Published in final edited form as:
Fungal Genet Biol. 2010 April ; 47(4): 297–309. doi:10.1016/j.fgb.2009.12.012.

PARP is involved in replicative aging in *Neurospora crassa*

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Abstract

Modification of proteins by the addition of poly(ADP-ribose) is carried out by poly(ADP-ribose) polymerases (PARPs). PARPs have been implicated in a wide range of biological processes in eukaryotes, but no universal function has been established. A study of the *Aspergillus nidulans* PARP ortholog (PrpA) revealed that the protein is essential and involved in DNA repair, reminiscent of findings using mammalian systems. We found that a Neurospora PARP orthologue (NPO) is dispensable for cell survival, DNA repair and epigenetic silencing but that replicative aging of mycelia is accelerated in an *npo* mutant strain. We propose that PARPs may control aging as proposed for Sirtuins, which also consume NAD+ and function either as mono(ADP-ribose) transferases or protein deacetylases. PARPs may regulate aging by impacting NAD+/NAM availability, thereby influencing Sirtuin activity, or they may function in alternative NAD+-dependent or NAD+-independent aging pathways.

INTRODUCTION

Poly(ADP-ribose) polymerases (PARPs) are ADP-ribose transferases that catalyze the formation of both linear and branched polymers of ADP-ribose (PAR) on target proteins. PAR is covalently linked to the γ–carboxy group of glutamic acid residues at acceptor sites (BURZIO *et al.* 1979; RIQUELME *et al.* 1979). Poly(ADP-ribosylation) (PARylation) consumes nicotinamide adenine dinucleotide (NAD+) and generates nicotinamide (NAM). The addition of PAR to proteins is thought to have dramatic effects on their catalytic activities, as well as on potential protein-protein and protein-nucleic acid interactions (BURKLE 2000; D'AMOURS *et al.* 1999; KRAUS and LIS 2003). Recently a number of different proteins have been identified that bind to PAR both *in vitro* and *in vivo*, including proteins containing Macro domains and proteins containing novel poly(ADP-ribose)-binding zinc finger (PBZ) motifs (AHEL *et al.* 2008; KARRAS *et al.* 2005). In higher eukaryotes PARylation is reversible through the action of PAR glycohydrolases (PARG), which are active in a variety of subcellular compartments, and are thought to be important in regulation of cell death after DNA damage

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(AME *et al.* 2009a; AME *et al.* 2009b). Thus, the principle players in PARylation thus far identified are the PARPs, PARG and PAR binding proteins.

PARP homologs have been identified in plants, metazoans, protists and filamentous fungi, but not in the yeasts, while PARG homologs have been identified in all eukaryotes, excluding fungi. PARPs and PARylation impact a variety of biological processes including development, transcriptional regulation, chromatin structure, epigenetic phenomena, DNA repair, mitosis, genome stability, neuronal function, cell death and aging (BENEKE and BURKLE 2004; BENEKE and BURKLE 2007; BOUCHARD *et al.* 2003; BOULU *et al.* 2001; BURKLE 2000; BURKLE 2001a; BURKLE *et al.* 2005; CHIARUGI and MOSKOWITZ 2002; D'AMOURS *et al.* 1999; HERCEG and WANG 2001; HONG *et al.* 2004; JEGGO 1998; KIM *et al.* 2005; KRAUS and LIS 2003; PIEPER *et al.* 1999; SMULSON *et al.* 2000).

The canonical PARP enzyme from mammals, PARP-1, has been implicated in both double and single strand break repair (DSB and SSB), as well as base excision repair (BER) (BURKLE 2001b; DANTZER *et al.* 1999; MASUTANI *et al.* 2003). In human and mouse cells, the majority of PARylation involves auto-modification of PARP-1 in response to DNA damage and PARP-1 has been described as a DNA damage sensor (D'AMOURS *et al.* 1999; DE MURCIA *et al.* 1997; HULETSKY *et al.* 1989; OGATA *et al.* 1981). Residual PARylation is detectable in mouse embryonic fibroblast homozygous for PARP-1 null mutations (PARP-$1^{-/-}$) (SHIEH *et al.* 1998) and this may reflect PARP-2, which has also been shown to PARylate in response to DNA damage (AME *et al.* 1999). Both PARP-$1^{-/-}$ and PARP-$2^{-/-}$ mice are viable, but are sensitive to DNA damaging agents, and PARP-$1^{-/-}$ mice have inherent genomic instability (DE MURCIA *et al.* 1997; MENISSIER DE MURCIA *et al.* 2003; TRUCCO *et al.* 1998; WANG *et al.* 1995; WANG *et al.* 1997). PARP-$1^{-/-}$/PARP-$2^{-/-}$ mice die as embryos prior to E8.0, and PARP-$1^{+/-}$/PARP-$2^{-/-}$ female mice exhibit X-chromosome instability, infertility, and higher levels of embryonic lethality (MENISSIER DE MURCIA *et al.* 2003). These results suggest that PARylation may be essential in higher eukaryotes.

A recent investigation using the filamentous fungus *Aspergillus nidulans* revealed the presence of a single PARP ortholog (PrpA) (SEMIGHINI *et al.* 2006). Disruption of the *prpA* gene was found to be lethal in haploid strains, and diploid strains carrying only a single copy of *prpA* had severe growth restrictions and were found to be sensitive to several mutagenic compounds (SEMIGHINI *et al.* 2006). These results suggest that the requirement of PARP for DNA repair and viability is conserved between animals and filamentous fungi.

In addition to evidence that PARPs and PARylation control diverse aspects of gene expression, DNA repair and genome stability, there are suggestions that PARP-1 is involved in controlling aging in metazoans. GRUBE and BURKLE (1992) found a strong positive correlation between lifespan and the degree of PARP activity in leukocytes of 13 mammalian species. Long-lived species had higher levels of PARylation, but similar levels of PARP protein, implying greater enzyme activity (GRUBE and BURKLE 1992). In addition, the WRN protein, which is defective in individuals with the premature aging disorder Werner's syndrome, was found to physically and functionally interact with PARP-1 (LI *et al.* 2004; VON KOBBE *et al.* 2004).

Research using microorganisms as models for aging has been dominated by studies in *Saccharomyces cerevisiae*. Replicative lifespan in *S. cerevisiae* is measured by determining the number of daughter cells an individual mother cell can produce (MORTIMER and JOHNSTON 1959). Mutations in SIR (Silent Information Regulator) complex components were isolated in a genetic screen designed to identify genes that control this form of aging (KENNEDY *et al.* 1995). In particular, the NAD+-dependent histone deacetylase Sir2 was shown to be a key regulator, acting to suppress recombination between rDNA repeats, thereby blocking the formation of extrachromosomal rDNA circles (ERCs), which are antagonistic to

long replicative lifespan in budding yeast (KAEBERLEIN *et al.* 1999; SINCLAIR and GUARENTE 1997).

Although Sir2-like proteins (Sirtuins) have been implicated in controlling lifespan in metazoans, regulation of ERC production is thought to be a yeast-specific aging mechanism (ROGINA and HELFAND 2004; TISSENBAUM and GUARENTE 2001). Like Sir2 itself, Sirtuins are NAD+-dependent enzymes. Some Sirtuins act as mono(ADP-ribose) transferases (ARTS), others function as protein deacetylases, and some have both activities (BELENKY *et al.* 2007). Genetic and biochemical investigations using *S. cerevisiae* have established that NAD+ and NAM levels impact replicative aging through regulation of Sir2 deacetylase activity (GALLO *et al.* 2004; SANDMEIER *et al.* 2002). Additional studies have shown that lifespan extension by calorie restriction (CR) in *S. cerevisiae* involves Sir2, as well as the NAD+-dependent deacetylase Hst2, and is thus regulated by NAD+ and NAM levels as well (ANDERSON *et al.* 2003; LAMMING *et al.* 2005; LIN *et al.* 2000; LIN *et al.* 2004). In addition, a yeast pathway for Sirtuin-independent lifespan extension by CR is also influenced by NAD+ and NAM availability (TSUCHIYA *et al.* 2006). The fact that CR extends lifespan in higher eukaryotes, and that Sirtuins have been implicated in controlling aging in flies and worms suggests that NAD+ and NAM metabolism may be of general importance in the regulation of lifespan. While Sirtuins are present in all eukaryotes including the yeasts, additional ARTS, along with PARPs and cADP-ribose synthases exist in metazoans and filamentous fungi (BELENKY *et al.* 2007). All of these enzymes are major consumers of NAD +, and might therefore be expected to impact aging. While aging studies in *S. cerevisiae* have provided many valuable insights, the involvement of certain key biological regulatory pathways that are common to many eukaryotic organisms, but absent from yeast, have not been adequately investigated. Research directed at understanding the roles of PARP and PARylation in aging of higher eukaryotes may be hindered by functional redundancy of multiple PARP enzymes and lethality of PARP mutants. Thus we chose to explore the function of PARP in the filamentous fungus *N. crassa*, which only has a single gene encoding this enzyme.

MATERIALS AND METHODS

Media and culturing conditions

N. crassa was cultured as described previously (DAVIS and DESERRES 1970). Strains were grown in liquid Vogel's minimal media with 1.5% sucrose or 2% glucose and supplements were added where indicated. Solid media was the same but with 2% agar. Strains were grown on FGS (0.05% fructose, 0.05% glucose, 2% sorbose, 1X Vogel's salts, 2% agar) to induce colonial growth. The concentrations of supplements were as follows: 1X alanine (1 mg/ml), 1X anthranillic acid (140 μg/ml), 1X histidine (0.5 mg/ml), 1X lysine (0.6 mg/ml), 1X nicotinamide (10 μg/ml). Hygromycin was used in the range of 200 μg/ml-1.5 mg/ml. Crosses were carried out on synthetic crossing medium with glucose or sucrose concentrations at 0.5% or 2.0%.

Southern and northern blots

DNA was isolated from *N. crassa* and Southern blots performed as previously described (LUO *et al.* 1995; MIAO *et al.* 2000). RNA extraction and northern blots were performed as previously described (ROUNTREE and SELKER 1997).

Analysis of PARP, MacroD and zf-PARP sequences

Fungal PARP-like proteins, MacroD-like, zf-PARP-like and their ORFs were identified using the BLASTP and TBLASTN programs at NCBI (http://www.ncbi.nih.gov). The BLASTP program was also used at the Broad Neurospora Genome Project database (http://www.broad.mit.edu/annotation/genome/neurospora) to identify the NPO and MacroD

proteins, and the NGP genome browser was used to identify their ORFs. Protein motifs and domains were verified in the SMART database (http://smart.embl-heidelberg.de/). All sequence alignments and analysis was performed with programs at the SDSC Biology Workbench. The BL2SEQ program was used to compare NPO with human PARP-1 and PARP-2. The *npo* gene and amino acid sequence shown in Figure 3 was generated with the Publish program using Genetics Computer Group (GCG) software.

Analysis of subcellular localization of GFP tagged proteins

PCR products of the *hp1*, *rap1*, *mcd* (MacroD) and *npo* genes amplified from wild type Neurospora were cloned into *pMF272* (HONDA and SELKER 2009) to allow *his-3* targeting of GFP tagged fusions expressed from the Neurospora *ccg-1* promoter. These PCR products spanned the start and stop codons of these genes, excluding five prime and three prime UTR sequences. The details of all cloning steps are available upon request. The Neurospora *yph1* and *zfp* (zf-PARP) genes were cloned, along with 2 kb of upstream sequences, as *NotI-PacI* PCR fragments into *NotI-PacI* digested *pMF272*. The *NotI-PacI pMF272* restriction fragment lacks the *ccg-1* promoter. All GFP fusion constructs were used to transform a *his-3* targeting strain p49 (relevant genotype: *his-3*; *inl*; npo^{+}) obtained by crossing the original *npo* KO strain (14-6-1-1A) with FGSC 7508. Conidia from transformants were imaged using a Zeiss LSM 510 confocal microscope at 630× magnification. Images were taken as Z-sections and analyzed using the program ImageJ. Individual slices were selected and saved as JPEGs.

Knockout of *npo* by homologous replacement

The *npo* gene was amplified by PCR from wild type *N. crassa* using the following primers: 5'-CAAATGGACGAAAGAGGAGA-3' and 5'-TGGTGAAAGGAAGGATGGAA-3'. The 6.5kb PCR product was digested with *Eco*RI and *Sac*I, and cloned into pBluescript SK+. This construct was then digested with *Xho*I to remove the *npo* ORF and the hygromycin B-resistant gene (*hph*), derived from pCB1003 (CARROLL *et al.* 1994), was cloned in its place. The resulting plasmid (pP1) contains the *hph* gene flanked by 1.9 kb and 1.0 kb of *npo* upstream and downstream sequences, respectively. To knockout the *npo* gene, wild type *N. crassa* (74-OR31-14a) was transformed with an *Eco*RI –*Sac*I fragment from pP1. Transformation was carried out by electroporation as described (NINOMIYA *et al.* 2004) and hygromycin resistant transformants were crossed to a wild type *N. crassa* strain of opposite mating type (74-OR31-16A) to render the integrations homozygous. Finally, *npo* knockout mutants were identified by PCR and Southern hybridization.

Cloning and mutation of *npo* by RIP

npo was cloned by PCR amplification from wild type *N. crassa* (N150, 74-OR23-IVA) using the following primers: (2653F) 5'-TCGAATTCATGCCGCCCAGACGAGCAAAG-3'; (2653R) 5'-CTGCGGCCGCTCATACGCAATGTACTCGTTG-3'. The PCR product was digested with *Eco*RI and *Not*I and cloned into *pBM61* (MARGOLIN 1997) to generate *pGK111*. This construct was linearized with *Dra*I, and targeted to the *his-3* locus in strain N1674. Ten transformants were isolated, and correct integrations were confirmed by Southern hybridization. Four of the transformants (pGK111-T1, T2, T3, T4) were crossed with strain N1444 and DNAs from ten histidine prototrophic progeny from each of the four crosses were analyzed for evidence of mutation of *npo*. Probing of Southern blots of *Dpn*II/*Sau*3A digested DNAs with *npo* sequences revealed RFLPs and heavy methylation in progeny 11 (P11), among others. P11 was obtained from a cross of strain pGK111-T2 with N1444. From here on this strain is referred to as N3180. The endogenous *npo* gene was cloned by PCR from strain N3180 using the following primers: (2653F2) 5'-CTTCACACACTTCACACCTTTGTTTC-3'; (2653R2) 5'-GCTATCTTGACACGGAAAAG-3'. Digestion of the PCR product with *Dpn*II confirmed the presence of the RFLPs detected by Southern blot, and the PCR product was gel

isolated and sent for sequencing using primer 2653F. The *npo* allele present in N3180 is designated *npo*^{RIP1}.

Testing for genetic interactions between *npo*^{RIP1} and *N. crassa* Sirtuins (*nsts*)

For the purpose of isolating the *npo*^{RIP1} allele in a *mat a* background, and to look for possible genetic interaction between *npo* and *nst-1*, N3180 was crossed with N1983 (*mat a*; *mtr col4; nst-1*^{RIP1} *trp-2*). No obvious defects in growth or development were observed in double mutant progeny. Strain N3181 (*mat a*; *npo*^{RIP1}; *nst-1*$^{+}$) was obtained from this cross. To isolate *npo*^{RIP1} in a background with a TPE marker and both *nst-1* and *nst-3* mutations, N3181 was crossed with N2636 (*mat A nst-3*^{RIP1}; *mtr col4*; *telVR::hph::T*; *nst-1*^{RIP1} *trp-2*). Numerous progeny were isolated from this cross and Southern blots were used to determine their genotypes. Among the progeny were P6 (*nst-3*$^{+}$; *npo*$^{+}$ *telVR::hph::T*; *nst-1*$^{+}$), P80 (*nst-3*$^{+}$; *npo*^{RIP1} *telVR::hph::T*; *nst-1*$^{+}$) and P23 (*nst-3*^{RIP1}; *npo*$^{+}$ *telVR::hph::T*; *nst-1*$^{+}$) which were tested for TPE (see Fig. 7) along with others. No obvious defects in growth or development were observed for triple mutant progeny.

TPE assays

Progeny from the cross of N3181 with N2636 were spot-tested on hygromycin to assay the effects of mutation of *npo*, *nst-1* and *nst-3* on TPE in genetic backgrounds with *telVR::hph::T* (SMITH *et al.* 2008). All possible combinations of alleles were analyzed. Approximately 1000 conidia were spot-tested on FGS plates containing 600 μg/ml or 1.5 mg/ml hygromycin and supplemented with alanine, lysine, inositol and anthranillic acid. Spot-tests were also done on identical plates with no hygromycin as a control for growth.

Mutagen sensitivity assays

For mutagen sensitivity assays, progeny from the cross of N3181 with N2636 were spot-tested on the same media used in the TPE assays, but containing either MMS (0.03%), MNNG (0.5 μg/ml), EMS (0.3%) or CPT (0.3 μg/ml). As with the TPE assay approximately 1000 conidia were spot-tested, and identical control plates with no mutagen were used as a control for growth. Mutagen sensitivity of the *npo* KO strain was tested as previously described (WATANABE *et al.* 1997).

PARylation assay

Crude *N. crassa* extracts were incubated in 50 mM Tri-HCl (pH8.0), 10 mM $MgCl_2$, 1 mM dithiothreitol, 10 μM (74 KBq/nmol) ^{32}P-NAD (Du Pont), 20 μg/ml activated DNA (Sigma) and 20 μg/ml calf thymus type II-A histones (Sigma H9250) at 25°C for 30 min. To stop the reaction, the PARP inhibitor 3-aminobenzamide was added to 5 mM and unincorporated NAD was removed using spin columns containing Sephadex G-50 resin (GE Healthcare). *E. coli* extracts expressing human recombinant PARP-1 (IKEJIMA *et al.* 1990) were used as positive controls. After centrifugation at 300×*g* for 4 min, the eluent containing ^{32}P-PARylated proteins was treated with 0.1 M NaOH at 37°C for 30 min. to detach ^{32}P-PAR, and the solution was neutralized by addition of Tri-HCl (pH7.5) to 50 mM and HCl to 0.1 N. After extraction with water-saturated phenol and chloroform-isoamyl alcohol (49:1 (v/v)), ammonium acetate was added to 2 M and ^{32}P-PAR was ethanol-precipitated. After washing with 70% ethanol, the fraction was dried and dissolved in a loading dye containing urea (PANZETER and ALTHAUS 1990). The fraction was then analyzed by 20% polyacrylamide gel electrophoresis as described elsewhere (PANZETER and ALTHAUS 1990). The gel was exposed and analyzed with BAS2500 (Fuji Film). The radioactive area containing ^{32}P-PAR was cut out and further analyzed. The gel fragments were rinsed with water and crushed. The radioactive material was eluted and digested by incubation overnight at 25°C in 100 μl of a PARG buffer containing 20 mM potassium phosphate (pH7.5), 10 mM β-mercaptoethanol, 0.05% triton X-100 (Sigma),

0.1% bovine serum albumin and rat PARG-conjugated with glutathione-*S*-transferase (GST-PARG) (SHIMOKAWA *et al.* 1999). Treatment with GST-PARG digested PAR to ADP-ribose, and the reaction mixture was treated with perchloric acid at 0.5 N on ice for 20 min and neutralized with 0.7 M glycyl-glycine-3 M potassium hydroxide and centrifuged at 15,000×*g* for 5 min at 4°C. The supernatant was subjected to high performance liquid chromatography (HPLC). HPLC was carried out using Develosil columns (C30-UG-5, Ø46×250 mm, Nomura Chemicals). UV absorbance was monitored at 254 nm (Toso, UV-8000). A linear gradient elution for 100 min. using buffer A (0.1 M ammonium acetate) and buffer B (50 mM ammonium acetate–50% acetonitrile) was performed, ranging from 2% to 100% buffer B at a flow rate of 0.5 ml/min. The retention time of ADP-ribose was 17–19 min. Each 0.5 ml fraction between 13–20 min. was concentrated and spotted on DE81 paper (Whatman) and analyzed by BAS2500.

Telomere erosion assay

Genomic DNAs from wild type and the *npo* strain were digested with *Cla*I and *Hin*dIII. Electrophoresis was carried out in a 2.5% agarose gel for 5 hrs at 50mV and Southern blots performed as previously described (LUO *et al.* 1995; MIAO *et al.* 2000). These blots were then probed with a non-isotopically labeled oligo composed of 7 direct tandem copies of the telomere repeat sequence [5'-CCCTAA-3'].

RESULTS AND DISCUSSION

There are two classes of fungal PARP-like proteins

SEMIGHINI *et al.* (2006) observed that PARP homologs exist in fungi that have multicellular hyphae and sophisticated developmental structures, but lack a prominent yeast-like budding growth phase. The canonical PARP enzyme from mammals, PARP-1, contains an N-terminal zinc finger DNA binding domain (zf-PARP), a BRCT motif that is the major target for auto-modification, a WGR motif, and a core catalytic domain (AME *et al.* 2004; KIM *et al.* 2005). We performed TBLASTN searches through the NCBI (http://www.ncbi.nih.gov) fungal genome databases using the human PARP-1 catalytic domain as the query. We then analyzed the hits using the SMART database (http://smart.embl-heidelberg.de/) to confirm the presence of a PARP catalytic domain (pfam 00644). Our analysis revealed two classes of PARP-like proteins: 1. homologous to *A. nidulans* PrpA, containing BRCT (pfam 00533) and WGR motifs (pfam 05406), and 2. those with a catalytic domain most similar to mammalian PARP-6/PARP-8 family members and having a carboxyl terminal extension showing homology to the catalytic domain (SMART 00212) of ubiquitin conjugating enzyme E2 (Fig. 1A). This domain organization seems to be specific to filamentous fungi. We refer to this second class of fungal PARP-like proteins as PARP/E2. Like the PrpA class, the PARP/E2 proteins are broadly distributed in the euascomycetes. In fact, *N. crassa* is the only euascomycete represented in the NCBI fungal genome databases (25 species) that does not have a PARP/E2 homolog, raising the possibility that a *N. crassa* homolog is in a sequencing gap. Homologs in the PARP/E2 class were also found in the basidomycetes *Coprinus cinereus* (EAU83704.1) and *Phanerochaete chrysosporium* (unannotated protein, contig accession: AADS01000086, gi: 46851846, approx. coordinates 71000–75000).

N. crassa has a single PARP homolog of the PrpA class

Fungal PARP proteins of the PrpA class lack an amino terminal zinc finger DNA-binding domain (zf-PARP), but have both an N-terminal BRCT motif and a WGR motif (SEMIGHINI *et al*. 2006). A BLASTP search with PARP-1 sequences through the Broad Institute Neurospora genome database (http://www.broad.mit.edu/annotation/genome/neurospora) identified a single ORF encoding a predicted protein of 592 amino acids with a WGR motif, but lacking a BRCT motif (NCU08852.3, EAA31746, GI:157070000, accession AABX02000063.1). We

feel that the most likely start codon for this ORF is 235 nucleotides upstream of that suggested by the Broad annotation, which would predict a protein of 670 amino acids with both WGR and BRCT motifs, as expected for a member of the PrpA class (Fig. 1A). We refer to this protein as NPO (Neurospora PARP Ortholog). The presence of BRCT motifs in the PrpA class of fungal PARPs, and their absence from PARP-2 homologs, suggests that proteins of the PrpA class are more closely related to PARP-1. However, comparison of NPO with human PARP-1 and PARP-2 using the BL2SEQ program suggests a closer relationship with PARP-2 [NPO:PARP-1, e $=10^{-79}$, identities = 216/681 (31%), similarities = 335/681 (49%), gaps = 67/681 (9%); NPO:PARP-2, e = 3×10^{-82}, identities = 187/464 (40%), similarities = 259/464 (55%), gaps = 39/464 (8%)]. In agreement with our analysis SEMIGHINI *et al.* (2006) observed that PrpA-like PARPs belong to a microbial clade more similar to PARP-2 than PARP-1.

Fungal zf-PARP proteins, Macro domain proteins, and nuclear localization of NPO

Fungal PARP-like proteins, including NPO, lack any obvious DNA binding domain, raising the question of whether these proteins are principally associated with chromatin, like their metazoan counterparts. NPO might contain a cryptic DNA-binding domain or could require a partner for DNA binding. Because PARP-1 contains a highly characteristic amino terminal zinc finger DNA-binding domain (zf-PARP), we sought to identify fungal proteins containing a similar motif. To this end we performed TBLASTN searches through the NCBI fungal genome database using the PARP-1 zinc finger as query. These searches identified a single Neurospora ORF encoding a protein of 404 amino acids, containing a single zinc finger of the zf-PARP class (Fig. 1B). We refer to this protein as ZFP. Although this ORF has not been annotated with an NCU number in the Broad database, we believe that it represents a functional gene, as a GFP tagged form, expressed via its own promoter, has a punctate nuclear staining pattern, similar to the heterochromatin associated protein, HP1 (Fig. 2) (FREITAG *et al.* 2004a). To determine the subcellular location of NPO we tagged the protein with GFP at its carboxyl terminus, and expressed the fusion protein in aerial hyphae and conidia using the developmentally regulated *ccg-1* promoter (Fig. 2) (FREITAG *et al.* 2004b;LOROS *et al.* 1989;MCNALLY and FREE 1988). GFP tagging of ectopically expressed NPO verifies that this protein is also localized primarily to nuclei (Fig. 2), and thus has a functional nuclear localization signal. The subnuclear distribution of NPO seems to be essentially uniform, in comparison to proteins localized specifically to heterochromatin (HP1), telomeres (RAP1) and rDNA (YPH1) (Fig. 2).

We have not been able to identify any PARG-like protein in any filamentous fungal database, and thus PAR may be a more stable posttranslational modification in fungi than in higher eukaryotes. Although we were unable to identify fungal proteins with the PAR-binding C2H2 zinc finger (PBZ) domain (AHEL *et al.* 2008), we did identified one ORF encoding a protein of 277 amino acids with a single Macrodomain (also designated as A1pp) (Fig. 1C). Macrodomains have also been shown to bind PAR both *in vivo* and *in vitro* (KARRAS *et al.* 2005). The Neurospora Macrodomain protein is annotated in the Broad database as NCU07925.3, and we refer to it as MCD (Macrodomain). An over-expressed GFP tagged form of MCD has essentially uniform cytoplasmic and nuclear distributions, but is slightly more concentrated in nuclei than in cytoplasm (Fig. 2). Thus, while NPO has an autonomous nuclear localization signal, it may be brought to DNA via association with other proteins such as ZFP. Furthermore, while fungi are unlikely to remove PAR via a glycohydrolase activity, as mammals do, they are likely to recognize PAR via nuclear localized Macrodomain proteins such as MCD.

npo is a nonessential gene in *N. crassa*

After verifying the nuclear distribution of NPO we then isolated *N. crassa* stains with mutations in the *npo* gene using Repeat Induced Point mutation (RIP) (SELKER 1990) and made

knockout strains by replacing the *npo* coding sequence with the bacterial hygromycin phosphotransferase gene (*hph*) (Fig. 3, Fig. 4A and B). Both homozygous and heterozygous crosses of strains carrying duplications of *npo* at the *his-3* locus were fully fertile. These results suggest that *npo* is not required in the brief diploid phase for completion of meiosis, as heterozygous duplications would be expected to trigger meiotic silencing by unpaired DNA (MSUD) (ARAMAYO and METZENBERG 1996; SHIU *et al.* 2001). However, it is also possible that there is enough transcript or protein present in ascogenous hyphae to override the effect of MSUD during the diploid phase.

We confirmed the presence of mutations by RIP in progeny from these crosses by Southern hybridization and DNA sequencing. Clear evidence of RIP was detected by Southern hybridization in six out of forty progeny. None of the six progeny exhibited any gross morphological or developmental phenotypes. Sequencing of a PCR product amplified from progeny number 11 (P11) identified 21 C:G to T:A transition mutations in a 591 base pair segment of the endogenous *npo* gene (Fig. 2). All G to A mutations were found on the coding strand, spanning the first and second exons. The 21 mutations affected 15 codons, with five mutations occurring in the intron. Of the 16 mutation occurring in codons, four were in 3rd position and silent (V53, L106, L203and Q205), seven were in 1st position, resulting in conservative substitutions (V59→I, E101→K, D121→N, D152→N, V181→M, V197→I and D200→N), two were in 1st position resulting in nonconservative substitutions (G78→R and A105→T) and one was in second position producing a stop codon (W209→stop). The conservative substitution at position 185 (V185→I) resulted from G to A transitions in both the 1st and 3rd positions. The introduction of a stop codon at W209 is very likely to eliminate NPO function, as it occurs in the amino terminal region of the WGR motif, upstream of the PARP catalytic domain (Fig. 3). We refer to this allele as *npo^{RIP1}* and the original progeny harboring the allele (P11) as N3180.

All tested strains carrying *npo^{RIP1}* were fully fertile as males or females, and homozygous crosses appeared normal as well. Knockouts of *npo* were made by homologous replacement of the *npo* coding region with *hph* in a wild type background. Proper replacements were confirmed by PCR analysis and Southern blots (Fig. 4A and B). Strains with the *npo* KO, like strains with the *npo^{RIP1}* allele, did not exhibit gross morphological or developmental phenotypes, and were fully fertile in heterozygous and homozygous crosses. We conclude that *npo* is a nonessential gene in *N. crassa* and is not required for normal growth or development. These results stand in contrast to what was reported for a *prpA* knockout in Aspergillus, which was lethal in haploid strains, and produced severe growth restrictions and developmental phenotypes in heterozygous diploid strains (Δ*prpA*/+), described as haplo-insufficient (SEMIGHINI *et al.* 2006).

NPO is a PAR-polymerase

We assayed PARylation activity in extracts from both wild type and *npo* KO strains to determine if NPO functions as a protein PAR-polymerase. To our knowledge, results from PARylation assays have only been reported for mammalian systems. To assay PARylation, crude extracts from wild type and the *npo* KO strain were prepared from conidia that had either been treated or not treated with MMS for 60 min. The crude extracts were incubated with ^{32}P-NAD, sheared DNA and histones. As a positive control, an assay was also performed on extracts from *E. coli* cells expressing recombinant human PARP-1. PAR was detached from proteins by alkaline treatment and analyzed on 20% PAGE. As shown in Fig. 5A, the MMS-treated wild type strain produced a PAR-ladder like the human PARP-1 control (right-most lane), but the *npo* KO strain did not. To confirm that the ladder observed with the MMS-treated wild type strain reflected PAR, the radioactive material was eluted from the gel, digested with PARG (PAR-glycohydrolase), which specifically cleaves PAR into ADP-ribose, and analyzed

by HPLC. As shown in Fig. 5B, the radioactivity that eluted at the retention time of ADP-ribose, namely at 18–19 min., is higher in the PARG-treated sample than in the untreated control. It is possible that the radioactivity detected at 18–19 min in the PARG untreated control is due to degradation of PAR to ADP-ribose during PARG-treatment or due to unrelated products generated during the ^{32}P-NAD incorporation reaction. The control extract containing human PARP-1 also showed high intensity spots at 18–19 min., corresponding to ADP-ribose. We conclude that *N. crassa* PARylation increases in response to MMS treatment, and that this activity depends on NPO, which is likely responsible for most or all PARylation in *N. crassa*.

npo transcription is induced by MMS treatment

It is well established that auto-modification of mammalian PARP-1 increases dramatically with the binding of the protein to double and single-strand DNA breaks. Although the transcriptional response of the mammalian PARP-1 gene to DNA damaging agents has not been reported, plant PARP-1 and PARP-2 gene transcription is highly induced by DNA damage (DOUCET-CHABEAUD *et al.* 2001). In addition, SEMIGHINI *et al.* (2006) found *prpA* steady-state transcript levels increased in response to MMS, BLM and 4-NQO treatments. Our results from PARylation assays demonstrated a dramatic increase in NPO activity in response to MMS treatment. To determine if this reflected a change in *npo* transcript levels or enzyme activity, we performed northern blots of RNAs isolated from wild type and *npo* KO strains that had either been treated or not treated with MMS. The blots were probed with *npo* sequences, and *cox-5* sequences as a control for the loading. In untreated wild type cells, *npo* transcripts were undetectable by northern blot, but a large accumulation of *npo* transcript was detected 30 min. after treatment of wild type cells with MMS, and high levels of transcript were still detectable 120 min. after treatment (Fig. 6). As expected, no *npo* transcripts were detectable in the *npo* KO strain (Fig. 6). Thus *npo* transcription is likely to be regulated in response to DNA damage, like the *A. nidulans prpA* gene.

npo mutant strains are not sensitive to DNA damaging agents

Genetic and biochemical studies of mammals established roles for PARP-1 in DNA repair and genome stability (MASUTANI *et al.* 2003; WATANABE *et al.* 2004). The fact that the steady-state transcript levels of *npo* were regulated by exposure to MMS suggested that NPO may play a role in a DNA damage response. We tested the effects of a number of DNA damaging agents on *N. crassa* strains carrying either the npo^{RIP1} allele or the *npo* KO. We tested CPT, EMS, H_2O_2, HU, MMS, MNNG and UV (Fig. 7), as well as BLM (data not shown). Neither mutant showed sensitivity to any of these compounds. SEMIGHINI *et al.* (2006) found the haplo-insufficient Δ*prpA*/+ mutant to be extremely sensitive to both phleomycin (PLM), which induces double-strand breaks, and the UV-mimetic agent 4-NQO. While we did not test PLM, the *npo* mutants were not sensitive to BLM, which also induces DNA double-strand breaks (POVIRK *et al.* 1977). Because *npo* transcript levels increase in response to MMS, it is likely that NPO function is connected with a DNA damage response. The fact that *npo* mutants are not sensitive to DNA damaging agents suggests the function may be redundant, or it may impact a nonessential aspect of repair. Alternatively, NPO may function in related processes such as regulating expression of genes controlled by DNA damage. The fact that *A. nidulans* PrpA is necessary for normal repair reveals divergence in DNA damage response pathways between Neurospora and Aspergillus.

NPO is not a global regulator of TPE

In metazoans, PARP enzymes are involved in chromatin-mediated regulation of transcription (KRISHNAKUMAR *et al.* 2008). Although considerable progress has been made in understanding the role of PARPs in regulating chromatin structure, simple genetic studies to

test their possible involvement in epigenetic position effects, such as Telomere Position Effect (TPE), are lacking. We recently developed *N. crassa* strains with markers at subtelomeric positions to examine TPE (SMITH *et al.* 2008). This system allowed us to identify factors that control TPE, including several Sirtuins, termed NSTs (Neurospora Sirtuins). To analyze the effect of mutations in *npo* on TPE, we crossed the npo^{RIP11} allele into a background with the *E. coli hph* gene targeted to telomere VR (*telVR::hph::T*). We found significant derepression of *hph* at telomere VR in a strain with mutations in the *N. crassa* Sirtuin gene *nst-3* ($nst\text{-}3^{RIP1}$), but not in a strain with npo^{RIP1} (Fig. 8).

NPO is not involved in DNA methylation or DNA methylation-dependent silencing

In mammals it has been reported that PARP-1 is antagonistic to DNA methylation. Treatment of mouse fibroblasts with the competitive PARP inhibitor 3-aminobenzamide (3-AB) resulted in DNA hypermethylation and PAR has been shown to inhibit the activity of the maintenance DNA methylase, DNMT1 (REALE *et al.* 2005). *N. crassa* is the simplest genetically tractable system used to study DNA methylation. In *N. crassa* virtually all DNA methylation occurs in transposons that have been mutated by RIP (SELKER *et al.* 2003) and this methylation is not confined to symmetrical positions (SELKER *et al.* 1993). Numerous viable *N. crassa* mutants with reduced methylation have been described, including *dim-2*, *dim-5*, and *hpo*. Mutation in any of these genes completely abolishes all detectable DNA methylation (FREITAG *et al.* 2004a; KOUZMINOVA and SELKER 2001; TAMARU and SELKER 2001). Although no mutants with hypermethylation have been described in *N. crassa* thus far, strains of *Ascobolus immersus* carrying silenced copies of the histone H1 gene (*hH1*) were shown to have elevated levels of DNA methylation (BARRA *et al.* 2000). This hypermethylated DNA could be detected globally on ethidium bromide stained agarose gels, as higher molecular weight fragments after digestion with methylation sensitive restriction enzymes.

As a first test of whether inhibition of NPO effects DNA methylation we treated wild type *N. crassa* cells with high concentrations of nicotinamide (NAM) and looked at global DNA methylation by analyzing *Sau*3A- and *Dpn*II-digested DNAs on agarose gels (Fig. 9A). NAM acts as a strong noncompetitive inhibitor of both Sirtuins and PARPs, and we had previously shown that treatment of *N. crassa* with NAM dramatically reduces silencing of *telVR::hph*, but has no effect on silencing of the methylated transgene $am^{RIP}\text{::}hph\text{::}am^{RIP}$ (SMITH *et al.* 2008). No effect on global DNA methylation was observed after NAM treatment (Fig. 9A). The fact that NAM treatment did not relieve silencing of $am^{RIP}\text{::}hph\text{::}am^{RIP}$, suggests that neither NPO nor NSTs are involved in methylation-dependent silencing at this locus. Because it was conceivable that NPO is resistant to NAM, we also tested if mutation of *npo* would affect DNA methylation. Southern blots of *Sau*3A- and *Dpn*II-digested DNAs from progeny with mutations by RIP in the *npo* gene, including strain P11, which is likely to be a null mutant, revealed heavy DNA methylation when probed with *npo* sequences (data not shown). We probed the same Southern blots with Ψ63 sequences, which are normally methylated (MARGOLIN *et al.* 1998), and did not see any change in DNA methylation at this locus (Fig. 9B). We also looked at global DNA methylation levels by ethidium bromide staining in *N. crassa* strains with the npo^{RIP1} allele and saw no effect (data not shown). In addition, presence of the npo^{RIP1} allele, or quelling experiments with *npo* sequences, had no effect on silencing of $am^{RIP}\text{::}hph\text{::}am^{RIP}$ (data not shown), indicating that NPO is not involved in methylation-dependent silencing. We conclude that *npo* is not involved in DNA methylation.

The *npo* knockout causes acceleration of replicative aging

Studies of aging in filamentous fungi have focused largely on replicative aging associated with mitochondrial DNA (mtDNA) rearrangements triggered by mitochondrial plasmid/intron mobilizations (OSIEWACZ 2002). Replicative lifespan is a measure of the number of mitotic divisions a cell undergoes before senescence. Analogous to the ERC situation in yeast, these

mechanisms seem to be specific to filamentous fungi. BARRA *et al.* (2000) reported that strains of *Ascobolus immersus* with silenced copies of the *hH1* gene exhibited a decreased replicative lifespan, along with DNA hypermethylation. Such strains were found to initiate growth normally, but to senesce between 6 and 13 days after germination, whereas strains with unsilenced *hH1* continued with a linear rate of growth for up to 40 days. We observed a similar phenotype for our *npo* KO strain, although the replicative lifespan of *N. crassa* mycelia is considerably longer than that of *A. immersus* (500 days versus 35–40 days, respectively). We grew both wild type and *npo* KO strains on minimal medium in 30 cm race tubes at 34°C with 12 hr. dark/light cycles, and were careful to transfer only mycelial fragments upon inoculation (Fig. 10A). The *npo* KO strain had a linear growth rate indistinguishable from wild type for the first 140 days of growth (6 cm/day), at which point the growth rate started to decrease gradually, culminating in senescence at around 300 days (Fig. 10B).

Telomere erosion does not occur in the *npo* knockout strain

Eukaryotic microorganisms must maintain telomere length in every proliferating cell type, either by telomerase activity or by recombination. We were therefore interested to test if the increased replicative aging observed in the *npo* strain reflected defective maintenance of telomeres. To determine if mutation of *npo* affects telomere length in *N. crassa*, DNA was isolated from young cultures (~80 hours) and old cultures (~8000 hours) of both wild type and *npo* KO strains. The DNAs were digested with *Cla*I and *Hin*dIII and Southern blots were probed with telomere repeat sequences. The 8000 hour time point was chosen because this is when the *npo* KO strain begins to senesce (Fig. 11A). The Southern blots did not reveal any obvious change in the length of the *npo* KO telomeres, even after 8000 hours of culture time (Fig. 11B). Therefore, regulation of telomere length does not appear to be a factor in lifespan reduction for the *npo* KO strain.

PARylation is not universally required for viability or DNA repair

PARP orthologs have been identified in all eukaryotes, excluding yeast. Both plants and animals typically have multiple PARP orthologs, making genetic characterization difficult. Lethality of PARP-$1^{-/-}$/PARP-$2^{-/-}$ mice and evidence linking PARylation with DNA repair and genomic stability support a view that PARylation impacts nuclear functions essential for higher eukaryotic development or survival. Unsuccessful attempts to generate PARP-1/PARP-2 double knockouts in mouse embryonic fibroblasts (MEDER *et al.* 2005) suggest that these functions may be critical for cellular survival. Mutation of dPARP in Drosophila results in larval lethality at the second instar stage, with disruption of heterochromatin organization and elimination of nucleoli (TULIN *et al.* 2002), again supporting the hypothesis that PARPs provide nuclear functions essential to the cell. Recent work on PARP in the filamentous fungus *A. nidulans* extends this view to PARylating lower eukaryotes (SEMIGHINI *et al.* 2006). Our work in *N. crassa* stands in contrast to what has been found for mammals and *A. nidulans*, as *N.crassa npo* mutants are viable and do not show sensitivity to mutagens, establishing that PARylation is dispensable for both viability and DNA-repair in certain eukaryotes with PARP orthologs. The fact that transcription of PARP genes is induced by DNA damage in both plants (DOUCET-CHABEAUD *et al.* 2001) and filamentous fungi (SEMIGHINI *et al.* 2006) does support the idea that there is a universal function for PARylation in DNA repair, but this function may be redundant in *N. crassa*, but not *A. nidulans*.

PARylation is not required for heterochromatin formation in *N. crassa*

Two major heterochromatin silencing pathways described in *N. crassa* are TPE (SMITH *et al.* 2008) and cytosine methylation (SELKER 2004). Our analysis indicates that neither pathway is significantly affected by mutation of *npo*. The histone H3 K9 methylase, DIM-5 (TAMARU and SELKER 2001), and the HP1 ortholog, HPO (FREITAG *et al.* 2004a), are

necessary for silencing at all tested *N. crassa* telomeres (SMITH *et al.* 2008), as well as for all detectable DNA methylation. It is formally possible, however, that NPO might regulate TPE at telomeres other than VR or DNA methylation at a subset of unanalyzed genomic loci, although we have no reason to expect this to be so. We have shown that treatment of *N. crassa* with NAM dramatically reduces silencing of *telVR::hph*, but has no effect on silencing of the methylated transgene am^{RIP}*::hph::*am^{RIP}. Before our analysis of the effect of the npo^{RIP1} allele on silencing of *telVR::hph*, we could not fully interpret these data. Our genetic studies now suggest that the mechanism of action of NAM on TPE involves inhibition of NSTs, but not NPO. The fact that NAM treatment did not relieve silencing of am^{RIP}*::hph::*am^{RIP}, strongly suggests that neither NPO nor NSTs are involved in methylation or methylation-dependent silencing at this locus. The observation that PARP-1 activity impacts DNA methylation in mammals implies divergence in pathways that regulate methylation between mammals and filamentous fungi. This is not surprising considering that the activity of DNMT1, which is the primary maintenance methylase in mammals, is inhibited by PAR (REALE *et al.* 2005). *N. crassa* lacks this form of maintenance methylation, which acts specifically on hemimethylated CpG dinucleotides in conjunction with DNA replication. In *N. crassa*, both maintenance and *de novo* methylation are carried out by a single methyltransferase, DIM-2 (KOUZMINOVA and SELKER 2001), which does not require a symmetrical sequence (SELKER *et al.* 1993). It would be interesting to know whether PARP inhibitors or silencing of a PARP ortholog affect DNA methylation in *A immersus*, as this species may have a maintenance methylation system that is more similar to that in mammals.

The NPO aging pathway does not involve telomere length maintenance

Some current models for regulation of aging in humans consider telomere maintenance potentially important, as somatic human cells lack telomerase activity, and thus have a finite replicative lifespan (CAMPISI 2005; VERDUN and KARLSEDER 2007). Recently, SIRT6 has been shown to function as a telomere-specific histone H3 K9 deacetylase, which is necessary for normal telomere maintenance and for prevention of premature cellular senescence in human fibroblasts (MICHISHITA *et al.* 2008). In addition to playing a role in replicative cellular aging, SIRT6 has also been shown to impact chronological aging in mice, as $SIRT6^{-/-}$ animals exhibit phenotypes characteristic of progeroid disorders (MOSTOSLAVSKY *et al.* 2006). We did not observe any effect on telomere length in an *npo* mutant strain. These results do not rule out the possibility, however, that mutation of *npo* might affect other aspects of telomere maintenance or stability. In fact, the aberrations seen at telomeres in *SIRT6* knockdown fibroblasts are similar to those seen in Werner syndrome cells, such as telomere deletions, duplications and fusions, with no obvious effect on the length of intact telomeres (MICHISHITA *et al.* 2008). Importantly, *N. crassa* has a homolog of SIRT6, termed NST-7 (Neurospora Sirtuin 7), not found in either *S. cerevisiae* or *S. pombe* (SMITH *et al.* 2008). It would be interesting to know whether NST-7 functions in the same aging pathway as NPO, and whether maintenance of telomere integrity is involved.

The NPO aging pathway and histone H1

The replicative aging phenotype that we observed in the *npo* mutant is novel for *N. crassa* but similar to that reported for a strain of the filamentous fungus *Ascobolus immerses* carrying a silenced epi-allele of the histone H1 (*hH1*) gene, that confers a DNA hypermethylation phenotype (BARRA *et al.* 2000). Although *N. crassa hH1* mutants do not display hypermethylation (FOLCO *et al.* 2003), it would be interesting to know whether Neurospora *hH1* mutants show a decreased replicative lifespan, and if so, whether this involves NPO. Conversely, one could ask whether PARP inhibitors or mutation/silencing of a PARP ortholog would affect replicative aging in *A. immersus*, and if so, whether the pathway is independent of the established hH1 pathway and/or DNA methylation. KIM *et al*. (2004) showed that PARP-1 associates with chromatin in a manner very similar to hH1: PARP-1 increases the

nucleosome repeat length and competes with hH1 in nucleosome assembly reactions. Like hH1, binding of PARP-1 to chromatin *in vitro* triggers condensation and transcriptional repression. Unlike hH1, however, PARP-1 dissociates from chromatin in the presence of NAD +, and it has been suggested that localized NAD+ levels in nuclei might control chromatin structure and transcription (KIM *et al.* 2004). Results of ChIP-chip experiments have shown that actively transcribed promoters have high levels of PARP-1 and low levels of hH1, and that hH1 occupancy is excluded by PARP-1 binding (KRISHNAKUMAR *et al.* 2008). An attractive hypothesis is that PARPs and hH1 provide related functions associated with nuclear NAD+ levels, genome stability and aging. Consistent with this possibility, dramatic loss of hH1 accompanies cellular senescence of human fibroblasts (FUNAYAMA *et al.* 2006). While it is intriguing that both PARP and hH1 orthologs have been implicated in replicative aging in filamentous fungi, there is currently no evidence that fungal PARPs of the PrpA class have the linker histone-like properties of PARP-1. Furthermore, they lack an amino terminal DNA binding domain, which is required for PARP-1 chromatin association. It remains possible, however, that fungal PARPs interact with DNA binding proteins that target them to chromatin.

NPO might regulate aging in a pathway with Sirtuins

The possible function of NSTs in regulation of lifespan in *N. crassa* has not been investigated. If PARPs impact aging exclusively through indirect effects on the activity of Sirtuins, then our observation that NPO is necessary for normal replicative lifespan in *N. crassa* is difficult to reconcile with current models on how Sirtuins regulate aging in yeast and higher organisms. Current models from yeast that assume Sirtuins function exclusively to promote longevity would predict that when NAD+ is limiting, PARylation would inhibit long lifespan, because NAD+-dependent deacetylation and PARylation both consume NAD+ and produce NAM. Thus, an important question is whether localized NAD+ levels in nuclei are in fact limiting. If they are not, then NSTs and NPO could presumably act in the same or parallel pathways, with both functioning to promote longevity. ANDERSON *et al.* (2002) found that increasing the levels of NAD+ salvage pathway proteins in *S. cerevisiae* increased telomere and rDNA silencing in a Sir2-dependent manner. Although sir2 deletion mutants were not found to have elevated levels of total cellular NAD+, the authors argue that most or all of NAD+ salvage in *S. cerevisiae* occurs in nuclei, and that nuclear NAD+ salvage pathway flux is important in regulation of Sir2 deacetylase activity (ANDERSON *et al.* 2002).

Unlike Sir2, PARP-1 can dramatically reduce total cellular NAD+ levels in response to DNA damage (ZONG *et al.* 2004). If NPO is as robust as PARP-1, and if NAD+ availability within nuclei is limiting in *N. crassa*, then NSTs and NPO may compete for NAD+, and thus function antagonistically in the same aging pathway. However, if it is also assumed that Sirtuins act exclusively to promote longevity, as some models suggest, then PARylation should have a negative affect on lifespan, and mutation of *npo* should increase longevity, which is contrary to our observations.

Recently FABRIZIO *et al.* (2005) have shown that while Sir2 has a positive impact on replicative lifespan in *S. cerevisiae*, it actually has a negative impact on chronological lifespan, which is a measure of how long a non-dividing cell or organism survives. In addition, while it is generally accepted that Sirtuins positively regulate longevity in metazoans, SIRT1 may actually function in a pro-aging pathway (FABRIZIO *et al.* 2005), as $sirt1^{-/-}$ mice manifest many phenotypes of long-lived IGF-I-deficient dwarf mice (MCBURNEY *et al.* 2003). Furthermore, SIRT1 represses the DAF-16 homolog FOXO3 (MOTTA *et al.* 2004), and this is presumably antagonistic to longevity (LIN *et al.* 1997). If the activities of NSTs negatively regulate replicative lifespan in *N. crassa*, then competition between NSTs and NPO for NAD + could occur, with NPO acting to promote longevity through inhibition of NSTs.

Regardless of whether Sirtuins promote or inhibit longevity, the general observation that NAD +-dependent deacetylases impact aging in both yeast and metazoans suggests conservation of this role during evolution. It is therefore reasonable to expect that NSTs may play a role in *N. crassa* as well. Until such a role has been definitively established, however, it is not possible to draw conclusions about the involvement of NSTs in the NPO pathway. Analysis of the aging phenotypes of *nst* mutants, individually and in combination with each other and the *npo* mutant, would provide an answer to these mechanistic questions.

Acknowledgments

We would like to thank Melissa Hemphill for helping to analyze *npo/nst* genetic interaction and Wendy Hanna-Rose for comments on the manuscript. Thanks to Melissa Rolls for expert advice in confocal imaging. This work was supported by grant GM025690 from the National Institutes of Health to EUS and by Rational Evolutionary Design of Advanced Biomolecules, Saitama Prefecture Collaboration of Regional Entities for the Advancement of Technological Excellence, Japan Science and Technology Agency to HI.

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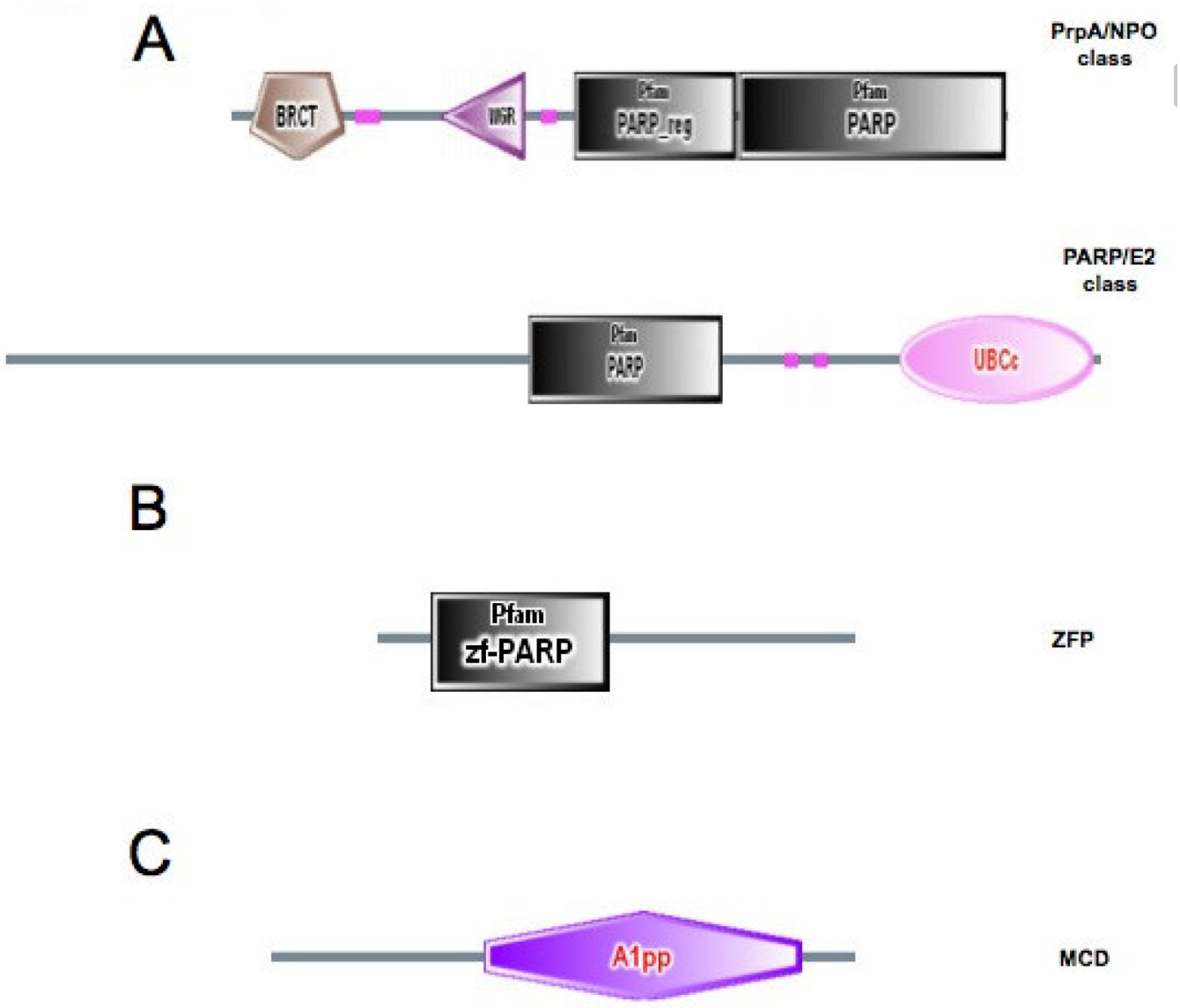

FIGURE 1. Domain organization of fungal PARP-like proteins and associated DNA and PAR binding proteins

(A) Schematic representation of the domain organization of the two classes of fungal PARP proteins. The complete amino acid sequences of NPO. *Neurospora crassa* PARP ortholog [CAD21266] and *Aspergillus nidulans* AN0482.2 [xm_652994.1] were used as queries to search the SMART database (smart.embl-heidelberg.de). Searching with NPO identified BRCT [IPR001357], WGR [IPR008893]. PARP-regulatory [PF02877], and PARP-catalytic [PF00644] domains, defining the PrpA/NPO class. Searching with AN0482.2 identified PARP-catalytic [PF00644] and Ubiquitin-conjugating enzyme E2 catalytic domains [SM00212], defining the PARP/E2 class. (B) Domain organization of the Neurospora MacroD protein. The complete amino acid sequence encoded by Neurospora ORF NCU07925.3 was used to search the SMART database identifying the A1pp domain [SM00506]. (C) Domain organization of the Neurospora zf-PARP protein. The complete amino acid sequence of a Neurospora hypothetical protein [Broad coordinates LGI, contig 2:447973-449833+] was used to search the SMART database identifying a single zf-PARP Pam domain [PF00645].

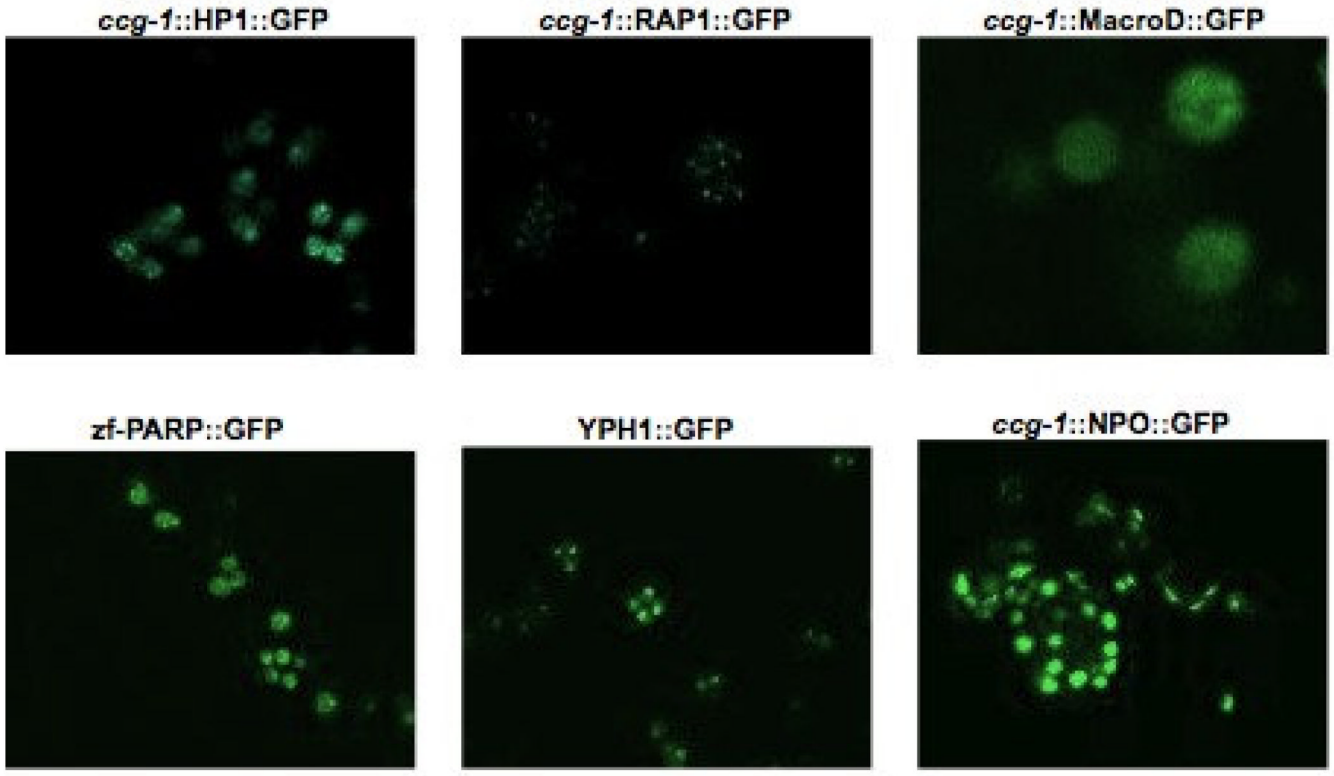

FIGURE 2. Confocal Images of GFP-tagged Neurospora proteins

Expression of heterochromatin protein 1 (HP1::GFP), a telomere repeat binding protein (RAP1::GFP), MacroD::GFP and NPO::GFP was driven by the *ccg-1* promoter. An rDNA associated protein (YPH1::GFP) and zf-PARP::GFP were expressed via their endogenous promoters.

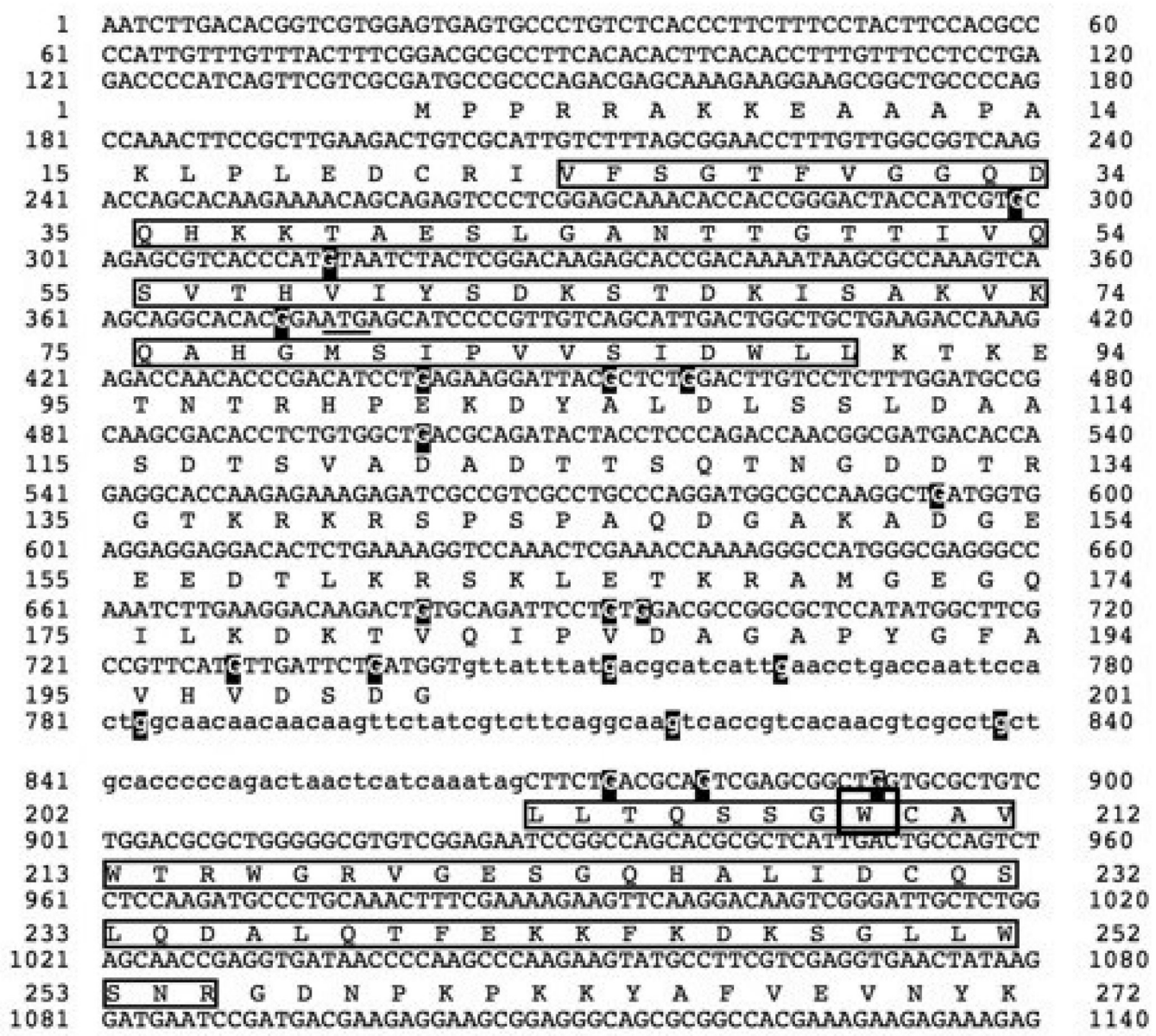

FIGURE 3. Sequence of the *npo*^{RIP1} allele

The NPO protein sequence is indicated beneath the upper case nucleotide coding sequence. Lower case nucleotides represent intron sequences. The boxed amino acids in the first exon indicate the BRCT domain and the boxed amino acids in the second exon indicate the WGR motif. Guanine residues mutated to adenines are highlighted, and the tryptophan codon that was mutated to a stop codon is boxed. The sequence of the *npo*^{RIP1} allele has been deposited in Genbank with the accession number EU869543.

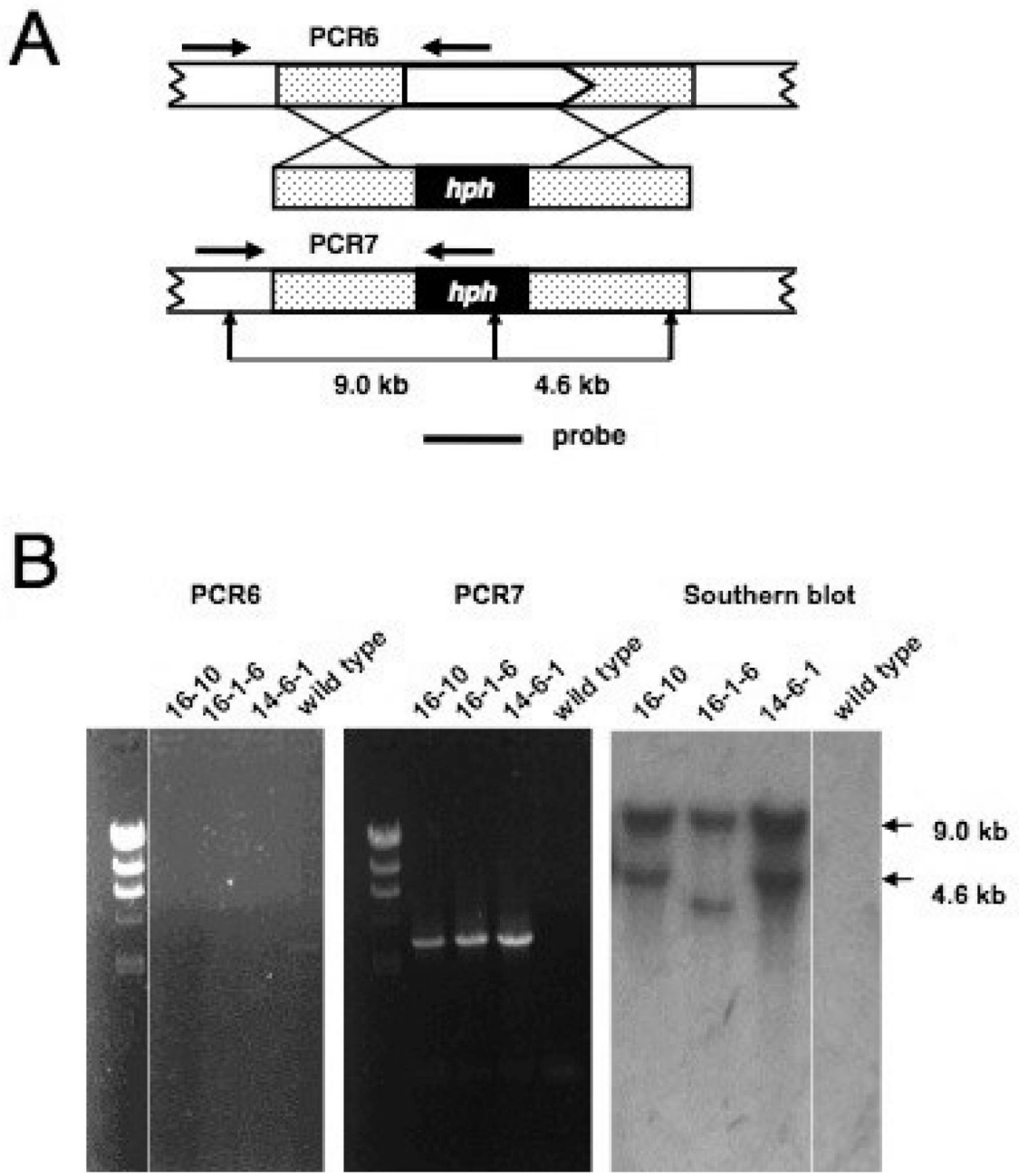

FIGURE 4. Disruption of the *N. crassa npo* gene by homologous recombination

(A) Schematic illustration of knockout strategy for the *npo* gene. A white arrow box represents the *npo* gene and shows the direction of transcription. Stippled boxes indicate immediate flanking sequences. The knockout construct is shown below the genomic sequence with the *E. coli hph* gene represented by a black box. The genomic sequence with the *E. coli hph* gene represented by a black box. The genomic sequence resulting from correct replacement is shown beneath the knockout construct. Horizontal black arrows indicate the positions of PCR primers used to analyze the transformants. Vertical black arrows indicate restriction sites used to characterize the transformants by Southern hybridization A horizontal black line represents the probe used in the Southern blot.(B) The images labeled PCR6 and PCR7 are ethidium

bromide-stained agarose gels with size markers run in the left-most lanes. The next three lanes contained PCR products that had been amplified from template DNAs isolated from the indicated transformants. The right-most lanes show PCR products amplified from wild type *N.crasse* DNA, as controls. The position of primers for the PCR6 and PCR7 reactions are shown in panel A. The right-most image shows an autoradiograph of a Southern blot probed with *hph* sequences. DNAs from the indicated transformations were digested with *Ncol*. DNA from wild type *N.crassa* was run in the right-most lane as a control.

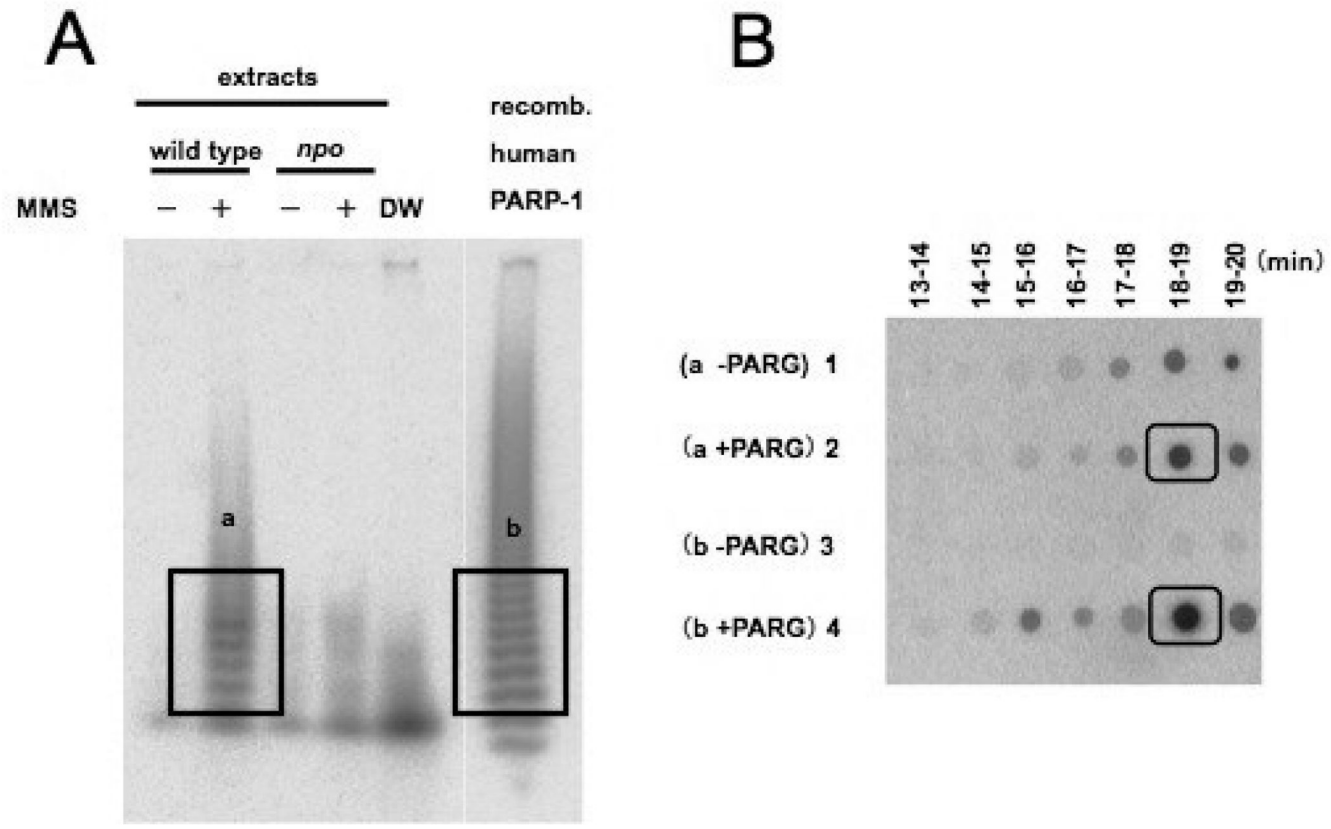

FIGURE 5. Verification of NPO PARylation activity

(A) Autoradiogram of a 20% polyacrylamide gel showing ^{32}P-PAR ladder. PARylation reactions with extracts from wild type *N.crassa* cells treated (+) or not treated (−) with MMS were run alongside reactions with extracts from *npo* KO cells treated (+) or not treated (−) with MMS. The lane labeled DW is a negative control reaction using distilled water in place of extract. The right most lane contains a positive control reaction with recombinant human PARP-1 expressed in *E. coli*. The boxed regions labeled **a** and **b** were excised and the radioactivity was eluted for analysis by HPLC.(B)An autoradiogram (BAS2500) of fractions from HPLC blotted onto DE81 paper (Whatman) with retention times indicated above and sample designations on the left. Eluents of ^{32}P-PAR from these gel slices were either treated with recombinant PARG, or not, and fractionated by HPLC as described in Materials and Methods. The boxed regions show the peak signals eludated at the retention time for ADP-ribose.

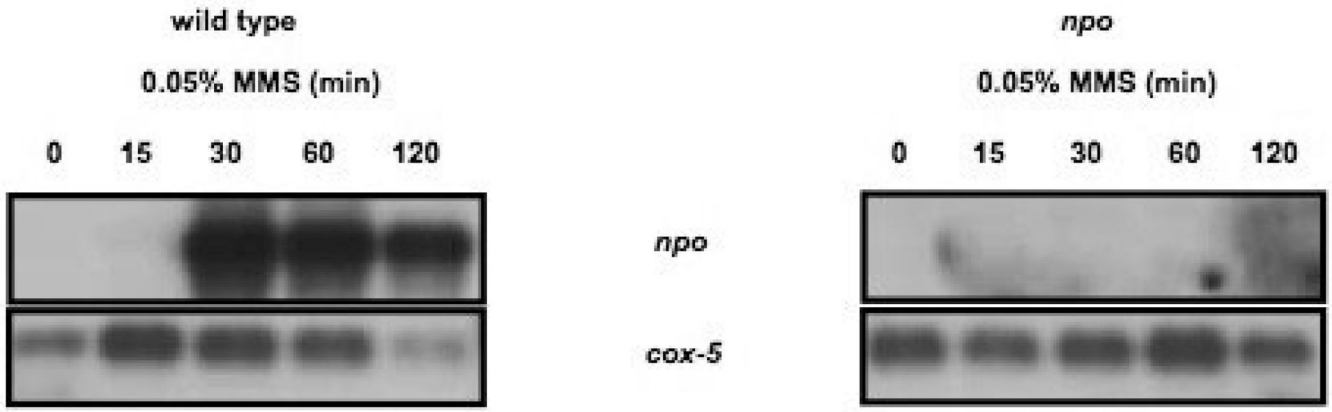

FIGURE 6. Analysis of *npo* transcription by northern blot

The left panel shows an autoradiogram of a northern blot of RNAs extracted from wild type *N. crassa* after the indicated duration of MMS treatment. The upper panel shows results of probing the blot with *npo* sequence and in the lower panel shows results of probing with *cox-5* sequences as a control for loading. The right panel shows the same for the *npo* strain.

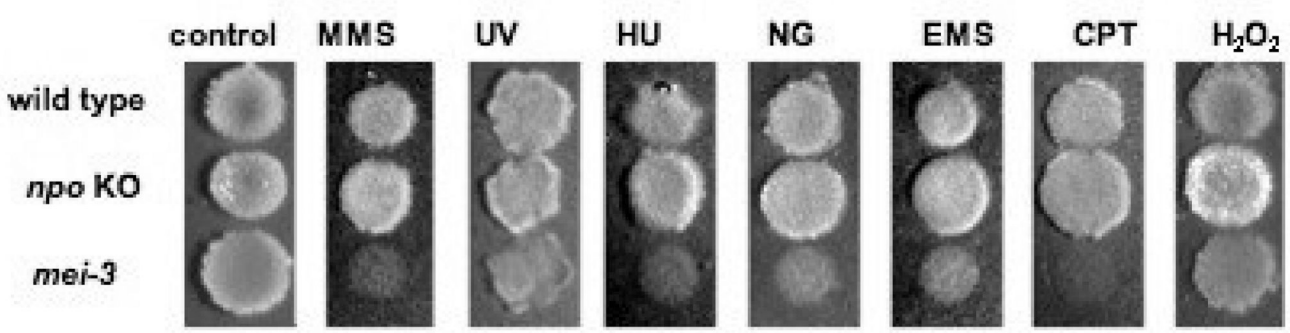

FIGURE 7. Mutagen sensitivity of the *npo* KO strain

Spot-tests of conidia on FGS plates for wild type *N. crassa* (top), the *npo* (middle) and *mel-3* strains (bottom) were done as described in Materials and Methods. The *mel-3* strain was used as a positive control for mutagen sensitivity. Panels from left to right are as follows: no mutagen; 0.015% methyl methane sulfonate (MMS); conidia pretreated with 450 J/MF UV; 30 mM hydroxy urea (HU); 0.05μg/ml N-methyl-N-nitro-N-nitrosoguanidine (MNNG); 0.3% ethyl methane sulfonate (EMS); 0.3μg/ml camptothecin (CPT) and 0.0015% H_2O_2.

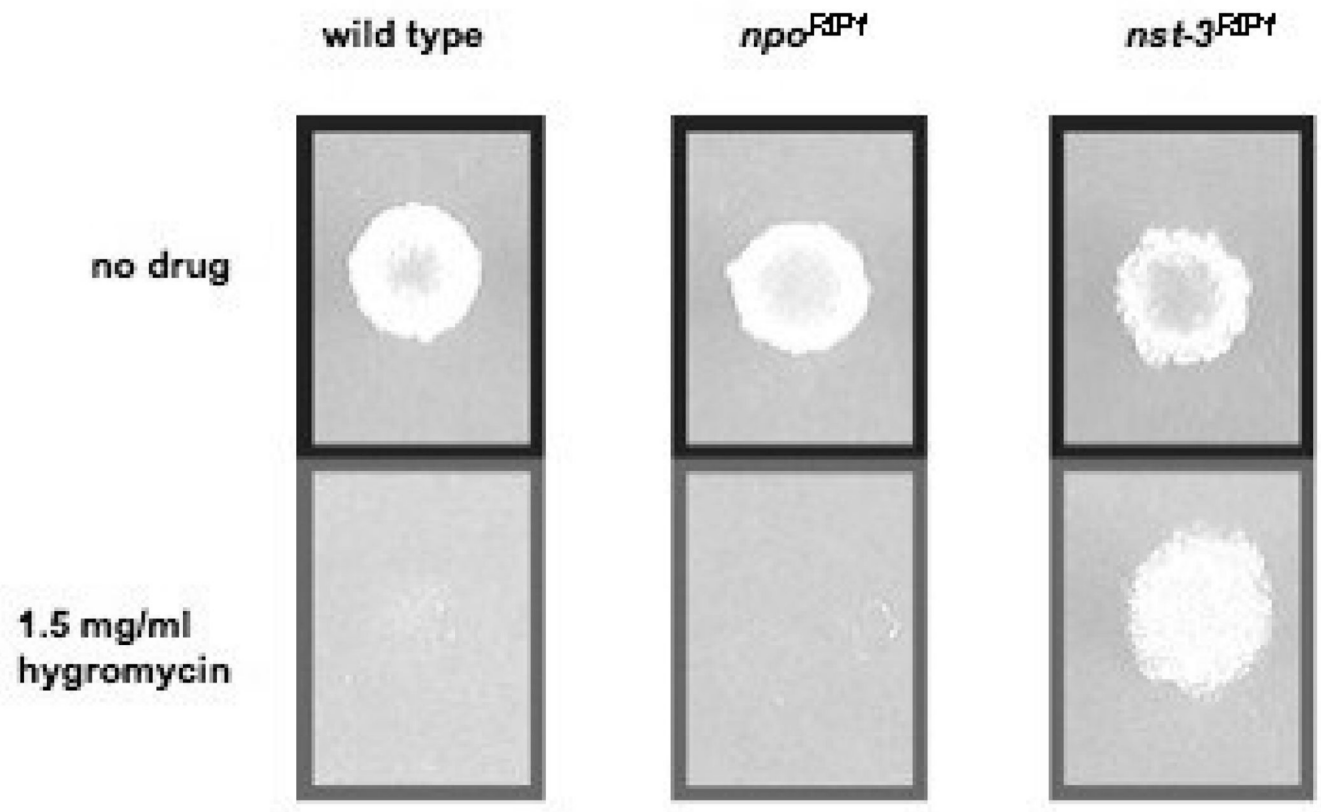

FIGURE 8. Telomere position effect assay

Spot-tests of conidia on FGS plates for wild type *N. crassa*, *npo*^{RIP1}, and *nst-3*^{RIP1} strains on media with 1.5 mg/ml hygromycin or no hygromycin, as described in Materials and Methods.

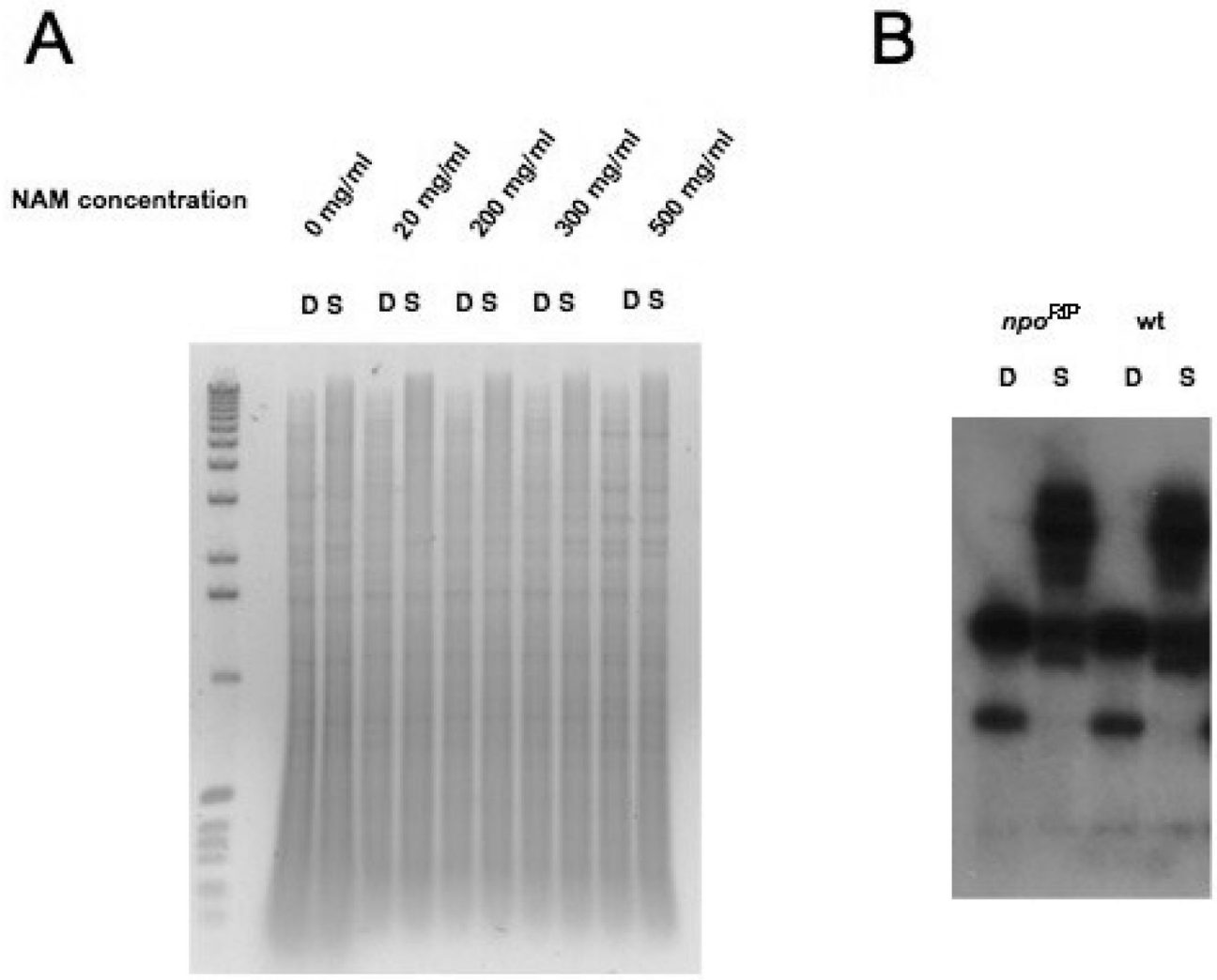

FIGURE 9. Absence of effects of NAM treatment and *npo* mutation on DNA methylation

(A) Approximately 1 μg samples of chromosomal DNA, isolated from wild type *N. crassa* (N 150) grown for 3 days in Vogels minimal media with the indicated concentrations of NAM, were digested either with *Dpn*II (D) or *Sau*3A (s) and fractionated on a 1× TAE/0.8% agarose gel containing 1 μg/ml ethidium bromide. The left-most lane contains 0.5 μg of 1 kilobase DNA ladder (Invitrogen). (B) A Southern blot of chromosomal DNAs from wild type and *npo*^{RP} mutant strains digested with *Dpn*II (D) or *Sau*3A (S), as described for panel A and in Materials and Methods. The Southern blot was probed with ψ*63* sequences.

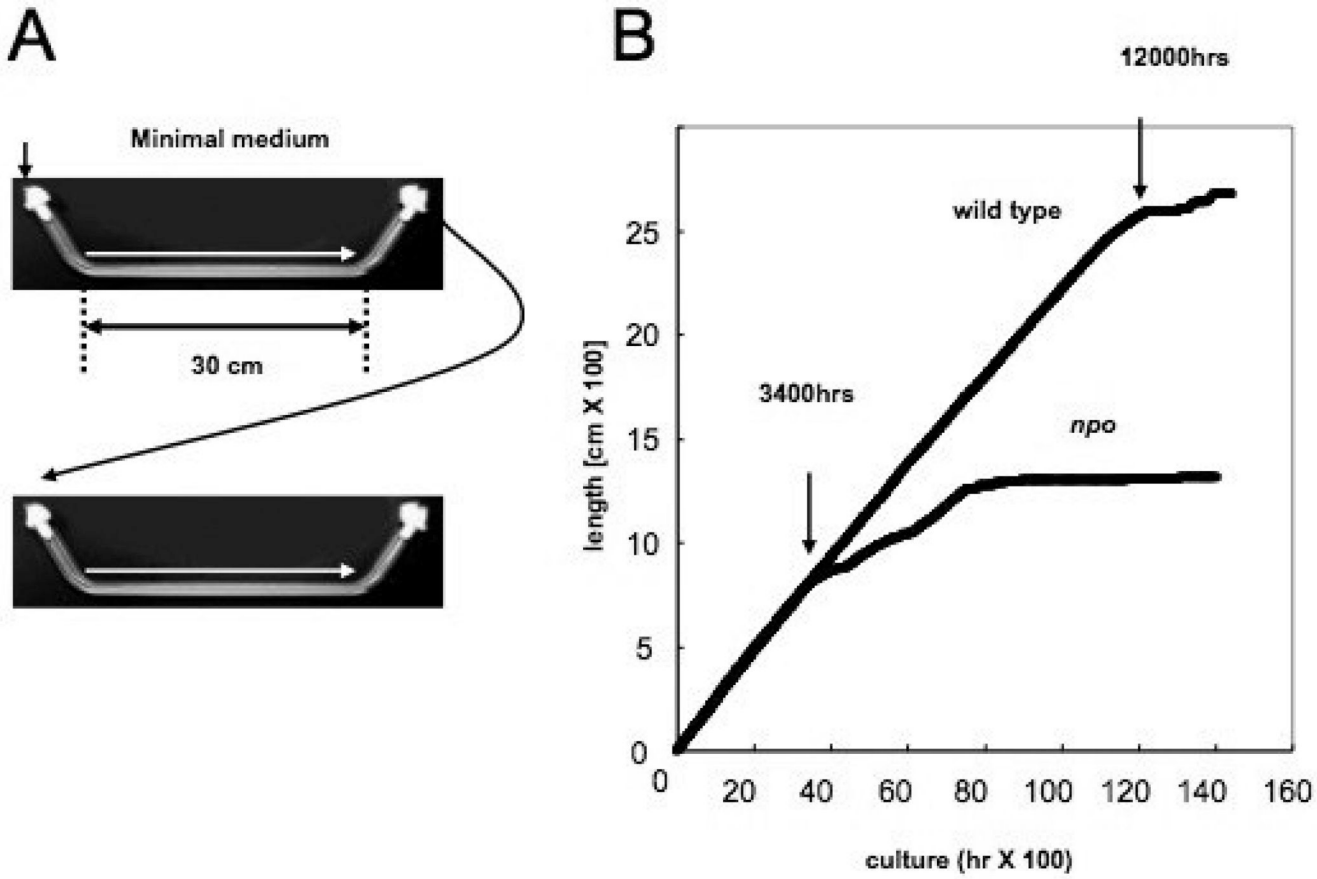

FIGURE 10. Method and results of senescence assay

(A) Schematic of race tube strategy for measuring long-term linear extension rate. (B) Plot of growth (cm/hr) for wild type *N. crassa* and *npo* strain. Arrows at 3,400 hrs and 12,000 hrs indicate entry into senescence for *npo* and wild type *N. crassa* strains, respectively.

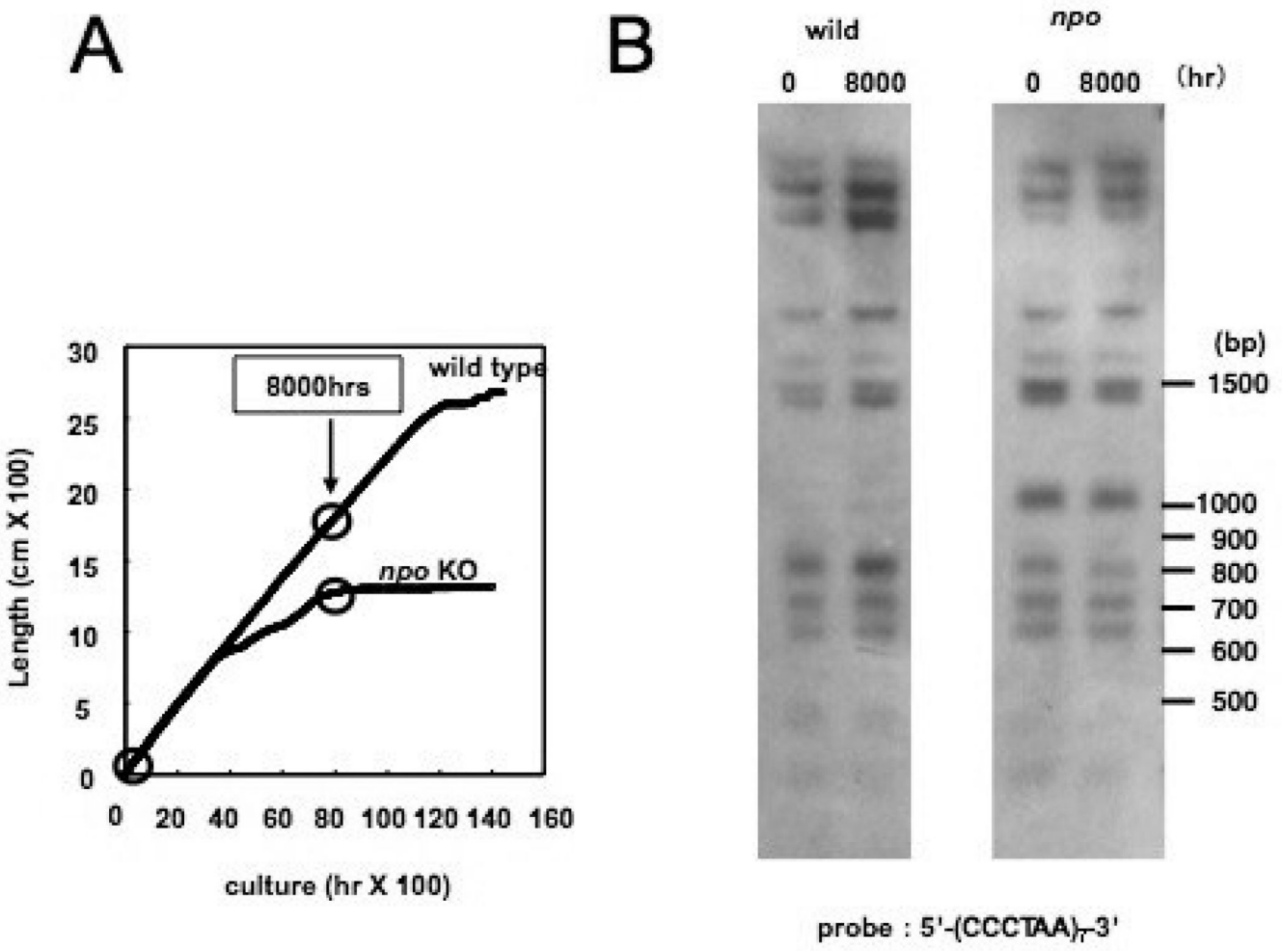

FIGURE 11. Telomere stability in wild type and *npo* mutant strains

(A) Arrows on plot shows time points used in telomere erosion assay. (B) Chromosomal DNAs were isolated from wild type and the *npo* mutant at the time points indicated in panel A. The DNAs were digested with *Hae*III, blotted as described in Materials and probed with telomere repeat sequences.

TABLE 1

NEUROSPORA CRASSA STRAINS USED IN THIS STUDY

Strain number	Genotype	Source
N150	*mat A*	FGSC 2489
N1444	*mat a his-3; am^{132}*	this study
N1674	*mat A his-3; lys-1 am^{132} inl; am^{RIP}::hph::am^{RIP}*	(HAYS *et al.* 2002)
N1983	*mat a; mtr col4; nst-1^{RIP1} trp-2*	this study
N2636	*mat A nst-3^{RIP1}; mtr col-4; telVR::hph::T; nst-1 ^{RIP1} trp-2*	(SMITH *et al.* 2008)
N3180	*mat A his-3::npo^{RIP0}; am^{132} npo^{RIP1}*	this study
N3181	*mat a; npo^{RIP1}*	this study
74-OR31-16A	*mat A al-2; pan-2;cot-1*	(DE SERRES 1980)
74-OR31-14a	*mat a al-2; pan-2; cot-1*	(DE SERRES 1980)
MKI-1411A	*mat A al-2; pan-2; cot-1; npoKO*	this study
MKI-1414a	*mat a al-2; pan-2; cot-1; npoKO*	this study
14-6-1-1A	*mat A al-2; pan-2; cot-1; npoKO*	this study
G1	*mat A his-3 cyh-1 al-1; mtr; inl*	FGSC 7508
P49	*mat A his-3 cyh-1 al-1; inl*	this study