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TORC1, Tel1/Mec1, and Mpk1 regulate autophagy induction after DNA damage in budding yeast

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ABSTRACT

Target of rapamycin complex 1 (TORC1) protein kinase responds to various stresses including genotoxic stress. However, its molecular mechanism is poorly understood. Here, we show that DNA damage induces nonselective and selective autophagy in budding yeast. DNA damage caused the attenuation of TORC1 activity, dephosphorylation of Atg13, and autophagy induction. The TORC1-upstream Rag GTPase Gtr1 was not required for TORC1 inactivation and autophagy induction after DNA damage. Furthermore, DNA damage responsive protein kinases Mec1/ATM and Tel1/ATR, and stress-responsive mitogen-activated protein kinase Mpk1/Slt2 were required for the full induction of autophagy. Autophagic proteolysis was required for DNA damage tolerance in TORC1 inactive conditions. This study revealed that multiple protein kinases regulate DNA damage-induced autophagy.

1. Introduction

Macroautophagy (hereafter autophagy) is a catabolic process that degrades cytoplasmic constituents and organelles in the lysosome/vacuole, and it is induced for nutrient recycling in nutrient starvation conditions [1–3]. Autophagy induction is regulated by target of rapamycin complex 1 (TORC1) protein kinase, which is a central controller of cell growth in response to nutrient availability [4–6]. TORC1 phosphorylates Atg13 to repress macroautophagy induction in nutrient-rich conditions in the budding yeast *Saccharomyces cerevisiae* [7,8]. When TORC1 is inactivated by nutrient depletion or rapamycin treatment, Atg13 is promptly dephosphorylated in a manner dependent on PP2A and Cdc14 [9,10], which in turn promote formation of the Atg1 kinase complex (containing Atg1 and Atg13) triggering isolation membrane formation and autophagy induction [1,11]. Thus, the TORC1–Atg13–Atg1 axis is a critical pathway for starvation-induced autophagy. In addition to nutrient depletion, various stresses such as DNA damage (genotoxic stress) attenuate TORC1 activity and autophagy induction [12,13].

In budding yeast, DNA damage activates the PI3 kinase-related kinases Mec1/ATM and Tel1/ATR [14,15]. These protein kinases phosphorylate and activate downstream protein kinases, inducing DNA repair and cell cycle arrest. This DNA damage response (DDR) signaling is critical for cell survival during genotoxic stress. In mammalian cells, upon DNA damage the DDR signaling pathway attenuates mTORC1 activity via ATM/ATR–p53-mediated induction of upstream repressors of mTORC1, PTEN, AMPK, Sestrins, and TSC2, inducing autophagy [16–18]. Autophagy is required for resistance to DNA damage in mammalian cells, but the physiological meaning of autophagy remains elusive [19–23].

In budding yeast, whether and how DNA damage induces autophagy is largely unknown. Cells defective in autophagy showed no hypersensitivity to DNA damage [24], although autophagic degradation of some proteins involved in the DDR after DNA damage was reported;

Abbreviations: CDK, cyclin-dependent kinase; Cvt, cytoplasm to vacuole targeting; DDR, DNA damage response; GFP, green fluorescent protein; HU, hydroxyurea; MAPK, mitogen-activated protein kinase; MMS, methyl methanesulfonate; PAS, pre-autophagosomal structure; TORC1, target of rapamycin complex 1; UV, ultraviolet light.

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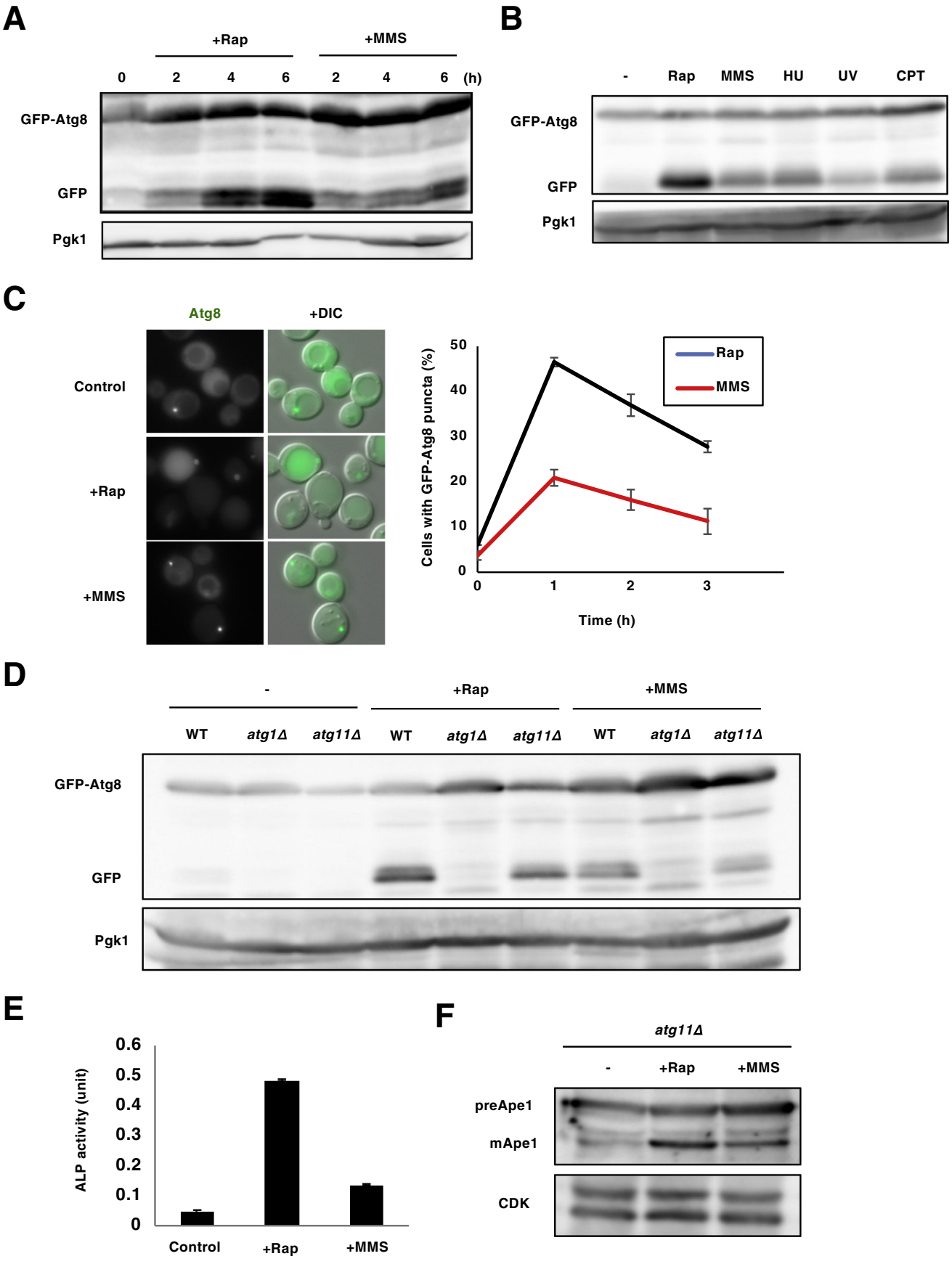

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Fig. 1. DNA damage induces autophagy in budding yeast.
(A) Exponentially growing cells of strain SCU893 (wild-type) harboring plasmid pSCU1998 (pGFP-ATG8) were treated with 0.1% methyl methanesulfonate (MMS) for the indicated times. Whole cell extracts were subjected to western blotting. Cells treated with 200 ng/ml rapamycin (Rap) were used as the control. (B) Cells of strain SCU893 (wild-type; WT) harboring plasmid pSCU1998 (pGFP-ATG8) were treated with 200 ng/ml rapamycin, 0.1% MMS, hydroxyurea (HU), and camptothecin (CPT) for 6 h. Alternatively, cells were incubated for 6 h after irradiation with 200 J/m^2 ultraviolet light (UV). Whole cell extracts were subjected to western blotting using an GFP antibody. Pgk1 was detected as the loading control using an anti-Pgk1 antibody. (C) Exponentially growing cells of strain SCU893 (wild-type) harboring plasmid pSCU1998 (pGFP-ATG8) were treated with MMS. Cells treated with rapamycin were used as the control. Cells with GFP-Atg8 puncta were counted and are expressed as percentages. Data obtained from three independent experiments are shown as average ± SD. (D) Cells of strains SCU893 (wild-type), SCU3365 (*atg1*Δ), and SCU3464 (*atg11*Δ) were treated with rapamycin or MMS for 6 h. Whole cell extracts were subjected to western blotting. (E) Cells of strain SCU3973 (*pho8Δ60 pho13*Δ) were treated with 200 ng/ml rapamycin or 0.1% MMS for 6 h. Whole cell extracts were subjected to the Pho8Δ60 assay. Data obtained from two independent experiments are shown as average ± error. (F) Cells of strain SCU3464 (*atg11*Δ) were treated with MMS 6 h. Whole cell extracts were subjected to western blotting using an anti-Ape1 antibody in order to detect production of mature Ape1 (mApe1) from premature Ape1 (preApe1) in *atg11*Δ cells in the course of nonselective autophagy. Whole cell extracts were subjected to western blotting. Cyclin-dependent kinase (CDK) was detected as the loading control using an anti-CDK antibody.

ribonucleotide reductase Rnr1, the mitotic regulator securin/Pds1, the nuclear metalloprotease Wss1, and the JmjC-domain containing histone demethylase Rph1 [24–27]. Here, we show that DNA damage attenuates TORC1 activity and induces autophagy via Atg13 dephosphorylation in a manner independent of DDR signaling and the mitogen-activated protein kinase (MAPK) Mpk1/Slt2 in budding yeast. We will discuss the physiological meaning of autophagy induction after DNA damage.

2. Materials and methods

2.1. Strains, plasmids, and media

The *S. cerevisiae* strains and plasmids used are listed in Supplemental Table S1 and Table S2, respectively. Glucose-containing YPAD (YPD containing 0.01% adenine) and synthetic minimal medium (SD) complemented with the appropriate nutrients for plasmid maintenance were prepared using standard methods. For assessment of autophagy, when cells harbor plasmids, cells were precultured in SD with the appropriate nutrients, and then cultured in YPAD. For nitrogen-starvation experiments, cells were transferred into SD-N without ammonium sulfate or amino acids. For carbon-starvation experiments, cells were transferred into S−C without glucose.

2.2. Western blotting analysis

Proteins were extracted using a post-alkaline extraction method in accordance with a previous report [28]. Briefly, cells (10 ml culture, OD_{600} = 0.2–0.8) were treated with 200 μl of 0.1 M NaOH for 5 min and then the pellet was collected by centrifugation. The pellet was resuspended in sample buffer (60 mM Tris-HCl [pH 6.8], 5% glycerol, 2% SDS, 4% 2-mercaptoethanol, and 0.0025% bromophenol blue) at 95 °C for 5 min. Crude extracts were cleared by centrifugation and the supernatant was used for western blotting analysis. We used the following antibodies: anti-green fluorescent protein (GFP) mouse monoclonal antibody (Santa Cruz, #sc-9996), anti-Ape1 rabbit polyclonal antibody (a gift from D. Klionsky), anti-Atg13 rabbit polyclonal antibody (a gift from Y. Kamada), anti-Rad53 goat polyclonal antibody (Santa Cruz, #sc-6749), anti-phospho-Sch9-S758 rabbit or mouse polyclonal antibody (a gift from R. Loewith) [29], anti-Tor1 goat polyclonal antibody (Santa Cruz, #sc-11,900), anti-HA mouse monoclonal antibody (Wako Pure Chemical, Osaka, Japan, #014021881), anti-cyclin-dependent kinase (CDK) rabbit polyclonal antibody (Santa Cruz, #sc-53), and anti-Pgk1 mouse monoclonal antibody (Thermo Fisher Scientific, #A-6457). Chemiluminescence signals from Western BLoT Quant HRP Substrate (Takara, #DS-T7102) for horseradish peroxidase and Immuno Shot (Cosmo Bio, #IS-012-250) as an immunoreaction enhancer solution were detected using an image analyser (Fuji LAS3000mini or LAS4000mini). For detection of the phosphorylation statuses of Atg13 and Rad53, 7.5% acrylamide gels were used for SDS-PAGE. All western blotting experiments were performed at least twice independently to confirm reproducibility of the results.

2.3. Microscope observations

Cell and GFP images were captured using a Carl Zeiss Axio Imager M1 microscope with a cooled CCD camera (Carl Zeiss AxioCam MRm). For examination of pre-autophagosomal structure (PAS) formation, more than 100 cells were counted and scored. All microscope observations were performed at least twice independently to confirm the reproducibility of the results. Data are shown as averages ± errors/SDs.

2.4. Pho8Δ60 assay

Exponentially growing cells expressing Pho8Δ60 were used. Whole cell extracts were subjected to determination of vacuolar alkaline phosphatase activity incorporated by nonselective autophagy. The activity was determined using a spectrophotometer in accordance with a previous report [30].

3. Results

3.1. DNA damage induces autophagy in budding yeast

First, to assess whether DNA damage induces autophagy in budding yeast, we examined overall autophagy flux after treatment with the alkylating agent methyl methanesulfonate (MMS) using the GFP-Atg8 processing assay [31]. As a control, we treated cells with the specific TORC1 inhibitor rapamycin. Free GFP generation from GFP-Atg8 increased drastically after rapamycin treatment (Fig. 1A). When cells were treated with MMS, free GFP was produced from GFP-Atg8, although to a lesser extent compared with the rapamycin-treated control. Autophagy induction occurred in the presence of a lower concentration of MMS (0.025%) to a lesser extent (Supplemental Fig. S1). This suggested that autophagy was induced in response to the amount of DNA damage, although higher concentrations (0.2% and 0.4%) of MMS compromised autophagy induction. Similarly, autophagy was moderately inducted after treatment with hydroxyurea (an inhibitor of ribonucleotide reductase), camptothecin (an inhibitor of topoisomerase I), and ultraviolet light (UV) (Fig. 1B). These findings indicated that DNA damage moderately (not massively) induces autophagy. Nutrient starvation and TORC1 inactivation promoted the formation of perivacuolar PASs, which are putative sites that produce isolation membranes in budding yeast [1,11]. Many ATG proteins including Atg8 are recruited at PASs near the vacuole to form foci. Consistent with the fact that MMS treatment moderately induced autophagy, MMS treatment moderately increased PAS formation (monitored using GFP-Atg8) compared with rapamycin treatment (Fig. 1C).

Atg1 protein kinase is essential for autophagy induction after TORC1 inactivation by rapamycin treatment [7] (see also Fig. 1D). Loss of Atg1 also canceled MMS-induced autophagy. Nutrient starvation and TORC1 inactivation provoke not only nonselective (bulk) autophagy

but also selective autophagy [32]. Loss of Atg11, a receptor required for selective autophagy such as mitophagy [33], canceled selective autophagy and hence partially reduced free GFP production from GFP-Atg8 after rapamycin treatment (Fig. 1D). Similarly, we found that MMS-induced autophagy was partially, but not completely, reduced in *atg11*Δ cells. This indicated that DNA damage induces both nonselective and selective autophagy. We confirmed using the Pho8Δ60 assay [30] and the Ape1 maturation assay in *atg11*Δ cells [34] that nonselective autophagy was induced after genotoxic stress (Fig. 1E, F).

3.2. Nuclear DNA damage signal induces autophagy

MMS and UV treatment injure not only DNA but also other cellular components. To confirm that genuine DNA damage elicits autophagy, we used cells accumulating DNA damage, cells defective in Cdc9 and Cdc13. Cdc9 is an essential DNA ligase I that joins Okazaki fragments during DNA replication [35]. Cells defective in Cdc9 accumulated DNA damage [36]. Cdc13, a telomeric single DNA-binding protein, plays multiple roles in telomere replication and protection [37]. Cdc13 defects result in abnormal uncapped telomeres with long exposed G-strands and activate the DNA damage response pathway [38–40]. Autophagy was prominently invoked in *cdc9-2* and *cdc13-1* cells after transfer from a permissive to semi-permissive temperature (Fig. 2A). Consistently, PAS formation increased in these mutants after the temperature shift (Fig. 2B). These findings supported the notion that DNA damage evokes autophagy. Because *CDC9* encodes a nuclear and a mitochondrial form of DNA ligase I, both nuclear DNA and mitochondrial DNA are damaged in Cdc9-deficient cells. By contrast, only nuclear DNA (but not mitochondrial DNA) is damaged in Cdc13-deficient cells. The fact that autophagy is induced in *cdc13-1* cells indicated that nuclear DNA damage signals stimulate autophagy.

DNA damage and *cdc* mutations cause cell cycle arrest. It might be wondered whether cell cycle arrest itself induces autophagy. We tested this idea using α-factor (G1 arrest) and the microtubule poison nocodazole (metaphase arrest). Treatment with these drugs did not promote autophagy induction (Supplemental Fig. S2). A similar result was reported previously using nocodazole [41]. These findings suggested that cell cycle arrest per se does not induce autophagy.

3.3. DNA damage attenuates TORC1 activity

In mammalian cells, TORC1 activity is reduced after DNA damage, resulting in autophagy induction [16,18] (see “Introduction”). We examined whether TORC1 activity is also reduced by DNA damage in budding yeast. Indeed, TORC1 activity (monitored by the phosphorylation status of Ser758 of Sch9) [42] was moderately reduced in MMS-treated cells, compared with that found in rapamycin-treated cells (Fig. 3A). A reduction in TORC1 inactivation was clearly observed at 2 h after MMS treatment (Fig. 3A), although appreciable changes in TORC1 activity were not significantly observed within 1 h after MMS treatment (Supplemental Fig. S3). Thus, DNA damage did not promptly compromise TORC1 activity, which was different from the fact that nutrient starvation and rapamycin treatment immediately inhibit TORC1 activity [43].

MMS treatment mildly dephosphorylated hyperphosphorylated Atg13 compared with rapamycin treatment (Fig. 3B). Thus, DNA damage caused moderate TORC1 attenuation and Atg13 dephosphorylation in budding yeast. In addition to this, the fact that Atg1 is required for DNA damage-induced autophagy (Fig. 1B) indicated that the TORC1–Atg13–Atg1 axis mediates DNA damage-induced autophagy. This idea was supported by observations that DNA damage caused by dysfunction of Cdc9 or Cdc13 at a semi-permissive temperature partially caused dephosphorylation of hyperphosphorylated Atg13 (Fig. 3C, D). Furthermore, we found that levels of Tor1 protein slightly but reproducibly decreased after MMS treatment (Fig. 3E). The decrease in the Tor1 protein levels should reduce TORC1 activity and helped to explain how DNA damage attenuates TORC1 activity in budding yeast.

3.4. Gtr1 does not mediate TORC1 inactivation and autophagy induction after DNA damage

TORC1 localizes on the vacuolar membrane and obtains nutrient signals via the EGO complex consisting of Rag GTPases (Gtr1 and Gtr2) and Ragulator (Ego1–3) complex [4,44–46]. The vacuolar localization of TORC1 is essential for its activity [47]. In mammalian cells, Rag GTPases mediate TORC1 inactivation after DNA damage [18]. We wondered whether Rag GTPases also mediates TORC1 inactivation and autophagy induction after DNA damage in budding yeast. However, autophagy induction and TORC1 inactivation after DNA damage still occurred in *gtr1*Δ cells (Fig. 4A, B). These findings indicated that Rag GTPases does not mediate TORC1 inactivation and autophagy induction after DNA damage in budding yeast, which is a different feature from that found in mammalian cells.

3.5. TORC1 and DDR independently mediate DNA damage-induced autophagy

How does TORC1 respond to DNA damage? In mammalian cells, ATM/ATR DDR signaling causes TORC1 attenuation and autophagy induction after genotoxic stress (see “Introduction”). We suspected a similar scenario in budding yeast. To test this idea, we examined autophagy induction after DNA damage in *tel1*Δ *mec1*Δ cells defective in the DDR signaling response. Autophagy induction after DNA damage was prominently reduced in this double mutant (Fig. 5A). This indicated that the DDR pathway is required for optimal autophagy induction after DNA damage in budding yeast. Autophagy induction after MMS treatment was slightly impaired in *tel1*Δ cells, while impairment of autophagy induction was marginal in *mec1*Δ cells (Fig. 5A). These findings indicated that Tel1 mainly regulates DNA damage-induced autophagy, although Mec1 redundantly participated in its regulation. In sharp contrast to MMS treatment, autophagy induction after rapamycin treatment was not impeded in *tel1*Δ *mec1*Δ cells (Supplemental Fig. S4). This demonstrated that the DDR signaling was not involved in autophagy induction by TORC1 inactivation after rapamycin treatment.

Next, to assess whether TORC1 inactivation after DNA damage is mediated by the DDR signaling pathway in yeast cells, we examined TORC1 activity after DNA damage in *tel1*Δ *mec1*Δ cells. However, TORC1 inactivation (Sch9 dephosphorylation) after MMS treatment occurred in *tel1*Δ *mec1*Δ cells (Fig. 5B). In addition, Atg13 dephosphorylation after DNA damage was not also impaired in this mutant (Fig. 5C). These findings indicated that the DDR signaling did not mediate TORC1 inactivation after DNA damage. Consistently, the decrease in Tor1 protein levels after DNA damage was still observed in *tel1*Δ *mec1*Δ cells, indicating that DDR signaling did not mediate the decrease in Tor1 protein levels after DNA damage (Fig. 5D). We noted that both total and phosphorylated Sch9 protein levels were reduced in *tel1*Δ *mec1*Δ cells in normal conditions (Fig. 5B), as described previously [48]. This indicated that DDR signaling is involved in the maintenance of Sch9 protein levels in normal conditions.

Conversely, TORC1 did not affect the DDR signaling pathway: rapamycin treatment and nitrogen and carbon depletion, which all inactivate TORC1, did not activate DDR signals (monitored using the phosphorylation status of Rad53; [49,50]) (Fig. 5E). In addition, rapamycin treatment did not compromise DDR activation after DNA damage (Fig. 5F). These findings indicated that the DDR signaling pathway is not regulated by TORC1. Collectively, the TORC1 and DDR pathways independently promote autophagy after DNA damage.

3.6. Mpk1 is required for proper autophagy induction after DNA damage

Next, we examined whether Mpk1/Slt2, a DNA damage-responsive

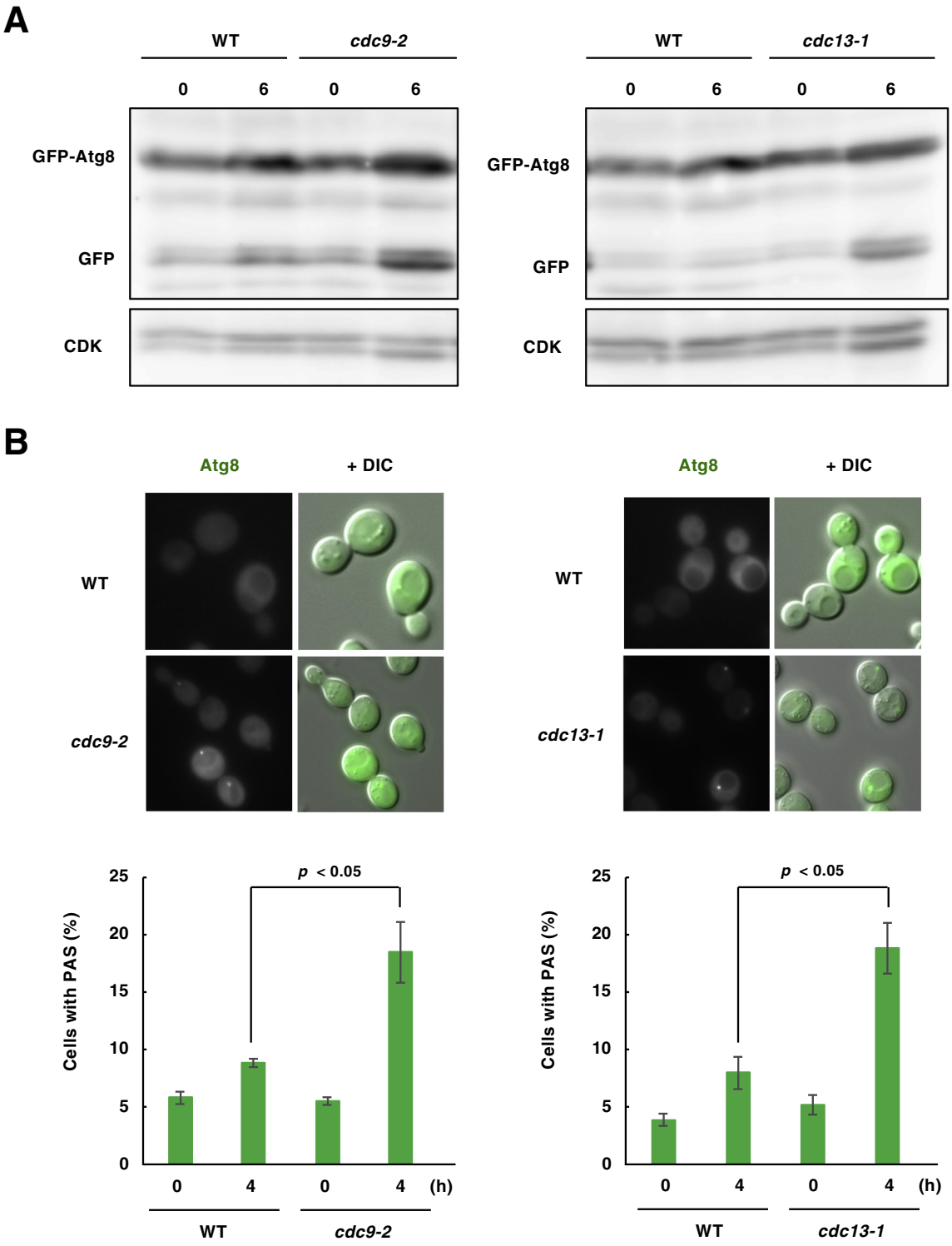

Fig. 2. Nuclear DNA damage induces autophagy.
(A) Cells of strains SCU3866 (*CDC9*), SCU3868 (*cdc9-2*), SCU893 (*CDC13*), and SCU47 (*cdc13-1*) harboring plasmid pSCU1978 (pGFP-ATG8) preincubated at a permissive temperature (25 °C) were transferred to a semi-permissive temperature (30 °C) for 6 h. Whole cell extracts were subjected to western blotting. CDK was detected as the loading control. (B) The same strains as those used in panel (A) were used. Cells were incubated at a permissive temperature (25 °C) (time 0) and transferred to a semi-permissive temperature (30 °C) for 4 h. Cells with GFP-Atg8 puncta were counted and are expressed as percentages. Data obtained from two independent experiments are shown as average ± SD. The *p*-values were calculated using two-tailed Student's *t*-test.

MAPK, is involved in DNA damage-induced autophagy. Mpk1 and the DDR pathway are independently activated by DNA damage, and loss of Mpk1 reduces cell viability in genotoxic conditions [51,52]. Rapamycin-induced autophagy was not impeded by loss of Mpk1 (Fig. 6A), indicating that TORC1 downregulates autophagy in a manner independent of Mpk1. In contrast, autophagy induction after DNA damage by MMS and hydroxyurea treatments was partially reduced by loss of Mpk1 (Fig. 6A). This indicated that Mpk1 was required for

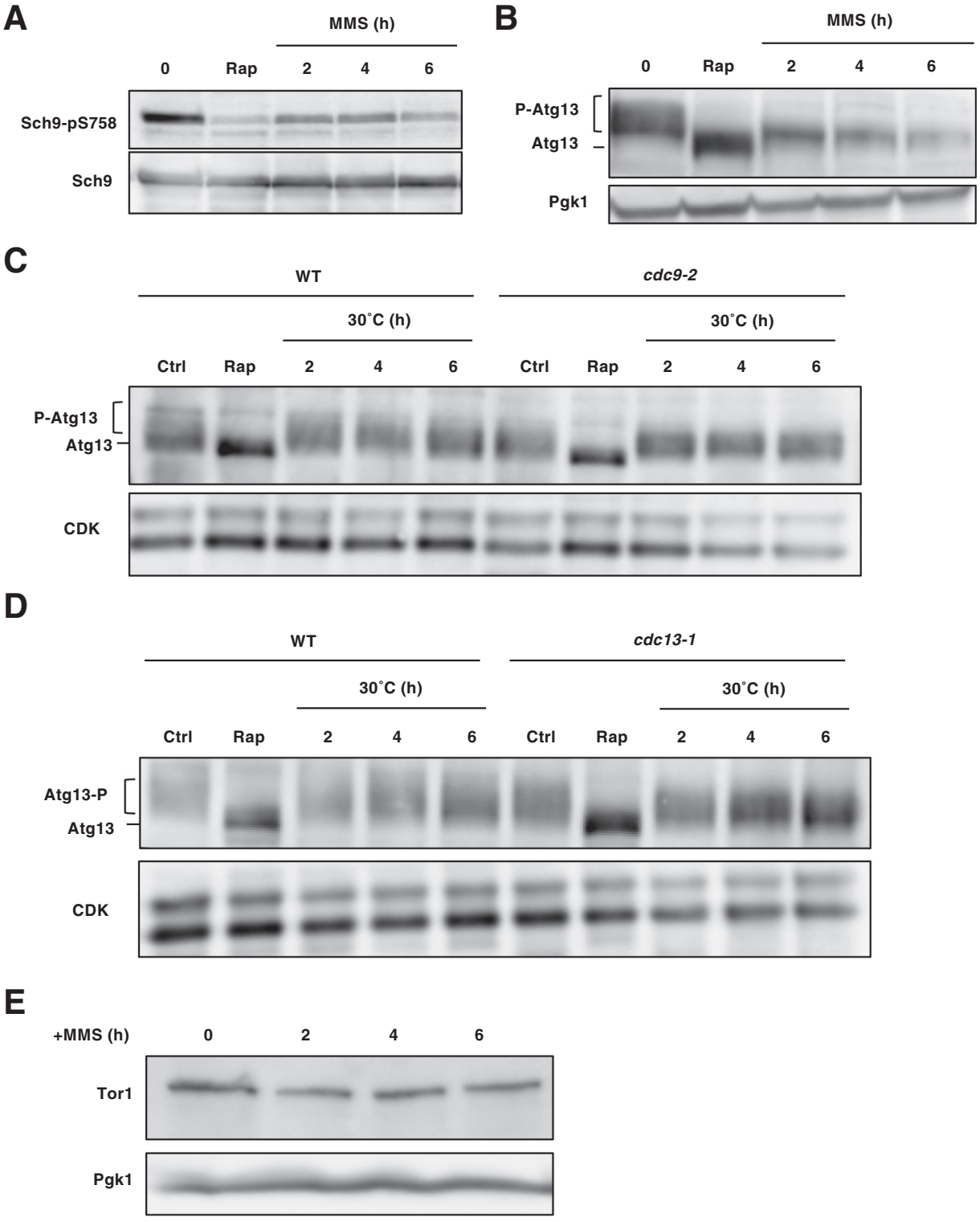

Fig. 3. DNA damage attenuates TORC1 activity.
(A) Cells of strain SCU2959 (*3HA-SCH9*) were treated with MMS for the indicated times. Whole cell extracts were subjected to western blotting using an anti-phospho-Sch9-S758 antibody. Total amounts of Sch9 were detected using an anti-HA antibody. Cells treated with rapamycin for 30 min were used as the control. (B) Cells of strain SCU893 (wild-type) and harboring plasmid pSCU1875 (pATG13) were treated with MMS for the indicated times. Whole cell extracts were subjected to western blotting using an anti-Atg13 antibody. Cells treated with rapamycin for 1 h were used as the control. (C, D) Cells of strains SCU3866 (*CDC9*), SCU3868 (*cdc9-2*), SCU893 (*CDC13*), and SCU47 (*cdc13-1*) harboring plasmid pSCU1875 (pATG13) preincubated at a permissive temperature (25 ˚C) were transferred to a semi-permissive temperature (30 ˚C) for the indicated times. Whole cell extracts were subjected to western blotting as described in panel (B). Cells treated with rapamycin at 25 ˚C for 30 min were used as the control. (E) Cells of strain SCU893 (wild-type) were treated with MMS for the indicated times. Whole cell extracts were subjected to western blotting using an anti-Tor1 antibody.

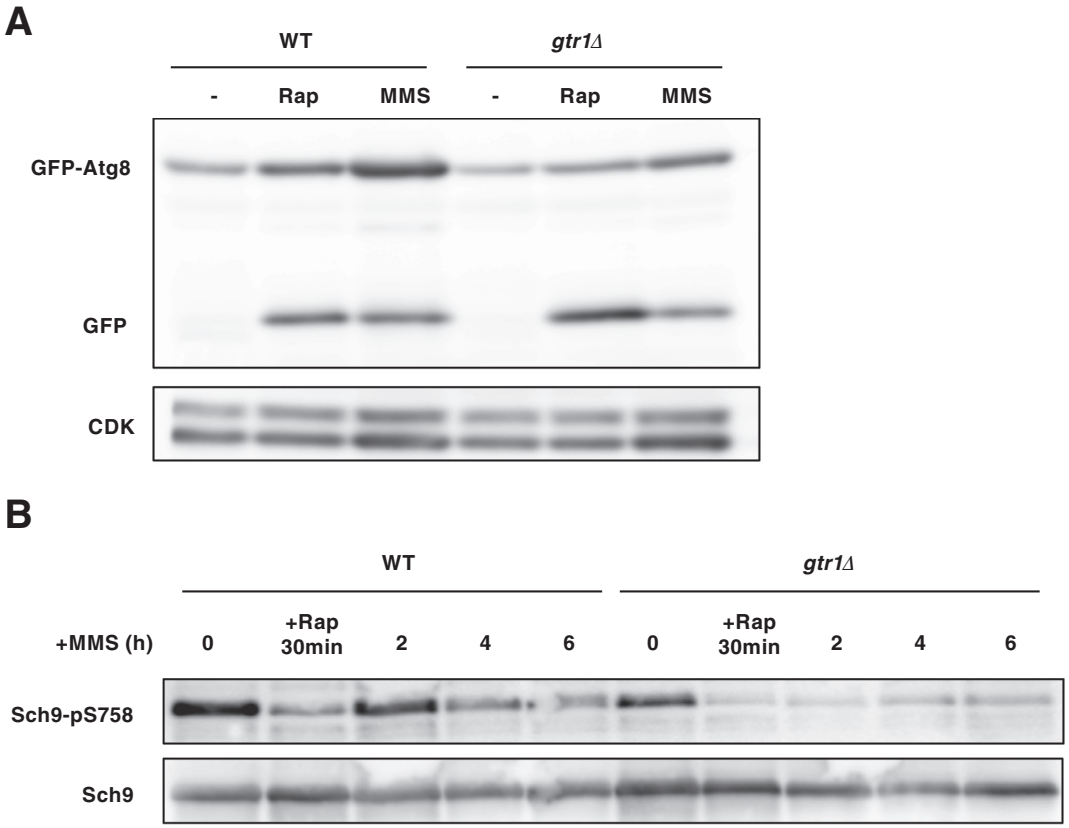

Fig. 4. Gtr1 does not mediate TORC1 inactivation and autophagy induction after DNA damage.
(A) Cells of strains SCU2959 (*3HA-SCH9*) and SCU4171 (*3HA-SCH9 gtr1*Δ) harboring plasmid pSCU1998 (pGFP-ATG8) were treated with MMS or rapamycin for 6 h. Whole cell extracts were subjected to western blotting. (B) Cells of strains SCU2959 (*3HA-SCH9*) and SCU4171 (*3HA-SCH9 gtr1*Δ) were treated with MMS for the indicated times. Whole cell extracts were subjected to western blotting. Cells treated with rapamycin 30 min were used as the control.

proper autophagy induction after DNA damage. Nevertheless, TORC1 inactivation (Sch9 dephosphorylation) and Atg13 dephosphorylation occurred similarly after DNA damage not only in wild-type but also in *mpk1*Δ cells (Fig. 6B, C). These findings suggested that TORC1 inactivation and Atg13 dephosphorylation after DNA damage are independent of Mpk1. It is likely that TORC1 and Mpk1 independently provoke autophagy after DNA damage. We noted that TORC1 activity was lower in *mpk1*Δ cells in normal conditions (Fig. 6B). This indicated that Mpk1 is required for the maintenance of TORC1 activity in normal conditions.

3.7. Autophagy is required for DNA damage tolerance in TORC1 inactive conditions

The physiological importance/meaning of DNA damage-induced autophagy remains elusive in both yeast and mammals. To assess whether autophagy is involved in the activation of DDR signaling, we monitored the activation of DDR signaling (monitored using Rad53 phosphorylation status) after DNA damage in autophagy-deficient *atg1*Δ, *atg5*Δ, and *atg8*Δ cells. We found that phosphorylated Rad53 levels after MMS treatment were slightly but reproducibly increased in these *atg*Δ mutants, especially in *atg1*Δ mutant cells (Fig. 7A). This indicated that autophagy was required for proper the activation of DDR signaling after genotoxic stress.

We detected no changes in the sensitivity to MMS treatment in an autophagy-deficient *atg1*Δ mutant (defective in all types of macroautophagy) (Fig. 7B, +MMS, Supplemental Fig. S5). Furthermore, MMS sensitivity in cells defective in nonselective macroautophagy (*atg17*Δ, *atg29*Δ, and *atg31*Δ) and in the autophagy-related cytoplasm to vacuole targeting (Cvt) pathway (*atg19*Δ, *atg20*Δ, and *atg21*Δ) [1,53] was not altered (Supplemental Fig. S5). Furthermore, Pep4, a vacuolar protease [32], and Rny1, a vacuolar ribonuclease required for RNA degradation in the vacuole [54,55], were not also required for resistance to DNA damage (Fig. 7B, +MMS). These findings indicated that autophagy and vacuolar proteolytic and ribonucleolytic activities in vacuoles are not involved in genotoxic resistance in these experimental conditions, although autophagy is induced after DNA damage.

It has been reported that autophagy-deficient cells had increased MMS sensitivity in the presence of rapamycin [24]. We also observed that loss of Atg1 caused slight MMS hypersensitivity in the presence of rapamycin (Fig. 7B). In addition, loss of Pep4, but not Rny1, elevated DNA damage sensitivity in the presence of rapamycin, although *pep4*Δ cells showed slow growth in rapamycin-containing media even without MMS treatment. These findings suggested that autophagy and vacuolar proteolysis become critical for tolerance to DNA damage in TORC1 inactive (nutrient starvation) conditions.

4. Discussion

In this study, we showed that the TORC1–Atg13–Atg1 axis mediates DNA damage-induced autophagy, similar to nutrient starvation-induced autophagy. However, the molecular mechanism of TORC1 inactivation after DNA damage was seemingly different from that after nutrient starvation and rapamycin treatment: (1) Autophagy induction after DNA damage was modest (not strong) compared with that after rapamycin treatment (this study). (2) TORC1 inactivation occurs promptly after nutrient depletion and rapamycin treatment, but not after DNA damage (this study) [43]. (3) The EGO complex regulates TORC1 activity in response to nutrient availability, but not DNA damage (this study). (4) Tor1 protein levels were reduced after DNA damage, but not

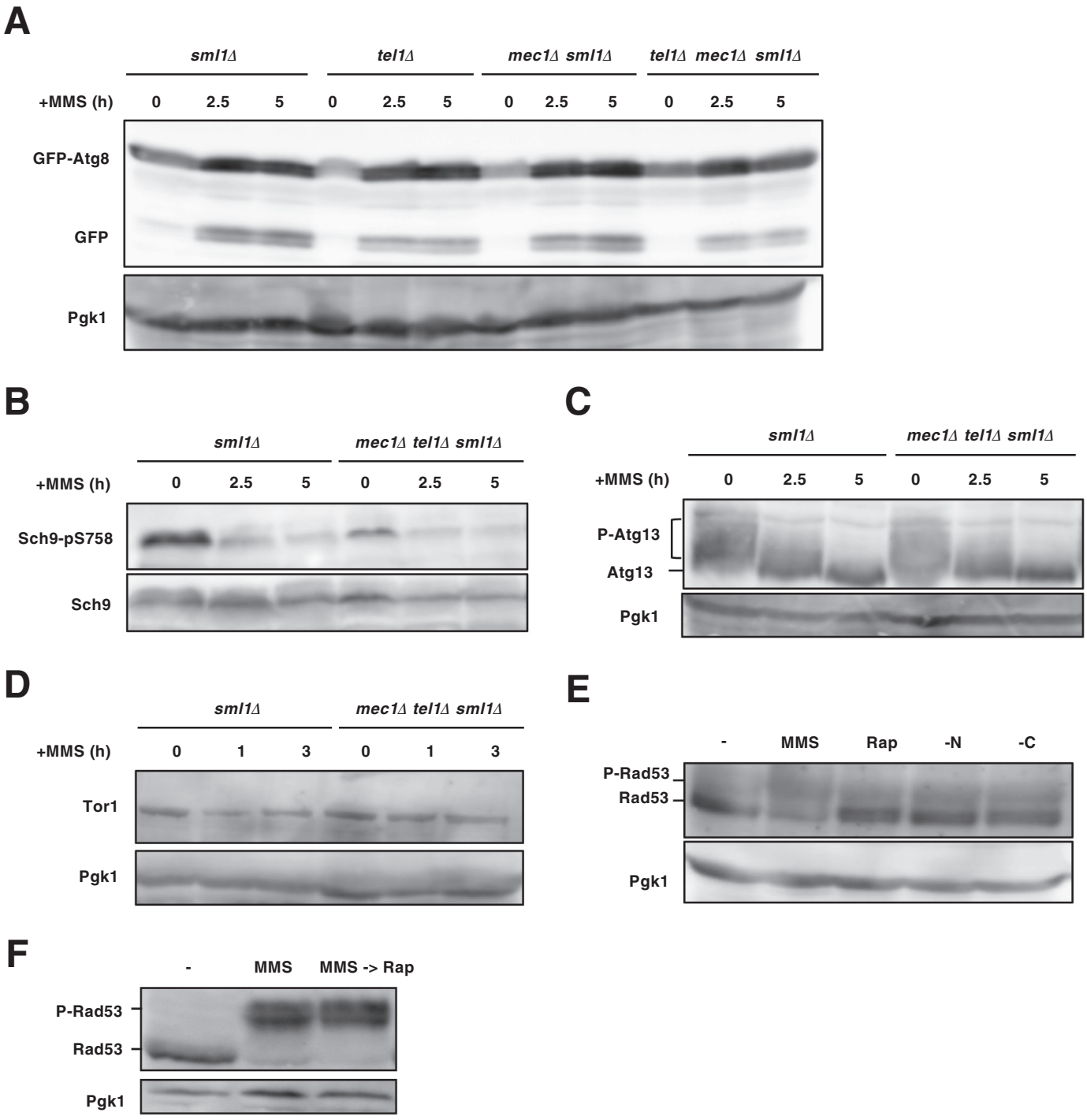

Fig. 5. The TORC1 and DDR pathways independently promote autophagy after DNA damage.
(A) Cells of strains SCU5626 (*sml1*Δ), SCU5966 (*tel1*Δ), SCU5967 (*tel1*Δ *sml1*Δ), and SCU5703 (*mec1*Δ *tel1*Δ *sml1*Δ) harboring plasmid pSCU1998 (pGFP-ATG8) were treated with MMS for the indicated times. Whole cell extracts were subjected to western blotting. (B) Cells of strains SCU5626 (*sml1*Δ) and SCU5703 (*mec1*Δ *tel1*Δ *sml1*Δ) harboring plasmid pSCU2345 (p3HA-SCH9) were treated with MMS for the indicated times. Whole cell extracts were subjected to western blotting. (C) Cells of strains SCU5626 (*sml1*Δ) and SCU5703 (*mec1*Δ *tel1*Δ *sml1*Δ) harboring plasmid pSCU1875 (pATG13) were treated with MMS for the indicated times. Whole cell extracts were subjected to western blotting as described in Fig. 3B. (D) Cells of strains SCU5626 (*sml1*Δ) and SCU5703 (*mec1*Δ *tel1*Δ *sml1*Δ) were treated with MMS for the indicated times. Whole cell extracts were subjected to western blotting using the anti-Tor1 antibody. (E) Cells of strain SCU893 (wild-type) were treated with rapamycin, or transferred to nitrogen- or carbon-depleted media for 4 h. Cells treated with MMS for 4 h were used as the control. Whole cell extracts were subjected to western blotting using an anti-Rad53 antibody. (F) Cells of strain SCU2000 (*RAD53-3HA*) were treated with MMS for 4 h and then further incubated for 1 h in the presence of rapamycin. Whole cell extracts were subjected to western blotting using an anti-HA antibody.

after nitrogen starvation (this study) [56]. Thus, it is unlikely that DNA damage induces nutrition depletion, leading to TORC1 inactivation. How Tor1 protein levels are reduced after DNA damage is unknown at present and it will be important for this to be addressed in the future.

Here, we showed that the TORC1 and DDR signaling pathways independently promoted DNA damage-induced autophagy (Fig. 7C). During preparation of this work, it was independently reported that DNA damage induced autophagy in budding yeast [48]. Eapen and

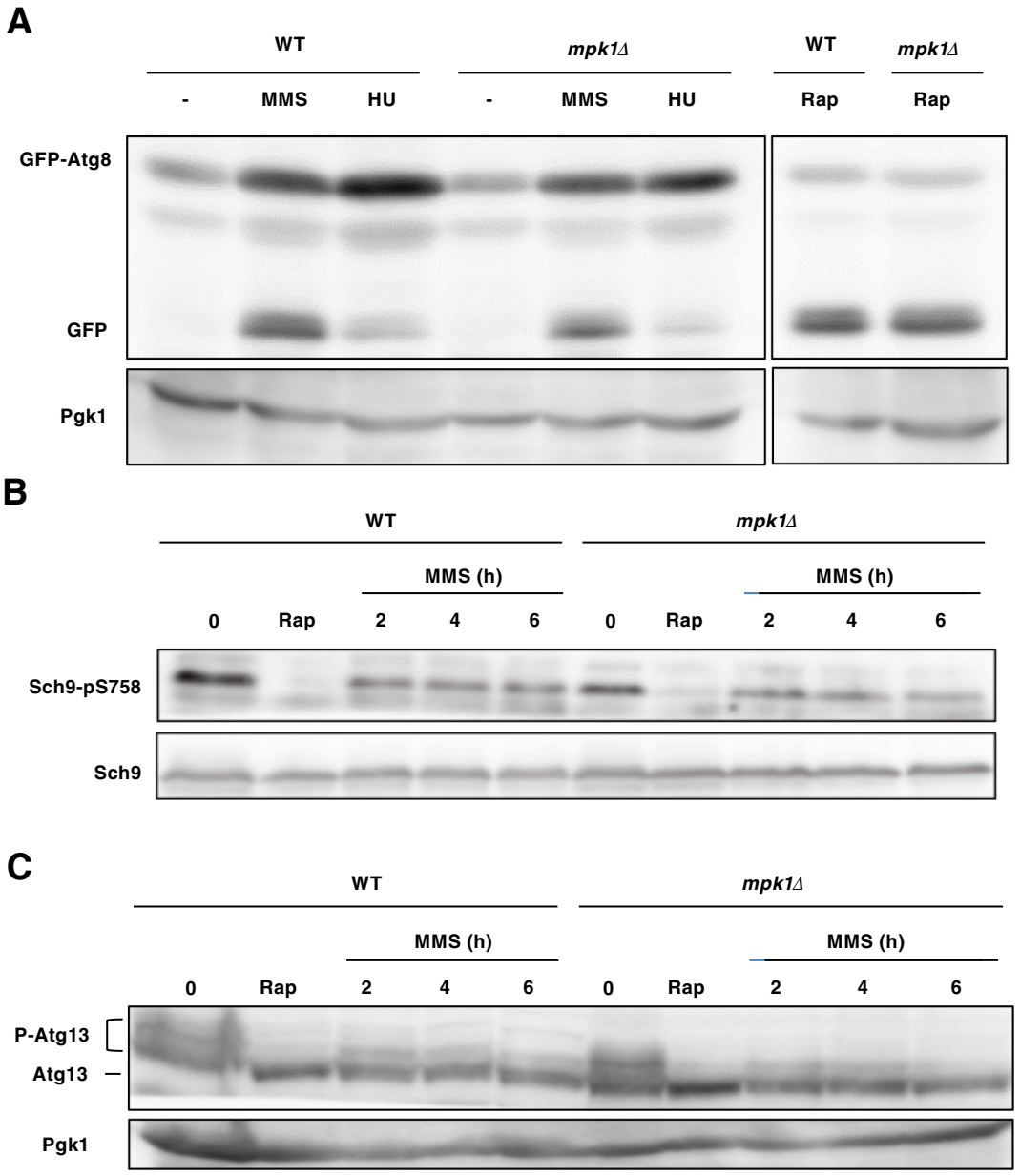

Fig. 6. Mpk1 is required for proper autophagy induction after DNA damage.
(A) Cells of strains SCU2684 (wild-type) and SCU2908 (*mpk1*Δ) harboring plasmid pSCU1998 (pGFP-ATG8) were treated with MMS or HU for 6 h. Cells treated with rapamycin for 3 h were used as the control. Whole cell extracts were subjected to western blotting. (B) Cells of strains SCU2959 (*3HA-SCH9*) and SCU4071 (*3HA-SCH9 mpk1*Δ) were treated with MMS for the indicated times. Whole cell extracts were subjected to western blotting as described in Fig. 3A. Cells treated with rapamycin for 30 min were used as the control. (C) Cells of strains SCU2684 (wild-type) and SCU2908 (*mpk1*Δ) harboring plasmid pSCU1875 (pATG13) were treated with MMS for the indicated times. Cells treated with rapamycin for 30 min were used as the control. Whole cell extracts were subjected to western blotting.

colleagues also showed that MMS-induced autophagy was canceled in *mec1*Δ *tel1*Δ double mutant cells, although Atg13 and Sch9 dephosphorylation still occurred in the mutant cells. This supported our finding that DDR signaling regulates autophagy induction after DNA damage in a TORC1-independent manner. Thus, TORC1 and DDR signaling collaboratively induces autophagy after DNA damage. The DDR protein kinases Tel1 and Mec1 complementarily mediate autophagy induction after DNA damage (this study) [48]. On the other hand, levels of total and phosphorylated Sch9 protein were reduced in *tel1*Δ *mec1*Δ cells in normal conditions (this study) [48], indicating that DDR signaling is required for maintenance of the TORC1–Sch9 signaling branch in normal conditions. Furthermore, we demonstrated that the DNA damage-responsive MAPK Mpk1 is a novel protein kinase that independently mediates DNA damage-induced autophagy and is required for optimal autophagy induction, although how Mpk1 regulates autophagy induction is unclear currently. Thus, multiple protein kinases, TORC1, Tel1/Mec1, and Mpk1 independently promoted DNA damage-induced autophagy (Fig. 7C). We demonstrated that DNA damage induces both selective and nonselective autophagy using multiple assays (Fig. 1D–F), whereas Eapen and colleagues showed that MMS-induced autophagy was completely abolished by loss of Atg11 and demonstrated that MMS treatment induces selective autophagy [48]. These differences might result from differences in the experimental conditions and/or genetic backgrounds of the strains used.

The JmjC-domain-containing histone demethylase Rph1 repressed the expression of genes for autophagy induction and DNA repair in normal conditions [57,58]. After DNA damage, the DDR kinase Rad53 phosphorylates Rph1 to release it from chromatin, inducing autophagy-

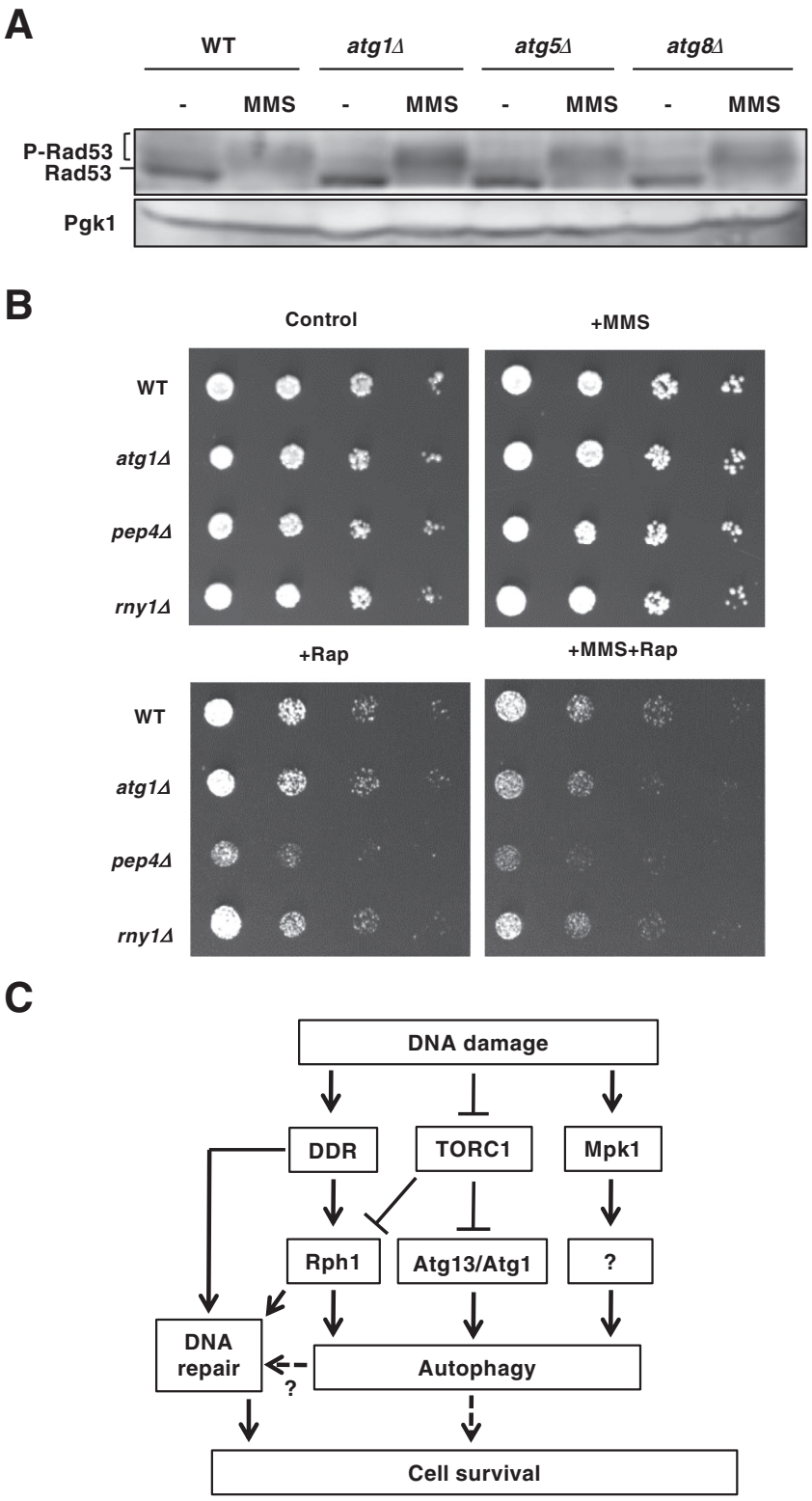

Fig. 7. The roles of autophagy in genotoxic stress conditions. (A) Cells of strains SCU2684 (wild-type), SCU3365 (*atg1*Δ), SCU4261 (*atg5*Δ), and SCU3366 (*atg8*Δ) were treated with MMS for 1 h. Whole cell extracts were subjected to western blotting. (B) Cells of strains SCU2684 (wild-type), SCU3365 (*atg1*Δ), SCU1690 (*pep4*Δ), and SCU4296 (*rny1*Δ) were used. Serially 5-fold diluted cells of each strain were spotted on plates with or without 0.01% MMS and/or 25 ng/ml rapamycin. The plates without and with rapamycin were incubated at 30 °C for 2 days and 4 days, respectively. (C) Model for autophagy induction after DNA damage. DNA damage signaling independently inactivates TORC1 and activates DDR signaling and Mpk1. TORC1 inactivation evokes autophagy via the Atg13–Atg1 module and inhibits Rph1 function to induce genes for autophagy induction and DNA repair. The DDR pathway also promotes autophagy and DNA repair partially via Rph1. Autophagy is required for DNA damage resistance in TORC1 inactive (nutrient-depleted) conditions. For details, see the text.

related *ATG* genes [59,60]. Meanwhile, upon nutrient starvation and TORC1 inactivation, activated Rim15 protein kinase, which is repressed by TORC1 in normal conditions, phosphorylates and inhibits Rph1 function [58]. This suggested that attenuation of TORC1 activity after genotoxic stress also inhibits Rph1 via Rim15, inducing *ATG* genes. Taken together, both the TORC1 and DDR signaling pathways promote autophagy and DNA repair via Rph1 after DNA damage (Fig. 7C).

Genotoxic stress induces autophagy in budding yeast, as found in mammalian cells. However, there are some differences in the systems of DNA damage-induced autophagy between the two species: (1) After DNA damage, DDR signaling mediates TORC1 inactivation, promoting autophagy induction in mammalian cells. By contrast, in yeast DDR signaling is also required for optimal DNA damage-induced autophagy, but it does not mediate TORC1 inactivation after DNA damage (this study) [48]. Rather, TORC1 and DDR signaling cooperatively but independently induce autophagy after DNA damage in yeast cells. (2) Rag

GTPases (Gtr1/2) mediate DNA damage-induced TORC1 inactivation in mammalian cells, but not in yeast cells (this study) [18]. In mammalian cells, mTORC1 is regulated by many upstream regulators, of which some are not conserved in budding yeast (e.g., TSC1/2, CASTOR and SAMTOR) [6,61]. It is likely that DNA damage stress attenuates TORC1 activity via an unidentified pathway in budding yeast.

In yeast cells, DNA damage induces autophagic degradation of various proteins involved in the response to DNA damage [24–27]. However, the autophagic degradation of these proteins should not be critical for tolerance to DNA damage, because autophagy deficiency does not increase sensitivity to DNA damage [24] (this study). In addition, mutation rates monitored using a *can1* mutation assay [62] were not changed by loss of Atg1 in the presence or absence of UV treatment (our unpublished data). Thus, autophagy mutants do not appreciably compromise cell survival or genome integrity in genotoxic stress conditions. Autophagy becomes required for cell survival in genotoxic stress conditions only when TORC1 activity is reduced by the presence of rapamycin (mimicking nutrient starvation) [24] (this study). It is likely that nutrient supply by autophagy in starved conditions is essential for de novo protein synthesis involved in DNA damage repair. Likewise, autophagy might be needed for tolerance to DNA damage in other stressful conditions. In human cells, autophagy is required for DNA damage resistance. This study provides new insights into precise molecular mechanisms and roles of autophagy induction in genotoxic stress conditions in human cells.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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Author contributions

TU designed the experiments. SU and RO performed most experiments. AK, RA and HK performed some experiments. A. Miura and A. Matsuura provided invaluable materials. A. Matsuura helped to design some experiments. TU wrote the paper. RO helped to write the paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellsig.2019.109344.

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