The Ccl1-Kin28 kinase complex regulates autophagy under nitrogen starvation.

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Abbreviations: Atg, autophagy-related; GFP, green fluorescent protein; Cvt, cytoplasm-to-vacuole targeting; PAS, phagophore assembly site.

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Summary statement

Screening of known kinases in the yeast genome revealed the Ccl1-Kin28 complex as a novel regulator of autophagy. Proteasomal turnover of Ccl1 limits autophagy during prolonged starvation.

Abstract

Starvation triggers global alterations in the synthesis and turnover of proteins. Under such conditions, the recycling of essential nutrients by autophagy is indispensable for survival. By screening known kinases in the yeast genome, we identified a novel regulator of autophagy, the Ccl1-Kin28 kinase complex (homologue of 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 critical in the assembly of the Atg1 kinase complex. The identification of this novel regulation pathway shed new lights on the complex signaling network governing autophagy activity.

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/lysosomal compartments (in animal cells) or the vacuole (in yeast and plant cells). By adjusting the specificity and magnitude of autophagy response, cells can adopt the same basic molecular machinery to accommodate diverse environmental challenges. Conversely, failure to achieve proper specificity or magnitude of autophagy lead 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 PAS (phagophore assembly site, also known as the preautophagosomal 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) The Atg1 kinase complex, with Atg13 as a subunit (note that the aforementioned PAS scaffold proteins are considered subunits of this complex in certain context). The Atg1 complex is a major signaling hub responsible for the initiation of autophagosome biogenesis. (3) 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) The phosphatidylinositol 3-kinase complex, with Atg14 as the autophagy specific subunit. (5) The Atg2-Atg18 complex, involved in retrograde trafficking of Atg9 complex. (6) Two conjugation systems, ultimately responsible for forming Atg8-phosphatidylethanolamine (PE) conjugate on autophagosomal membranes. The Atg12-Atg5-Atg16 complex acts in an E3-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; Suzuki et al., 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 level (Fullgrabe et al., 2014), or at post-translational level (Wani et al., 2015). In the latter scenario, the role of several protein kinases has been well documented. TOR, AMPK and PKA all target the Atg1 complex (or the ULK1 complex in mammals)(Egan et al., 2011; He and Klionsky, 2009; Kamada et al., 2000; Kamada et al., 2010; Kim et al., 2011; Stephan et al., 2009; Wong et al., 2013). AMPK also phosphorylate Beclin-1 (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 may regulate the autophagy machinery indirectly through TOR signaling (Gwinn et al., 2008; Inoki et al., 2003; Umekawa and Klionsky, 2012; Yorimitsu et al., 2007). In recent years, the involvements of several other kinases have 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 novel kinase regulators of autophagy, we screened 123 mutants of known kinases or their subunits in the yeast genome, 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 by 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). Under 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 the GFP-Atg8 processing assay and the Pho8Δ60 assay (Fig. 1B-C). In wild type cells, GFP-Atg8 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 microscopic observation, a significant 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 resulting activittheautopha3(im)-3 (mun9 12 Tf204.02

defect in *ccl1-ts4*. The results from the GFP-Atg8 processing assay and Pho8Δ60 assay were consistent with our microscopic analysis (Fig. 2B-C). Furthermore, polymerase II mutant defective in CTD Ser 5 phosporylation (S5A) also displayed severe autophagy impairment (Fig. 2D). These data indicate that Ccl1 acts upstream of Kin28 in autophagy, possibly through 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). Two hours after starvation, the expression level of Ccl1 was substantially reduced. By four hours, the amount of Ccl1 was barely detectable. The expression of Ccl1 was restored upon shifting of yeast cells back to 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 the *GPD1* promoter did not prevent its degradation *per se*, it brought the residual amount of Ccl1 after starvation to a level comparable to 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

Ccl1 degradation (data not shown), indicating the existence of redundant E3s mediating 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

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depletion (Nishimura et al., 2009). Each of the three subunits could be efficiently depleted using this method (Fig. S1A), which in turn lead to block of autophagy (Fig. S1B). Inhibition of TOR activity is a critical step in the induction of autophagy under starvation conditions(Diaz-Troya et al., 2008; Jung et al., 2010; Kamada et al., 2004; Kamada et al., 2010; Noda and Ohsumi, 1998). Therefore, we first examined whether Ccl1 interferes with TOR signaling. In wild type cells, starvation resulted in the migration of Npr1 (a TOR substrate) towards lower molecular weight species (Fig. S1D), reflecting the dephosphorylation of this protein. In a control sample, only higher molecular weight species were present when cells were treated with cycloheximide (CHX) to activate TOR (Watanabe-Asano et al., 2014). Similar downshifts were detected when Ccl1 was depleted, indicating that Ccl1 is not essential for TOR inhibition.

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, 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 found comparable reductions in both Atg29 and Atg31 (Fig. 5B). The regulation of *ATG29* and *ATG31* appeared to be post-transcriptional, as we observed elevated levels of their mRNA upon Ccl1 depletion (Fig. S3C). Further analysis revealed that Atg29 and Atg31 were less stable than the rest of Atg1 complex components (Fig. 5C). Ccl1 depletion also led to reduction of free Atg5, although no substantial impact on the amounts of Atg12-Atg5 conjugate 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 by 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 TOR 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

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 was 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 might affect 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 1x or 2x GFP 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)(Li et al., 2011). The original auxin-inducible degron plas-9 (p)-(e)27 (e)4 (e)27 (e)4 (S)-3 (a)(e)4 (pop)-(eb)-11 6 (

Culturing of yeast cells

Unless otherwise noted, cells were inoculated into YPD (1% yeast extract, 2% Peptone, 2% glucose) medium and gown overnight. For experiments that do not involve temperature sensitive mutants, the culture was incubated at 30℃. For those involving temperature sensitive mutants, the culture was initially grown at 24℃. Upon reaching OD_{600} 0.6, the culture was shifted to non-permissive temperature for 2 h to inactivate the mutant allele. For nitrogen starvation treatment, the culture was then shifted to SD-N (2% glucose, 0.17% yeast nitrogen base without amino acids and ammonium sulfate) medium. For experiments involving temperature sensitive mutants, starvation treatment was performed at non-permissive temperature unless otherwise noted. The non-permissive temperature for *kin28-ts* and *ccl1-ts4* was 39℃.The non-permissive temperature for *uba1-1* and *cdc34-1* was 37℃.

Depletion of AID-tagged proteins

The turnover of AID-tagged proteins was induced by addition of 500 μM IAA (Indole-3-Acetec Acid) to the media 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 ubiquitination of IAA proteins and their subsequent degradation in plant. 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 a non-radioactive pulse chase method (Stelter et al., 2012). In this system, the synthesis of target protein is controlled by three independent mechanisms: (1) galactose-inducible transcription, (2) aptamer-based tetracycline repressible translation, and (3) amber codon within ORF plus an orthogonal suppressor tRNA/aminoacyl-tRNA synthetase pair for the utilization of an unnatural amino acid. WT, *atg1Δ* and Ccl1-AID strains were transformed with plasmids resulting in the expression of Protein A tagged prApe1 under the aforementioned principle. Cells were first inoculated into SD-Trp-Leu medium and grown to saturation. Cells were then switched to YP-Raffinose medium at a starting concentration of 0.15 OD. Upon reaching 0.5 OD, 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, 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 volume of liquid culture was collected from each sample and processed for subsequent immunoblotting.

Fluorescent microscopy

Glass-bottom 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, stepping size was 0.5 μm. To visualize vacuoles with FM4-64, cells were incubated in rich medium containing 64μM 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 *ATG1*: AGACCATACACAAGCCGTAG, AGCGAGGATATAGACAAGCG; *ATG11*: CGCCTTTGGATGCTATGTCT, CTGAAACCAAACTGAGCCCT; *ATG13*: TGATGACGAGAATGACCGTT, TGAAATTTCGCCTGAGCTTG; *ATG17*: GAGCTGTTTAAGGTGGTACA, TCCTTTCTCCTCTTTGCTTC; *ATG29*: TAAATGTATCCGCAAGCCCA, GCTTCTTCCAACGCAGATTT; *ATG31*: TCACACTAATCAGCGACCAA, AGAAAAGGAGACAGATCGCA; *ACT1*: TGGTCGGTATGGGTCAAAAA, CCATCACCGGAATCCAAAAC.

Other methods

Yeast protein sample extraction, immunoblotting, and the Pho8Δ60 (ALP) assay were performed as described previously (Huang et al., 2014; Noda and Klionsky, 2008). Except 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: AID (Cosmo Bio, BRS-APC004AM), GFP (Roche, 11814460001), HA (Abmart, M20003L), Myc (Roche, 11667149001), Pgk1 (Nordic Immunology, NE130/7S), Protein A (Sigma, 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 the 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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Figures

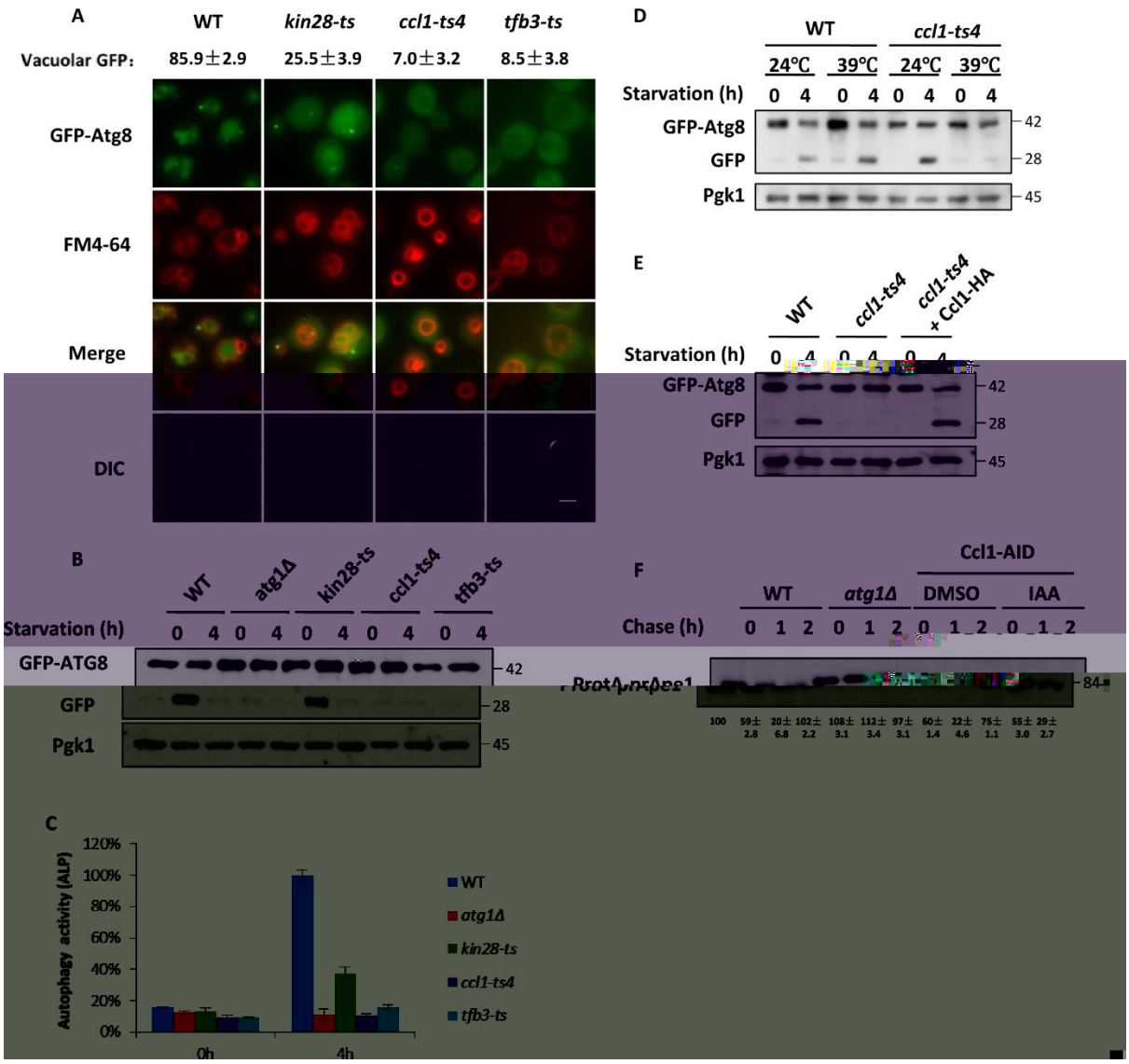

Figure 1. Ccl1 is required for starvation induced autophagy.

(A) Translocation of GFP-Atg8 into the vacuole. Wild type, *kin28-ts*, *ccl1-ts4* and *tfb3-ts* cells expressing GFP-Atg8 were grown to mid-log phase under permissive temperature, shifted to non-permissive temperature for 2 h, and then starved for 4 h under 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 by fluorescent microscopy. The limiting membrane of vacuoles was stained by FM4-64 prior to 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 on top of the images (mean ± standard deviation, n>100). DIC, differential interference contrast. Scale bar, 2 μm.

(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. 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 by the Pho8Δ60 assay. All values are normalized against the ALP activity in wild type cells after 4 h of starvation. Error bar, standard deviation, n=3.

(D) Autophagy in *ccl1-ts4* cells was normal under permissive temperature. Wild type and *ccl1-ts4* cells expressing GFP-Atg8 were treated and analyzed as in (B), with or without shifting to non-permissive temperature.

(E) Autophagy defect in *ccl1-ts4* cells was rescued by ectopic expression of Ccl1-HA. Ccl1-HA was expressed by a centromeric plasmid under the control of its own promoter. GFP-Atg8 expressing cells carrying the indicated genotype were treated and analyzed as in (B).

(F) Ccl1 was not essential for the Cvt pathway. The kinetics of precursor Ape1 (prApe1) processing was monitored by a non-radioactive pulse-chase method (see Materials and Methods for details). ProtA-(amber)-prApe1 was expressed on a 2-micron plasmid under the control of *GAL1* promoter and a tetracycline-repressible riboswitch. Loading of Ccl1-AID samples was 2x(DMSO)/5x(IAA) that of the rest to compensate for its lower expression. *atg1Δ* was included as a negative control.

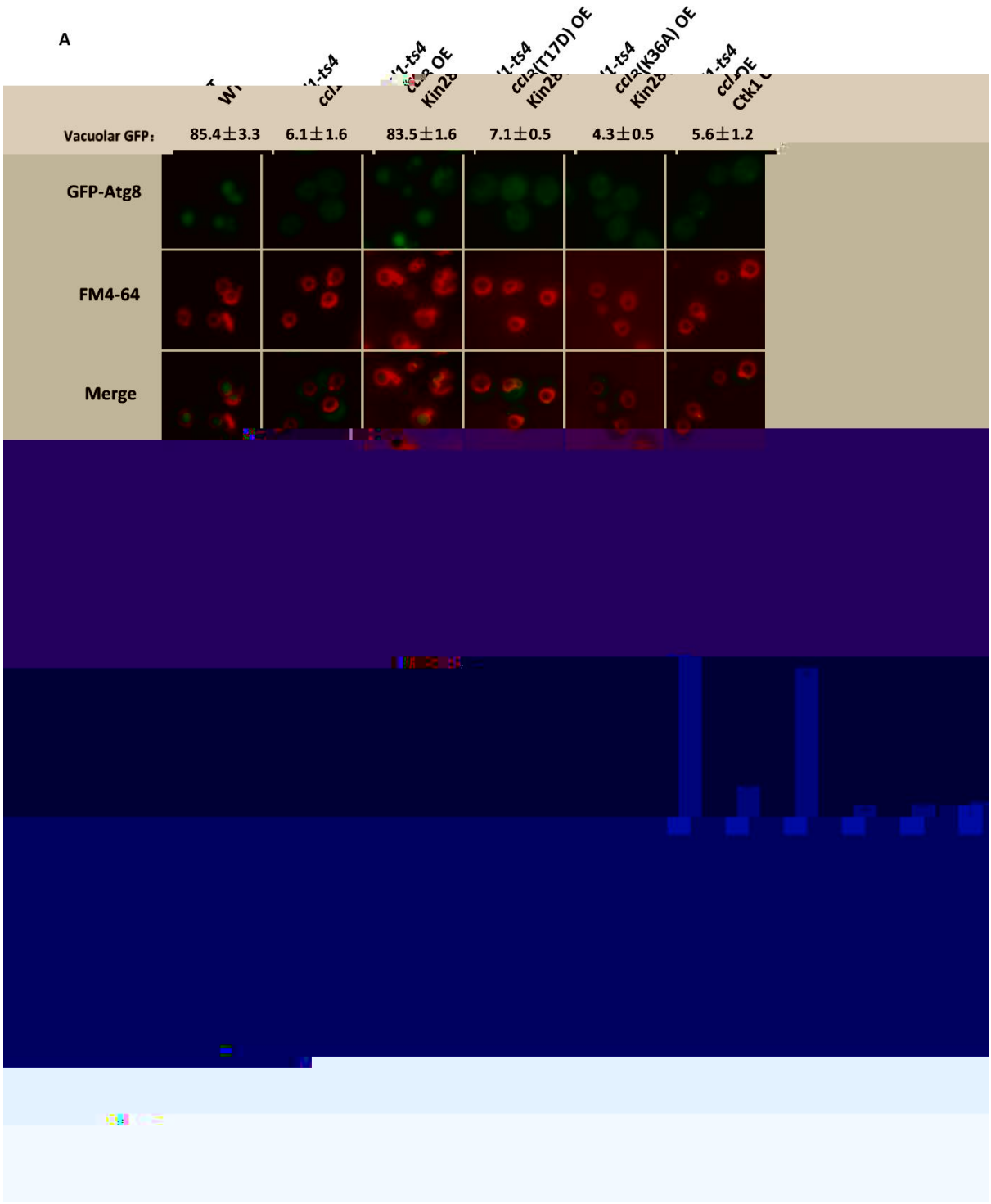

Figure 2. Ccl1 acts upstream of Kin28 in autophagy.

(A-C) Autophagy defects in *ccl1-ts4* cells were rescued by overexpression of wild type, but not kinase dead variants of Kin28. Overexpression of Ctk1, another kinase that targets Kin28 substrate RNA polymerase II, also failed to rescue the defects. Autophagy in cells with the indicated genotype was measured by 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 as in Fig. 1A-1C, and the results are presented as therein. OE, over-expression by 2-micron plasmids expressing the indicated constructs under the control of *GPD1* promoter.

(D) CTD S5A mutant failed to rescue the autophagy defect caused by Rbp1 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 centrimeric plasmids expressing WT or CTD mutants of Rpb1 under the control of its own promoter, or empty control plasmids. The processing GFP-Atg8 before and after 4 h of starvation was analyzed by immunoblotting.

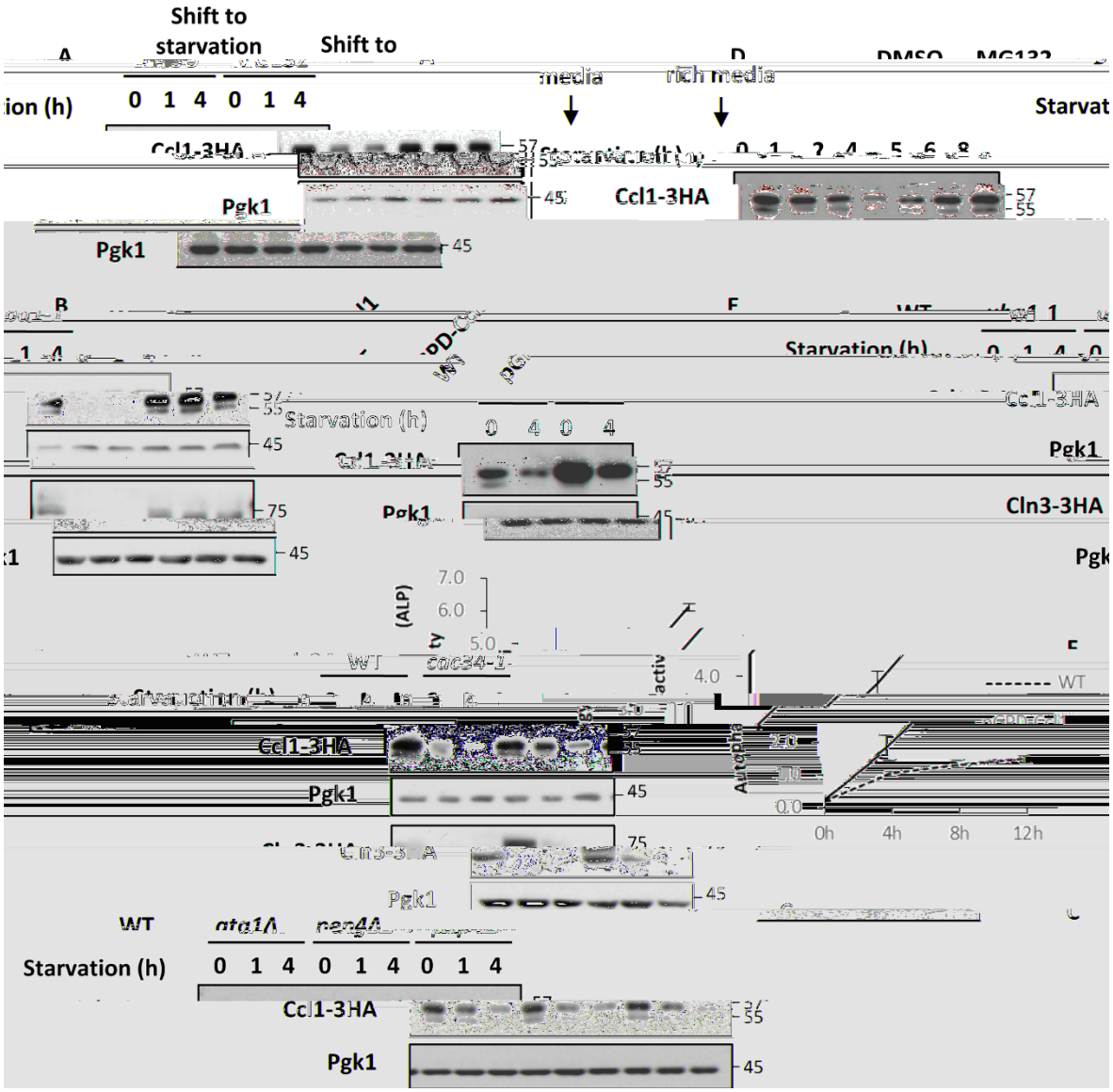

Figure 3. Proteasomal turnover of Ccl1 limits the autophagy response during prolonged starvation.

(A) Turnover of Ccl1 under starvation and its recovery in rich medium. Wild type cells expressing Ccl1-3HA were grown to mid-log phase, shifted to starvation medium for 4 h, then shift 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 GPD1 promoter on a 2-micron plasmid 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 higher level of

autophagy after starvation, as monitored by the pho8Δ60 assay. Error bar, standard deviation, n=3.

(C) Autophagy is not required for Ccl1 turnover under starvation. Ccl1-3HA expressing cells carrying the indicated genotype 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 MG132 (D), a proteasomal inhibitor, or by using mutants of E1 (E) and E2 (F) enzymes. The experiment in panel D was conducted as in panel C, except that cells were either mock treated with DMSO, or with MG132. For panel E and F, cells were treated and analyzed as Fig. 1B, using 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.

Figure 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 2xGFP at the C-termini, except for Atg8, which was tagged at the N-terminus. Wild type and Ccl1-AID expressing cells were either mock treated with DMSO, or with IAA 2 h prior to starvation. At 1 h after starvation, the formation of perivacuolar GFP puncta was examined by fluorescent microscopy. The experiment was repeated three times. (A) Representative images for GFP-Atg8, and Atg1/11/13/17/29/31-2xGFP expressing cells. Scale bar, 2 μm. (B, C) Quantification of GFP puncta per cell. Error bar, standard deviation, n>100. Representative images for strains listed in panel C are shown in Figure S2A. (D) Trafficking of Atg9, Atg23 and Atg27. Experiments were performed as in (A) except that a second strain set with *ATG1*

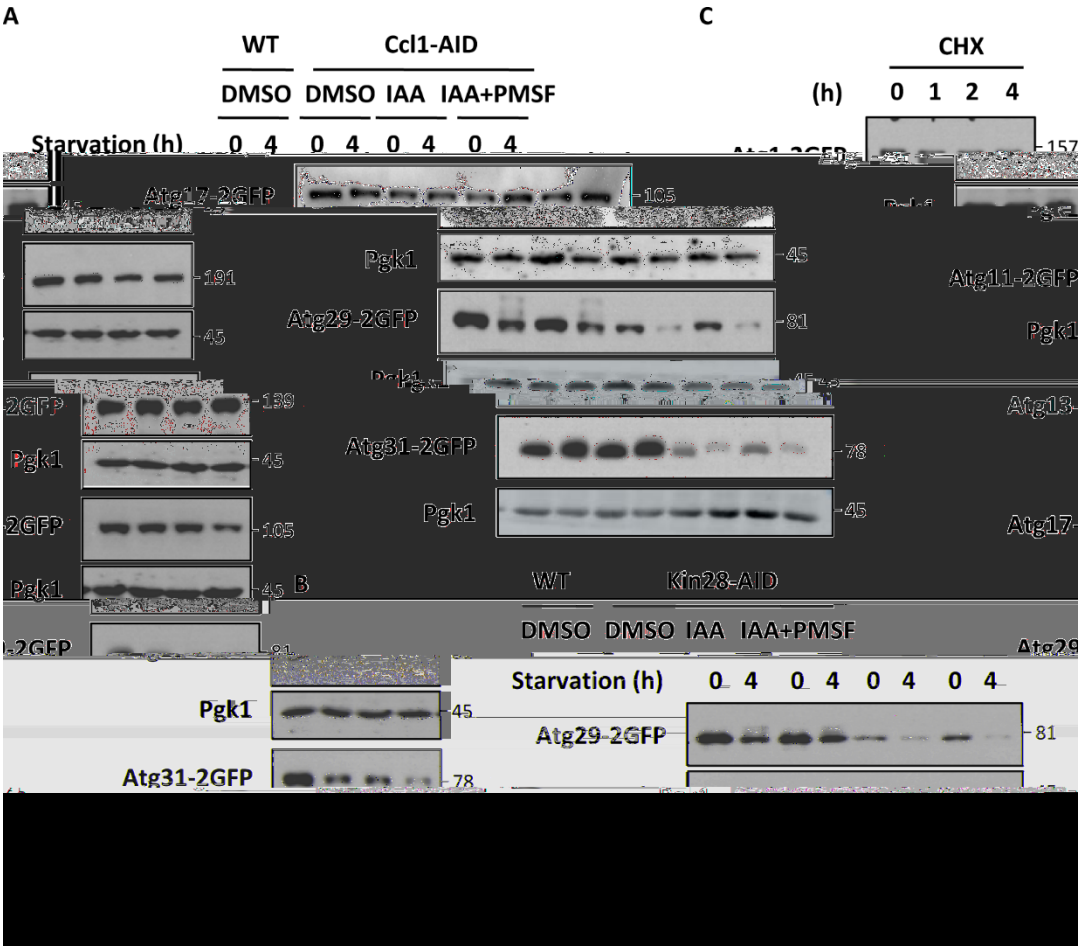

Figure 5. Ccl1 maintains the expression of Atg29 and Atg31.

(A, B) Depletion of Ccl1 or Kin28 results in reduced expression of Atg29 and Atg31. 2 h before starvation, wild type cells were mock treated with DMSO; Ccl1-AID (A) or Kin28-AID (B) cells were treated with a combination of IAA or PMSF, or mock treated with DMSO. Cells were then shifted to starvation media with the same additives for 4 h. The expression of GFP-tagged Atg proteins was examined by immunoblotting. Representative images for Atg17/29/31-2xGFP are shown. Images for 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 indicated proteins was assessed by cycloheximide (CHX) treatment that inhibits protein translation. Samples were analyzed by immunoblotting.