Nicotinamide Suppresses the DNA Damage Sensitivity of *Saccharomyces cerevisiae* Independently of Sirtuin Deacetylases

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ABSTRACT Nicotinamide is both a reaction product and an inhibitor of the conserved sirtuin family of deacetylases, which have been implicated in a broad range of cellular functions in eukaryotes from yeast to humans. Phenotypes observed following treatment with nicotinamide are most often assumed to stem from inhibition of one or more of these enzymes. Here, we used this small molecule to inhibit multiple sirtuins at once during treatment with DNA damaging agents in the *Saccharomyces cerevisiae* model system. Since sirtuins have been previously implicated in the DNA damage response, we were surprised to observe that nicotinamide actually increased the survival of yeast cells exposed to the DNA damage agent MMS. Remarkably, we found that enhanced resistance to MMS in the presence of nicotinamide was independent of all five yeast sirtuins. Enhanced resistance was also independent of the nicotinamide salvage pathway, which uses nicotinamide as a substrate to generate NAD+, and of a DNA damage-induced increase in the salvage enzyme Pnc1. Our data suggest a novel and unexpected function for nicotinamide that has broad implications for its use in the study of sirtuin biology across model systems.

KEYWORDS nicotinamide; sirtuins; DNA damage; checkpoint; Pnc1; NAD+

THE DNA damage checkpoint is a highly conserved signaling cascade initiated in response to DNA lesions. In the budding yeast *Saccharomyces cerevisiae*, checkpoint activation begins with the exposure of single-stranded DNA (ssDNA), either from exonuclease-resected DNA double-strand breaks (DSBs), or from stalled replication forks during S phase. Resected DNA coated by the ssDNA binding protein RPA is thought to act as a landing pad for Mec1-Ddc2 complexes (Melo and Toczyski 2002; Gobbini *et al.* 2013; Edenberg *et al.* 2014a). Mec1 is a sensor kinase that, in concert with adaptor proteins such as Rad9 or Mrc1, phosphorylates downstream checkpoint targets, including the Rad53 and Chk1 transducing kinases (Melo and Toczyski 2002; Gobbini *et al.* 2013; Bastos de Oliveira *et al.* 2015). Following autophosphorylation and release from adaptors, Rad53 is thought to move throughout the cell to phosphorylate targets that promote cell cycle arrest, the inhibition of late-firing origins of replication, and a global transcriptional response (Melo and Toczyski 2002; Jaehnig *et al.* 2013; Edenberg *et al.* 2014a). While the DNA damage response is traditionally associated with phosphorylation-based signaling cascades, it has recently emerged that other post-translational modifications including ubiquitylation, sumoylation, and acetylation play prominent roles in the response in both yeast and other eukaryotes (Downey and Durocher 2006a; Psakhye and Jentsch 2012; Panier and Durocher 2013; Edenberg *et al.* 2014a; Elia *et al.* 2015).

Acetylation of lysine residues is catalyzed by histone acetyltransferases (HATs) and reversed by histone deacetylases (HDACs). Despite their names, these enzymes also have non-histone targets that play critical roles in maintaining cellular homeostasis in organisms from bacteria to humans (Choudhary *et al.* 2009; Weinert *et al.* 2011; Henriksen *et al.* 2012; Choudhary *et al.* 2014; Downey *et al.* 2015). In *S. cerevisiae*, for example, acetylation sites have been mapped on over a third of all proteins (Downey and Baetz 2016). HAT and HDAC enzymes modulating these protein substrates can

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be organized into families based on common biochemical and structural characteristics. The largest family of HDACs in yeast is the sirtuins, which includes Sir2 and Hst1–Hst4 (Brachmann *et al.* 1995; Wierman and Smith 2014). Four of the five sirtuins have reported roles in responding to or preventing enhanced accumulation of DNA damage. Hst3 and Hst4 deacetylate histone H3 on K56, which is deposited during DNA replication, and this deacetylation is prevented in the presence of DNA damage (Celic *et al.* 2006; Maas *et al.* 2006; Miller *et al.* 2006; Edenberg *et al.* 2014b). Levels of Hst3 and Hst4 are tightly controlled (Thaminy *et al.* 2007; Delgoshaie *et al.* 2014; Edenberg *et al.* 2014b) and their absence results in persistent H3 K56 acetylation. Deregulation of H3 K56 acetylation is associated with spontaneous activation of the DNA damage response and defects in DNA repair (Celic *et al.* 2006, 2008; Maas *et al.* 2006; Muñoz-Galván *et al.* 2013; Che *et al.* 2015; Simoneau *et al.* 2015).

Sir2, with the SIR silencing complex, is recruited to locations in the genome that have endured DSBs and is thought to promote DNA repair via the nonhomologous end-joining pathway (Martin *et al.* 1999; Mills *et al.* 1999; Tamburini and Tyler 2005). The impact of Sir2 on DNA repair could be largely indirect, as the SIR complex represses *HMRa* and *HML*α, which in turn impacts the expression of DNA repair factors (Lee *et al.* 1999; Frank-Vaillant and Marcand 2001; Valencia *et al.* 2001). Hst1 also localizes to DNA DSBs (Tamburini and Tyler 2005) and regulates accumulation of H2A phosphorylated on serine 129 (Szilard *et al.* 2010). This modification is catalyzed by Mec1 and the related Tel1 protein kinase to serve as a marker of DNA damage and a checkpoint maintenance signal (Rogakou *et al.* 1999; Downey and Durocher 2006b; Keogh *et al.* 2006). The function of Hst2 is unclear. Although most Hst2 localizes to the cytoplasm, it enters the nucleus under some circumstances to interact with histones and regulate chromosome condensation, which is required for DNA segregation during cell division (Wilson *et al.* 2006, 2014). Sirtuins are highly conserved and human homologs (SIRT1–SIRT7) also have critical roles in the DNA damage response (Yuan *et al.* 2007; Kaidi *et al.* 2010; Serrano *et al.* 2013; Zhang *et al.* 2013; Liu *et al.* 2014).

All sirtuins require nicotinamide adenine dinucleotide (NAD+) as a cosubstrate, which is cleaved to produce nicotinamide and 2′-O-acetyl-ADP-ribose (Yuan and Marmorstein 2012). Nicotinamide functions to inhibit sirtuins in a negative feedback loop (Sauve *et al.* 2001; Bitterman *et al.* 2002; Jackson *et al.* 2003; Yuan and Marmorstein 2012). It is normally cleared by the nicotinamide salvage pathway, which also contributes to the maintenance of NAD+ levels within the cell (Gallo *et al.* 2004; Sauve *et al.* 2005; McClure *et al.* 2008). NAD+ can also be produced *de novo* from tryptophan in a pathway requiring Bna2 (Panozzo *et al.* 2002). Exogenously added nicotinamide is used to inhibit sirtuins at concentrations of 5–25 mM (Bitterman *et al.* 2002; Tsuchiya *et al.* 2006). In yeast, nicotinamide and/or salvage pathway intermediates and enzymes can impact transcriptional silencing (Belenky *et al.* 2007; McClure *et al.* 2008), replicative lifespan (Belenky *et al.* 2007), and the clearing of protein aggregates (Ocampo *et al.* 2013). Nicotinamide and other regulators of sirtuins have been proposed for use in the treatment of cancer and other diseases (Demarin *et al.* 2004; Lara *et al.* 2009; Chopra *et al.* 2012; Chen *et al.* 2015; Cheon *et al.* 2015), but little is known about the specificity of these drugs or the consequences of inhibiting multiple sirtuins simultaneously.

To investigate sirtuin redundancy in the cellular response to genotoxic agents, we examined the growth characteristics of yeast cells treated with DNA damaging agents in the presence or absence of nicotinamide. Despite a documented role for sirtuins in the DNA damage response, we observed that treatment of cells with nicotinamide actually improved resistance to high concentrations (>0.03%) of the alkylating agent MMS on both solid agar plates and in liquid media. Surprisingly, this protective effect occurred independently of all five sirtuins and the nicotinamide salvage pathway. These findings suggest a novel function for this compound in countering the growth inhibiting effects of DNA alkylating agents. Our work has important implications for the use of nicotinamide as a therapeutic agent and as a tool in acetylation research.

Materials and Methods

Yeast strains and growth conditions

Yeast strains were generated using standard techniques as described previously (Downey *et al.* 2013), and genotypes for all strains are indicated in Supplemental Material, Table S1. ORF disruptions were confirmed using PCR to test for (1) the correct positioning of knock-out cassettes, and (2) for the absence of the wild-type ORF. Epitope-tagged strains were confirmed via immunoblotting and by PCR to check for the correct location of the tagging construct. This includes strains sourced from the GFP-tagged collection. Sirtuin quintuple mutant strains generated for this study were made as follows: First, an *hst3*::*URA3 hst4*::*LEU2* strain (YMD006) was crossed to an *hst1*::*kanMX* strain from the MAT*a* haploid gene deletion collection. *HST2* and *SIR2* were then sequentially replaced in the resulting diploid with *sp*HisMX and HYGMX cassettes via transformation. Strains were sporulated and dissected to recover haploid sirtuin quintuple mutants as well as all other combinations. These slow growing strains retain temperature sensitivity associated with mutation of both *HST3* and *HST4* (Figure S5A) (Brachmann *et al.* 1995; Maas *et al.* 2006). Unless indicated, strains were grown in standard YPD media supplemented with 0.005% adenine and tryptophan.

Drug treatments

MMS was purchased from Sigma Chemical (St. Louis, MO) (129925). Plates with MMS were used within 24 hr, after which the effective concentration of the drug appeared to

diminish. For all figures shown, nicotinamide used was from Sigma Chemical (N3376). The positive impact of nicotinamide on MMS sensitivity was also observed with nicotinamide purchased from ACROS Organics (128271000). Nicotinamide at low concentrations did not dramatically impact the pH of the media used (Figure S5B). Cycloheximide was purchased from Sigma Chemical (#018105). Cycloheximide was stored as a stock solution of 100 mg/ml, which was used at a 1:2000 dilution.

Spot tests and plating assays

Fivefold serial dilutions of the indicated strains (from freshly grown plates) were made in sterile water and 4 μl of each dilution was spotted on the indicated media. Unless indicated otherwise, plates were dried at room temperature before being incubated at 30° for 48 hr. For quantitative measurement of survival in the presence of chronic MMS exposure, strains were grown overnight in liquid YPD media prior to dilution to an OD600 reading of 0.2 (determined using an Eppendorf BioPhotometer) and grown for an additional 2 hr at 30°. Appropriate dilutions were plated on YPD plates with and without MMS and nicotinamide to yield a countable number of colonies, and plates were incubated for 5 days at 30°. All visible colonies were counted, regardless of size.

Liquid growth curves

Overnight cultures of wild-type yeast (YMD0963) were inoculated to an OD600 value of 0.2 (determined using an Eppendorf BioPhotometer) in 50 ml YPD and incubated at 30° with shaking for exactly 2 hr. Cells were again diluted to achieve a final OD600 value of 0.027 in 10 ml YPD. In a 100-well honeycomb plate, reactions matching the indicated conditions were set in a final volume of 200 μl. The plate was incubated in a Bioscreen C plate reader (Oy Growth Curves Ab) set at 30° and without shaking. A wideband wavelength range of 420–580 nm was used to measure optical density every 15 min for up to 4 days. Plates were monitored for contamination by plating aliquots of selected wells on YPD plates and by observation under the microscope.

Immunoblotting

For immunoblotting, 3–6 OD600 equivalents of cells in log phase were lysed using acid-washed glass beads in 300 μl 20% trichloroacetic acid (TCA). Supernatants (S1) were removed prior to washing of beads in an additional 300 μl 5% TCA to generate supernatant S2. S1 and S2 supernatants were combined and spun at 17,000 *g* for 4 min. Pellets were resuspended in 3× SDS-PAGE sample buffer (buffer was supplemented with 100 μl Tris-HCl, pH 8.8, 1.5 M, per ml and 100 μl 1 M DTT per ml) and boiled for 5 min before clarification via centrifugation at 17,000 *g* for 4 min. Typically, 10–20 μl of each sample was loaded per lane. SDS-PAGE gels were 8% (with the exception of Pnc1-flag experiments which used 10% gels) with 37.5:1 acrylamide:bisacrylamide (BioRad). Gels were transferred to PVDF membrane, blocked in 5% nonfat dry milk in TBST (0.1% tween) and incubated with anti-Rad53 antibody (1:2000 dilution, Santa Cruz, sc-6749) overnight at 4°. Secondary antibody was donkey anti-goat HRP-coupled antibody (Santa Cruz) and used at a dilution of 1:10,000. For anti-GFP blots, a monoclonal antibody from Abcam (JL-8) was used at a dilution of 1:2000. 3Flag was detected with M2 anti-flag from Sigma Chemical at a dilution of 1:2000. Secondary detection was with HRP-coupled goat anti-mouse antibody (BioRad) at a dilution of 1:10,000. Polyclonal rabbit antibodies provided by Adam Rudner were used to detect Cdc55 (1:10,000) and Clb2 (1:2500). These were detected with goat anti-rabbit secondary antibody coupled to HRP (BioRad). ECL reagent was from Perkin Elmer. Blots were exposed to autoradiography film purchased from Progene (Cat # 39-20810).

Data availability

Primer sequences and strains are available upon request. Strain genotypes are detailed in Table S1.

Results

Nicotinamide enhances MMS resistance

We initially set out to reexamine the role of sirtuins in regulating the response to DNA damage. Since we previously found that multiple sirtuins regulate acetylations on the same substrates (Downey *et al.* 2013, 2015), we used nicotinamide to inhibit these enzymes as a group (Figure 1A). We found that plates that included nicotinamide at a concentration of 5 mM allowed for increased growth in the presence of high concentrations of MMS (*i.e.*, 0.03% and above; Figure 1B). Interestingly, we did not observe nicotinamide-induced rescue of strains treated with the UV mimetic 4-nitroquinoline 1-oxide or replication fork-stalling agent hydroxyurea, suggesting that the suppressive effect of nicotinamide is specific to certain types of DNA damage (Figure S1, A and B). We also assayed the hyper-phosphorylation of Rad53, an essential checkpoint kinase, following MMS treatment in the presence or absence of nicotinamide. As shown in Figure 1C, Rad53 phosphorylation was equal under both conditions. Thus, nicotinamide does not allow for better growth following MMS treatment by preventing checkpoint activation.

Quantitative analysis of nicotinamide-induced MMS resistance

We turned to a liquid growth assay to confirm our initial observations and better understand the dynamic range of the observed effect. We used a Bioscreen C plate reader system to measure growth of wild-type yeast in the presence and absence of MMS and nicotinamide for >72 hr. A 5 mM concentration of nicotinamide had no impact on cell growth in the absence of DNA damage (Figure 1D). At the lowest concentrations of MMS tested (0.02%), nicotinamide also had minimal impact on growth, with curves appearing very similar in both the presence and absence of nicotinamide treatment. However, as we increased the concentration of MMS (0.025–0.0325%), nicotinamide improved growth

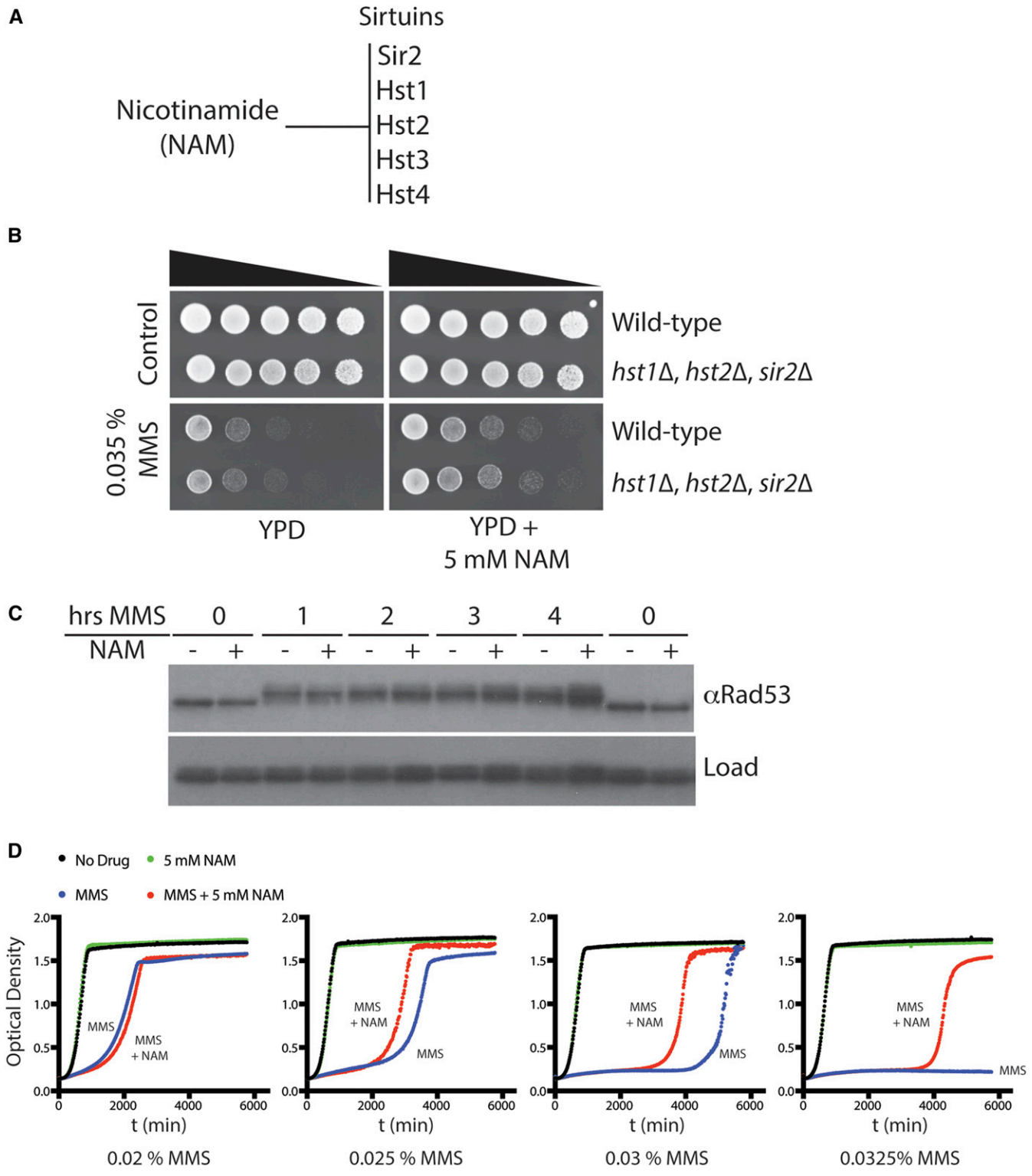

Figure 1 (A) Nicotinamide is an inhibitor of yeast sirtuins. Nicotinamide is a noncompetitive inhibitor of the five yeast sirtuins, Sir2 and Hst1–Hst4. (B) Nicotinamide suppresses MMS sensitivity. Serial dilutions (fivefold) of the indicated strains were spotted on YPD media with or without 0.035% MMS and 5 mM nicotinamide. Plates were grown at 30° for 48 hr. (C) Nicotinamide treatment does not affect checkpoint activation. MMS (0.035%) was added to control cells or cells grown in the presence of nicotinamide (5 mM, added 1 hr before MMS treatment). At the indicated time points, proteins were extracted as described in *Materials and Methods* prior to SDS-PAGE and immunoblotting with an antibody against Rad53, which is hyper-phosphorylated in response to DNA damage. An antibody against Cdc55 was used as a loading control. (D) Nicotinamide suppression of MMS sensitivity depends on MMS concentration. Growth of wild-type yeast (BY474) was monitored over 96 hr in the presence and absence of 5 mM nicotinamide and the indicated concentrations of MMS. Some variation was observed in the exact shape of the curves in different trials but graphs are representative of at least five repeats. NAM, nicotinamide.

(Figure 1D). The most dramatic effect was observed at the highest concentrations of MMS used (0.03 and 0.0325%). In these experiments, MMS-treated strains failed to grow for almost 48 hr. After that time, however, cultures also treated with nicotinamide began to grow, while nonnicotinamide-treated cultures remained arrested for another ~24 hr (0.03% MMS) or failed to grow at all during the experiment (0.0325% MMS) (Figure 1D). We also assayed the impact of varied nicotinamide concentrations on growth of strains treated with 0.03% MMS. Although the observed suppression of MMS sensitivity was most robust at 5 mM nicotinamide, concentrations of 10–20 mM had a similar effect in this assay (Figure S1C).

Nicotinamide suppresses MMS sensitivity independently of sirtuins

Since cells lacking *HST3* and *HST4* are reported to display increased sensitivity to DNA damaging agents (Celic *et al.* 2006; Maas *et al.* 2006), we reasoned that the positive effects of nicotinamide observed in our assays were likely due to inhibition of the remaining sirtuins, Hst1, Hst2, and Sir2, which impact the acetylation status of many proteins within the cell (Downey *et al.* 2013, 2015; Downey and Baetz 2016). However, an *hst1*Δ *hst2*Δ *sir2*Δ triple mutant failed to phenocopy treatment with nicotinamide (Figure 1B). Moreover, treatment with 5 mM nicotinamide allowed for suppression of MMS sensitivity of this strain in a manner similar to what we observed for wild type (Figure 1B). These same observations held true for various combinations of these mutants (data not shown), suggesting that nicotinamide does not promote viability on MMS via its inhibition of Hst1, Hst2, or Sir2.

We next considered whether Hst3 and Hst4 could be contributing to our observed effect, despite their well-documented sensitivity to DNA damaging agents (Celic *et al.* 2006; Maas *et al.* 2006; Celic *et al.* 2008). To test this, we examined the MMS sensitivity of strains mutated for all five sirtuins at once. These strains grew slowly and were substantially more sensitive to MMS treatment than their wild-type counterparts, in both the presence and absence of nicotinamide. Nevertheless, at 0.035% MMS, prolonged incubation revealed colony growth on plates with MMS and nicotinamide but not on plates with MMS alone (Figure 2A). A qualitatively similar effect was observed on lower concentrations of MMS that still significantly inhibited the growth of sirtuin quintuple mutant strains in the absence of nicotinamide treatment (Figure 2B). In addition to an apparent increase in the growth rate of colonies on MMS-containing plates with nicotinamide, a quantitative survival assay uncovered an approximately fivefold increase in overall viability (Figure 2C). Finally, we assayed an independently generated sirtuin quintuple mutant, derived from a different strain background (YPH499) (Brachmann *et al.* 1995). Here, we again observed that nicotinamide increased resistance to concentrations of MMS that significantly inhibited the growth of sirtuin quintuple mutants on their own (Figure S2). Thus, unexpectedly, nicotinamide can act independently

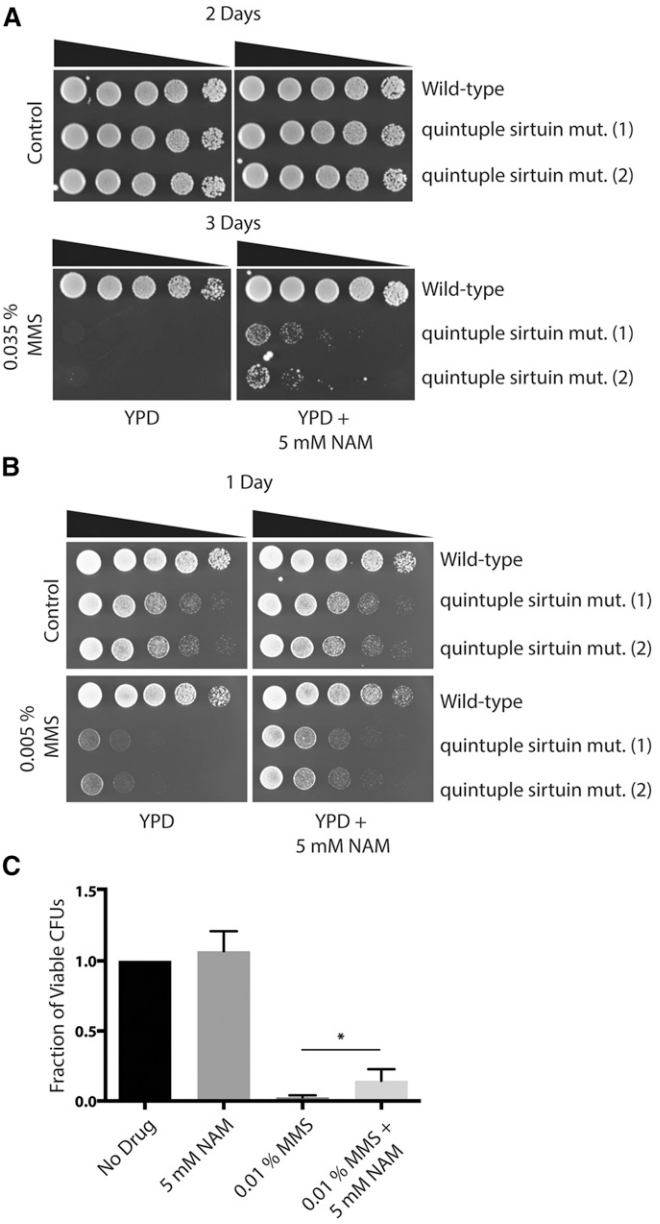

Figure 2 (A–B) Nicotinamide increases resistance to MMS independently of sirtuins. Serial dilutions (fivefold) of the indicated strains were spotted on YPD media with or without the indicated concentrations of MMS and 5 mM nicotinamide (NAM). Plates were grown at 30° for the indicated periods of time. Two isolates of the sirtuin quintuple mutants are shown. (C) Quantitation of MMS resistance in sirtuin mutants. Cells were plated on the indicated media to yield a countable number of viable colonies and CFUs were counted after 4 days. Viability is expressed as a fraction of CFUs on YPD control plates. *, statistically significant using ratio paired two-tailed Student's *t*-test ($P = 0.022$, $n = 4$ independent isolates, calculated using GraphPad Prism software). NAM, nicotinamide; mut., mutant.

of sirtuin HDACs to regulate survival in the presence of MMS.

Pnc1 protein levels are regulated by DNA damage

In yeast, nicotinamide can be converted to NAD+ via the Pnc1-dependent nicotinamide salvage pathway (Figure 3A)

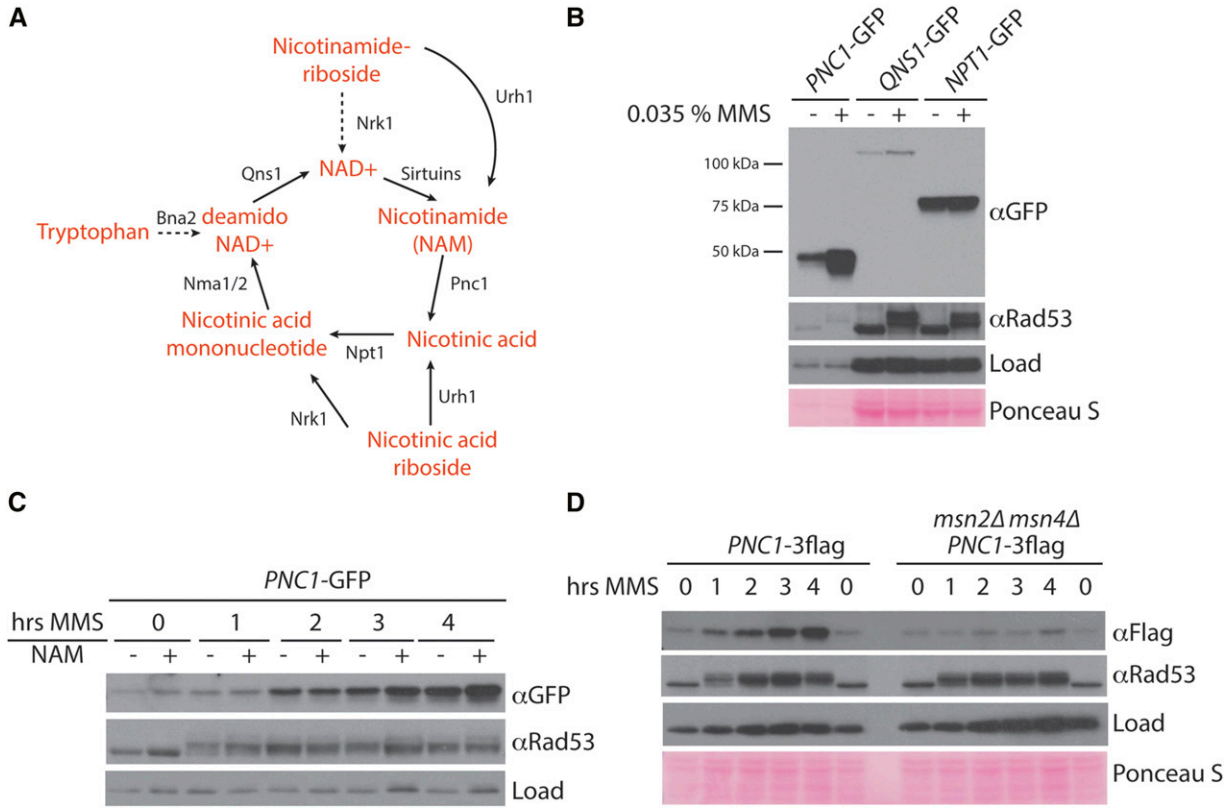

Figure 3 Pnc1 is regulated by DNA damage. (A) NAD+ biosynthesis in yeast. NAD+ can be produced *de novo* from tryptophan in a pathway requiring Bna2. NAD+ can also be made from exogenously added nicotinamide or that produced via sirtuin-mediated deacetylation reactions via the NAD+ salvage pathway in a stepwise reaction requiring Pnc1 and Npt1. Urh1 contributes to NAD+ synthesis by participating in the conversion of nicotinamide riboside to nicotinamide and catalyzing the conversion nicotinic acid riboside to nicotinic acid, which can enter the NAD+ salvage pathway. Solid arrows represent direct enzymatic action on an indicated metabolite, while broken arrows indicate one enzyme functioning within a larger chain of events not shown in this schematic (see Kato and Lin 2014). (B) Pnc1 levels increase after treatment with MMS. Strains expressing the indicated GFP-tagged proteins were treated with or without 0.035% MMS for 2 hr prior to protein extraction, SDS-PAGE, and immunoblotting with an antibody against GFP. For this figure, less protein was loaded for samples with Pnc1-GFP, which is very abundant, so that all NAD+ salvage enzymes could be assayed on the same blot. An antibody against Rad53 was used to assay checkpoint activation in damage-treated strains. An antibody against Cdc55 was used as a loading control. (C) Pnc1 levels are not affected by nicotinamide treatment. Pnc1 levels were assayed as in (B) in either the presence or absence of nicotinamide treatment (added 1 hr before MMS time course). Blot against Rad53 is from samples rerun on a different gel to achieve maximum separation of Rad53 isoforms. (D) Increased Pnc1 levels after MMS treatment require transcription factors Msn2 and Msn4. MMS at 0.035% was added to the indicated strains expressing flag-tagged Pnc1 and proteins were extracted at the indicated time points and subjected to immunoblot analysis with anti-flag antibody. As a control, Rad53 phosphorylation was assayed to monitor checkpoint activation. An antibody against Cdc55 was used as loading control. To allow proper visualization of highly expressed Pnc1-3flag, it was necessary to run diluted samples on a separate gel for this portion of the blot.

(Kato and Lin 2014). Therefore, we next sought to investigate whether flux through the salvage pathway impacted DNA damage sensitivity. We first tested whether salvage enzymes themselves might be regulated by DNA damage. Indeed, Pnc1 protein levels were previously reported to increase during various cellular stresses, including high salt, low glucose, and amino acid starvation (Medvedik *et al.* 2007). We observed that the levels of GFP-tagged Pnc1 increased after MMS treatment (Figure 3B), but this increase was not influenced by nicotinamide (Figure 3C). Levels of Qns1-GFP, which catalyzes the last step in the salvage pathway and is required for *de novo* NAD+ synthesis from tryptophan (Figure 3A), also increased. However, this effect was marginal compared to that observed for Pnc1-GFP (Figure 3B). *PNC1* transcription during stress is regulated by cooperative action of transcription factors Msn2 and Msn4 (Medvedik *et al.* 2007). To test the role of these in regulating Pnc1 levels during DNA damage, we generated strains expressing 3flag-tagged Pnc1 in wild type or *msn2*Δ *msn4*Δ double mutants. While Pnc1-3flag levels increased dramatically following MMS treatment of wild-type strains, only basal levels of Pnc1-3flag expression were detected in the *msn2*Δ *msn4*Δ mutants, even after 4 hr of MMS treatment (Figure 3D). We also assayed to determine whether the half-life of Pnc1-GFP was altered during treatment of cells with DNA damaging agents by using a cycloheximide chase assay (Figure S3). Pnc1-GFP appeared to have a half-life of >40 min and this turnover rate was not impacted by treatment of cells with MMS. Therefore, Msn2/4-regulated transcription of the *PNC1* gene appears to be the critical means of controlling Pnc1 levels in response to genotoxic stress.

MMS sensitivity of NAD+ biosynthetic mutants

The impact of nicotinamide on the MMS sensitivity of NAD+ biosynthetic mutants varied considerably. In strains deleted for *PNC1* or *NPT1*, which are encoding enzymes catalyzing the first two steps of nicotinamide salvage, exogenous nicotinamide failed to increase the growth of strains on plates with 0.0375% MMS (Figure 4, A and B). In fact, in most of our assays, nicotinamide reduced growth at these concentrations, contrary to what we observed previously for wild-type strains. At 0.04% MMS, *pnc1*Δ mutants grew at least equally well with nicotinamide as without. Nicotinamide-induced resistance was observed in some assays (*e.g.*, Figure 4B), but not others. Strains lacking Urh1, which promotes NAD+ synthesis from the precursors nicotinamide riboside or nicotinic acid riboside (Kato and Lin 2014), were hypersensitive to MMS, but grew similar to wild-type cells on plates with both MMS and nicotinamide (Figure 4A). The sensitivity of *urh1*Δ mutants to DNA damage may be attributable to its role in salvaging pyrimidine deoxyribonucleosides (Kurtz *et al.* 2002; Mitterbauer *et al.* 2002), high levels of which may facilitate DNA repair (Sirbu and Cortez 2013). Nicotinamide had no effect on the MMS sensitivity of a *bna2*Δ mutant, which is defective in *de novo* synthesis of NAD+ from tryptophan (Figure 4A) (Panozzo *et al.* 2002). Notably, nicotinamide enhanced the MMS resistance of *msn2*Δ *msn4*Δ strains (Figure 4B), wherein Pnc1 levels remained at basal levels after MMS treatment (Figure 3D).

Nicotinamide can promote resistance to MMS independently of PNC1 and NPT1

The failure of nicotinamide to robustly rescue sensitivity to MMS in *pnc1*Δ and *npt1*Δ mutants initially suggested that this novel effect required an intact nicotinamide salvage pathway. We wondered whether nicotinamide could be having both positive and negative impacts in these salvage pathway mutants. For example, negative impacts of nicotinamide in *pnc1*Δ mutants could stem from inhibition of sirtuins Hst3 and Hst4 in the absence of nicotinamide clearance and its subsequent conversion to NAD+ (see *Discussion*) (Figure 3A). Therefore, we generated strains lacking *NPT1* or *PNC1* in two different backgrounds mutated for all sirtuins. In this context, 5 mM nicotinamide clearly and consistently enhanced resistance to MMS (Figure 5, A and B, and Figure S4). These results indicate that nicotinamide promotes MMS resistance independently of both sirtuins and its conversion to NAD+ via the salvage pathway.

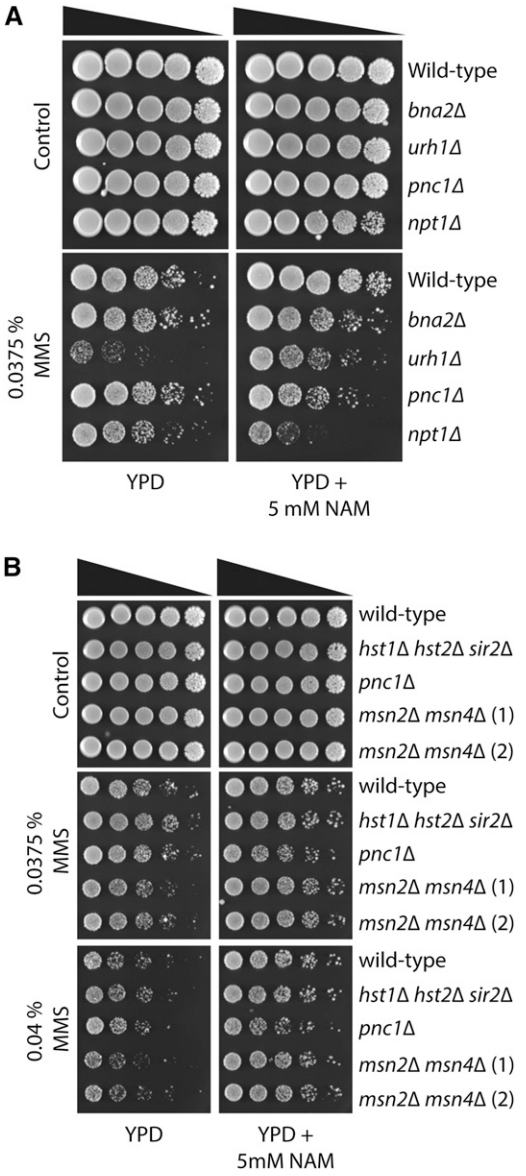

Figure 4 (A) MMS sensitivity of NAD+ biosynthetic mutants. The indicated strains were spotted in fivefold dilutions on media with or without MMS and nicotinamide, and incubated at 30° for 72 hr before being photographed. (B) Nicotinamide suppresses MMS sensitivity in strains lacking Pnc1 or Msn2/4. Strains with the indicated genotypes were spotted in a fivefold serial dilution series in the presence or absence of the indicated concentrations of MMS and nicotinamide. Plates were incubated at 30° for 72 hr prior to photographs being taken. NAM, nicotinamide.

Discussion

Nicotinamide promotes MMS resistance independently of sirtuins and Pnc1-mediated NAD+ synthesis

Phenotypes resulting from the addition of nicotinamide to cells are usually thought to stem from inhibition of the sirtuin family of deacetylases or the modulation of NAD+ levels in the cell. In this study, we found that nicotinamide can promote resistance to the DNA damaging agent MMS. Unexpectedly, our careful analysis of strains mutated for all five sirtuin-encoding genes allowed us to rule out these HDACs as the downstream targets mediating this effect. Critically, we were also able to demonstrate that this function of nicotinamide cannot be explained by its role in promoting NAD+ synthesis via the salvage pathway.

The addition of exogenous nicotinamide was also previously reported to decrease replicative lifespan—defined

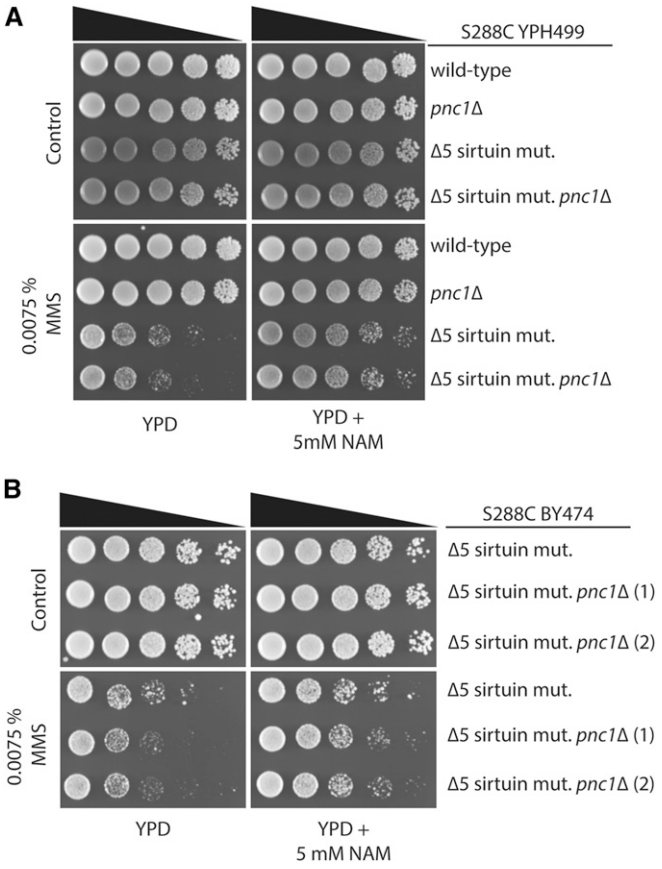

Figure 5 (A and B) Nicotinamide suppresses MMS sensitivity independently of both the salvage pathway and sirtuins. The indicated strains were spotted on media with or without MMS and nicotinamide, and incubated at 30° for 48 hr before being photographed. NAM, nicotinamide; mut., mutant.

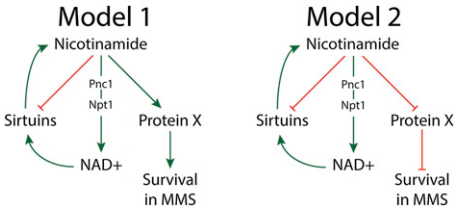

Figure 6 The complex role of nicotinamide in the DNA damage response. Nicotinamide regulates the sirtuin family of HDACs, which have important roles in the DNA damage response. This nicotinamide is converted to NAD+ via Pnc1 and Npt1 action in the salvage pathway. NAD+ is a required cosubstrate for sirtuins and has roles in diverse metabolic processes in the cell. We have identified a third branch of this pathway wherein nicotinamide promotes resistance to MMS treatment independently of sirtuins and the Pnc1-dependent salvage of nicotinamide into NAD+. Nicotinamide may promote the activity of enzymes required for DNA repair or the clearance of alkylated protein aggregates (model 1) or may serve to inhibit proteins that negatively regulate these processes (model 2).

as the number of times a mother cell can bud to give rise to a daughter—independently of sirtuins (Tsuchiya *et al.* 2006). However, it was not possible to examine this phenomenon in a strain lacking all five sirtuins and the role of the nicotinamide salvage pathway was not tested (Tsuchiya *et al.* 2006). While the mechanism behind this effect has remained elusive, one suggestion put forth by the authors was that enhanced accumulation of DNA damage in nicotinamide-treated strains could be responsible. Our observation that nicotinamide allows for increased growth on plates with MMS now suggests that this hypothesis may be incorrect. However, common downstream effectors could affect both replicative lifespan and survival during MMS treatment.

MMS sensitivity in nicotinamide salvage pathway mutants

We initially found that nicotinamide struggles to promote MMS resistance in salvage pathway mutants (Figure 4). However, in strains lacking sirtuin enzymes, nicotinamide improved resistance equally well in presence or absence of *PNC1* and *NPT1* (Figure 5 and Figure S4). We suggest that nicotinamide simultaneously both promotes and inhibits MMS resistance via different pathways. In *pnc1*Δ cells, the inhibitory effect could be mediated by a failure to properly clear exogenously added nicotinamide and convert it to NAD+ (McClure *et al.* 2008). This could negatively regulate functions of sirtuins that promote DNA repair. However, the same cannot be said for *npt1*Δ mutants, which also show a net sensitivity to MMS at 0.0375% in the presence of nicotinamide (Figure 4A), since they would be expected to clear nicotinamide properly (Figure 3A). Notably *npt1*Δ mutants are more sensitive to MMS than are *pnc1*Δ cells (Figure 4A), suggesting that the accumulation of the nicotinic acid intermediate (Figure 3A) may be particularly problematic for cell viability under these circumstances. Testing the impact of *npt1*Δ and *pnc1*Δ mutations on the levels of nicotinamide and salvage pathway intermediates will require the direct measurement of these metabolites using mass spectrometry.

Of all enzymes required for conversion of nicotinamide to NAD+, the level of Pnc1-GFP was most dramatically increased following treatment with MMS (Figure 3B). This increase, which is consistent with a previously reported increase in *PNC1* transcripts following MMS treatment (Gallo *et al.* 2004; Rahat *et al.* 2011), was fully dependent on Msn2 and Msn4 (Figure 3D). However, this transcriptional regulation of *PNC1* is not required for the positive effect of nicotinamide on cell viability, as nicotinamide still improved MMS resistance in *msn2*Δ *msn4*Δ mutants (Figure 4B). Yet, Pnc1 and other salvage enzymes are likely to play important roles in regulating sirtuin-dependent pathways that operate in parallel to the effect we have described here (Figure 6). Intriguingly, Pnc1-GFP levels were not affected by nicotinamide itself (Figure 3C), suggesting that increasing the cell's capacity for clearing nicotinamide may actually require stresses such as DNA damage. It is unclear if Msn2 and Msn4 are regulated by the DNA damage checkpoint or whether they respond to a more general stress response that might be activated in the presence of MMS. Notably, previous work identified Rad53-dependent phosphorylation sites on

Msn4 (Jaehnig *et al.* 2013), although it is not known if Msn4 is a direct target.

Models for the role of nicotinamide in promoting MMS resistance

A previous study found that at high molar concentrations (*i.e.*, 50 mol nicotinamide:1 mol MMS), nicotinamide can react with MMS *in vitro* (Hirayama *et al.* 1992). However, our data cannot be explained by a model wherein nicotinamide prevents DNA damage by reacting with MMS. First, an inactivation model is inconsistent with our observation that the positive contribution of nicotinamide to MMS resistance was most dramatic at the highest MMS concentrations tested (0.03 and 0.0325% in our liquid growth assays) and completely absent at a lower concentration (0.02%), where the nicotinamide:MMS molar ratio is highest (Figure 1D). Second, as shown in Figure 1C, we found that the activation of checkpoint kinase Rad53 was equal in both control and nicotinamide-treated strains in the presence of 0.035% MMS (also used for growth experiments in Figure 1B). These data suggest that the level of DNA damage endured under both conditions was equal and that nicotinamide acts downstream or in parallel to the initial stages of checkpoint activation.

Normally, the checkpoint response prevents cell division until damage is repaired (Melo and Toczyski 2002). Nicotinamide could promote DNA repair or help to turn off the checkpoint to allow resumption of the cell cycle once repair has been completed. Alternatively, nicotinamide could promote checkpoint adaptation, allowing for cell division in the presence of persistent damage (Toczyski *et al.* 1997). Finally, in addition to DNA damage, MMS may result in alkylation of proteins (Boffa and Bolognesi 1985), RNA (Drablos *et al.* 2004), or other cellular components and nicotinamide may promote cell survival by aiding in the clearance of these damaged molecules. To this end, it is intriguing that both Sir2 (Erjavec *et al.* 2007; Orlandi *et al.* 2010; Song *et al.* 2014) and NAD+ metabolic enzymes (Ocampo *et al.* 2013) have also been implicated in the response to proteotoxic stress. Thus, whether nicotinamide impacts DNA repair or protein dynamics (or both) in response to alkylation stress, it is very likely to function as part of a larger, complex, and interconnected response that includes both positive and negative regulators of cell survival.

Novel targets for nicotinamide?

At the molecular level, nicotinamide could either stimulate the activity of a protein that promotes cell survival in MMS or it could inhibit a negative regulator (Figure 6). Since nicotinamide forms part of the NAD+ molecule, NAD+-requiring enzymes are particularly attractive targets for its direct action. The *Saccharomyces* Genome Database (http://yeastgenome.org) (Chan and Cherry 2012) annotates 27 proteins with NAD+ binding capabilities (including the five sirtuins). Future work will focus on the role of these individual factors in coordinating a response to DNA damage and mediating the downstream effects of nicotinamide treatment in both yeast and in higher eukaryotes.

As part of the B-vitamin group, nicotinamide has important implications for human health. Both the *in vivo* modulation of NAD+ metabolites and inhibition of sirtuins have been proposed for the treatment of cancers and neurological conditions such as Alzheimer's disease (Demarin *et al.* 2004; Chopra *et al.* 2012; Martinez-Pastor and Mostoslavsky 2012; Zhang *et al.* 2015). Similarly, in rat models, nicotinamide has been observed to inhibit alkylation or ketamine-induced apoptotic neurodegeneration (Ullah *et al.* 2011, 2012). Our results highlight that nicotinamide and related metabolites may have unintended consequences and suggest that their application in clinical settings should be approached with care. This is also true for basic science work wherein nicotinamide has been used extensively to inhibit sirtuins with the intention of gaining novel insights into their functions. Our study cautions that conclusions derived from such experiments may require additional scrutiny. In particular, two recent studies have examined sensitivity of yeast deletion mutants to nicotinamide on a genome-wide scale, identifying a plethora of strains that are both sensitive and resistant to concentrations of 20 mM or higher (Choy *et al.* 2015; Simoneau *et al.* 2016). While these results were discussed in the context of sirtuin biology, our novel study suggests that sirtuin-independent effects should also be considered.

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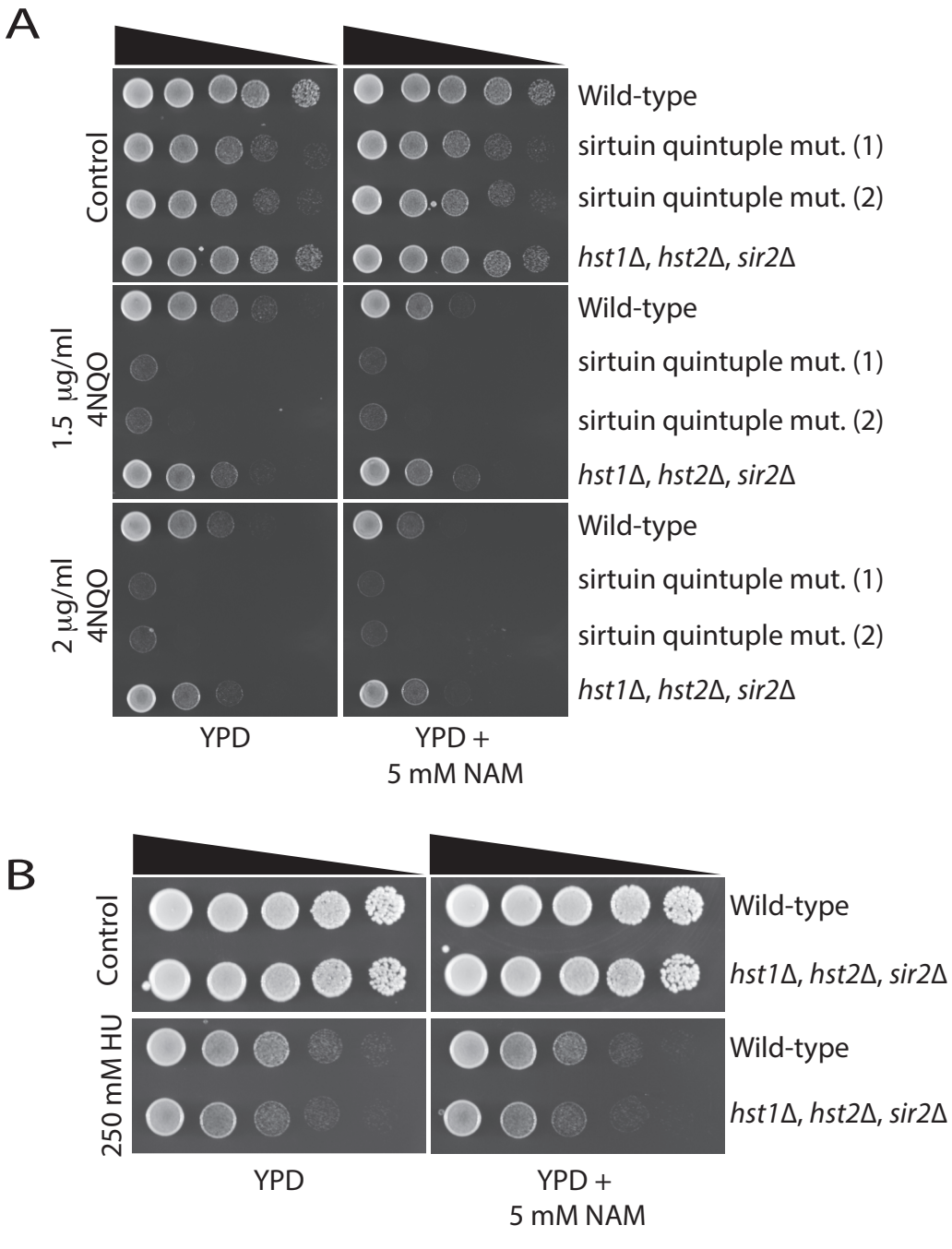


Figure S1- a)-b) Nicotinamide does not increase resistance to the UV-mimetic 4NQO or hydroxyurea (HU). The indicated strains were spotted in five-fold dilution series with or without 4NQO or HU at the indicated concentrations and nicotinamide at a concentration of 5 mM. Plates were incubated at 30 °C for 24-48 hours prior to taking photographs. **c) Impact of nicotinamide concentration on suppression of MMS sensitivity.** Growth of wild-type yeast (BY474) was monitored over 96 hours in the presence and absence of 0.03 % MMS and the indicated concentrations of nicotinamide. Some variation was observed in the exact shape of the curves in different trials (see variations observed here for MMS alone), but graphs are representative of at least 5 repeats.

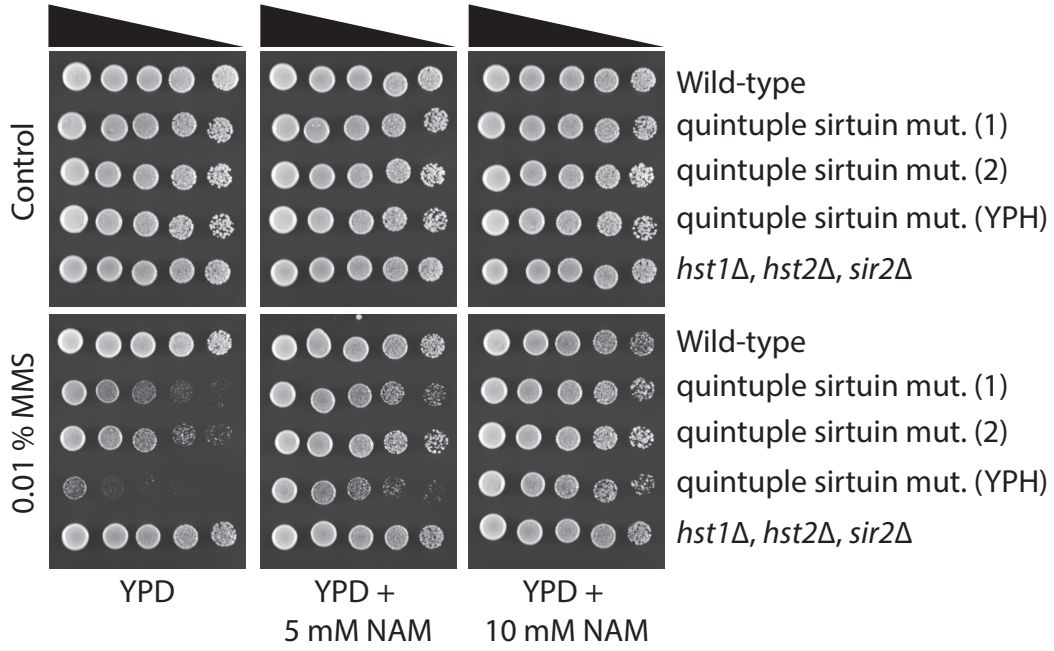


Figure S2 - **Suppression of MMS sensitivity by nicotinamide occurs independently of sirtuins.** The indicated strains were spotted on media with or without MMS and nicotinamide at the indicated concentrations and incubated at 30 °C for 48 hours before being photographed.

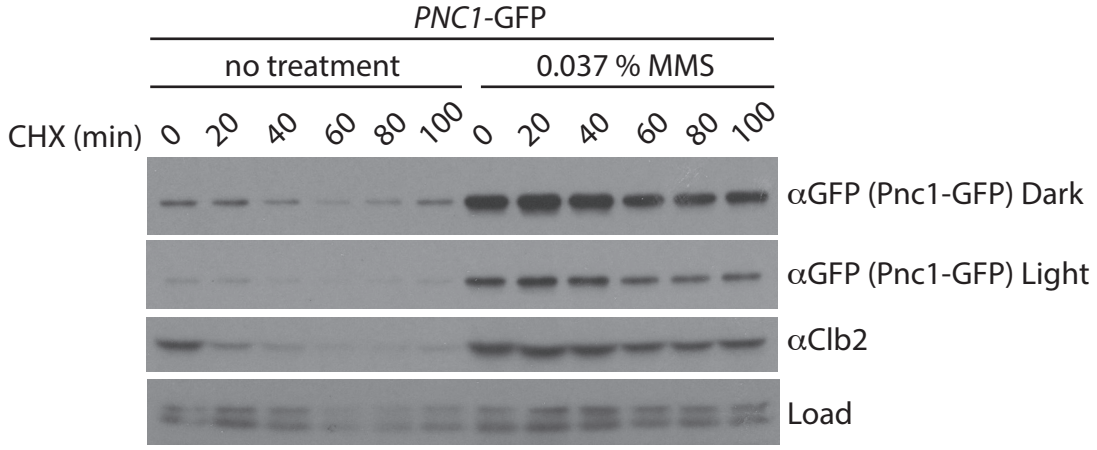


Figure S3 - **Pnc1-GFP turnover is not altered during DNA damage.** Cells expressing Pnc1-GFP were grown with or without 0.037 % MMS for 2 hours prior to the addition of cycloheximide (CHX) to stop protein translation. Samples were recovered every 20 minutes prior to protein extraction using TCA lysis, SDS-PAGE and immunoblotting with the indicated antibodies. Clb2 is an unstable protein that accumulates during DNA damage and is used here as a control. Since Pnc1-GFP is very highly expressed, diluted protein samples were run on a separate SDS-PAGE gel from that used for anti-Clb2 and loading control blots. Multiple exposures of the anti-GFP immunoblot are included so that Pnc1-GFP turnover in the presence and absence of damage can be judged on the basis of matched starting levels at time zero. An antibody against Cdc55 was used as a loading control.

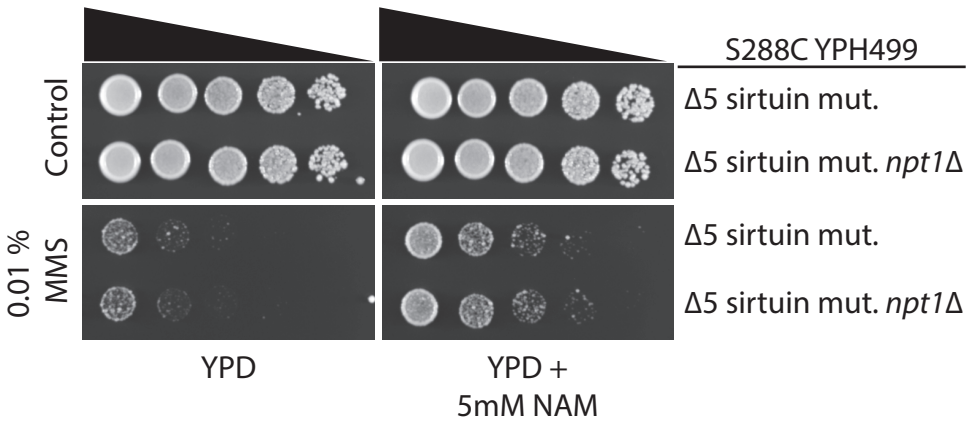
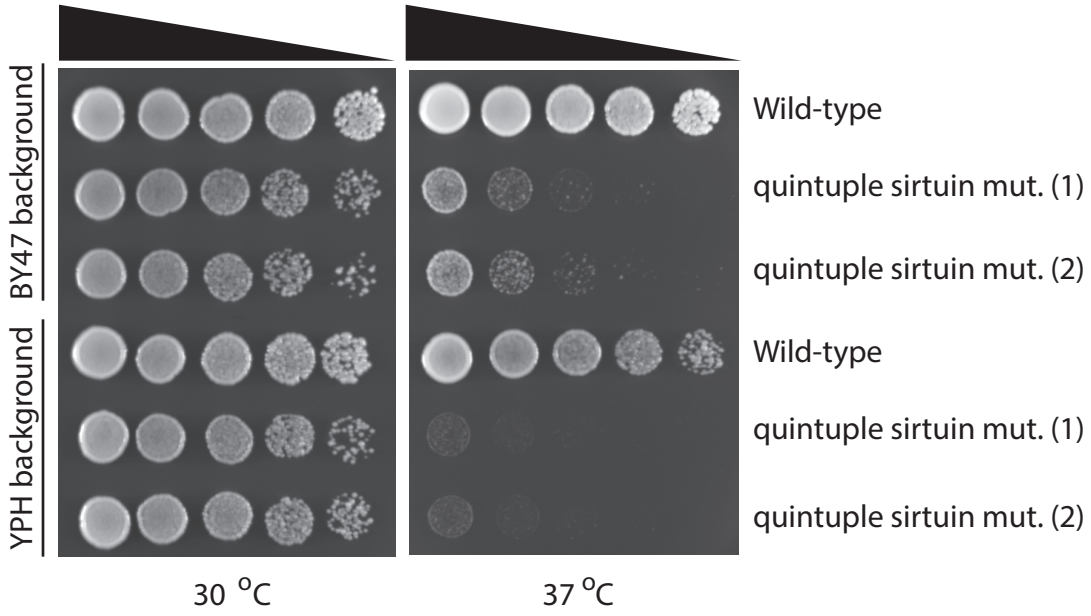


Figure S4 - **Nicotinamide promotes resistance to MMS independently of sirtuins and the salvage pathway.** The indicated strains were spotted in a five-fold dilution series with or without MMS and the indicated concentrations of nicotinamide. Plates were incubated at 30 °C for 48 hours prior to taking photographs.

A



B

		START		1 DAY		2 DAY		
		- MMS	+ MMS	- MMS	+ MMS	- MMS	+ MMS	
NAM	0 mM	6.88	6.88	6.68	6.56	6.63	6.51	0.075 % MMS
	5 mM	6.88	6.88	6.63	6.56	6.62	6.56	

		START		1 DAY		2 DAY		
		- MMS	+ MMS	- MMS	+ MMS	- MMS	+ MMS	
NAM	0 mM	6.91	6.89	6.60	6.33	6.66	6.31	0.03 % MMS
	5 mM	6.88	6.88	6.62	6.32	6.66	6.37	
	10 mM	6.83	6.84	6.62	6.40	6.66	6.41	
	15 mM	6.86	6.84	6.58	6.37	6.67	6.45	
	20 mM	6.87	6.84	6.64	6.43	6.66	6.48	

Figure S5 - **a) Sirtuin quintuple mutants are temperature sensitive.** The indicated strains were spotted in five-fold dilutions series at the indicated temperatures and incubated for 48 hours prior to taking photographs. **b) Nicotinamide has minimal impact on pH of media used for experiments.** The pH of uninoculated media with the indicated concentrations of nicotinamide and MMS was taken immediately upon combination of the components or after 1 or 2 days of incubation 30 °C.

Table S1. Strain genotypes. (.xlsx, 20 KB)

Available for download as a .xlsx file at
www.genetics.org/lookup/suppl/doi:10.1534/genetics.116.193524/-/DC1/TableS1.xlsx