

The F-box Protein Rcy1 Is Involved in the Degradation of Histone H3 Variant Cse4 and Genome Maintenance*[§]

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Cse4, a histone H3-like centromeric protein, plays critical functions in chromosome segregation. Cse4 level is tightly regulated, but the underlying mechanism remains poorly understood. We employed a toxicity-based screen to look for the degradation components involved in Cse4 regulation. Here, we show that the F-box containing protein Rcy1 is required for efficient Cse4 turnover as Cse4 degradation is compromised in yeast cells lacking *RCY1*. Excessive Cse4 accumulation in *rcy1Δ* cells leads to growth retardation. Furthermore, the deletion of *RCY1* is tied to enhanced chromosome instability and temperature-sensitive cell growth. Our results reveal the involvement of Rcy1 in chromosome regulation and another regulatory pathway controlling the Cse4 level and activity.

Chromosome segregation is key to equally divide the duplicated DNA to daughter cells. Following DNA replication, sister chromatids are joined at a highly condensed and constricted chromosome region called centromere (1, 2). A distinct feature of the specialized centromeric chromatin is the incorporation of an essential histone H3 variant CENP-A (called Cse4 in yeast *Saccharomyces cerevisiae*), instead of histone H3 in nucleosomes (1, 3, 4).

As altered localization and expression of CENP-A and its homologues have been linked to genome instability and cancers (1, 5, 6), CENP-A activity must be tightly regulated. Human CENP-A and yeast Cse4 have been found to be subject to proteasome-mediated degradation (1, 7, 8). Proteasome substrates are selected by a ubiquitin ligase E3, which collaborates with a ubiquitin-activating enzyme (E1) and a ubiquitin-conjugating enzyme (E2) to covalently attach ubiquitin molecules onto substrates (9, 10). Ubiquitin-marked substrates are then delivered to and degraded by the proteasome. The key to understanding the role of proteolysis in CENP-A regulation is to identify E3 enzymes involved.

Two groups previously isolated Psh1 as a yeast Cse4-binding protein via the affinity purification-mass spectrometry coupled approach and further demonstrated that Psh1 acts as an E3

ligase for Cse4 ubiquitylation and degradation (11, 12). The mechanism underlying Psh1-mediated Cse4 turnover remains to be elucidated. Because Cse4 degradation is partially impaired in *psh1Δ* cells, other E3s likely also promote Cse4 turnover (11–15).

We have previously developed a synthetic dosage lethality screen to uncover physiological targets of several proteolytic pathways (16–18). The synthetic lethality screen can be easily adapted to isolate relevant components involved in the degradation of a given substrate. Because Cse4 overexpression leads to toxicity in *psh1Δ* cells (11, 12), we looked for the degradation mutants that grow slower upon Cse4 overexpression. Yeast cells lacking *RCY1* are found to be sensitive to Cse4 expression and exhibit compromised Cse4 degradation. *RCY1* encodes a F-box containing protein, which is a substrate recognition component of the Skp1-Cdc53-F box (SCF)² E3 complex (19). Rcy1 is one of the eight F-box proteins in yeast, but little is known about its degradation targets (19–21). Consistent with the involvement of Rcy1 in centromere regulation, *rcy1Δ* mutants display increased genome instability. Interestingly, Rcy1 and Psh1 appear to act in parallel pathways for Cse4 degradation. Our findings will help to unravel the intricate mechanisms that regulate Cse4/CENP-A level and activity.

Experimental Procedures

Yeast Strains and Plasmids—A collection of yeast *S. cerevisiae* strains lacking non-essential ubiquitylation or degradation components including various E2s and E3s in BY4741 background were obtained from Dr. Mark Hochstrasser (Yale University) (22). Haploid yeast mutant strains lacking *RCY1*, *TOM1*, *PSH1*, *ATG1*, *ATG9*, *ATG14*, *YPT31*, *YPT32*, and *SNC1* in the BY4741 strain background were obtained from Open Biosystems (Huntsville, AL). GFP- or GST-tagged Cdc53 strain was obtained from Open Biosystems and ThermoFisher Scientific Inc., respectively. Strain YHR305 (*psh1::LEU2 rcy1::KanMX4*) was constructed by replacing *PSH1* with *LEU2* in the *rcy1Δ* strain. Sectoring strain YPH278 was obtained from Dr. P. Hieter and was used to delete the *RCY1* gene by *LEU2* to make strain YHR313. Endogenous *RCY1* gene was tagged 9 Myc epitopes at its C-terminal using PCR-mediated homologous recombination to construct strain YHR297 in BY4741 background.

The plasmid pMB1458 bearing 3HA-tagged Cse4 and pYES2-HTH-HHT2 expressing HA-tagged histone H3 were previously described (12, 14, 23). The plasmid for V5-tagged Cse4 expression in *Escherichia coli* was kindly provided by Dr.

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[§] This article contains supplemental Table S1.

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² The abbreviation used is: SCF, Skp1-Cdc53-F box.

Biggins. *RCY1* was amplified by PCR to append the FLAG epitope at its C terminus and inserted into pRS425GAL1 to create the plasmid pRS425GAL1-Rcy1-Flag for FLAG-tagged Rcy1 expression in yeast. Yeast cells were grown in rich (YPD) or synthetic media containing standard ingredients and 2% glucose (SD medium), or 2% raffinose (SR medium), or 2% raffinose + 2% galactose (SRG medium).

Synthetic Dosage Lethal Screen—The plasmid pMB1458 was separately transformed into wild-type or 93 yeast mutants (see supplemental Table S1). Yeast cells bearing the plasmid were first grown in synthetic media containing glucose. Actively growing cells were spotted onto glucose-containing SD-ura and galactose-containing SG-ura media in serial 10-fold dilution. Cse4 expression is controlled by a *GAL1* promoter and induced by galactose. The plates were incubated at 30 °C for 3 days. Thirteen mutants that exhibited reduced growth on SG-ura plates were deemed possible candidates from the initial screen and further analyzed (18).

Antibodies—Antibodies against HA and myc were obtained from BioLegend Inc. (San Diego, CA). Antibodies for V5 and FLAG were purchased from ThermoFisher Scientific and Sigma, respectively. Rpt5 antibody was obtained from Enzo Life Sciences (Farmingdale, NY).

Expression Shut-off Assay—As previously described (17), yeast cells expressing HA-tagged Cse4 were grown at 30 °C to an A_{600} of ~1 in synthetic medium SR-uracil. The expression of HA-Cse4 was induced by galactose for 2 h (17). Protein translation was shut off by the addition of cycloheximide (150 μ g/ml). Samples were collected at the indicated time points. Proteins were extracted by glass bead lysis of cells, processed for immunoblotting with anti-HA (BioLegend, MMS101P), followed by detection with goat anti-mouse HRP conjugate using ECL reagents (GE Healthcare). The stable protein Rpt5 was used as a loading control in the expression shutoff experiments.

Sectoring Assay—Yeast YPH278 cells contained the defective *ade2-101* ochre mutation, which can be suppressed by the *SUP11* gene, carried on a non-essential, 90-kb chromosome fragment. YPH278 cells that maintained this chromosomal fragment produced white colonies, whereas the loss of it resulted in a red color or sectors (17). The *RCY1* gene was deleted in the YPH278 strain, and the resulting mutant YHR313 was compared with YPH278. As previously described (17), cells were first grown overnight in uracil-lacking medium that retained the *SUP11*-containing chromosome, followed by growing in non-selective rich YPD medium for 8 h and plating on YPD plates. The plates were incubated at 30 °C for 48 h and then kept at room temperature for ~5 days for color development.

Results

The involvement of the proteasome in Cse4 turnover was previously demonstrated using proteasome mutant *cim3-1* (7, 9), which has a number of defects that may potentially contribute to Cse4 stabilization indirectly. We assessed the effects of the proteasome inhibitor MG132 on Cse4 degradation. Cse4 turnover is impaired upon MG132 treatment (Fig. 1A), supporting the involvement of the proteasome in Cse4 regulation (7). Besides the proteasome, autophagy is the other major pro-

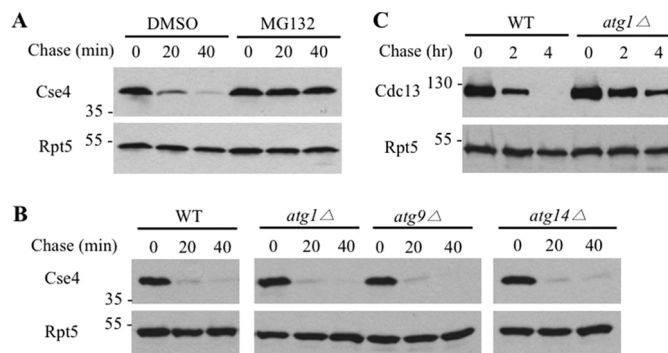


FIGURE 1. The involvement of the proteasome and autophagy in the degradation of Cse4. A, Cse4 turnover is compromised upon MG132 treatment. Wild-type yeast cells expressing HA-Cse4 were grown to an A_{600} of ~1 and then added with the proteasome inhibitor MG132 (75 μ M) or dimethyl sulfoxide (DMSO) control for 30 min. Samples were taken after expression shut-off by cycloheximide at the time points indicated and analyzed by Western blots using anti-HA antibody (BioLegend, MMS-101P). Equal amounts of samples were used and ascertained by blotting with Rpt5 antibody (Enzo Life Sciences, BML-PW8245) in all of the expression shutoff experiments (*lower panels*). Molecular mass standards (kDa) are indicated on the *left*. B, Cse4 is degraded efficiently in autophagy mutants. Cse4 turnover in wild-type and 3 mutant cells was determined as above. C, Cdc13 degradation is compromised in the *atg1* Δ mutant. GST-Cdc13 turnover was determined as previously described.

teolytic system in eukaryotes (24). We evaluated whether autophagy is involved in Cse4 turnover. Whereas Cdc13 was degraded by autophagy as previously reported (Fig. 1C), Cse4 stability was not altered significantly in yeast cells lacking one of the core autophagy components (*i.e.* *ATG1*, *ATG9*, *ATG14*) (Fig. 1B), suggesting that autophagy is not involved in Cse4 degradation.

As Cse4 is still degraded in yeast cells lacking Psh1 E3 (11–15), other ubiquitin ligases likely are involved in Cse4 turnover. To this end, we employed an array of yeast mutant cells lacking known or putative ubiquitylation/degradation enzymes (22) (supplemental Table S1). Reasoning that Cse4 accumulation may lead to toxicity, we adopted a synthetic lethal screen to look for yeast mutants that show growth retardation upon Cse4 expression. The plasmid bearing the *GAL1*-promoter regulated *CSE4* was introduced into wild-type and 93 yeast degradation mutants separately (Fig. 2A). Following the initial screen, seven mutants including *psh1* Δ were confirmed to show Cse4-induced slower growth by the serial spotting assay (Fig. 2B). It is worth noting that *PSH1* and *DOA1*, both known to promote Cse4 destruction (11, 12, 14), were recovered in the screen, validating our screening approach. *Doa1*, also called Ufd3, is involved in ubiquitin homeostasis albeit the underlying mechanism remains enigmatic (14, 25–28).

Next, we compared the degradation kinetics of Cse4 in wild-type and these mutants by an expression shut-off assay. Consistent with previous studies, Cse4 degradation is compromised in yeast cells missing *PSH1* or *DOA1* (11, 12, 14, 15). Four isolated mutants (*i.e.* *vps18* Δ , *doa4* Δ , *ufo1* Δ , *ubx7* Δ) did not exhibit Cse4 stabilization (Fig. 2C), indicating that they do not directly promote Cse4 turnover. The mechanisms underlying the Cse4-triggered toxicity in these mutants are unknown, but these genes may possibly be involved in a Cse4-related event indirectly.

Interestingly, Cse4 degradation is impaired in the *rcy1* Δ mutant (Fig. 2C). Rcy1 contains a F-box domain and can be

A Proteolytic Role of Rcy1 in Cse4 Regulation

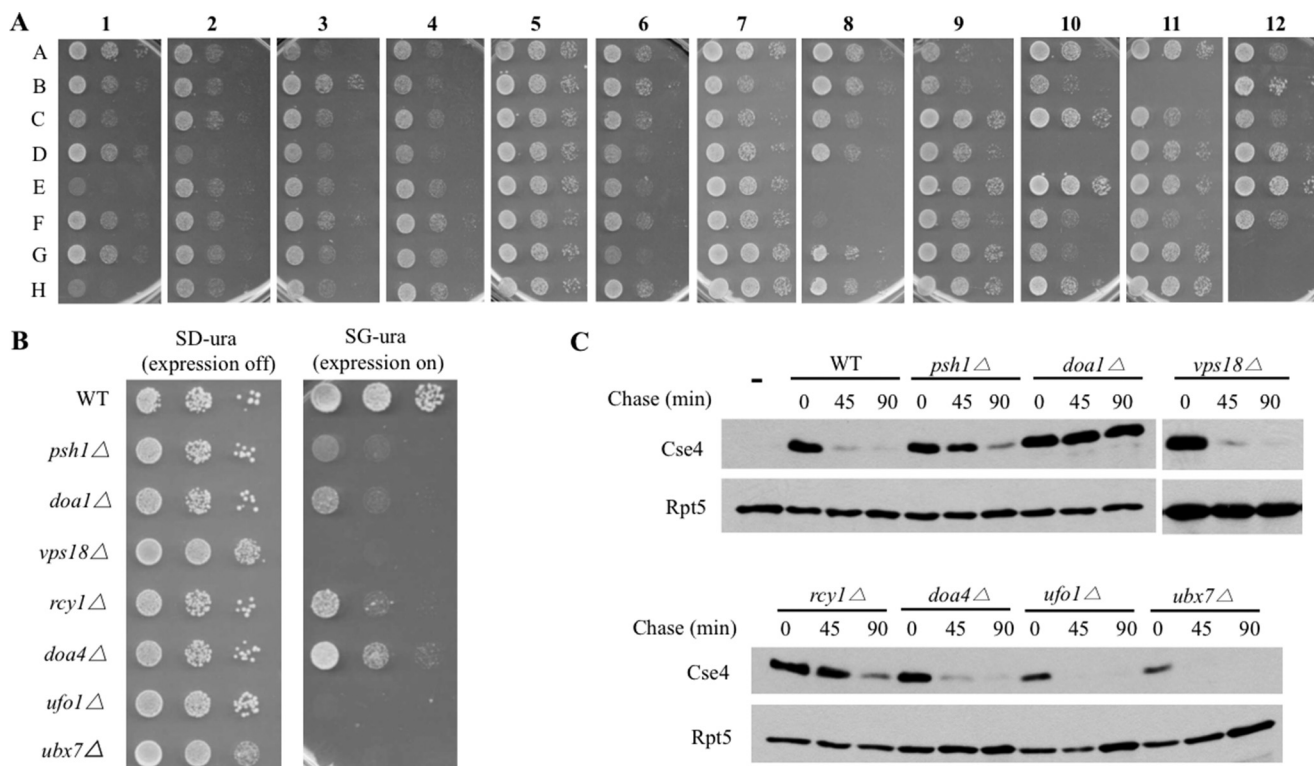


FIGURE 2. Identification of Rcy1 as a regulator of Cse4 turnover. *A*, isolation of yeast mutants sensitive to Cse4 overexpression. The plasmid bearing the *GAL1*-promoter-regulated Cse4 was transformed to wild-type and 93 mutants deficient in various ubiquitin pathways. Transformants were grown to the same density and spotted onto galactose-containing SG plates in 10-fold serial dilution. The labels (e.g. A–H, 1–12) indicate the corresponding positions of yeast mutants in the original 96-well plate provided by Dr. M. Hochstrasser. *B*, a spotting assay indicates Cse4 overexpression causes growth retardation in 7 mutants. The spotting assay was done as described above except yeast cells were spotted onto both glucose (*SD*, expression off) and galactose (*SG*, expression on) plates. The identities of these mutants are listed to the left of the panels. *C*, efficient Cse4 degradation requires Psh1, Rcy1, and Doa1. Cse4 turnover was determined as described in the legend to Fig. 1.

assembled into a Skp1-Cdc53-Rcy1 complex with demonstrated ubiquitin ligase activity (19). *RCY1* was originally identified in a screen for genes involved in an endocytic process and was shown to regulate protein trafficking from endosomes to the Golgi network (20, 21, 29, 30). Although yeast Rcy1 was shown to regulate Snc1 ubiquitylation (21) and the *Aspergillus nidulans* Rcy1 homologue promotes kinesin KipA degradation (31), the physiologic function of Rcy1 in the ubiquitin-proteasome pathway remains elusive. It is possible that the Rcy1-mediated protein trafficking may indirectly affect Cse4 turnover. To determine the specificity of Rcy1 in Cse4 regulation, we evaluated Cse4 degradation in yeast cells lacking Ypt31 and Ypt32, two GTPases that work with Rcy1 in the endosome-Golgi transport (21, 30, 32). Interestingly, Cse4 did not elicit toxicity and was not stabilized in *ypt31Δ* or *ypt32Δ* cells (Fig. 3, *A* and *B*). Moreover, Cse4 was unaffected in cells missing the SNARE Snc1 (Fig. 3, *A* and *B*), which interacts with Rcy1 and regulates protein trafficking (21). Combined, the data suggest that the role of Rcy1 in Cse4 regulation is likely separate from its involvement in protein trafficking outside of nucleus. Histone H3 was previously shown to be ubiquitylated and degraded by the ubiquitin ligase Tom1 pathway (23). We found that histone H3 degradation was compromised in *tom1Δ* (Fig. 3*C*) as expected (23), but was little affected in *rcy1Δ* cells (Fig. 3*C*), suggesting the specific involvement of Rcy1 in Cse4 turnover. Although Tom1 regulates histone H3, Tom1 was not involved in Cse4 turnover (Fig. 3*D*).

As the F-box protein is the substrate-recognition component of SCF E3s (9), we examined whether Cse4 associates with Rcy1 in yeast by the co-immunoprecipitation experiment (Fig. 4*A*). We found that endogenous Rcy1 immunoprecipitates Cse4 (Fig. 4*A*), supporting a close link between Rcy1 and Cse4 *in vivo*. Furthermore, we also carried out the *in vitro* binding experiment. Cse4 purified from *E. coli* can interact with Rcy1 (Fig. 4*B*), suggesting that Rcy1 recognizes Cse4. Consistent with the role of Rcy1 in Cse4 ubiquitylation, Cse4 ubiquitylation was reduced in *rcy1Δ* cells (Fig. 4, *C* and *D*). We also evaluated whether other SCF components are involved. We found that Cse4 interacts with Cdc53, a core component of SCF E3, *in vivo* and *in vitro* (Fig. 4, *E* and *F*).

Because both Psh1 and Rcy1 promote Cse4 destruction, we wondered whether they work the same or different pathways. To determine the functional relationship between *PSH1* and *RCY1*, we assessed the degradation of Cse4 in cells lacking either or both *RCY1* and *PSH1*. Consistent with earlier studies, deletion of *PSH1* slightly stabilized Cse4 (11, 12, 14, 15). *RCY1* deletion appeared to cause stronger Cse4 stabilization (Fig. 5, *A* and *B*). Interestingly, the double mutant *psh1Δ rcy1Δ* showed more compromised Cse4 degradation than single mutants, suggesting that Psh1 and Rcy1 likely work in parallel pathways in Cse4 regulation.

As Cse4 plays a critical role in chromosome segregation, altered Cse4 regulation may affect genome stability (5). To determine whether Rcy1 may regulate genome maintenance,

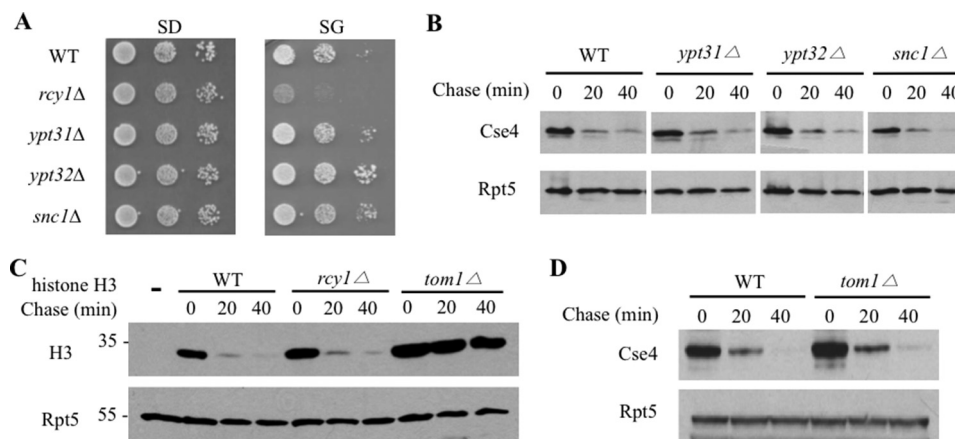


FIGURE 3. **Specific involvement of Rcy1 in Cse4 regulation.** *A*, Cse4 expression is not toxic in other protein trafficking mutants. The spotting assay was done as described in the legend to Fig. 2*B*. *B*, Cse4 is efficiently degraded in yeast cells deficient in protein transport. Cse4 stability in *ypt31Δ*, *ypt32Δ*, and *snc1Δ* was determined as described in the legend to Fig. 2*C*. *C*, histone H3 turnover is not affected in *rcy1Δ* cells. The empty vector (*lane 1*) or the plasmid expressing HA-tagged histone H3 was transformed into the indicated yeast cells. As the ubiquitin ligase Tom1 is known to be involved in histone H3 turnover, *tom1Δ* cell serves as a positive control. *D*, Tom1 is not involved in Cse4 degradation. Cse4 turnover in wild-type or *tom1Δ* was determined as described in the legend to Fig. 2*C*.

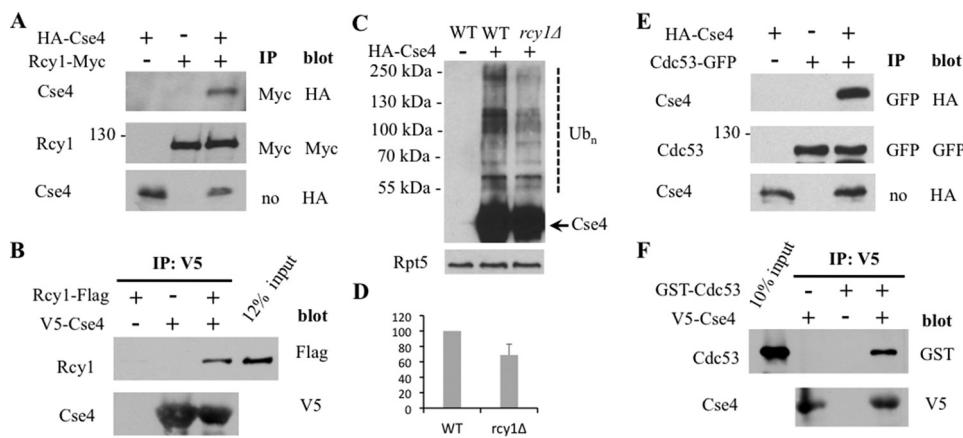


FIGURE 4. **Cse4 interacts with Rcy1 and Cdc53.** *A*, Cse4 binds endogenous Rcy1 *in vivo*. The plasmid bearing HA-Cse4 or a control vector was transformed into yeast cells expressing Rcy1-tagged with the Myc epitope at its chromosomal locus. Proteins were extracted from the cells indicated and incubated with the protein G beads coated with Myc antibody (BioLegend, MMS150P). Samples were resolved by SDS-PAGE and visualized by Western blotting using Myc or HA antibody as indicated. Cse4 levels in the extract were also determined. The identity of the bands is shown on the left of the panels. The antibodies for immunoprecipitation (IP) and Western blotting are indicated to the right. *B*, Cse4 interacts with Rcy1 *in vitro*. The V5 antibody (ThermoFisher Scientific, R960-25)-coated beads were mixed with V5-tagged Cse4 expressed in *E. coli* and yeast extracts with or without FLAG-tagged Rcy1 at 4 °C. The bound proteins were resolved by SDS-PAGE and immunoblotted with antibody to FLAG (*upper panel*; Sigma, F1804) or V5 (*lower panel*). *Lane 4* contains ~12% input extract expressing FLAG-Rcy1. *C*, reduced Cse4 ubiquitylation in *rcy1Δ* cells. The experiment was carried out in a similar previously described procedure (12, 37). Briefly, wild-type or *rcy1Δ* cells bearing a vector plasmid or a plasmid expressing Cse4 tagged with both HA and His₆ epitopes were grown in galactose media. Cse4 was enriched by incubating extracts with nickel-nitrilotriacetic acid-agarose. Cse4 and its ubiquitin conjugates were detected by immunoblotting with anti-HA. Ubiquitylated and unmodified Cse4 are indicated by a dashed line and an arrow, respectively. Rpt5 was employed as a loading control. *D*, quantification of the data in *C*. Values are derived from three independent experiments and SD is shown. *p* value is 0.062. *E*, Cse4 associates with Cdc53 *in vivo*. The experiment was carried out as described in the legend to Fig. 4*A* except the yeast strain bearing Cdc53 tagged with GFP at its chromosomal locus was used. *F*, Cse4 binds Cdc53 *in vitro*. Similar to the experiment described in Fig. 4*B*, Cse4 purified from *E. coli* was mixed with yeast extracts containing GST-tagged Cdc53. *Lane 1* contains 10% input extract expressing GST-Cdc53.

we employed a colony sectoring assay to compare the rate of chromosome loss in wild-type and *rcy1Δ* cells (Fig. 6*A*) (17). These cells contain a non-essential chromosome fragment bearing the *SUP11* gene, which can suppress the *ade2-101* mutation-induced red pigment accumulation. As expected, wild-type cells can efficiently maintain the non-essential *SUP11*-bearing chromosome, leading to few cells (less than 2%) with red sectors. In contrast, *RCY1* deletion leads to a significant increase of red sectors in *rcy1Δ* cells (~5%) (Fig. 6*A*), suggesting enhanced chromosome instability in *rcy1Δ* cells, a phenotype that is consistent with the *RCY1-CSE4* link.

We noticed that *rcy1Δ* cells grow slower than wild-type cells at room temperature. Indeed, upon closer examination, we found that *rcy1Δ* cells are sensitive to cold temperature (Fig.

6*B*). In line with temperature sensitivity, a drastic increase of chromosome loss was observed in *rcy1Δ* cells (~40%) that were grown at room temperature (data not shown).

Discussion

Here we identify another degradation component, Rcy1, involved in Cse4 regulation. Rcy1 partnered with Skp1 and Cdc53 exhibits the ubiquitin E3 ligase activity (19). Rcy1 was previously known to promote intracellular trafficking from the endosome to the Golgi (20, 21, 29, 30), an endocytic recycling pathway important for the localization of several proteins including a GTPase-activating protein Bem3, a Golgi resident Kex2 protease, and a SNARE protein Snc1 (21, 30, 33). Without *RCY1*, Snc1 ubiquitylation was impaired, leading to its entrap-

A Proteolytic Role of Rcy1 in Cse4 Regulation

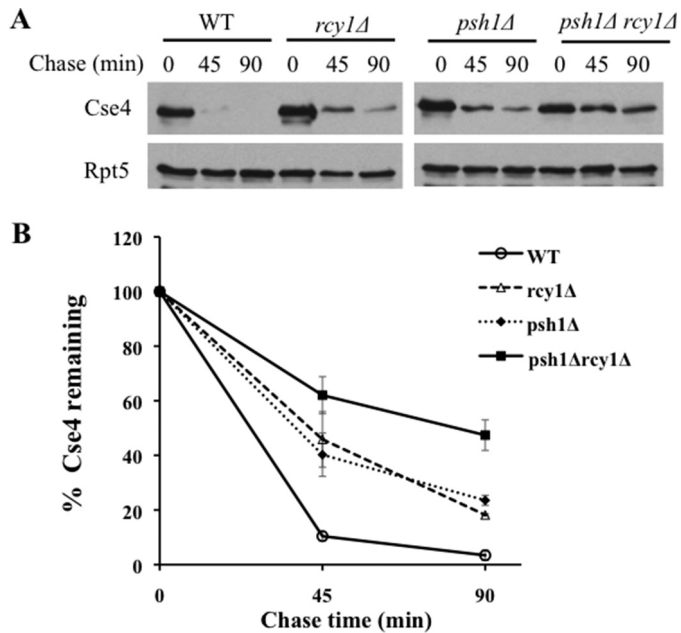


FIGURE 5. Stronger stabilization of Cse4 in *rcy1Δ psh1Δ* double mutant cells. *A*, Cse4 degradation in wild-type, *rcy1Δ*, *psh1Δ*, *rcy1Δ psh1Δ* cells was determined as described above. *B*, quantitation of the data in *A*. The experiments were done more than 3 times, and the average values with S.D. are shown.

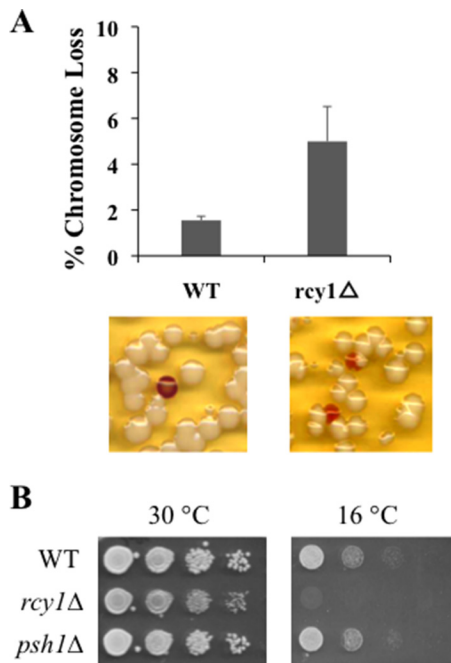


FIGURE 6. Phenotypes associated with *rcy1Δ* mutant. *A*, increased chromosome loss in *rcy1Δ* mutant cells. Sectoring assay was done as described (17). Actively growing wild-type or *rcy1Δ* mutant cells were plated onto YPD plates and incubated for 48 h at 30 °C. Quantification of three independent experiments is shown in the upper panel (*p* value 0.023), and representative images from the sectoring assay are shown in lower panels. *B*, *rcy1Δ* mutant is cold-sensitive. Mid-log phase yeast cultures were spotted onto the plates in serial 10-fold dilution and incubated at either normal temperature, 30 °C, or 16 °C as indicated. The genotypes of the strains are shown on the left of the panels.

ment on endosomes (21, 30). Nevertheless, the physiological functions of RCY1 remain far from clear. We found that Rcy1 is specifically involved in the degradation of Cse4, but not histone H3. Our study expands the function of Rcy1 in proteolysis, dis-

tinct from its involvement in protein trafficking, and reveals new phenotypes associated with the *RCY1* deletion (Fig. 3).

Cse4/CENP-A is crucial to establish a specialized chromatin structure at the centromere (1, 2), the site of kinetochore assembly, which is key to chromosome segregation that allows proper distribution of duplicated genetic information to daughter cells. Excessive or nonrestraint Cse4 can lead to its occupancy at the euchromatic region, which may cause aneuploidy and promote tumorigenesis (1, 5, 6, 11, 12). The discovery of enhanced chromosome instability in *rcy1Δ* cells suggest that altered regulation of the Rcy1-Cse4 pathway may be involved in cancer.

Doa1, but not Rcy1, was isolated in a similar synthetic dosage lethality screen employed to identify potential regulators of Cse4 (5). Whereas the early screen scanned the entire set of yeast deletion mutants available (>4000) (5), we probed a selected group of mutants linked to ubiquitin-mediated proteolysis. Our targeted approach allowed a more careful examination of mutant phenotypes. As Doa1 regulates intracellular levels of ubiquitin in general, Doa1 affects ubiquitin-mediated processes much more broadly than Rcy1 (14, 25–27). Thus, it is not surprising that Cse4 turnover is more severely compromised in *doa1Δ* than in *rcy1Δ*.

Our results suggest that Psh1 and Rcy1 likely work in parallel pathways to tune down the Cse4 level (Fig. 5, *A* and *B*). Because Cse4 remains partially degraded in *psh1Δ rcy1Δ* double mutant cells, other E3s involved in Cse4 degradation exist. It is not unusual for a protein targeted for degradation by multiple E3s. For example, histone H3 degradation has been shown to involve five E3s including Tom1, Snt2, Pep5, Hel1, and Hel2 (34). A more “extreme” case is the p53 tumor suppressor, for which more than 15 E3 enzymes have been implicated in its turnover under various conditions (35). The employment of multiple E3s in substrate degradation allows more regulation. Identification of additional E3s involved in the degradation of Cse4, especially its human homolog CENP-A, remains a future challenge.

Besides ubiquitin-mediated proteolysis, a variety of approaches are adopted to restrain Cse4 activity with the participations of casein kinase 2, the proline isomerase Fpr3, the H3 chaperones HIR and CAF-1, and several chromatin modulators (e.g. SWI/SNF remodeling enzymes, the FACT complex) (15, 36–39). Unraveling the functional cross-talks among these factors/pathways will be key to revealing the molecular basis underlying Cse4 regulation. Given the intimate tie between CENP-A and cancer, the insights gained on Cse4 may shed light into elusive principles governing human CENP-A regulation and potential strategies against CENP-A-triggered diseases.

Author Contributions—H. C. designed and carried out most of the experiments described. X. B. performed the experiments in Fig. 4. H. R. designed the experiments. All three authors were involved in data analysis and manuscript preparation.

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