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RESEARCH ARTICLE

The Ccl1–Kin28 kinase complex regulates autophagy under nitrogen starvation

Jing Zhu^{1,2,*}, Shuangsheng Deng^{3,*}, Puzhong Lu¹, Wenting Bu⁴, Tian Li³, Li Yu^{1,‡} and Zhiping Xie^{2,‡}

ABSTRACT

Starvation triggers global alterations in the synthesis and turnover of proteins. Under such conditions, the recycling of essential nutrients by using autophagy is indispensable for survival. By screening known kinases in the yeast genome, we newly identified a regulator of autophagy, the Ccl1–Kin28 kinase complex (the equivalent of the mammalian cyclin-H–Cdk7 complex), which is known to play key roles in RNA-polymerase-II-mediated transcription. We show that inactivation of Ccl1 caused complete block of autophagy. Interestingly, Ccl1 itself was subject to proteasomal degradation, limiting the level of autophagy during prolonged starvation. We present further evidence that the Ccl1–Kin28 complex regulates the expression of Atg29 and Atg31, which is crucial in the assembly of the Atg1 kinase complex. The identification of this previously unknown regulatory pathway sheds new light on the complex signaling network that governs autophagy activity.

KEY WORDS: Autophagy, Kinase, Regulation, Proteasome, Yeast

INTRODUCTION

Autophagy is a major intracellular degradation pathway (Mizushima and Komatsu, 2011; Mizushima et al., 2011). Instead of being directly digested in the cytoplasm, substrates of autophagy are first sequestered into double-membrane autophagosomes. Their degradation happens at a later time point after autophagosomes fuse with the endocytic and/or lysosomal compartments (in animal cells) or the vacuole (in yeast and plant cells). By adjusting the specificity and magnitude of the autophagy response, cells can adopt the same basic molecular machinery to accommodate diverse environmental challenges. Conversely, failure to achieve proper specificity or magnitude of the autophagy response leads to developmental defects, immunological disorders, tumorigenesis and neurodegeneration (Choi et al., 2013; Deretic et al., 2013; Komatsu et al., 2006; Metcalf et al., 2012; Mizushima and Komatsu, 2011).

In yeast, the formation of autophagosomes is mediated by the Atg proteins and occurs at the phagophore assembly site (PAS; also known as the pre-autophagosomal structure) (Nakatogawa et al., 2009; Reggiori and Klionsky, 2013; Xie and Klionsky, 2007). Based on our current knowledge, the Atg proteins can be further categorized into the following groups: (1) Scaffold proteins that act most upstream

in the assembly of Atg proteins at the PAS, including Atg11, Atg17, Atg29 and Atg31. (2) Those that form the Atg1 kinase complex, with Atg13 as a subunit (note that the aforementioned PAS scaffold proteins are considered subunits of this complex in certain contexts). The Atg1 complex is a major signaling hub that is responsible for the initiation of autophagosome biogenesis. (3) Those that comprise the Atg9 complex (including Atg23 and Atg27), which shuttles between the PAS and non-PAS locations in vesicles with a hypothetical role of membrane transport. (4) Those that form the phosphatidylinositol 3-kinase complex, with Atg14 as the autophagy-specific subunit. (5) The Atg2–Atg18 complex, which is involved in retrograde trafficking of the Atg9 complex. (6) Two conjugation systems that are ultimately responsible for forming Atg8–phosphatidylethanolamine (PE) conjugates on autophagosomal membranes. The Atg12–Atg5–Atg16 complex acts in an E3-ligase-like manner in the conjugation reaction. Atg8 itself functions in the membrane expansion process (Nakatogawa et al., 2007; Xie et al., 2008). Genetic evidence suggests that the assembly of Atg proteins at the PAS follows a hierarchy, with Atg8 being the most downstream (Kim et al., 2002; Suzuki et al., 2001, 2007). Mature autophagosomes carry small amounts of Atg8 on their inner membrane. Upon fusion with the vacuole and subsequent digestion of the inner vesicles, these Atg8 molecules are released into the vacuolar lumen (Huang et al., 2000; Kirisako et al., 1999; Xie et al., 2008).

As both insufficient and excess levels of autophagy are detrimental, cells must judiciously regulate the autophagy response under all circumstances. Autophagy can be tuned at the transcription (Füllgrabe et al., 2014) or post-translational levels (Wani et al., 2015). In the latter scenario, the role of several protein kinases has been well documented. TOR complex 1 (TORC1), AMPK and protein kinase A (PKA) all target the Atg1 complex (or the ULK1 complex in mammals) (Egan et al., 2011; He and Klionsky, 2009; Kamada et al., 2000, 2010; Kim et al., 2011; Stephan et al., 2009; Wong et al., 2013). AMPK also phosphorylates Beclin-1 (the counterpart of yeast Atg6), a key subunit of the phosphatidylinositol 3-kinase complex (Kim et al., 2013). In addition to targeting the Atg proteins, both AMPK and PKA can regulate the autophagy machinery indirectly through TORC1 signaling (Gwinn et al., 2008; Inoki et al., 2003; Umekawa and Klionsky, 2012; Yorimitsu et al., 2007). In recent years, the involvement of several other kinases has been also been reported (Cebollero and Reggiori, 2009; Wang et al., 2012; Yang et al., 2010). Nevertheless, it is clear that our current understanding of the autophagy regulatory network is far from complete and that additional autophagy regulators, including kinases, remain to be discovered.

RESULTS

The Ccl1–Kin28 complex is required for starvation-induced autophagy

To identify new kinase regulators of autophagy, we screened 123 mutants of known kinases or their subunits in the yeast genome,

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using either knockout or conditional alleles (Table S1). Mutant strains were transformed with a plasmid expressing GFP–Atg8. The translocation of cytosolic GFP–Atg8 into the vacuole under starvation conditions was monitored with fluorescent microscopy (Huang et al., 2000; Kirisako et al., 1999). Among all the mutants screened, we found that *ccl1-ts4* and *ctk1Δ* cells displayed the most severe defects in GFP–Atg8 translocation. Here, we focused on Ccl1 for the remaining part of this study. Ccl1 is an essential cyclin (homologue of mammalian cyclin H) (Feaver et al., 1994; Valay et al., 1996). It exists in a complex containing the Kin28 kinase and a third subunit, Tfb3 (homologues of mammalian Cdk7 and Mat1, respectively) (Feaver et al., 1997; Hsin and Manley, 2012; Jeronimo et al., 2013; Jeronimo and Robert, 2014; Keogh et al., 2002; Korsisaari and Makela, 2000; Larochelle et al., 1998; Rodriguez et al., 2000; Valay et al., 1996). At a non-permissive temperature, a significant amount of GFP was transported to the vacuole in wild-type cells after 4 h of starvation (Fig. 1A). In contrast, no translocation was observed in *ccl1-ts4* and *tfb3-ts* cells. The translocation of GFP–Atg8 was also defective in *kin28-ts* cells, albeit not as severe as that in *ccl1-ts4* cells. We further verified the autophagy defect in *ccl1-ts4*, *kin28-ts* and *tfb3-ts* cells by using the GFP–Atg8 processing assay and the Pho8Δ60 assay (Fig. 1B,C). In wild-type cells, GFP–Atg8 that has been released into the vacuole is processed by proteases into free GFP, which is relatively stable in the vacuole. Accordingly, a separate GFP band can be detected by immunoblotting (Huang et al., 2014; Shintani and Klionsky, 2004). Consistent with our microscopy observations, a substantial amount of free GFP was present in samples from wild-type cells (Fig. 1B). In contrast, only a faint GFP band was present in *kin28-ts* samples, and no band was detectable in *ccl1-ts4* and *tfb3-ts* samples. The Pho8Δ60 assay measures the autophagy-dependent activation of a cytosolic mutant zymogen (Noda and Klionsky, 2008). Compared with wild-type cells, the resultant activity in *kin28-ts* cells was substantially reduced, and that in *ccl1-ts4* and *tfb3-ts* cells was almost indistinguishable from background values (Fig. 1C). As control, we verified that the *ccl1-ts4* allele was functional at the permissive temperature (Fig. 1D) and that the defect in autophagy could be complemented through ectopic expression of Ccl1 (Fig. 1E). In addition, we found that Ccl1 is not essential for the cytoplasm-to-vacuole targeting (Cvt) pathway (Huang et al., 2014; Klionsky et al., 1992; Reggiori and Klionsky, 2013) as there was only a minor slowdown in the rate of Ape1 maturation in the absence of Ccl1 (Fig. 1F).

Next, we performed an epistasis analysis on Ccl1 and Kin28. Ccl1 functions as a regulatory subunit of the Kin28 complex in the phosphorylation of the C-terminal domain of RNA polymerase II (Cismowski et al., 1995; Feaver et al., 1997; Valay et al., 1995). To determine whether Ccl1 also acts upstream of Kin28 in autophagy, we overexpressed wild-type and kinase-dead variants (T17D and K36A) of Kin28 in *ccl1-ts4* cells. Only the overexpression of wild-type Kin28, but not the kinase-dead variants, restored the translocation of GFP–Atg8 at the non-permissive temperature (Fig. 2A). As a control, we tested the overexpression of Ctk1, the other kinase identified in our screen, and found that Ctk1 could not rescue the autophagy defect in *ccl1-ts4*. The results from the GFP–Atg8 processing assay and Pho8Δ60 assay were consistent with those of our microscopy analyses (Fig. 2B,C). Furthermore, an RNA polymerase II mutant defective in phosphorylation of the C-terminal domain at residue Ser5 (S5A) also displayed severe impairment of autophagy (Fig. 2D). These data indicate that Ccl1 acts upstream of Kin28 in autophagy, possibly by regulating RNA polymerase II.

Proteasomal turnover of Ccl1 limits the autophagy response during prolonged starvation

As Ccl1 is a cyclin, we examined whether the expression of Ccl1 is altered during starvation (Fig. 3A). At 2 h after starvation, the expression level of Ccl1 was substantially reduced. By 4 h, the amount of Ccl1 was barely detectable. The expression of Ccl1 was restored upon shifting of yeast cells back to nutrient-rich medium. The depletion of Ccl1 did not affect the amount of Kin28 (Fig. S1C), indicating that the phenomenon is specific. Although overexpression of Ccl1 by using the *GPD1* promoter did not prevent its degradation per se, it raised the residual amount of Ccl1 after starvation to a level comparable to that of the endogenous protein during vegetative growth (Fig. 3B). This strain displayed substantially higher autophagic flux during prolonged starvation (Fig. 3B), suggesting that the turnover of Ccl1 limits autophagy.

The downregulation of Ccl1 occurred normally in both *atg1Δ* and *pep4Δ* cells (Fig. 3C), indicating that neither macroautophagy nor the proteolytic activity of the vacuole is required for this process. In contrast, the degradation of Ccl1 was completely blocked upon treatment with a proteasomal inhibitor, MG132 (Fig. 3D). Consistently, we found that the downregulation of Ccl1 was significantly reduced in cells that carried a temperature-sensitive allele of the E1 activating enzyme Uba1 (Fig. 3E) (McGrath et al., 1991). Among 13 E2 enzyme mutants tested (Table S2), the turnover of Ccl1 was retarded only in *cdc34-1* at the non-permissive temperature (Fig. 3F), suggesting that Cdc34 is the main E2 enzyme responsible (Feldman et al., 1997; Goebel et al., 1988; Mathias et al., 1998; Skowyra et al., 1997). As a control, we examined the turnover of a known substrate of Cdc34, Cln3. The degradation of Ccl1 and Cln3 was blocked to similar extents in *uba1-1* and *cdc34-1* mutants (Fig. 3E,F). We subsequently searched for the E3 ligase(s) involved in the turnover of Ccl1. Among 87 mutants of E3 or E3-related genes (Table S3), we did not find any single mutant that blocked Ccl1 degradation (data not shown), indicating the existence of redundant E3 enzymes that mediate Ccl1 degradation.

Ccl1 regulates the assembly of Atg1 complex through Atg29 and Atg31

We then investigated how the Ccl1–Kin28 complex regulates autophagy. To avoid complicating data interpretation with the heat-shock treatment necessary to inactivate temperature-sensitive alleles, we switched to the ‘auxin degron’ system for targeted protein depletion (Nishimura et al., 2009). Each of the three subunits could be efficiently depleted using this method (Fig. S1A), which in turn lead to a block of autophagy (Fig. S1B). Inhibition of TORC1 activity is a crucial step in the induction of autophagy under starvation conditions (Díaz-Troya et al., 2008; Jung et al., 2010; Kamada et al., 2004, 2010; Noda and Ohsumi, 1998). Therefore, we first examined whether Ccl1 interferes with TORC1 signaling. In wild-type cells, starvation resulted in an Npr1 species of a lower molecular mass (a TORC1 substrate) (Fig. S1D), reflecting the dephosphorylation of this protein. In a control sample, only higher molecular mass species were present when cells were treated with cycloheximide (CHX) in order to activate TORC1 (Watanabe-Asano et al., 2014). Similar downshifts were detected when Ccl1 was depleted, indicating that Ccl1 is not essential for TORC1 inhibition.

Once autophagy is induced, the concerted actions of the Atg proteins lead to formation of autophagosomes. This step involves the hierarchical assembly of key Atg proteins at the PAS, in which Atg8 is the most downstream (Suzuki et al., 2007). After 1 h of starvation, GFP–Atg8 puncta could be clearly observed in wild-type cells (Fig. 4A,B). In contrast, few puncta were present when Ccl1

Fig. 1. Ccl1 is required for starvation-induced autophagy. (A) Translocation of GFP–Atg8 into the vacuole. Wild-type (WT), *kin28-ts*, *ccl1-ts4* and *tfb3-ts* cells expressing GFP–Atg8 were grown to mid-log phase at the permissive temperature, shifted to the non-permissive temperature for 2 h and then starved for 4 h at the non-permissive temperature (see Materials and Methods for details of temperature shifts). At this stage, the translocation of GFP–Atg8 into the vacuole was monitored with fluorescent microscopy. The limiting membrane of vacuoles was stained with FM4-64 before starvation. The experiment was repeated three times, and representative mid-section images are shown. The percentage of cells with vacuolar accumulation of GFP is labeled at the top of the images (mean ± s.d., $n > 100$). DIC, differential interference contrast. Scale bar: 2 μ m. (B) Processing of GFP–Atg8 into free GFP. The experiment was performed as in A, except that protein extracts were prepared and analyzed by immunoblotting. Blotting of Pgk1 was used as a loading control. (C) Activation of Pho8Δ60. Wild-type, *kin28-ts*, *ccl1-ts4* and *tfb3-ts* cells carrying *pho8Δ60 pho13Δ* alleles were treated as in A. Autophagic flux was measured with the Pho8Δ60 assay. All values are normalized against the ALP activity in wild-type cells after 4 h of starvation. Error bars represent the mean ± s.d., $n = 3$. (D) Autophagy in *ccl1-ts4* cells was normal at the permissive temperature. Wild-type and *ccl1-ts4* cells expressing GFP–Atg8 were treated and analyzed as described in B, with or without shifting to the non-permissive temperature. (E) Autophagy defects in *ccl1-ts4* cells were rescued by ectopic expression of Ccl1–HA. Ccl1–HA was expressed from a centromeric plasmid under the control of its own promoter. GFP–Atg8-expressing cells carrying the indicated genotype were treated and analyzed as described in B. (F) Ccl1 was not essential for the Cvt pathway. The kinetics of precursor Ape1 (prApe1) processing was monitored using a non-radioactive pulse–chase method (see Materials and Methods). ProtA–(amber)–prApe1 was expressed on a 2- μ plasmid under the control of the *GAL1* promoter and a tetracycline-repressible riboswitch; the amber stop codon was utilized to incorporate OMe-Tyr, an unnatural amino acid. Twice as much of the DMSO Ccl1–AID sample and five times as much of the IAA Ccl1–AID sample relative to that of the other samples were used to compensate for the lower expression. *atg1Δ* was included as a negative control. Quantification of the blots is shown underneath the image (mean ± s.d., $n = 3$).

had been depleted, indicating that Ccl1 acts somewhere upstream. We then systematically analyzed the subcellular localization of other key Atg proteins (Fig. 4A–D; Fig. S2). In addition to that of

Atg8, we found the recruitment of the following Atg proteins and/or complexes to the PAS to be severely defective: Atg5 and Atg16 into the E3-like complex that is involved in targeting of Atg8

Fig. 2. Ccl1 acts upstream of Kin28 in autophagy. (A–C) Autophagy defects in *cc1-ts4* cells were rescued by overexpressing wild-type (WT) but not kinase-dead variants of Kin28. Overexpression of Ctk1, another kinase that targets the Kin28 substrate RNA polymerase II, also failed to rescue the defects. Autophagy in cells with the indicated genotype was determined by measuring translocation of GFP–Atg8 into the vacuole (A), processing of GFP–Atg8 into free GFP (B) or the Pho8Δ60 assay (C). The experiments were performed and the results are presented as described in Fig. 1A–C. OE, overexpression using 2-μ plasmids expressing the indicated constructs under the control of the *GPD1* promoter. (D) The S5A C-terminal domain (CTD) mutant failed to rescue the autophagy defect caused by Rpb1 depletion. Depletion of Rpb1, the largest subunit of RNA polymerase II, was achieved through an auxin-inducible degron (AID) system (see Materials and Methods for details). The indicated strains were transformed with centromeric plasmids expressing WT or CTD mutants of Rpb1 under the control of its own promoter, or empty control plasmids. The processing of GFP–Atg8 before and after 4 h of starvation was analyzed by immunoblotting.

Fig. 3. Proteasomal turnover of Ccl1 limits the autophagy response during prolonged starvation. (A) Turnover of Ccl1 under starvation and its recovery in rich medium. Wild-type (WT) cells expressing Ccl1 tagged with three HA molecules (Ccl1–3HA) were grown to mid-log phase, shifted to starvation medium for 4 h, then shifted back to rich medium for 4 h. Samples were collected at the indicated time points and analyzed by immunoblotting. (B) Turnover of Ccl1 limited autophagy during prolonged starvation. Top panel, overexpression of Ccl1 under the control of the *GPD1* promoter resulted in the residual amount of Ccl1 after 4 h of starvation being comparable to the endogenous level before starvation. Bottom panel, overexpression of Ccl1 led to a higher level of autophagy after starvation, as monitored with the Pho8Δ60 assay. Error bars represent mean±s.d., $n=3$. (C) Autophagy is not required for Ccl1 turnover under starvation. Ccl1–3HA-expressing cells carrying the indicated genotypes were grown to mid-log phase and then shifted to starvation medium for 4 h. Samples were collected at the indicated time points and analyzed by immunoblotting. *ATG1* and *PEP4* were knocked out to examine the roles of autophagy and vacuolar proteases, respectively. (D–F) Turnover of Ccl1 was mediated by the proteasome. The role of the proteasome was examined by using 50 μ M MG132 (D), a proteasomal inhibitor, or by using mutants of E1 (E) and E2 (F) enzymes. The experiment in D was conducted as described in C, except that cells were either mock treated with DMSO, or with MG132. For E,F, cells were treated and analyzed as described in Fig. 1B, using the non-permissive temperature to inactivate the temperature-sensitive alleles. Cln3, a known substrate of Cdc34, was used as a control. Note that the elevation of temperature accelerated the degradation of Ccl1 in wild-type cells.

(Fig. 4C; Fig. S2A) (Hanada et al., 2007); the Atg2–Atg18 complex that is involved in the retrograde trafficking of Atg9 (Fig. 4C; Fig. S2A) (Reggiori et al., 2004); Atg14 into the autophagy-specific phosphatidylinositol 3-kinase complex (Fig. 4C; Fig. S2A) (Obara et al., 2006); and the Atg1 kinase complex and PAS scaffold proteins (except for Atg11) (Fig. 4A,B) (Wong et al., 2013). The trafficking of Atg9, as well as that of Atg23 and Atg27, appeared to be normal (Fig. 4D; Fig. S2B) (Reggiori et al., 2004; Yen et al., 2007). Our fluorescent microscopy data indicate that Ccl1 regulates

an early step of autophagy – the assembly of the Atg1-complex scaffolds.

Among the Atg proteins we examined, the expression of most proteins appeared to be independent of Ccl1 (Fig. 5A; Fig. S3A,B). In contrast, the amounts of both Atg29 and Atg31 were substantially reduced, which possibly explains the assembly defect of the Atg1 complex (Chew et al., 2013; Fujioka et al., 2014; Kawamata et al., 2008; Mao et al., 2013; Ragusa et al., 2012; Stjepanovic et al., 2014). We additionally tested the effect of Kin28 depletion and

Fig. 4. Ccl1 is involved in the assembly of the Atg1 complex at the PAS. (A–D) The recruitment of Atg proteins to the PAS depends on Ccl1. The indicated Atg proteins were tagged with GFP or 2×GFP at the C-termini, except for Atg8, which was tagged at the N-terminus. Wild-type (WT) and Ccl1-AID-expressing cells were either mock treated with DMSO, or with IAA 2 h before starvation. At 1 h after starvation, the formation of perivacuolar GFP puncta was examined with fluorescent microscopy. The experiment was repeated three times. (A) Representative images of cells expressing GFP-tagged Atg8, or 2×GFP-tagged Atg1, Atg11, Atg13, Atg17, Atg29 or Atg31. DIC, differential interference contrast. Scale bar: 2 μ m. (B,C) Quantification of GFP puncta per cell. Error bars represent mean $\pm$ s.d., $n > 100$. Representative images for strains listed in C are shown in Fig. S2A. (D) Trafficking of Atg9, Atg23 and Atg27. Experiments were performed as described in A, except that a second strain set with *ATG1* knocked out was included to examine the anterograde movement of indicated proteins to the PAS. Error bars represent mean $\pm$ s.d., $n > 100$. Representative images for strains listed in panel D are shown in Fig. S2B.

found comparable reductions in both Atg29 and Atg31 levels (Fig. 5B). The regulation of *ATG29* and *ATG31* appeared to be post-transcriptional because we observed elevated levels of their mRNA upon Ccl1 depletion (Fig. S3C). Further analysis revealed that Atg29 and Atg31 were less stable than the other Atg1-complex components (Fig. 5C). Ccl1 depletion also led to a reduction in free Atg5, although no substantial impact on the amount of Atg12–Atg5 conjugates and Atg8–PE conjugate was detected (Fig. S3A,B). Taken together, these data are consistent with a model in which Atg29 and Atg31 are the key effectors of Ccl1–Kin28 in the regulation of autophagy.

DISCUSSION

In this study, we screened for novel kinase regulators in the yeast genome and identified the Ccl1–Kin28 complex as an essential factor for starvation-induced autophagy. Both inactivation of temperature-sensitive alleles and depletion of the corresponding proteins caused defects in autophagy, as evidenced using multiple assays. Defects in *ccl1-ts4* cells could be rescued by overexpression of wild-type but not kinase-dead mutants of Kin28, indicating that Ccl1 acts upstream of Kin28 in autophagy. We further demonstrated that Ccl1 was gradually degraded by the proteasome, which in turn

limited the level of autophagy during prolonged starvation. In the absence of Ccl1, inhibition of TORC1 occurred normally. Systematic analysis of the localization of Atg proteins at the PAS indicated that Ccl1 acts to maintain Atg29 and Atg31, which are essential in the assembly of the Atg1 complex. These data reveal a new pathway of autophagy regulation, in which the Ccl1–Kin28 complex acts as a limiting factor in the initiation of autophagy.

Starvation is a potent trigger for autophagy. The subsequent replenishment of nutrients through autophagy is crucial for survival during starvation; however, cell death can result from excessive autophagy. Therefore, it is vital that the levels of autophagy be kept in check, which can be achieved through several non-exclusive mechanisms. For instance, S6K, an important downstream target of TORC1 can be a positive mediator of autophagy (Scott et al., 2004). Thus, dampening of S6K activity under conditions of TORC1 inhibition serves as a built-in brake. Secondly, TORC1 itself becomes reactivated as a result of the nutrient recycling process (Chen et al., 2014; Matsui et al., 2013; Yu et al., 2010). This feedback mechanism ensures that the levels of autophagy only go as high as needed. Once regenerated nutrients are available, autophagy is abrogated. Thirdly, some components of the autophagosome biogenesis machinery are subject to turnover by autophagy. The first

Fig. 5. Ccl1 maintains the expression of Atg29 and Atg31.

(A,B) Depletion of Ccl1 or Kin28 results in reduced expression of Atg29 and Atg31. At 2 h before starvation, wild-type cells were mock-treated with DMSO; Ccl1-AID- (A) or Kin28-AID-expressing (B) cells were treated with IAA alone or in combination with PMSF, or mock treated with DMSO. Cells were then shifted to starvation medium with the same additives for 4 h. The expression of GFP-tagged Atg proteins was examined by immunoblotting. Representative images for cells expressing 2×GFP (2GFP)-tagged Atg17, Atg29 and Atg31 are shown. Images of the remaining Atg proteins are shown in Fig. S3A. (C) Atg29 and Atg31 are less stable than the rest of Atg1-complex components. The turnover of the indicated proteins was assessed by using cycloheximide (CHX) treatment, which inhibits protein translation. Samples were analyzed by immunoblotting.

example in this category is Atg8, the turnover of which requires continuous production of new protein molecules in order to maintain autophagy (Huang et al., 2000; Kirisako et al., 1999). Recently, turnover of Atg1 has also been reported, which appears to involve an interaction with Atg8 (Alemu et al., 2012; Kraft et al., 2012; Nakatogawa et al., 2012; Suttangkakul et al., 2011). Here, we found that Ccl1 is gradually turned over under starvation conditions. Unlike the aforementioned Atg proteins, turnover of Ccl1 is mediated by the ubiquitin-proteasome pathway, not autophagy. This phenomenon could provide an additional mechanism to tame the autophagy response over extended periods of starvation.

The Atg1 complex has been shown to be the target of several key autophagy regulators, including TORC1 and PKA (He and Klionsky, 2009; Komatsu et al., 2006). Both TORC1 and PKA are negative regulators of autophagy, and their phosphorylation of Atg13, albeit at different sites, appears to be inhibitory for the function of the complex (Kamada et al., 2010; Stephan et al., 2009). Considering that the Ccl1–Kin28 complex is a positive regulator of autophagy, its mechanism of regulation is likely to be different from that of TORC1 and PKA. In particular, it is unclear how Ccl1–Kin28 targets Atg29 and Atg31. This complex was originally discovered as part of the ten-subunit general transcription factor TFIIF complex, which mainly participates in RNA-polymerase-II-mediated gene transcription (Cismowski et al., 1995; Feaver et al., 1997; Valay et al., 1995). In this context, Kin28 phosphorylates a repeating heptapeptide, YSPTSPS, located in the C-terminal domain of the largest subunit of RNA polymerase II (Huang et al., 2000). Notably, both Atg29 and Atg31 are phospho-proteins, and their phosphorylation has been shown to be important for autophagy

(Feng et al., 2015; Kabeya et al., 2009; Mao et al., 2013). Based on the patterns of band shifts in immunoblots, the phosphorylation of Atg31 appeared to be normal, whereas that of Atg29 was absent upon Ccl1 depletion (Fig. 5A). However, we did not find any obvious matches for the heptapeptide in Atg29. Recently, it has been found that the translation of *ATG31* is subject to interference from an upstream noncoding transcription unit (Korde et al., 2014). Whether this mechanism constitutes the missing link between the Ccl1–Kin28 complex, RNA polymerase II and Atg31, and whether similar mechanisms apply to Atg29 remain to be tested by further studies. It should also be noted that our current data do not rule out the possibility that Ccl1 affects the post-translational modification of other Atg proteins without affecting their protein levels.

In summary, here, we report the initial characterization of a novel kinase regulator of autophagy, the Ccl1–Kin28 complex. Our results provide new insights into the regulatory network of autophagy during starvation.

MATERIALS AND METHODS

Strains and plasmids

Yeast gene knockout and C-terminal epitope tagging were performed using the common PCR-based method. Tagging of Atg proteins with one or two GFP molecules was performed using a plasmid tool set (Li et al., 2015). For our initial screening, knockout mutants (in BY4741 background) were from the *Saccharomyces* Genome Deletion Project (Giaever et al., 2002); temperature-sensitive mutants were gifts from Dr Brenda Andrews (University of Toronto, ON, Canada) (Li et al., 2011). The original auxin-inducible degron plasmids made by the Kanemaki group were obtained from National BioResource Project (Japan, <http://yeast.lab.nig.ac.jp/>) (Nishimura et al., 2009). Plasmids for an optimized auxin-inducible

degron system were gifts from Dr Helle D. Ulrich (Institute of Molecular Biology, Mainz, Germany) (Morawska and Ulrich, 2013). Plasmids for the non-radioactive pulse–chase labeling system were gifts from Dr Ed Hurt (Biochemie-Zentrum der Universität Heidelberg, Heidelberg, Germany) (Stelter et al., 2012). Plasmids expressing wild-type and C-terminal-domain-mutants of Rbp1 were gifts from Dr François Robert (Institut de Recherches Cliniques de Montréal, QC, Canada) (Jeronimo and Robert, 2014). Additional strains used in this study are listed in Table S4.

Culturing of yeast cells

Unless otherwise noted, cells were inoculated into YPD medium (1% yeast extract, 2% peptone, 2% glucose) medium and grown overnight. For experiments that did not involve temperature-sensitive mutants, the culture was incubated at 30°C. For those involving temperature-sensitive mutants, the culture was initially grown at 24°C. Upon reaching OD₆₀₀=0.6, the culture was shifted to non-permissive temperature for 2 h to inactivate the mutant allele. For nitrogen-starvation conditions, the culture was then shifted to SD-N medium (2% glucose, 0.17% yeast nitrogen base without amino acids and ammonium sulfate). For experiments involving temperature-sensitive mutants, conditions of starvation were performed at the non-permissive temperature unless otherwise noted. The non-permissive temperature for *kin28-ts* and *cel1-ts4* was 39°C. The non-permissive temperature for *ubal-1* and *cdc34-1* was 37°C.

Depletion of auxin-inducible-degron-tagged proteins

The turnover of auxin-inducible degron (AID)-tagged proteins was induced through addition of 500 µM indole-3-acetic acid (IAA) to the medium 2 h before starvation. This method is based on the auxin-induced interaction between IAA-family transcription factors and F-box protein TIR1, which results in the ubiquitylation of IAA proteins and their subsequent degradation in plants. Tagging of target proteins with a degron domain from IAA17 in the presence of ectopically expressed TIR1 allows auxin-induced turnover of target proteins in yeast and animal cells (Nishimura et al., 2009).

Pulse–chase analysis of Ape1 maturation

Maturation of Ape1 in Ccl1-depleted cells was analyzed by using a non-radioactive pulse–chase method (Stelter et al., 2012). In this system, the synthesis of the target protein is controlled by three independent mechanisms: (1) galactose-inducible transcription, (2) aptamer-based tetracycline-repressible translation, and (3) amber codon within the open reading frame plus an orthogonal suppressor tRNA and aminoacyl-tRNA synthetase pair for the utilization of an unnatural amino acid. Wild-type, *atg1Δ* and strains expressing AID-tagged Ccl1 (Ccl1–AID) strains were transformed with plasmids resulting in the expression of Protein-A-tagged pApe1 under the aforementioned principle. Cells were first inoculated into synthetic dropout (SD) medium lacking Trp and Leu, and grown to saturation. Cells were then switched to YP raffinose medium at a starting density of 0.15 OD₆₀₀. Upon reaching 0.5 OD₆₀₀, IAA or DMSO vehicle were added to manipulate the turnover of Ccl1–AID. After 2 h, the cell culture was supplemented with 2% galactose to induce transcription for 25 min and then supplemented with 1 mM OMe-Tyr to allow translation for 25 min (the pulse phase). The chase phase was initiated by switching to YPD medium containing 350 µg/ml tetracycline. At the indicated time points, equal volumes of liquid culture were collected from each sample and processed for subsequent immunoblotting.

Fluorescent microscopy

Glass-bottomed live-cell chambers were coated with 1 mg/ml concanavalin A to attach yeast cells to the cover glass. Images were acquired on a DeltaVision imaging workstation (Applied Precision). For z-stacks, the stepping size was 0.5 µm. To visualize vacuoles with FM4-64, cells were incubated in rich medium containing 64 µM FM4-64 for 5 min before shifting to starvation medium.

Real-time PCR

For quantification of mRNA levels of ATG genes, the following primer pairs were used (written from 5' to 3'): *ATG1*: AGACCATACACAAGCCGTAG,

AGCGAGGATATAGACAAGCG; *ATG11*: CGCCTTTGGATGCTATGTCT, CTGAAACCAAACTGAGCCCT; *ATG13*: TGATGACGAGAATGACCGTT, TGAAATTTTCGCCTGAGCTTG; *ATG17*: GAGCTGTTTAAAGGTGGTACA, TCCTTTCTCCTCTTTGCTTC; *ATG29*: TAAATGTATCCGCAAGCCCA, GCTTCTTCCAACGCAGATTT; *ATG31*: TCACACTAATCAGCGACCAA, AGAAAAGGAGACAGATCGCA; *ACT1*: TGGTCGGTATGGGTCAAAAA, CCATCACCGGAATCCAAAAAC.

Other methods

Yeast protein sample extraction, immunoblotting and the Pho8Δ60 (alkaline phosphatase, ALP) assay were performed as described previously (Huang et al., 2014; Noda and Klionsky, 2008). Except when blotting for Pgk1, all immunoblots were performed against epitope-tagged proteins as indicated in the figures. Unless otherwise noted, tagging was achieved through C-terminal chromosomal insertion. Antibodies used in this study were against the following proteins and tags: AID (Cosmo Bio, BRS-APC004AM), GFP (Roche, 11814460001), hemagglutinin (HA) (Abmart, M20003L), Myc (Roche, 11667149001), Pgk1 (Nordic Immunology, NE130/7S), Protein A (Sigma-Aldrich, P3775). At least three independent repeats were performed for each experiment, with representative images shown in figures.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

P.L. constructed some of the strains and plasmids; W.B. and T.L. performed the E3-ligase screen; J.Z. and S.D. performed all the other experiments. J.Z. and Z.X. prepared the figures and manuscript. L.Y. and Z.X. designed and supervised the project.

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Supplementary information

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