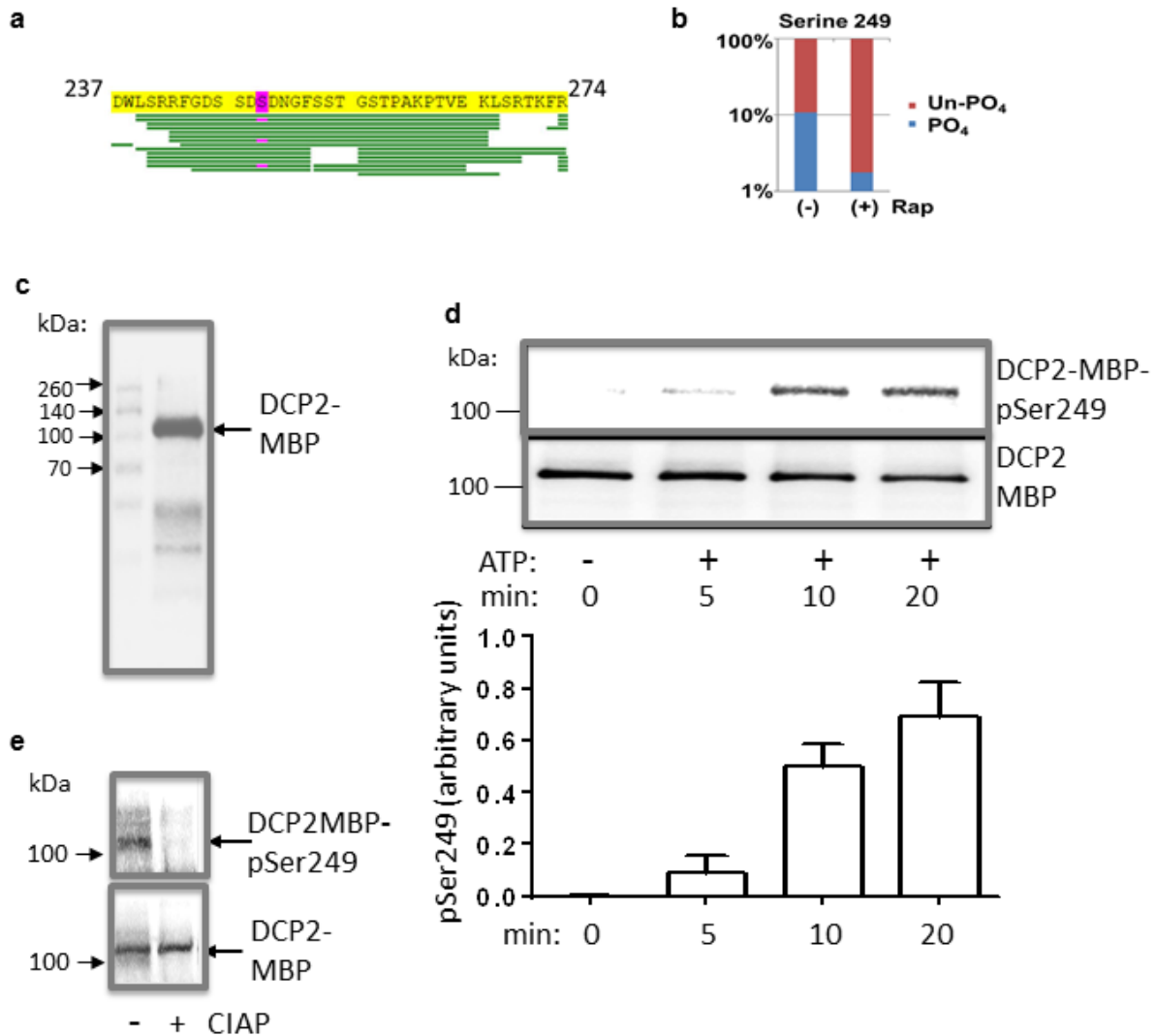
Supplementary Figure 5 *ATG8* and *ATG5* Transcripts Demonstrate Robust Translational Efficiency and Induction under Starvation Conditions, related to Figure 2 and 4. (a) Left panels: Indicated gene transcripts were localized using multiple Cy3-labeled oligonucleotide probes by single mRNA-sensitivity FISH (*ATG8*-Cy3, *ATG5*-Cy3) and P-bodies localized by a Vad1-GFP fusion protein. White arrows correspond to P-bodies, red arrows to the indicated transcripts. Right panels: Quantification of mid-log (Glucose +) or starvation conditions (Glucose -) of the indicated fluorescent transcript

puncta, Vad1-GFP labeled P-bodies (Vad1) and co-localized puncta on deconvolved images of 20 cells. Student's t-test, **** indicates $p < 0.0001$. Scale bar = 4 μ m. (b) Sucrose gradient sedimentation polysome profiles of extracts from *C. neoformans* cells under mid-log or starvation conditions. Results of polysome profile quantification of the indicated mRNA are shown below the corresponding sucrose fractions. Corresponding ribonuclear protein (RNP) 40S, 60/80S and polysome fractions as indicated. Error bars indicate the standard deviation of $n=3$ independent experiments +/- SD.



Supplementary Figure 6 The RCK Mammalian Homolog DDX6 is a Suppressor of Autophagy in Primary Embryonic Stem Cells, Related to Figure 5. (a) Schematic representation of *DDX6* WT and two independent *DDX6* gene trap insertion clones. (b) Decapping assay: The presence of capped *LC3* transcripts was assayed in *DDX6* WT, *DDX6* clone #1 (*DDX6*^{+/+} #1), and

DDX6 clone #2 (*DDX6*^{+/+} #2) by the method of Fig. 3 at the indicated times after transcriptional suppression. (c) Degradation assay: Quantification of *LC3* transcripts of the indicated cells after transcriptional suppression in n=3 independent experiments normalized to each respective time zero +/-SD. Student's t-test, * P < 0.05; ** p < 0.01.



Supplementary Figure 7 The Mammalian Dcp2 Homolog Is Phosphorylated by MTOR, Related to Figure 5. (a) Schematic of the identification of phosphorylated Ser249 of DCP2 by mass spectrometry. The peptides are represented by green lines and the phosphorylation site is indicated in purple. (b) Differential phosphorylation of DCP2 after treatment with rapamycin assayed by targeted ion mass spectrometry. (c) SDS-PAGE of recombinant DCP2-MBP fusion protein purified by amylose-agarose affinity chromatography. (d) Ex vivo phosphorylation of DCP2 by MTOR. MTOR

was immunoprecipitated from HeLa cells using an anti-MTOR antibody, washed, then incubated with 100 ng DCP2-MBP at 30°C for the indicated times, and subjected to western blot using a rabbit DCP2-pSer249 affinity-purified antibody. Lower panel, density quantification of pSer249 in n=3 independent experiments. Bar +/- SD. (e) DCP2-MBP phosphorylated with MTOR for 20 min as in in Fig. S3d, then subjected to treatment (+) or no treatment (-) with calf intestinal alkaline phosphatase (CIAP) prior to western blot using antigen-purified anti-DCP2-pSer249 antibody.

Name	Genotype
BY4742	<i>MATα his3Δ1 leu2Δ0 ura3Δ0</i>
<i>dcp2-7Δ</i>	BY4742, <i>dcp2-7Δ::KanMX6</i>
SEY6210	<i>MATα his3Δ200 leu2-3,112 lys2-801 suc2-Δ9 trp1Δ901 ura3-52</i>
WLY176	SEY6210 <i>pho13Δ pho8::pho8Δ60</i>
YAB269	SEY6210 <i>pho13Δ pho8::pho8Δ60 dhh1Δ::HIS5</i>
YAB278	<i>dcp2-7Δ, pho13Δ::LEU2 pho8::pho8Δ60::HIS3</i>
YAB281	WLY176, <i>DHH1-PA::HIS5</i>
YAB282	YAB281, <i>pGAL-DHH1::TRP1</i>
YTS158	BY4742, <i>pho13Δ::KanMX6 pho8::pho8Δ60</i>

Supplementary Table 1 *S. cerevisiae* strains used in this study.

SUPPLEMENTARY INFORMATION

Gene name	Forward primers (5'-3')	Purpose
ALG9 F	CACGGATAGTGGCTTTGGTGAACAATTAC	qPCR <i>S. cerevisiae</i>
ALG9 R	TATGATTATCTGGCAGCAGGAAAGAACTTGGG	qPCR <i>S. cerevisiae</i>
ATG1 F	ATCTAAGATGGCCGCACATATG	qPCR <i>S. cerevisiae</i>
ATG1 R	AGGGTAGTCACCATAGGCATTG	qPCR <i>S. cerevisiae</i>
ATG2 F	CCCACGGGTTTGAAGGAGGC	qPCR <i>S. cerevisiae</i>
ATG2 R	CGGCCCTCCCGTTTATCATC	qPCR <i>S. cerevisiae</i>
ATG3 F	TCTGTTTCCATTATCCATG	qPCR <i>S. cerevisiae</i>
ATG3 R	TGGTCTACCCGTAACGAATC	qPCR <i>S. cerevisiae</i>
ATG4 F	GGTGCAATATCCCACACACAAC	qPCR <i>S. cerevisiae</i>
ATG4 R	CTCTACAAGGACGCCTTCTAC	qPCR <i>S. cerevisiae</i>
ATG5 F	TCGGTCAACGAAGCTCGAAA	qPCR <i>S. cerevisiae</i>
ATG5 R	GATGAGCGGTATATGTCGCG	qPCR <i>S. cerevisiae</i>
ATG6 F	TCACTCGGCAGCTTCTACG	qPCR <i>S. cerevisiae</i>
ATG6 R	GGGTAGCTCGAGAATGGAAG	qPCR <i>S. cerevisiae</i>
ATG7 F	ATGAGCATTGTCCAGCATGTAG	qPCR <i>S. cerevisiae</i>
ATG7 R	GACCTCCTGCTTATGACTGAC	qPCR <i>S. cerevisiae</i>
ATG8 F	GAAGGCCATCTTCATTTTTGTC	qPCR <i>S. cerevisiae</i>
ATG8 R	TTCTCTGAGTAAGTGACATAC	qPCR <i>S. cerevisiae</i>
ATG9 F	CGTACTAACAGAGTCTTTCCTTG	qPCR <i>S. cerevisiae</i>
ATG9 R	CTAAGACACCACCTTATTGAG	qPCR <i>S. cerevisiae</i>
ATG10 F	GACCAAATTGATGCTCCCAAC	qPCR <i>S. cerevisiae</i>
ATG10 R	ACATGAACTCAGCCTGGTCAC	qPCR <i>S. cerevisiae</i>
ATG11 F	CTTTGGAACATTTGTCAGATAGCCC	qPCR <i>S. cerevisiae</i>
ATG11 R	CTCCCTGGTATGAAACCACAACCGC	qPCR <i>S. cerevisiae</i>
ATG12 F	TGGCACTTATGAACAGGAAGAG	qPCR <i>S. cerevisiae</i>
ATG12 R	AACTGCCCAATAGAACCAATG	qPCR <i>S. cerevisiae</i>
ATG13 F	CCGTTTGCTCAACCAATGGG	qPCR <i>S. cerevisiae</i>
ATG13 R	GCTTGAAAGGGCGCAGTAG	qPCR <i>S. cerevisiae</i>
ATG14 F	TACTGGACCAGTACGATGTG	qPCR <i>S. cerevisiae</i>
ATG14 R	TGCAGGATGTCCTTTGTG	qPCR <i>S. cerevisiae</i>
ATG15 F	CCAAGCAGAGACTGGGAATCC	qPCR <i>S. cerevisiae</i>
ATG15 R	CCAATTGCGGCCTACACAGG	qPCR <i>S. cerevisiae</i>
ATG16 F	GGTTGAATGACGAATTGATTAGT	qPCR <i>S. cerevisiae</i>
ATG16 R	CGCTGTTTCATGGCTTCTGTC	qPCR <i>S. cerevisiae</i>
ATG17 F	GCTCAGGACCAGGAAGAACG	qPCR <i>S. cerevisiae</i>
ATG17 R	TCGTCAATTTACCGGGCCA	qPCR <i>S. cerevisiae</i>
ATG18 F	TGGCAATCCGCAAGTACAGAGTCC	qPCR <i>S. cerevisiae</i>
ATG18 R	CGCCTCTCTCCGGTCCATAAC	qPCR <i>S. cerevisiae</i>
ATG19 F	TTAGAGGCCGGCTCCACTAT	qPCR <i>S. cerevisiae</i>
ATG19 R	GGCTCTTGGGATGCCTGTAA	qPCR <i>S. cerevisiae</i>
ATG20 F	CAGGTAGCGGTGGGAAATCA	qPCR <i>S. cerevisiae</i>
ATG20 R	TGCGGTATGCGGATCTGTTT	qPCR <i>S. cerevisiae</i>
ATG21 F	ACCAAACGAGAACCTGAGCC	qPCR <i>S. cerevisiae</i>
ATG21 R	GAGGCAATATAGACTTGGTTGGG	qPCR <i>S. cerevisiae</i>
ATG22 F	GCGGTTTTGTTCTCCAAGGC	qPCR <i>S. cerevisiae</i>
ATG22 R	GTCCACCGAAACTTTGTGGC	qPCR <i>S. cerevisiae</i>
ATG23 F	AGATGGGTCAAGCGCACTACC	qPCR <i>S. cerevisiae</i>
ATG23 R	CGCGTTTCGACCCTTATGGC	qPCR <i>S. cerevisiae</i>
ATG24 F	CGCTAAAAGGTGGCGGATG	qPCR <i>S. cerevisiae</i>
ATG24 R	ATGATGGTCCGCTAGTGAGC	qPCR <i>S. cerevisiae</i>
ATG27 F	GGCCATCTGGCTGTTTGAAG	qPCR <i>S. cerevisiae</i>
ATG27 R	TAGTGGCTTTTTCCCTCG	qPCR <i>S. cerevisiae</i>
ATG29 F	ATGAGGCGTTACAACATTTGC	qPCR <i>S. cerevisiae</i>
ATG29 R	TCGTATCTGAACCTACCGCAC	qPCR <i>S. cerevisiae</i>
ATG31 F	TCAAAGAGCACGACGGAAG	qPCR <i>S. cerevisiae</i>
ATG31 R	GCTGTCAAAGCAGCATCACT	qPCR <i>S. cerevisiae</i>
ATG32 F	GGGCAAAATGAATACTTTGTCTTGCATGC	qPCR <i>S. cerevisiae</i>
ATG32 R	CCCAGTGCCAAAATCCGATTAGATTCATC	qPCR <i>S. cerevisiae</i>
ATG34 F	AGCCTCATTTTCGACAGGCA	qPCR <i>S. cerevisiae</i>
ATG34 R	CTCGCCTAGAAAGGGAGTGC	qPCR <i>S. cerevisiae</i>
TAF10 F	ATATCCAGGATCAGGTCTTCCGTAGC	qPCR <i>S. cerevisiae</i>
TAR10 R	GTAGTCTTCTCATTCTGTTGATGTTGTTGTTG	qPCR <i>S. cerevisiae</i>
TFC1 F	GCTGGCACTCATATCTTATCGTTTCAATGG	qPCR <i>S. cerevisiae</i>
TFC1 R	GAACCTGCTGTCAATACCGCCTGGAG	qPCR <i>S. cerevisiae</i>

Supplementary Table 2 qPCR Primers used in this study.

Name	Genotype
H99	<i>MAT</i> α
<i>pkc1</i> Δ	<i>MAT</i> α <i>pkc1::NAT</i>
<i>vad1</i> Δ	<i>MAT</i> α <i>vad1</i> Δ :: <i>URA5</i>
YP501	<i>MAT</i> α <i>vad1</i> Δ <i>ura5ACT1</i> _{pro} - <i>MYC</i> - <i>VAD1</i> - <i>MYC</i> - <i>EF1</i> α _{ter} <i>URA5</i>
EV-1	<i>MAT</i> α <i>ura5</i> ::pKUTAP empty vector (<i>URA5</i>)
EV-2	<i>MAT</i> α <i>ura5</i> ::pKUTAP empty vector (<i>URA5</i>)
ATG8-Nat	<i>MAT</i> α <i>ura5</i> :: pKUT- <i>ATG8</i> _{pro} -GFPcneo- <i>ATG8</i> _{cod} - <i>ATG8</i> _{term} (<i>URA5</i>)
ATG8-EF1	<i>MAT</i> α <i>ura5</i> :: pKUT- <i>ATG8</i> _{pro} -GFPcneo- <i>ATG8</i> _{cod} - <i>EF1</i> α _{term} (<i>URA5</i>)
Voex-1	<i>MAT</i> α <i>ura5</i> :: pKUT- <i>ACT1</i> _{pro} - <i>ATG8</i> (<i>URA5</i>)
Voex-2	<i>MAT</i> α <i>ura5</i> ::pKUT- <i>ACT1</i> _{pro} - <i>ATG8</i> (<i>URA5</i>)
<i>atg8</i> Δ	<i>MAT</i> α <i>atg8</i> Δ :: <i>URA5</i>
Vad1-GFP	<i>MAT</i> α <i>ura5</i> ::pKUT- <i>VAD1</i> -GFPcneo (<i>URA5</i>)

Supplementary Table 3 *C. neoformans* yeast strains used in this study¹.

SUPPLEMENTARY INFORMATION

Name	Sequence (5'-3')	Restriction enzyme	Purpose
ATG8-S-cDNA	ATCCGTCCGAAGCAAGGATG		ATG8 probe
ATG8-A-cDNA	ATTCTTACTCGGAGATGGCGT		ATG8 probe
ATG5-381-S	CTCCGCAAGACCGTTTGTTA		ATG5 probe
ATG5-804-A	AGAAGTGGTAGATGGGCTG		ATG5 probe
ATG22-S	CCGTCTTTATTCGTGGAACAC		ATG22 probe
ATG22-A	CGTAATGTGAGGTCGGATGA		ATG22 probe
ATG3-1-S	CTACGCCAACTTCCGTTATG		ATG3 probe
ATG3-1160-A	CGTAGTACTGTCCACTTCGA		ATG3 probe
ATG9-11-S	TTGTATCGTATTAGCGCGCG		ATG9 probe
ATG9-2561-A	CTGCTGCAATAAACCCACCA		ATG9 probe
ATG8-574-S	GGATAGGATCCCGTGATCTG		ATG8 qPCR
ATG8-855-A	TGATCCGTTTCTGTGACATA		ATG8 qPCR
ACT1-S	ATGGAAGAAGAAGTCGCCGC		ACT1 qPCR
ACT1-A	TCGTCGTATTCGCTCTTCGC		ACT1 qPCR
CNAG-2823-S	ACCAACCCAATCACGTCTTCA		ribo prot S17n probe
CNAG-2823-A	CACATTTCTCTCGGCAACCT		ribo prot S17n probe
CNAG-4664-S	AAGACCCCTCACGACATTA		ribo prot I37 probe
CNAG-4664-A	CTATGCCGTTGACTTCAAGTA		ribo prot I37 probe
RIB-1-S	ATGCACCCACAGCCCTC		ribo prot S19 probe
RIB-247-A	TCCAAGGCCTCCTTGTAGG		ribo prot S19 probe
tRNA-377-S	AGAAAGTGGAGATCAGGCGA		tRNA meth transf probe
tRNA-842-A	AATCTCGTCTTCGGACAAGC		tRNA meth transf probe
4664-RNA-S	CCCGGAAGGGCTGTTGTTT		ribo prot I37 qPCR-RNA
4664-RNA-A	TTGTGCGATTTAAGTTTTCAACTT		ribo prot I37 qPCR-RNA
4664-DNA-S	AGTGTTCGAAAGTACGATCTT		ribo prot I37 qPCR-RNA
4664-DNA-A	CTGCCAGTTGTCATCGATCA		ribo prot I37 qPCR-RNA
2838-RNA-S	ACTGTCACTGTTGAACGAATAT		ribo prot S17 qPCR-RNA
2838-RNA-A	TGGGTGGGTATAGGGTCGT		ribo prot S17 qPCR-RNA
2838-DNA-S	AATGTCCCAGTTCAAAGATTTAA		ribo prot S17 qPCR-RNA
2838-DNA-A	TGGGTGGGTATAGGGTCGT		ribo prot S17 qPCR-RNA
VAD1-S-MunI	GGCGGCAATTGTTCTTCCCTCAACGTTAGCGAA	MunI	Voex
VAD1-A-MunI	GGCCCAATTGTTAGGCTTGAGCCTGTTGG	MunI	Voex
ATG8-PRO-BglII	ATATAAGATCTTAAAGTTCTCCTTCTTCGACCAATTAGG TA	BglII	ATG8-Nat, ATG8-EF1
ATG8-PRO-NdePstR	TATATAGATCTCATATGCTGCAGGGTGTCTACGAAATAG GTGGGATGTGATAGTTGCACGT	NdeI, PstI	ATG8-Nat, ATG8-EF1
GFPmyc-PstL	TTATACTGCAGATGTCCAAGGGTGAGGAGCTTTCAC CGGT	PstI	ATG8-Nat, ATG8-EF1
GFPmyc-PstR	TTATACTGCAGGAGTCTCCTCGGAGATGAGCTTCT GCTC	PstI	ATG8-Nat, ATG8-EF1
ATG8ORF-NdeL	TATATCATATGATGGTCCGAAGCAAGTTTAAAGGATGAG CAC	NdeI	ATG8-Nat, ATG8-EF1
ATG8ORF-NdeR	TATATCATATGTTACTCGGAGATGGCGTATTGTTCAAGG TC	NdeI	ATG8-Nat, ATG8-EF1
ATG8ORF-Ter-NdeR	TATATCATATGTCTGTTCCGACTTGGCCGGTCTGTGCG CA	NdeI	ATG8-Nat, ATG8-EF1
ATG8-62-S-XbaI	GCCGGCTCTAGATGTTGCCACCGAGTGA	XbaI	Δatg8
ATG8-572-A-R1	CCACGAATTCGCTTGTCTACCCACAG	EcoRI	Δatg8
ATG8-1541-S-BglII	GCCGCCAGATCTTTGATATGATAGAGGAAGC	BglII	Δatg8
ATG8-1866-A-XhoI	GCCGCCCTCGAGACTTGGTGTGATGAGCA	XhoI	Δatg8
URA5-S-R1	CCGGAATCCGGATGGTATTGAAGACGA	EcoRI	Δatg8
URA5-A-BglII	GAAGATCTTCGGACGCCTGCTGTAC	BglII	Δatg8

Supplementary Table 4 Primers for *C. neoformans* yeast studies.

NAME	PROBE REGION	SEQUENCE	LENGTH
ATG5 1-1R	29-47BP	ACGACCTTGCCCTTGACGT	19
ATG5 2-1R	48-64BP	CACCGCCGGTGACAAGG	17
ATG5 3-1R	65-83BP	GCTTTGCCGAGACCTGTGC	19
ATG5 4-1R	84-104BP	TGGACGAACCCGAGGAGATA	21
ATG5 5-1R	105-126BP	GATGTATACTTTGGCGCCATT	22
ATG5 6-1R	127-145BP	CCTGTTGACGGCCTCCTGT	19
ATG5 7-1R	146-167BP	TCCTTGGCAGCTTCTCAAGTA	22
ATG5 8-1R	168-187BP	GGATGATATCCCGCCAATT	20
ATG5 9-1R	188-211BP	TGGTAGAACATCCCCTTGAATAG	24
ATG5 10-1R	212-234BP	CGCAATAGCCTTACATCCTTCT	23
ATG5 11-1R	235-257BP	GACTCCTTGGCAGATAAAGCATC	23
ATG5 12-1R	258-277BP	TGATGAGGGCATCGACTTG	20
ATG5 13-1R	278-296BP	CGCATCACACCGCACAGT	19
ATG5 14-1R	297-322BP	GATCCTTGATAGTGTGTTTATAGGCT	26
ATG5 15-1R	323-343BP	CAACTTCGTCGGGTATTGT	21
ATG5 16-1R	344-364BP	GGCCTTCCCAAGAGCTTCT	21
ATG5 17-1R	365-389BP	GAATAGTTGAAGTCATCATGTCGT	25
ATG5 18-1R	390-410BP	CCATTGATGTTGATGGCGTTG	21
ATG5 19-1R	411-434BP	AAAGCACAGGTCATAAAGTAAGGG	24
ATG5 20-1R	435-456BP	GGACTTCCCAAAGAGGGATC	22
ATG5 21-1R	457-474BP	GACGACGCTGCGGAGGTC	18
ATG5 22-1R	475-493BP	CGGCGATGGAGGCGATAAT	19
ATG5 23-1R	494-510BP	TCGCTGTTGGCCAAGC	17
ATG5 24-1R	511-531BP	GTAAGAAACACTTCCCGTCGC	21
ATG5 25-1R	532-551BP	GCAGCAGCCTTGAGACACC	20
ATG5 26-1R	552-573BP	GAGGAGTTTACCAGGTGGATG	22
ATG5 27-1R	574-589BP	GGTGAAGACGGCCGGC	16
ATG5 28-1R	590-612BP	GGTGTTCACCTGAACTTCAAGG	23
ATG5 29-1R	613-634BP	AGGGGAAGATACAGGACAAAT	22
ATG5 30-1R	635-656BP	TCGTTCTTCCAGTCATCTCAG	22
ATG5 31-1R	657-679BP	CATATTCAGACCTTGACCAGCA	23
ATG5 32-1R	680-702BP	TTTGGTAGGGATTCTCCGATAT	23
ATG5 33-1R	703-722BP	ACGGTTGATCTTGGCAGC	20
ATG5 34-1R	723-739BP	GCAAACCGGACGACCGC	17
ATG5 35-1R	740-758BP	GGGCCGACAATCTCTCCG	19
ATG5 36-1R	759-781BP	CTTTACTGAAAGCAAGAGCACCA	23
ATG5 37-1R	782-801BP	TCGCTCAAAGTATCCTCCCG	20
ATG5 38-1R	802-821BP	CCGTCCACAGTAAGCATCGC	20
ATG5 39-1R	822-840BP	GGCACCATCAACCTTCCA	19
ATG5 40-1R	841-861BP	TCGGAGACCATCATGGATACC	21
ATG5 41-1R	862-884BP	CAAAGATACGATCCTCAGGCCAA	23
ATG8 1-1R	23-42BP	CTTGTGCAAGGGGTGCTCAT	20
ATG8 2-1R	43-58BP	CCGCTTCGGCCTTTCG	16
ATG8 3-1R	59-82BP	CCTGATACTTTTGCTGATCCTCT	24
ATG8 4-1R	83-105BP	CTCACAGATCACGGGAATCCTAT	23
ATG8 5-1R	106-128BP	GGGATACACTTCTTCAGCCTT	23
ATG8 6-1R	129-154BP	CGAGATACTTCTTATCAATCGTT	26
ATG8 7-1R	155-176BP	CCAACAGTAAGATCGGTGGTA	22
ATG8 8-1R	177-199BP	TCCTGATGACATAGACGAATTGC	23
ATG8 9-1R	200-217BP	GCGCGAGCTTGATCCGTT	18
ATG8 10-1R	218-240BP	AAAGATGAAAATGGCCTTTTCAG	23
ATG8 11-1R	241-264BP	TGTTGGAGGAAGGATATCATCAAC	24
ATG8 12-1R	265-286BP	AAATTGAACTCATAAGTGCCGC	22
ATG8 13-1R	287-308BP	TCTTCGCTTGTGCTCATCAT	22
ATG8 14-1R	309-332BP	GCATAGAGACGTAAGGAAACCA	24
ATG8 15-1R	333-353BP	TCGCCAAAGGTGTTCTCAGAA	21
ATG8 16-1R	354-375BP	GGAGATGGCGTATTGTTCAAGG	22
ATG8 17-1R	376-397BP	CGTACTGCCTGGTGTACTC	22
ATG8 18-1R	398-420BP	GATGCCGAATCAACAGTATTAT	23
ATG8 19-1R	421-444BP	AGTTTCTCAAAAAACAGATCCAA	24
ATG8 20-1R	445-466BP	CCCACAACGTTTGCAAAAAAC	22
ATG8 21-1R	467-489BP	AGTTGCTTGTAAAAAACGCT	23
ATG8 22-1R	490-509BP	TCGCCACCCGAGTTAAACAT	20
ATG8 23-1R	510-526BP	CCACCTTCTCCGCCCT	17
ATG8 24-1R	527-546BP	TTGCTACCGCCTTCCAAAAT	20
ATG8 25-1R	547-562BP	CCAGCCTCGGCTCCCA	16
ATG8 26-1R	563-584BP	TTAAGCAGTTTCTCCATTGA	22
ATG8 27-1R	585-605BP	GAAGCTCGTACTAGCCGATC	21
ATG8 28-1R	606-633BP	CCATTCAAATAATACAGATCATAACA	28
ATG8 29-1R	634-662BP	TGCTAGGGCTAGTTATATAAGTTCAAT	29
ATG8 30-1R	663-678BP	TGGACGGCCACCCAGG	16
ATG8 31-1R	679-705BP	TTCTCTATCATATAAAGATCTGAAG	27
ATG8 32-1R	706-729BP	GCAATAACTATCCACTCTTCAGC	24
ATG8 33-1R	730-754BP	AGGTCAATATTCTGTACCAAGCAAG	25

Supplementary Table 5 FISH probes used in this study.

Name	Sequence (5' to 3')	Method
Hs-Actin-S	TGAAGATCCTCACTGAGCGT	qPCR
Hs-Actin-A	ATGCTGTTGTAGGTGGTCTC	qPCR
Hs-RCK-A	ATGGGTCTGTCCAGTCAAATG	qPCR
Hs-RCK-S	GGTGGTCATACTCTGTGCTTG	qPCR
Hs-LC3-S	ATGCCGTCCGAGAAGACTTT	qPCR
Hs-LC3-A	TGTCCGTTCAACCAACAGGAA	qPCR
Mm-ACT-S	GGCTGTATCCCCTCCATCG	qPCR
Mm-ACT-A	CCAGTTGGTAACAATGCCATGT	qPCR
Mm-LC3B-S	ATAATCAGACGGCGCTTGCA	qPCR
Mm-LC3B-A	ATGTCTCCTGCGAGGCATAA	qPCR
Mm-RCK-A	TCTGTCCAGTCAGAACGGC	qPCR
Mm-RCK-S	GCCATTATTGATTGTGCTGGTGT	qPCR
Mm-RCK #1-S	CTTGAACACACAGAGATTGGA	Insert Verification
Mm-RCK #1-A	AATCCTAGTCACCACACCTG	Insert Verification
Mm-RCK #2-S	TTGGGAGCTTCGAGTCAACA	Insert Verification
Mm-RCK #2-A	TCGCACTCCCAACCTCACTC	Insert Verification
LTR-Insert-A	ATAAACCCCTCTTGCAAGTTGCA	Insert Verification
LTR-Insert-S	TCCATGCCTTGCAAATGGC	Insert Verification
pMAL-Dcp2F	AGGCCTGGATCCATGGAGACCAAACGGGTGGAGATTCC	r-Dcp2 protein
pMAL-Dcp2R	GCTTGCGATATCGTCTGACTCAAAGGTCCAAGATTTTCAT	r-Dcp2 protein

Supplementary Table 6 Primers for mammalian studies