Gcn5-mediated Rph1 acetylation regulates its autophagic degradation under DNA damage stress

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ABSTRACT

Histone modifiers regulate proper cellular activities in response to various environmental stress by modulating gene expression. In budding yeast, Rph1 transcriptionally represses many DNA damage or autophagy-related gene expression. However, little is known how Rph1 is regulated during these stress conditions. Here, we report that Rph1 is degraded upon DNA damage stress conditions. Notably, this degradation occurs via the autophagy pathway rather than through 26S proteasome proteolysis. Deletion of *ATG* genes or inhibition of vacuole protease activity compromises Rph1 turnover. We also determine that Rph1 and nuclear export protein Crm1 interact, which is required for Rph1 translocation from the nucleus to the cytoplasm. More importantly, Gcn5 directly acetylates Rph1 *in vitro* and *in vivo*, and Gcn5-containing complex, SAGA, is required for autophagic degradation of Rph1. Gcn5-mediated Rph1 acetylation is essential for the association of Rph1 with the nuclear pore protein Nup1. Finally, we show that sustaining high levels of Rph1 during DNA damage stress results in cell growth defects. Thus, we propose that Gcn5-mediated acetylation finely regulates Rph1 protein level and that autophagic degradation of Rph1 is important for cell homeostasis. Our findings may provide a general connection between DNA damage, protein acetylation and autophagy.

INTRODUCTION

Eukaryotic cells are constantly subjected to diverse forms of DNA damage (1,2). DNA lesions lead to genetic errors, and failure to repair DNA damage will cause severe genome instability and cell death (3). Proper cellular responses, such as alteration of chromatin architecture, activation of transcription factors and recruitment of DNA repair machinery, are required for maintaining homeostasis (4). Chromatin functions in regulating the cellular response to DNA damage (4). In the context of chromatin, histones and histone modifications contribute to DNA damage response (DDR) and repair (2,5). Transcription-associated histone modifications, including H3 lysine 4 methylation (H3K4me) and H3 lysine 79 methylation (H3K79me) have been reported to participate in DDR and repair (4). Phosphorylation of histone H2A serine 129 (γH2AX) occurs at an early stage in DDR, and is required for DNA damage signal amplification and the accumulation of many DDR proteins at DNA lesion sites (6). Recently, an increasing line of evidence has indicated that histone H3 lysine 36 tri-methylation (H3K36me3) and its methyltransferase Set2 play essential roles in checkpoint activation and DNA repair, from yeast to humans, reinforcing the importance of dynamic chromatin regulation in genome integrity (7–10).

The JmjC-domain-containing protein Rph1 has been characterized as a demethylase that catalyzes the removal of H3K36 di- and tri-methylation in budding yeast (11–13). Rph1 contains a JmjN domain in the N-terminus, which is required for its demethylase activity, and a zinc finger (ZF) domain in the C-terminus, which is responsible for DNA binding (11). In contrast, no *in vivo* demethylase activity has been observed for Gis1, a paralog of Rph1Rph1 (11,12). Rph1 and Gis1 were originally identified as repressors of the DNA repair gene *PHR1*, suggesting that they might be important mediators during DDR. Rph1 is considered as a major regulator of *PHR1* gene expression, because *RPH1* mRNA transcripts are approximately 3-fold more abundant than *GIS1* mRNA in yeast cells (14). Further study suggested that Rad53 kinase-catalyzed phosphorylation may be required for Rph1 dissociation from the *PHR1* promoter (14,15). Overexpression of Rph1 retards cell growth and increases sensitivity to UV irradiation, indicating its potential role in the DDR signaling pathway (11,13,16). Microarray analyses showed that the mRNA levels of approximately 70% genes were upregulated in cells deleted with *RPH1*, in which a group of stress-response genes primarily emerged,

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including DNA damage, environmental and oxidative stress genes (15,17). Recently, Rph1 has been unveiled as a master transcriptional repressor in regulating autophagy and environmental stress through binding the gene promoter regions of individuals (15,18). Upon nitrogen starvation or DNA damage stress, phosphorylation of Rph1 by Rim15 or Rad53, respectively, prevented Rph1's repressive role on gene expression (15,18). However, the regulation and cellular fate of Rph1 under stress conditions are not understood.

In this paper, we show that endogenous Rph1 is degraded upon methyl methanesulfonate (MMS) treatment or UV irradiation. Rph1 degradation is mediated by autophagic pathway instead of the 26S proteasome. Moreover, acetyltransferase Gcn5 acetylates Rph1 *in vivo* and *in vitro*. Gcn5-mediated Rph1 acetylation is required for its nuclear export and autophagic degradation under DNA damage stress conditions. We conclude that tight control of Rph1 protein levels by acetylation-dependent autophagic degradation is essential for response to DNA damage so that cellular homeostasis is well maintained.

MATERIALS AND METHODS

Yeast strains and plasmids

The yeast strains and plasmids used in this study are listed in Supplementary Tables S1 and S2. Gene disruptions or insertion with an integrated tag were performed as described previously (19). More detailed information for plasmids and strains can be found in the Supplementary Data.

Cell cultures and drug treatment

Yeast cells were grown to OD_{600} 0.6–0.8 at 30°C in YPD medium or synthetic complete medium with necessary additives unless otherwise indicated. Yeast cells were usually treated with certain concentrations of MMS or indicated drugs for 2 h unless otherwise indicated. Cells were 5-fold serially diluted and then spotted onto SC-Leu or SC-His plates with indicated drugs or exposure to UV irradiation. Plates were incubated at 30°C and imaged at 2–3 days.

Gene expression and chromatin immunoprecipitation (ChIP) analysis

The yeast cells were extracted using TRIzol (Invitrogen) and further purified via chloroform extraction methods. After drying, an aliquot of 1 μg RNA was subjected to reverse transcription using the cDNA synthesis kit (with genomic DNA removal, Tiangen Company) according to the manufacturer's protocol. The ChIP assays were performed as described previously using 10 μl IgG Sepharose beads or α-FLAG M2 resin for each sample. Quantitative PCR was performed as previously described (20), and the primer sets are listed in Supplementary Table S3.

Microscopy analysis

Yeast cells were cultured to mid-log phase. After washed with cold phosphate buffered saline once, the cells were re-suspended in phosphate buffered saline. An Olympus BX51 microscope (Tokyo, Japan) and a Retiga 2000R CCD camera (QImaging Corporation, Canada) were used to visualize cell morphology by differential interference contrast and fluorescent microscopy. A total of 100x oil immersion objective was used. A 150-ms exposure time for tdTomato and FM4-64, and a 250-ms exposure time for Green Fluorescent Protein (GFP) were fixed in all experiments. Images were acquired using QCapture Suite (QImaging Corporation, Canada). The merged color Images were generated by Photoshop CS5.

FM4-64 staining

Yeast cells were grown to an OD_{600} of 0.6–0.8 in SC-Ura medium at 30°C. Two-milliliter cultures were collected and cell pellets were re-suspended in 100 μl YPD with 1 μl FM4-64 stock (1.6 mM, Santa Cruz) dissolved in Dimethyl Sulphoxide (DMSO). Cells were kept in the dark and incubated at 30°C for 30 min. Free FM4-64 dye in cultures were washed out and cells were re-suspended in Yeast Peptone Dextrose (YPD) and were shaken at 30°C for 1 h. An aliquot of 5 μl cells were dropped onto glass slides and the fluorescent signals were observed using the Olympus Microscope.

TAP purification and *in vitro* acetyltransferase assay

Gcn5-TAP purifications were performed as described previously (20). The *in vitro* acetyltransferase assays were performed at 30°C for 6 h using 1 μg recombinant GST-Rph1 protein incubated with or without 100 ng Gcn5-TAP eluates or 2 mg bacterial purified yAda3Δ2HIS-yAda2Δ1-Gcn5 complex in the presence of 5 μCi radioactive ^{3}H-labeled acetyl-CoA (Perkin Elmer) in buffer (200 mM NaCl, 200 mM Tris-HCl at pH 8.0, 0.4 mM ethylenediaminetetraacetic acid at pH 8.0, 20% glycerol, 40 mM sodium butyrate, 10 mM fresh Dithiotheritol (DTT)). A half volume of each reaction (25 μl) was used to conduct the liquid scintillation counting assay described previously by a liquid scintillation analyzer (Tri-carb 2910TR, PerkinElmer) (21). The other half volume of each sample was quenched with an equal volume of 2x sodium dodecyl sulphate sample buffer and was loaded onto an sodium dodecyl sulphate-polyacrylamide gel electrophoresis gel. After Coomassie blue staining, the gel was exposed to X-ray film at −80°C freezer for a month. The radioactive ^{3}H-acetyl signals were detected by autography.

Quantification and statistical analysis

For quantification of the western blot data, Image J software was used to measure the relative intensity of each band, and the relative Rph1 protein levels were normalized to the relative G6PDH levels. Quantification data were presented as the mean ± SD (standard deviation) from at least three independent experiments. Statistical differences were determined by two-tailed unpaired *t*-test, and a *P*-value of <0.05 was considered statistically significant and marked as '*', a *P* value < 0.01, 0.001 or 1×10^{-4} was marked as '**', '***', '****', respectively. ' ns' indicates 'not significant'.

RESULTS

Rph1 protein was degraded under DNA damage stress conditions

To investigate if Rph1 is regulated under DNA damage stress conditions, the changes in Rph1 in both transcript levels and protein levels upon MMS treatment were determined. To directly monitor the endogenous protein levels of Rph1 and Set2, rabbit α-Rph1 and α-Set2 polyclonal antibodies were generated. The specificity of these antibodies were examined using the *rph1Δ* and *set2Δ* strains (Supplementary Figure S1A and B). Although *RPH1* transcript levels significantly increased (see Figure 7D), Rph1 protein was degraded in cells treated with 0.1% MMS for 2 h. In contrast, histone H3K36 methyltransferase Set2 protein levels were unchanged in the same experimental condition (Figure 1A). The addition of the translational inhibitor cycloheximide and MMS shortened the half-life of Rph1 protein from ∼60 min to ∼30 min, suggesting that endogenous Rph1 is unstable in this stress condition (Figure 1B). To rule out the possibility that degradation of Rph1 only occurred upon MMS treatment, the status of Rph1 protein was examined under various stress conditions. Rph1 was significantly degraded under UV irradiation following different recovery times (1 h or 2 h). However, Rph1 levels were unchanged in the presence of hydrogen peroxide (H_2O_2, an oxidative stress reagent), or hydroxyurea (HU, a DNA synthesis inhibitor) or doxycycline (DOX, an inhibitor of matrix metalloprotease), which is likely due to the failure of activating the DDR pathway as judged by the intensities of γH2AX (Figure 1C and Supplementary Figure S2A). Impairment of the DDR pathway by adding the histone deacetylase inhibitor valproic acid counteracted the H2AX phosphorylation and Rad53 activation and also hampered Rph1 degradation even in the presence of MMS ((22) and Figure 1D, supplementary Figure S2B). Overall, our results support the idea that Rph1 protein must be degraded when exposed to genotoxic stress.

Degradation of Rph1 upon DNA damage was mediated by the vacuole

Two main degradation systems are utilized by the eukaryotic cell to regulate protein stability: the proteasome and the lysosome (the counterpart in *S. cerevisiae* is the vacuole) (23). To explore which degradation pathway controls Rph1 turnover in response to DNA damage, a 26S proteasome inhibitor, MG132, was added to cells in combination with MMS treatment or UV irradiation. Surprisingly, MG132 treatment restored Gis1 protein levels but did not inhibit Rph1 degradation (Figure 2A, B and F). Accordingly, the abundance of Rph1 treated with cycloheximide in a temperature-sensitive proteasome-deficient strain (*cim3-1*) at the non-permissive temperature still showed decreased stability, whereas Set2 protein was stable under the same conditions (Figure 2C and D). Consistent with our results, Gis1 and Set2 have been reported as well-characterized substrates of the 26S proteasome (24,25). These data suggested that the mechanism controlling Rph1 protein turnover differs from that of Gis1 and Set2, which suggests that Rph1 protein degradation is likely mediated by autophagy.

Autophagy is a lysosome-dependent (vacuole-dependent in yeast) cellular catabolic process that degrades cell components, toxic proteins and damaged organelles to maintain cell homeostasis, especially when cells in certain stresses (26). Autophagy is mainly regulated by a step-wise process including the formation of autophagosome (a double-membrane organelle), docking and fusion of autophagosome with lysosome/vacuole and proteases-mediated degradation of the cargos (27). A set of autophagy-related (ATG) proteins have been reported to function in different stages of autophagy (27,28). To test whether autophagy pathway regulates Rph1 degradation upon DNA damage stress, cells were treated with a protease inhibitor, Phenylmethanesulfonyl fluoride (PMSF), to block vacuolar proteolysis. Rph1 protein levels were restored by the addition of PMSF even in the presence of MMS or UV irradiation exposure (Figure 2E–G). Rapamycin, an mTOR kinase inhibitor, has been widely used to activate autophagy. Cells treated with rapamycin exhibited Rph1 degradation (Figure 2A, lane 5 and B, H). Interestingly, Rph1 degradation could not be attenuated by adding MG132 (Figure 2A, lane 6 and B), providing additional evidence that Rph1 degradation is not mediated by the 26S proteasome. Moreover, Rph1 protein levels diminished faster under MMS and rapamycin treatment than with MMS alone (Figure 2I). The degradation of Rph1 caused by rapamycin treatment could be rescued by the addition of PMSF (Figure 2H). Thus, these results indicated that Rph1 degradation upon DNA damage stress is mediated by the vacuole, but not by the 26S proteasome.

MMS-induced Rph1 degradation was regulated by Crm1-mediated nuclear export

It is known that Rph1 executes its function of transcriptional regulation in the nucleus. To be degraded by the vacuole, Rph1 must be shuttled to the cytoplasm. Thus, we hypothesized that an exportin protein carries Rph1 from the nucleus to the cytoplasm. In our work to identify the interacting proteins of Rph1 by immunoprecipitation of the endogenous Rph1 protein following LC-MS/MS analysis from an integrated 3xFlag-tagged Rph1 strain, we unexpectedly discovered that Crm1, a highly conserved and Ran GTPase-driven exportin, co-immunoprecipitated with Rph1 (Supplementary Figure S3A and B). Reciprocal co-immunoprecipitation results further confirmed that endogenous Rph1 protein interacts with endogenous Crm1 protein *in vivo* (Figure 3A). Next, we want to test whether *CRM1* deletion would attenuate the degradation of Rph1 after MMS treatment. As *CRM1* is essential to cells, we took advantage of the strain bearing a *Tet*-titratable promoter-driven *CRM1* gene (*crm1-tet*). *CRM1* gene expression was shut off by adding doxycycline (Dox) just before treating cells with MMS (Figure 3C). As shown in Figure 3B, shut-down of *CRM1* expression impeded the MMS-induced degradation of Rph1, indicating that the export of Rph1 from the nucleus to the cytoplasm is a prerequisite for its destruction upon DNA damage stress.

To determine the localization of Rph1 in different environmental conditions, we took advantage of a strain deleting *PEP4* gene, which encodes a major vacuolar protease, as
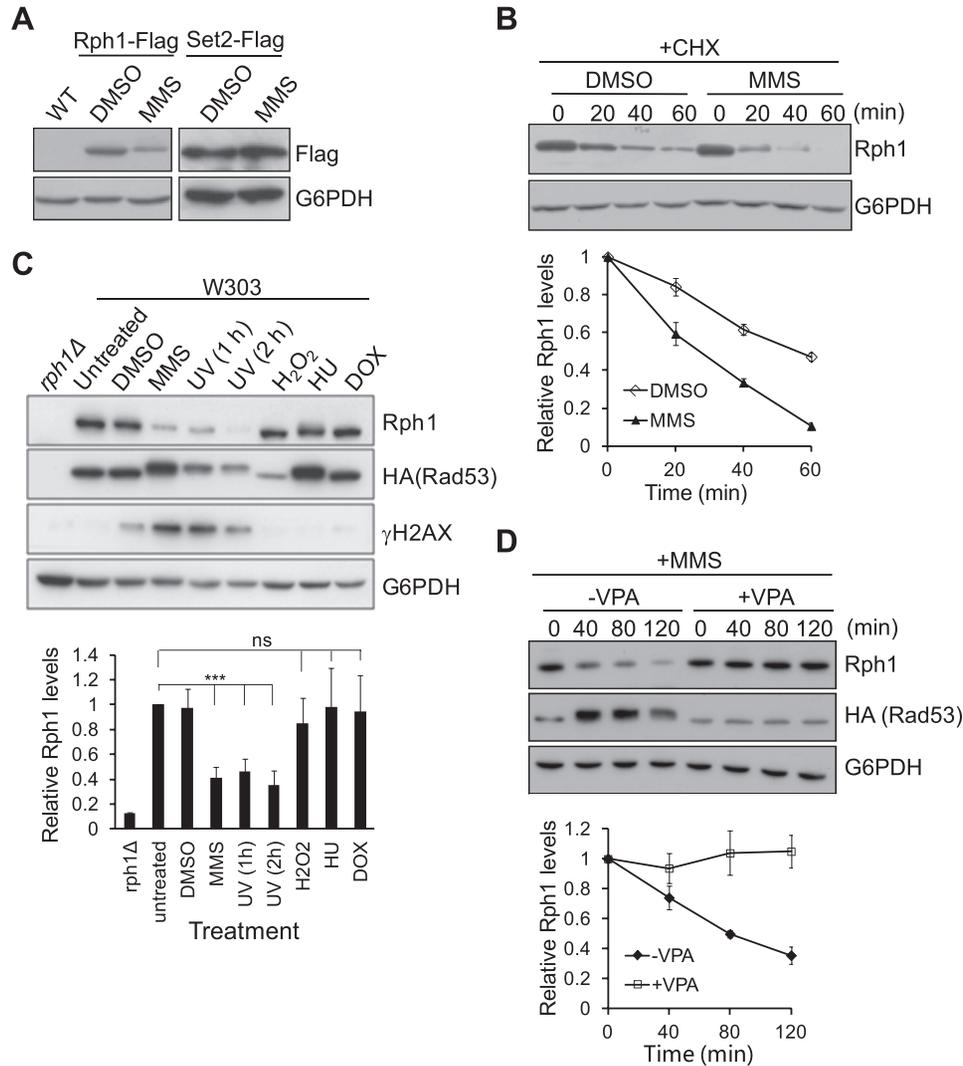
Figure 1. Rph1 protein was degraded under DNA damage stress conditions. (**A**) Strains with integrated 3xFlag-Rph1 or Set2 were treated with DMSO or methyl methanesulfonate (MMS). Rph1 or Set2 protein levels were examined using α-Flag antibody. (**B**) In the presence of DMSO or MMS, Rph1 protein stability was examined by a time course experiment with addition of cycloheximide (CHX), probing with α-Rph1 antibody. (**C**) The W303-strain cells were treated with the indicated DNA damage reagents or exposed to 100 mJ/cm^2 UV irradiation following cell recovery for 1 h or 2 h. Western blotting analysis was performed by probing with the indicated antibodies. (**D**) In the presence of MMS, Rph1 and Rad53 protein levels were examined by a time course experiment ± valproic acid (VPA). (B–D) Representative data were shown (top), and relative Rph1 abundance compared with the internal control protein G6PDH was plotted with the indicated time points by densitometric western blot analysis using Image J software (bottom). The error bars were generated by a standard deviation (SD) form three biological repeats.

the background strain to perform all the following fluorescent experiments (29). In addition, given the undetectable signals of endogenous Rph1, we constructed an endogenous promoter-driven Rph1 plasmid with a C-terminal tandem 2xGFP-tag (Rph1-2xGFP). We have confirmed that, under an MMS-untreated condition, cells expressing Rph1-2xGFP exhibited a relatively normal cell growth phenotype, and GFP signals are only detected in the nucleus, which indicated that exogenous expression of this native promoter-driven plasmid does not produce deleterious effects on cells (data not shown). Using this optimized system, *pep4Δ* cells or *pep4Δcrm1-tet* double mutant cells exogenously expressing Rph1-2xGFP were treated with DMSO or MMS in the presence of Dox. Rph1-2xGFP signals were examined by

western blot analysis or by the fluorescence microscopy. The integrated tdTomato-tagged histone H2A (red) represented the nuclear localization. Under normal conditions, *pep4Δ* cells expressing Rph1-2xGFP showed GFP nuclear foci in the majority of yeast cells (91%). With 1-h MMS treatment, GFP signals were diffused into the cytoplasm significantly increased from ∼2% to 28%. In contrast, *pep4Δcrm1-tet* cells expressing Rph1-2xGFP did not display obvious GFP diffusion from the nucleus to the cytoplasm (Figure 3D–F). These results reflect the cytosolic turnover of Rph1, suggesting that Rph1 degradation is regulated by the vacuole, most likely through autophagy.
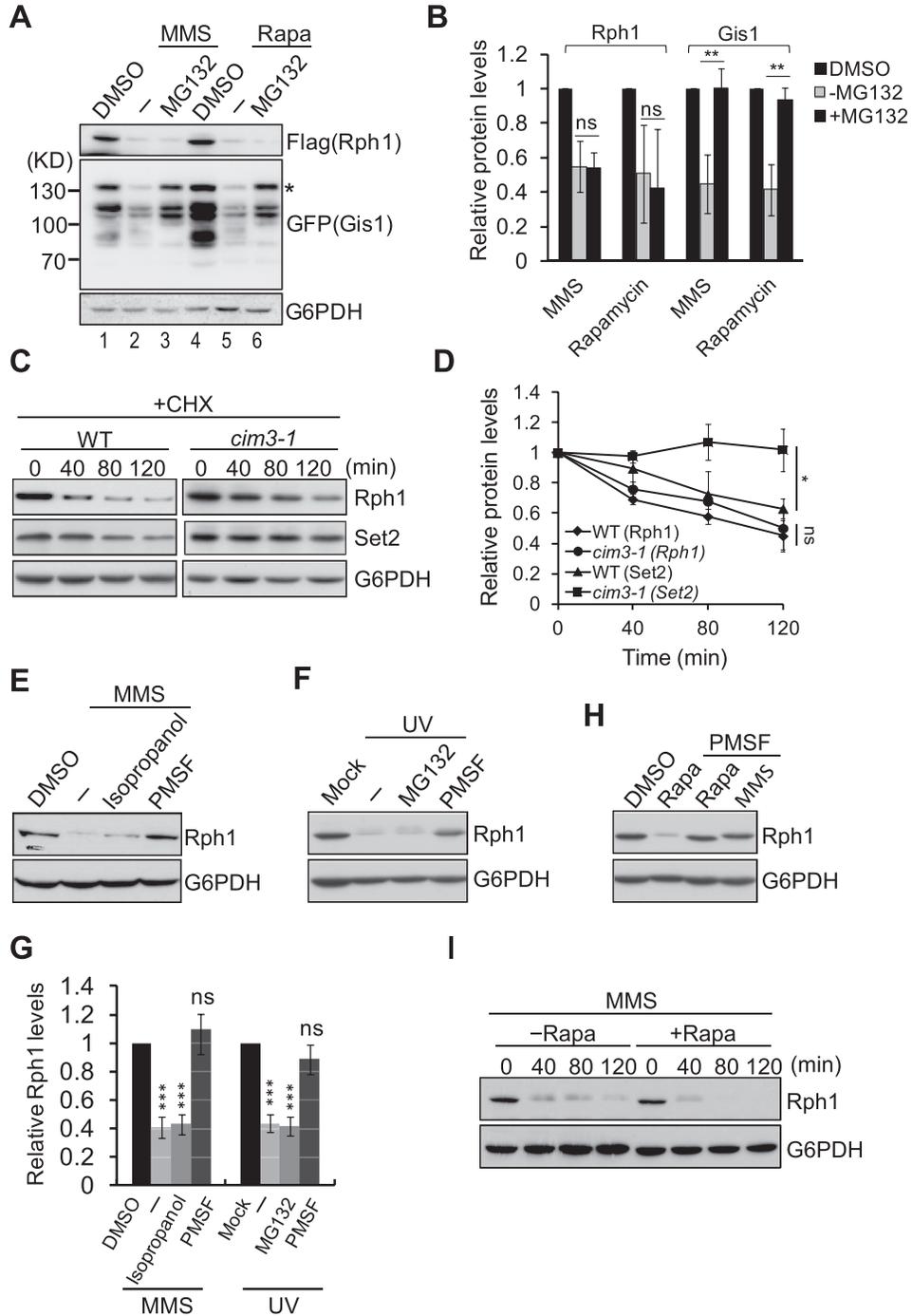
Figure 2. Degradation of Rph1 protein upon DNA damage was mediated by vacuole, but not 26S proteasome. (**A** and **B**) In the presence of MMS or rapamycin (Rapa), *pdr5Δ* cells integrated with 3xFlag-Rph1 were treated ± MG132. The protein levels of endogenous Rph1 or exogenous expressed GFP-Gis1 were immunoblotted with α-Flag or α-GFP antibodies, respectively. (**C** and **D**) Rph1 or Set2 protein levels in WT or a proteasome-deficient (*cim3-1*) strain grown at 37°C were immunoblotted with α-Rph1 antibody at the indicated time points in the presence of CHX. (**E–I**) Cells were treated with the indicated reagents, UV irradiation or combination. Rph1 protein levels were immunoblotted with an α-Rph1 antibody. Representative data were shown in panel A, C, E, F and relative Rph1 levels were quantified.
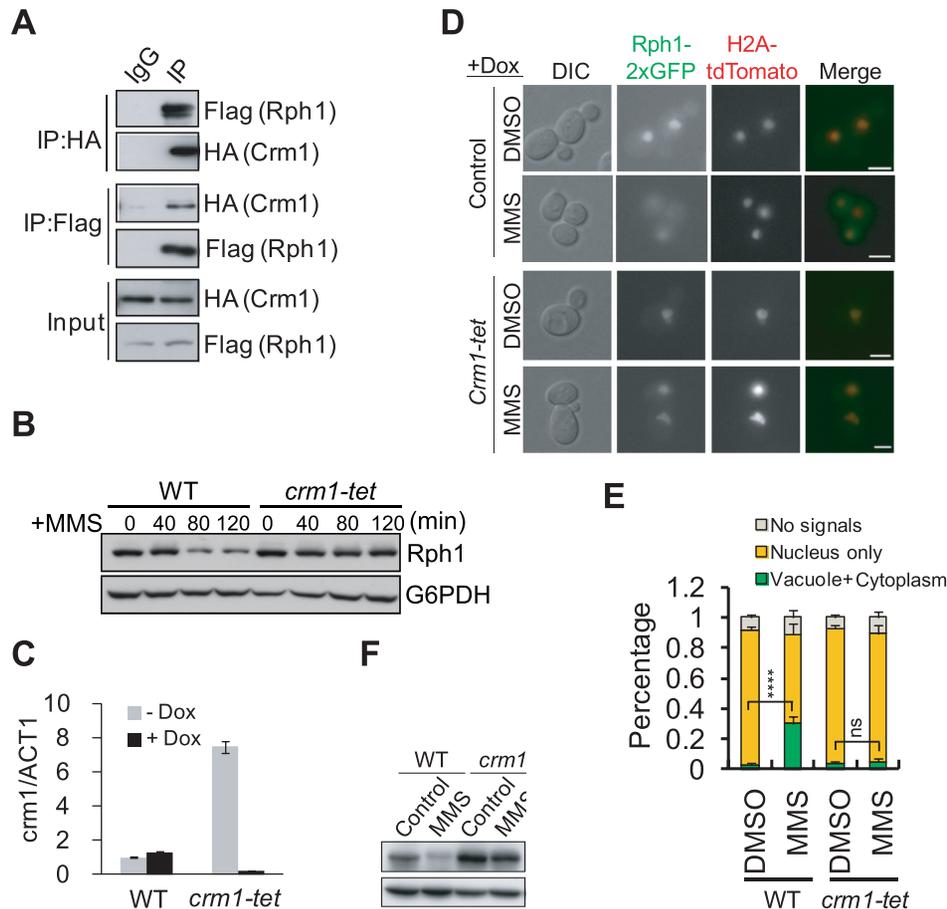
Figure 3. MMS-induced Rph1 degradation was regulated by Crm1-mediated nuclear export. (**A**) WT or *crm1-tet* cells were pre-treated with Dox before adding MMS. Rph1 protein levels were immunoblotted with α-Rph1 antibody at the indicated time points. (**B**) Relative *CRM1* mRNA levels were examined in WT or *crm1-tet* cells treated ± Dox. (**C**) Reciprocal co-IP assays were performed using α-HA resins or α-Flag M2 resins from cells integrated with C-terminal 3xFlag-Rph1 and 6xHA-Crm1. (**D**) Representative fluorescent images showed the distribution of Rph1-2xGFP (green) in WT cells treated as indicated. The H2A-tdTomato (red) represented the nuclear DNA. The cell morphology was shown by differential interference contrast. *Scale bar,* 5 μm. (**E**) Quantification of Rph1-2xGFP localization in cells displayed in (D). The bar graphs represent the percentages of cells exhibiting Rph1-GFP localized at vacuole and cytoplasm (green), nucleus only (yellow) and no GFP signals (gray). Data show mean±SD from at least three experiments, with ∼50 cells counted for each strain per experiment. (**F**) Protein expressions of GFP-tagged Rph1 in the indicated cells shown in (D) were immunoblotted with an α-Rph1 antibody.

MMS-induced Rph1 degradation was dependent on autophagy

Previous study indicated that DNA damage checkpoint signaling can stimulate autophagy (29). In agreement of this finding, we noticed that autophagy pathway was induced by the DNA damage reagent MMS, as free GFP moieties cleaved from overexpressed GFP-Atg8 proteins were produced by the vacuole in the wild-type (WT) cells, but not in the *ATG*s-deficient cells (Supplementary Figure S4A). Cells pretreated with autophagy inhibitors 3-methyladenine (3-MA) and chloroquine (CQ), prevented Rph1 turnover but did not affect the Rad53 checkpoint activation (Figure 4A). To determine whether ATG proteins regulates Rph1 degradation, we examined the status of Rph1 protein levels in *ATG* deletion strains following MMS treatment. In particular, deletion of *ATG2, ATG6, ATG8, ATG11* and *ATG12* genes significantly inhibited degradation of Rph1, whereas deletion of *ATG1* or *ATG7* showed a modest inhibition

(Supplementary Figure S4B and C). The half-life of Rph1 was prolonged at least 1.5-fold in *atg2Δ* and *atg12Δ* strains when compared to the WT strain (Figure 3C). This divergence suggested that different autophagy pathways may be involved in regulation of Rph1 degradation. In addition, deletion of *PEP4* gene, also dramatically compromised endogenous Rph1 protein turnover (Figure 4D and E). Furthermore, we utilized fluorescent imaging to observe the localization changes of Rph1 under the stress conditions. The red fluorescence from H2A-tdTomato or from FM4-64 dye staining represented a nucleus localization or the vacuolar membrane, respectively. Exogenous expression of Rph1-2xGFP in *pep4Δ* cells displayed increased vacuolar and cytoplasmic accumulations of GFP signals upon MMS treatment (Figure 4F–I). In agreement of previous study, deletion of the JmjN or ZF domain could not prevent Rph1 degradation with MMS treatment, indicating that demethylase activity and DNA-binding ability are not involved in regulating Rph1 stability (Supplementary Figure S4D and
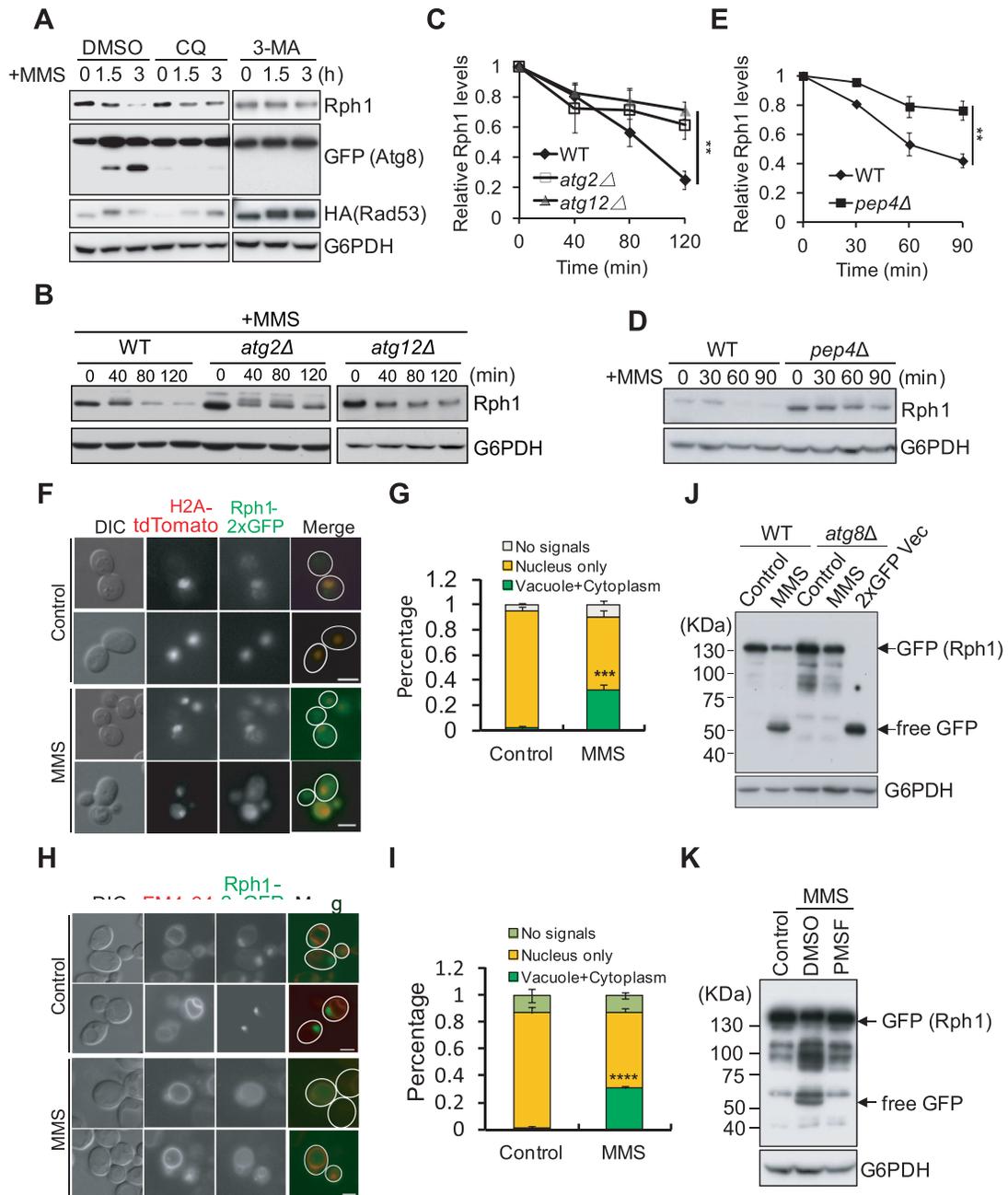
Figure 4. MMS-induced Rph1 degradation was mediated by autophagy. (**A**) Cells bearing integrated HA-tagged Rad53 and exogenous GFP-Atg8 were treated with DMSO or chloroquine (CQ) or 3-methyladenine (3-MA) ± MMS at the indicated time points. Cell extracts were immunoblotted with α-Rph1, α-GFP, α-HA and α-G6PDH (control) antibodies. (**B** or **D**) Rph1 protein levels in WT or autophagy-deficient (*atg2Δ*, *atg12Δ* or *pep4Δ*) cells were immunoblotted with an α-Rph1 antibody in the presence of MMS, and (**C** or **E**) relative Rph1 abundance was quantified. The error bars were generated by a standard deviation from three biological repeats. (**F** and **H**) Representative fluorescent images showed the distribution of Rph1-2xGFP (green) in *pep4Δ* cells ± MMS. The H2A-tdTomato or FM4-64 staining represented the (**F**) nuclear DNA or (**H**) the vacuole, respectively. Each single cell shape was circled out in the merged images. (**G** and **I**) Quantification of Rph1-2xGFP localization in cells displayed in (F) or (H), respectively. The bar graphs represent the percentages of cells exhibiting Rph1-GFP localized at vacuole and cytoplasm (green), nucleus only (yellow) and no GFP signals (gray). Data show mean ± SD from at least three experiments, with ∼50 cells counted for each strain per experiment. (**J** and **K**) WT or *atg8Δ* cells expressing Rph1-2xGFP were either with (**J**) MMS treatment or with (**K**) MMS treatment addition of PMSF. Free GFP moieties pointed out by arrows were detected by immunoblotting with an α-GFP antibody. Cells expressing a 2xGFP empty vector served as a control.

([15])). To further confirm that Rph1 was indeed delivered to the yeast vacuole, Rph1-2xGFP plasmid was transformed into different strains and the free GFP moieties in the vacuole upon MMS treatment were examined using western blot analysis probing with an α-GFP antibody. As expected, Rph1-2xGFP was degraded and free GFP signal was detected in the WT cells only in the precence of MMS, but not in the *atg8Δ* cells in the same culture condition (Figure [4]J). Upon MMS treatment, free GFP moieties were observed in the WT cells but not in the *pep4Δ* cells (Supplementary Figure S4E). Moreover, addition of PMSF blocked the production of free GFP moiety in cells treated with MMS (Figure [4]K). Collectively, these results demonstrated that DNA damage-induced degradation of Rph1 was mediated by autophagy pathway.

Gcn5-dependent acetylation, but not phosphorylation, of Rph1 was required for Rph1 degradation upon DNA damage

Next, we sought to determine what action triggers Rph1 degradation processes upon DNA damage stress. The phosphorylation of Rph1 by Rad53 has been suggested to promote its degradation in response to DNA damage ([15]). To clarify that possibility, *mec1Δsml1Δ* and *rad53Δsml1Δ* strains were treated with MMS and Rph1 protein levels were examined. Surprisingly, deleting Rad53 or the Rad53 upstream kinase Mec1 did not inhibit Rph1 degradation (Supplementary Figure S5A). The strain deleted *SML1* gene alone (suppressor of Mec1 or Rad53 lethality) served as a control. To test the possibility that other Rph1-related kinases might participate in this event, we generated a *tel1Δmec1Δsml1Δ* strain, which mimics deletion of the human homologs of ATM and ATR kinases. However, this triple deletion mutant also did not attenuate Rph1 degradation upon MMS treatment (Supplementary Figure S5A). Recently, Bernard *et al*. reported that Rim15 kinase mediated Rph1 phosphorylation in nutrient starvation ([18]). Therefore, Rph1 protein levels in a *rim15Δ* strain were examined. The results indicated that Rim15 also did not regulate Rph1 degradation upon DNA damage stress (Supplementary Figure S5B). Moreover, an *rph1* mutant (S459AS652A) that was predicted to impede its phosphorylation and that was hypersensitive to UV irradiation also did not inhibit its protein degradation under the same stress condition (Supplementary Figure S5C). Thus, we concluded that phosphorylation of Rph1 is not required for its degradation upon DNA damage.

Protein acetylation has been implicated in promoting protein degradation through autophagy ([22,30,31]). To determine if Rph1 degradation is mediated by protein acetylation, the acetylation status in WT or *crm1-tet* cells expressing HA-tagged Rph1 was examined upon DNA damage. To avoid the degradation of the majority of Rph1 after a longer treatment period, cells were only treated with MMS for 1 h. After immunoprecipitation of Rph1 using an HA antibody, the intensities of acetylated Rph1 were dramatically enhanced in the MMS-treated samples compared to controls, especially in the *crm1-tet* strain (Figure [5]A). This evidence suggests that Rph1 is acetylated in the nucleus upon MMS treatment. To explore which acetyltransferase is responsible for Rph1 acetylation and its degrada-

tion, eight non-essential acetyltransferase deletion strains were analyzed. We found that *GCN5* deletion was able to attenuate Rph1 degradation upon MMS treatment (Supplementary Figure S6A), and the deletion of other two subunits of the Gcn5 complex, *ADA2* and *ADA3*, also inhibited Rph1 turnover (Supplementary Figure S6B). To verify whether Rph1 degradation is associated with enzymatic activity of Gcn5, a rescue experiment was performed using *gcn5Δ* strains expressing wild-type or enzymatically inactive (E173Q) *GCN5* constructs. In agreement with our data described above, *GCN5* deletion attenuated the Rph1 degradation rate and adding back wild-type *GCN5* but not the *GCN5-E173Q* mutant rescued this phenotype in response to MMS treatment or UV irradiation (Figure [5]B and data not shown). To determine if Gcn5 could directly acetylate Rph1, *in vitro* acetyltransferase assays were performed using recombinant GST-Rph1 incubated with bacterial purified yeast Gcn5/Ada2/Ada3 subcomplex. Total ^{3}H-acetyl incorporation was measured by gel and fluorography or by filter binding and liquid scintillation counting. Consistent with our previous results, Gcn5 demonstrates a robust acetyltransferase activity on Rph1 *in vitro* (Figure [5]C and Supplementary Figure S6C, lane 3). In addition, we also noticed that GST-Rph1 protein was acetylated by yeast Gcn5 complex purified from the WT strain but not from the *ada2Δ* strain *in vitro* by liquid scintillation counting (Supplementary Figure S6D). More importantly, exogenous GFP-Rph1 protein can be acetylated in the WT strain but not in the *ada2Δ* strain upon MMS treatment *in vivo* (Figure [5]D). Altogether, these data indicated that DNA damage-induced Rph1 degradation is regulated by the Gcn5-containing complex, most likely dependent on Gcn5-mediated acetylation.

The Gcn5/Ada2/Ada3 core components have been identified to exist in three distinct acetyltransferase complexes, named as SAGA, SLIK and ADA, respectively ([32–34]). Although they all possess a Gcn5-dependent acetyltransferase activity, some unique subunit in every complex is critical for the integrity of those complexes or for regulating their distinct biological functions. To figure out which Gcn5-containing complex is responsible for Rph1 degradation, a unique subunit in each complex, including *SPT20* in SAGA, *AHC1* in ADA and *RTG2* in SLIK, were deleted, and Rph1 protein levels were examined upon MMS treatment in the corresponding strains (Figure [5]E and F). Intriguingly, only deletion of *SPT20*, but not the others, compromised the degradation of Rph1, suggesting that SAGA complex is essential for Rph1 degradation under DNA damage stress conditions.

Gcn5-mediated Rph1 acetylation regulated its nuclear export

How does Gcn5-mediated Rph1 acetylation regulate Rph1 protein degradation upon DNA damage? We speculated that acetylation of Rph1 likely plays roles at three different steps: (i) releasing Rph1 from chromatin; (ii) regulating the translocation of Rph1 from the nucleus to the cytoplasm; and (iii) regulating Rph1 trafficking to the vacuole. To determine which step is modulated by Rph1 acetylation, we performed the following experiments. First, ChIP of integrated 3xFlag-tagged Rph1 following quantitative real-time
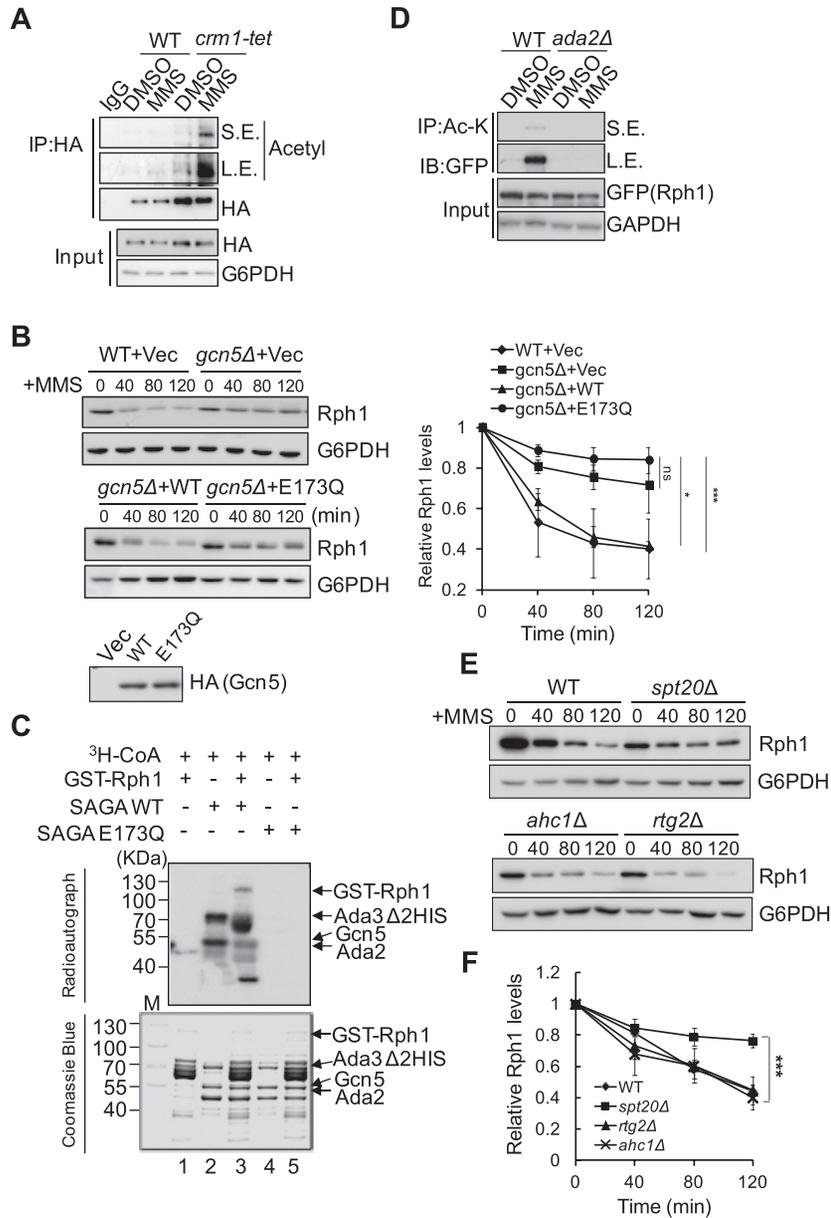
Figure 5. Gcn5-dependent Rph1 acetylation was required for Rph1 protein degradation upon DNA damage. (**A**) WT or *crm1-tet* cells bearing endogenous promoter-driven HA-tagged Rph1 construct were treated ± MMS and subjected to protein immunoprecipitation (IP) with α-HA resin followed by immunoblotting with α-acetylated-lysine antibody (Ac-K). Both short exposure (S.E.) and long exposure (L.E.) results were shown. Protein levels of HA-Rph1 in whole-extract were examined by probing with α-HA antibody. (**B**) Rph1 protein levels in the indicated cells with exposure to MMS were immunoblotted with α-Rph1 antibody at the indicated time points (left upper panel). Relative Rph1 levels were quantified (right panel). The expressions of the WT or catalytically inactive (E173Q) Gcn5 constructs were examined (left bottom panel). (**C**) *In vitro* acetyltransferase assays were performed (details see the main content). The reaction samples were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and analyzed by radioautography (^{3}H-acetyl) (top) and Coomassie blue staining (bottom). (**D**) Acetylation of overexpressed GFP-Rph1 proteins in either the WT or the *ada2Δ* cells treated ± MMS was detected by IP with α-acetyl-lysine resin (α-Ac-K) followed by immunoblotting (IB) of Rph1 with α-GFP antibody. The expression levels of GFP-Rph1 and GAPDH (Input) served as the loading control. (**E** and **F**) Rph1 protein levels in the indicated strains treated with MMS were immunoblotted with an α-Rph1 antibody, and relative Rph1 abundance from three biological repeats was quantified.

PCR (RT-qPCR) analysis was performed. Several previously identified DDR-related genes repressed by Rph1 that can be induced by MMS treatment were chosen as the potential Rph1-bound targets (17). Consistent with previous study, Rph1 was found bound to the promoters of *STP4*, *PHR1* and *RNR2* under physiological conditions. Upon MMS treatment, Rph1 was released from these regions. Remarkably, *Gcn5* deletion did not alter the binding capacities of Rph1 with these genes even in the presence or absence of MMS treatment, suggesting that Gcn5-mediated acetylation of Rph1 does not affect its chromatin binding (Figure 6A). In agreement with ChIP data, deletion of *GCN5* did not obviously affect mRNA expression of these genes, even though basal expression of those genes decreased significantly in the *gcn5Δ* cells compared to the WT cells (Figure 6B). Moreover, *CRM1*-deficient mutant also did not alter mRNA levels of these indicated genes compared to the normal cells except *MAG1* (Supplementary Figure S7A). Combined with the previous reports that phosphorylation of Rph1 is sufficient to mediate the release of Rph1 from chromatin, we assumed that acetylation of Rph1 is not required for its dissociation from regulatory gene promoters.

Next, we tested whether the acetylation of Rph1 altered its cellular localization. Because the *gcn5Δ* cells exhibited a severe growth defect and their cell size was much smaller than that of the WT cells, the *ada2Δ* strain was chosen to monitor Rph1 shuttling between the nucleus and the cytoplasm. In agreement with our previous observations, ∼25% portion of Rph1-2xGFP can be exported from the nucleus to the cytoplasm upon 1 h MMS treatment in the *pep4Δ* cells, but not in the *ada2Δpep4Δ* cells (Figure 6C, D and Supplementary Figure S7B). These data implied that the abolishment of Rph1 acetylation may prevent its nuclear export.

It has been reported that SAGA complex physically interacted with the nuclear pore complex (NPC), and their association is necessary for anchoring the actively transcribed *GAL* genes to the nuclear pore (35,36). Thus, we wondered if SAGA complex is also required for Rph1 targeting the NPC and subsequent vacuolar degradation. Consistent with the results by shutdown of *CRM1* expression, deletion of the NPC subunits, *NUP60* or *MLP1,* abolished Rph1 degradation upon MMS treatment (data not shown). More interestingly, we detected the physical interaction between endogenous Rph1 and the core component of NPC, Nup1 in the WT cells, but not in the *gcn5Δ* cells. The association between Rph1 and Nup1 is evidently enhanced under the DNA damage stress condition (Figure 6E). Based on these results, we proposed that SAGA complex is necessary for anchoring Rph1 to the NPC without affecting gene activation upon MMS treatment.

Proper Rph1 levels are critical for maintaining cellular homeostasis

Because Gcn5-mediated Rph1 acetylation and subsequent degradation is not necessary for transcriptional activation of the DNA damage-related genes, the autophagic degradation of Rph1 may be dispensable. To address this issue, we generated a series of WT or mutant Rph1 constructs, which constitutively expressed N-terminal GFP tag under

the control of the *MET25* promoter. Cells expressing these constructs were tested for DNA-damage sensitivity. Consistent with previous reports, under the control of the constitutive *MET25* promoter, relative higher exogenous expression of WT Rph1 and Gis1, but not Rph1 with deletions of the JmjN or ZF domain, caused a severe growth defects in both normal or stress conditions (Supplementary Figure S8A and B) (11,16). To see if proper Rph1 protein level is critical for cellular response to DNA damage, cells with relative lower exogenous expression of WT Rph1 that is under the control of endogenous promoter showed sensitivity to UV irradiation, MMS or camptothecin treatment, but showed minor slow growth defects when untreated (Figure 7A and B). The effects of Rph1 on cell growth and the cellular DNA-damage response were independent of its histone demethylase activity, as the catalytically inactive mutant of Rph1 (H235A) displayed defects similar to those observed with the wild-type Rph1 (Figure 7A and Supplementary Figure S8A). Gis1 mutated in its putative catalytic site also showed similar defects (Figure 7A and Supplementary Figure S8A).

To verify if excessive Rph1 protein interfered with the DNA damage signaling pathway, we examined the status of several DNA damage checkpoint proteins upon DNA damage stress. In the presence of MMS, overexpression of endogenous promoter-driven Rph1 did not alter Ser129 phosphorylation levels of histone H2A (γH2AX) but dramatically inhibited accumulation of Rad53 proteins, suggesting that Rph1 negatively regulates Rad53 activation under DNA-damage stress conditions (Figure 7C). Furthermore, RT-qPCR analyses showed that MMS treatment of cells was accompanied by an increased expression of several DDR-related genes that have been identified in the microarray analyses, whereas overexpressing Rph1 significantly inhibited gene expression compared to the WT strain even in the absence of MMS treatment (Figure 7D) (15,17). In contrast, cells lacking *RPH1* moderately increased mRNA levels of the indicated genes compared to the WT cells when grown in the rich YPD medium (Supplementary Figure 8C). These data further suggested that fine-tuning proper Rph1 protein levels in different cellular conditions is critical for maintaining cell homeostasis. Finally, we found that *nup60Δ* cells exhibited sensitivity to MMS treatment or UV exposure. However, deletion of *RPH1* gene in the *nup60Δ* cells nearly rescued the slow growth defect (Figure 7E). Therefore, our findings of acetylation-mediated Rph1 nuclear export and autophagic degradation shows that cells must maintain proper Rph1 protein levels in the DNA damage stress conditions.

DISCUSSION

Our studies provide genetic and biochemical evidence that Rph1 protein is required to be dynamically regulated in order to be appropriate response to different environmental conditions. Specifically, we show that Rph1 is a negative regulator of the expression of various genes under physiological conditions, including *DUN1, MAG1* and *RNR2,* which are involved in the DNA checkpoint signaling pathway (37). Consistently, microarray data and previous studies have identified several environmental stress response genes
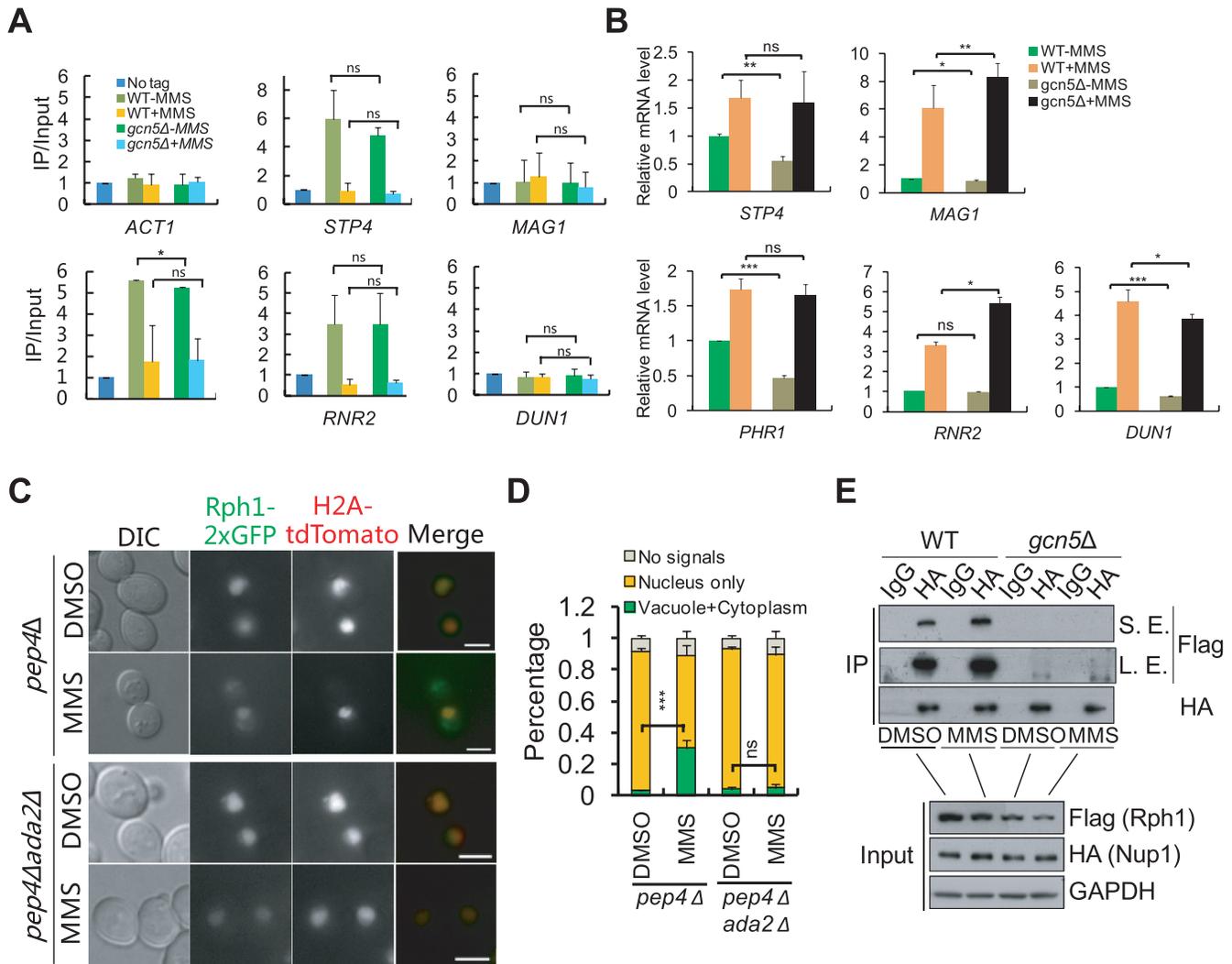
Figure 6. MMS-induced Rph1 acetylation regulated its nuclear export. (**A**) ChIP assays from the indicated strains ± MMS treatment were performed using α-Flag antibody. No Tag: cells without Flag tag; WT: WT cells integrated with 3xFlag-Rph1; *gcn5Δ*: *gcn5Δ* cells integrated with 3xFlag-Rph1. qRT-PCR was performed using primer sets each probing the promoter regions of individual genes above. The ChIP data were normalized to input levels. Data show mean ± SD represent three independent experiments. (**B**) The relative mRNA levels of the indicated genes were quantified by RT-qPCR in WT or *gcn5Δ* cells ± MMS treatment. Error bars indicate the SD of three independent experiments. (**C**) The distribution of Rph1-2xGFP in the indicated cells ± MMS treatment was observed by fluorescence microscopy. Representative fluorescent images showed the distribution of Rph1-2xGFP in the indicated cells ± MMS treatment as described above. *Scale bar*, 5 μm. (**D**) Quantification of Rph1-2xGFP localization in cells displayed in (**C**). The bar graphs represent the percentages of cells exhibiting Rph1-GFP localized at vacuole and cytoplasm (green), nucleus only (yellow) and no GFP signals (gray). Data show mean ± SD from at least three experiments, with ∼50 cells counted for each strain per experiment. (**E**) Co-IP assays were performed in WT or *gcn5Δ* cells integrated with 3xFlag-Rph1 and 6xHA-Nup1 ± MMS treatment using an α-HA resin. The precipitated proteins or cell lysates were immunoblotted with the indicated antibodies.

(*AMS1*, *GTT1*, *UGX2*) and *ATG* genes (*ATG2*, *ATG7*, *ATG24* and *ATG30*) (*ATG*s) which are negatively regulated by Rph1 as well (15,18). In contrast, some evidence show that excessive Rph1 in yeast cells is not only deleterious to cell growth under normal growth conditions, but also causes hypersensitivity to DNA damaging agents (Figure 7, Supplementary Figure S8 and (11) and (16)). In order to prevent casual cell death resulted from excessive Rph1, autophagy-mediated Rph1 protein degradation is executed to maintain a proper cellular environment. Interestingly, we found that Rph1 is acetylated in a Gcn5-dependent manner upon DNA damage stress and that this acetylation is accompanied by a degradation of the protein. The preven-

tion of Rph1 acetylation leads to an inhibition of protein destruction. Rph1 degradation is primarily mediated by the autophagy pathway, as disruption of autophagy-related genes attenuated Rph1 turnover. Moreover, we uncovered that only SAGA complex, but not SLIK or ADA complex, is able to regulate Rph1 protein stability. This is likely due to its unique function in which SAGA complex is capable to anchoring the active transcripts and acetylated proteins (such as Rph1) to the nuclear pore, which is required for nuclear export of those factors. Taken together, we propose a model in which Rph1 maintains DNA damage checkpoint inactivation under physiological conditions by repressing the expression of DDR- and repair-related genes. Upon
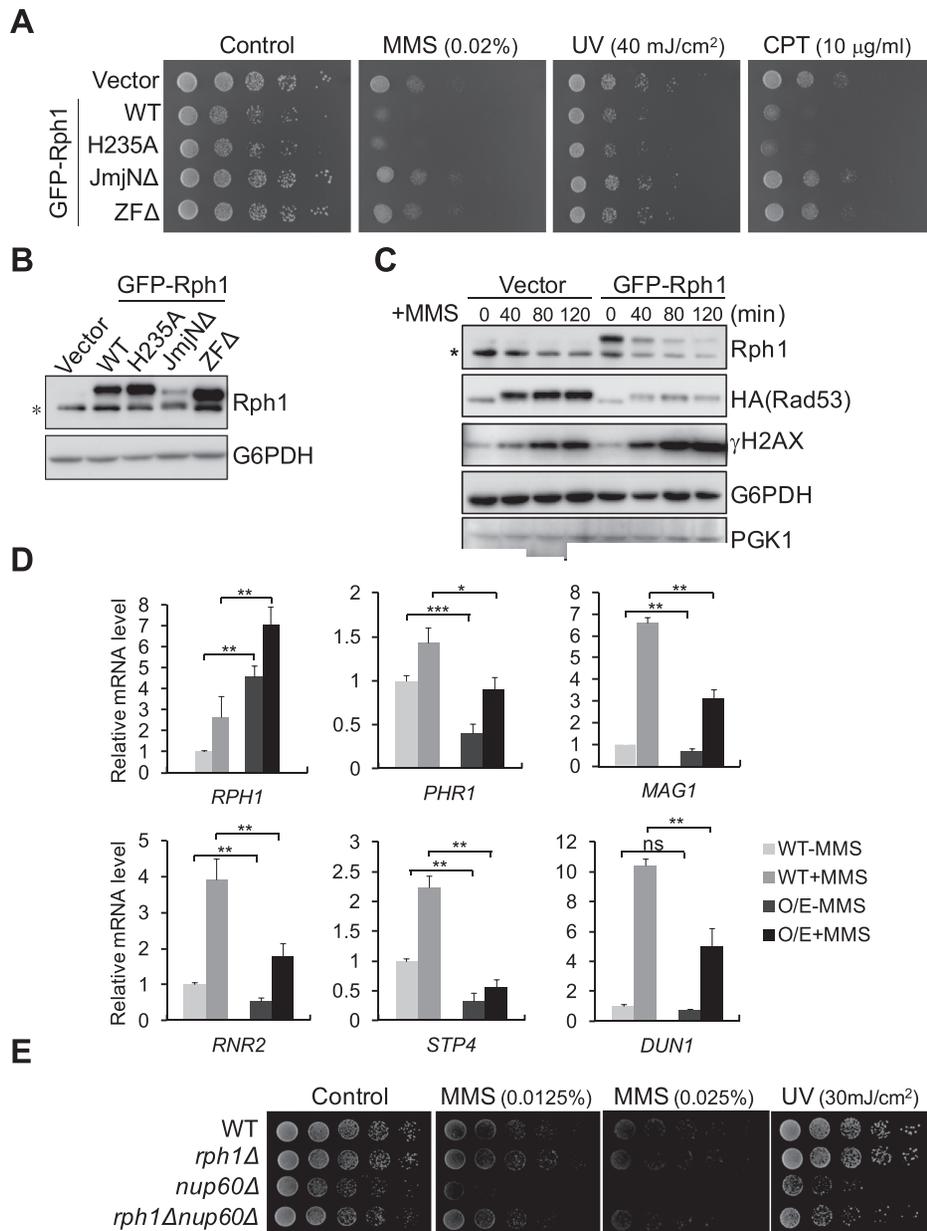
Figure 7. Maintaining proper Rph1 levels are critical for cell homeostasis. (**A**) Yeast spot assays were performed using cells expressing endogenous promoter-driven either WT or the indicated Rph1 mutant plasmids bearing a GFP tag. Cell growth was monitored in SC-Leu plates with or without exposure to UV irradiation, MMS or camptothecin (CPT). (**B**) The protein levels expressed in the indicated strains described in (A) were immunoblotted with α-Rph1 and α-G6PDH (loading control) antibodies. The asterisk represented endogenous Rph1 protein. (**C**) W303 strains integrated with C-terminal 6xHA-Rad53 were transformed with either empty vector or an endogenous-promoter driven GFP-Rph1 construct. Those strains were treated with MMS at the indicated time points and immunoblotted with α-HA, α-H2A phospho-S129 (γH2AX) or α-G6PDH and α-PGK1 (loading control) antibodies. The asterisk represented endogenous Rph1 protein. (**D**) The relative mRNA levels of the indicated genes were quantified by RT-qPCR in WT or overexpression (OE) of GFP-Rph1 strains ± MMS treatment for 1 h. Error bars indicate mean ± SD of three independent experiments. (**E**) Spot assays were performed using the indicated cells with or without exposure to MMS or UV irradiation.

DNA damage stress, following Rad53-mediated phosphorylation of Rph1 and chromatin release, Gcn5-mediated acetylation of Rph1 regulate the export of Rph1 from the nucleus to the cytoplasm. The acetylated Rph1 will be delivered to yeast vacuole and subsequently destroyed by autophagy (Figure 8).

Abundant evidence has demonstrated that the protein levels of many critical transcription factors are regulated by protein phosphorylation and proteasomal degradation (38). Several histone methyltransferases and demethylases, such as Set2, Jhd2 and Gis1, are degraded by the 26S proteasome system (Figure 2) (24,25,39). A previous study showed that Rad53, the Rph1 kinase, only modestly regu-
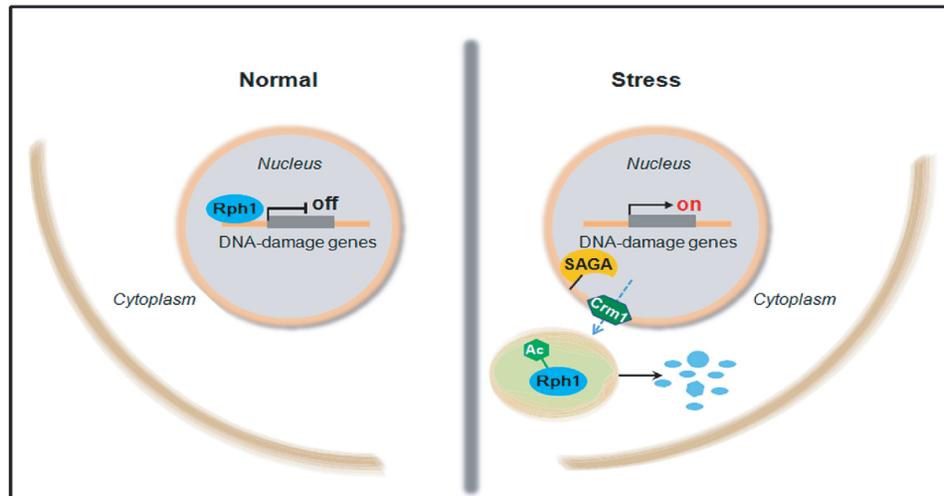
Figure 8. A proposed model for Gcn5-mediated acetylation of Rph1 necessary for its cytoplasmic translocation and protein degradation by the autophagosome. Under normal growth conditions, Rph1 binds the promoters of multiple genes to inhibit their gene expression. In response to DNA damage stress, Rph1 is phosphorylated, which allows it to be released from chromatin. Meanwhile, Rph1 is acetylated by Gcn5-containing SAGA complex in the nucleus upon DNA damage stress. Acetylated Rph1 is required for its association with the NPC and translocation of Rph1 from the nucleus to the cytoplasm by Crm1. The acetylated Rph1 eventually entered into the vacuole and is degraded via autophagy.

late Rph1 protein degradation, suggesting that additional factors may be involved in the regulation of Rph1 protein in response to DNA damage (15). Indeed, we demonstrate that Rph1 is predominantly degraded, at least under DDR conditions, by an acetylation-dependent autophagy system. Notably, the deletion of several potential Rph1-related kinases, including Rad53, Rim15, Mec1 and Tel1, did not attenuate endogenous Rph1 protein degradation (Supplementary Figure S5). This discrepancy between previous data and our data is likely due to different experimental conditions. It is possible that Rph1 phosphorylation is involved in regulating protein turnover under other environmental conditions.

Protein acetylation generally influences chromatin structure, protein turnover and the DNA damage response (40). Recently, the importance of acetylation in autophagy regulation has been recognized. On one hand, many ATG proteins are directly acetylated by acetyltransferases, and the p300-mediated acetylation of ATG proteins appears to have an inhibitory role in autophagy (41). For example, the acetylation of Beclin 1 (the mammalian ortholog of yeast Atg6) blocks autophagosome maturation (42). In budding yeast, Esa1 acetylates Atg3, which facilitates Atg3-Atg8 interaction and autophagy (43). In this scenario, protein acetylation is involved in different steps of the autophagy pathway. On the other hand, protein acetylation is required for the clearance of damaged proteins by autophagy. It has been shown that the Gcn5-mediated acetylation of a key yeast checkpoint protein, Sae2 (human CtIP), promotes its destruction by autophagy (22). In this paper, we illustrated another case in which Rph1, is also degraded through the acetylation-dependent autophagy pathway, specifically by Gcn5. Very recently, two other DNA damage checkpoint proteins, Chk1 and Rnr1, have been reported to be degraded by autophagy upon DDR in eukaryotes (44,45). Therefore, it would be interesting to explore whether these

proteins are also acetylated and whether the acetylated proteins are also targeted to the vacuole for protein degradation.

Gcn5 is the catalytic acetyltransferase subunit of the SAGA complex that plays essential roles in diverse chromatin-based cellular processes, including DDR (46). Cells lacking Gcn5 are sensitive to DNA-damaging agents, indicating that Gcn5 positively influences the DDR pathway (47). Ada2 and Ada3, together with Gcn5, form the catalytic core of the SAGA complex and are also required for *in vivo* acetyltransferase activity (48). In our manuscript, we showed that deletion of *GCN5*, *ADA2* or *ADA3* attenuated Rph1 degradation in response to MMS treatment, and that the acetyltransferase activity is necessary for Rph1 degradation (Figure 5B and supplementary Figure S6B). Given that numerous histone and non-histone substrates of the Gcn5 complex have been identified, it was plausible that Gcn5 affected Rph1 protein stability indirectly (49). To rule out this possibility, we tested whether Gcn5 directly acetylates Rph1. Our results demonstrated that Gcn5 can acetylate Rph1 both *in vivo* and *in vitro* (Figure 5C and D). To identify which lysine residue of Rph1 is critical for Rph1 degradation, endogenous Rph1 was immunoprecipitated from yeast cells or recombinant GST-Rph1 protein was acetylated by yeast purified Gcn5 complex *in vitro*, and the enriched Rph1 proteins were subjected to mass spectrometry analysis. A total of eight potential acetylated lysine sites (K658, K665, K694, K705, K470, K422, K717 and K759) on Rph1 were identified. We mutated either a single lysine residue or a combination of multiple lysine residues, and tested the protein stability upon MMS treatment. Although some single mutant slightly prevented Rph1 turnover, we could not verify either single site or a combination sites of Rph1 (including all eight lysine mutated to arginine) can dramatically inhibit Rph1 destruction (data not shown). Given that a line of evidence showed various protein acetylated sites were cat-

alyzed by acetyltransferases, it is possible that other unidentified sites on Rph1 are required for its turnover.

Autophagy is classified into three primary types: macroautophagy, microautophagy and chaperone-mediated autophagy (26). Chaperone-mediated autophagy has been reported to only occur in mammalian cells. In budding yeast, both macroautophagy and micro-autophagy can be selective and non-selective (26). Non-selective autophagy is used for the turnover of bulk cytoplasm, whereas selective autophagy specifically targets damaged or superfluous materials, including mitophagy, pexophagy, reticulophagy, ribophagy, lipophagy, aggrephagy, xenophagy and nucleophagy, depending on the cargos (50). To date, many *ATG* genes have been identified that are essential for macroautophagy, whereas molecules involved in microautophagy have been less explored (27). Particularly, the molecular processes and key regulators involved in nucleophagy (piecemeal microautophagy of the nucleus), which selectively degrades nuclear materials, remain largely unknown (50). Rph1 is a nuclear protein, so it is possible that degradation of Rph1 is regulated via nucleophagy. Since several ATG proteins participate in both macroautophagy and microautophagy processes, it is difficult to determine which particular pathway mediates turnover of nuclear Rph1 protein (51). However, our data clearly showed that the turnover of Rph1 was blocked by the protease inhibitor PMSF or in the *pep4Δ* strain. Combined with our other results, these evidence can allow us to certainly draw a conclusion that degradation of Rph1 is mediated by autophagy upon DNA damage stress (Figure 2 and 4).

In summary, acetylation-dependent autophagic degradation is vital to dynamically regulate the DNA-damage checkpoint pathway (52). When checkpoint activation is impaired upon valproic acid treatment, the recombinant protein Sae2 and the nuclease Exo1, which function positively in DDR and repair, are degraded by autophagy in an acetylation-dependent manner, whereas Rph1, which functions negatively in DDR and repair, remains stable even in the presence of MMS. In contrast, when this pathway is activated upon MMS treatment, the fate of these proteins is completely reversed. These observations suggest that the acetylation-dependent autophagic degradation of proteins contributes to a general regulatory role in the DNA damage signaling pathway.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

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