Abundance of the RSC nucleosome-remodeling complex is important for the cells to tolerate DNA damage in *Saccharomyces cerevisiae*

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Abstract

The essential Nps1p/Sth1p is a catalytic subunit of the nucleosome-remodeling complex, RSC, of *Saccharomyces cerevisiae* that can alter nucleosome structure by using the energy of ATP hydrolysis. Besides the ATPase domain, Nps1p harbors the bromodomain, of which function(s) have not yet been defined. We have isolated a temperature sensitive mutant allele of *NPS1*, *nps1-13*, which has amino acid substitutions within the bromodomain. This mutation perturbed the interaction between the RSC components and enhanced the sensitivity of the cells to several DNA-damaging treatments at the permissive temperature. Reduced expression of *NPS1* also caused DNA-damage sensitivity. These results suggest the importance of the Nps1p bromodomain in RSC integrity and a model in which high amounts of RSC would be required for the cells to overcome DNA damage.

1. Introduction

In eukaryotes, the wrapping of DNA around histone octamers to form nucleosomes blocks access of DNA binding factors and/or advancing polymerases, resulting in inhibition of transcription, replication, repair and recombination. Recent studies revealed that a number of specialized ATP-dependent nucleosome-remodeling enzymes have evolved to overcome this inhibition [1, 2]. The conservation of this type of enzymes from yeast to humans indicates the evolutionary importance of the nucleosome-remodeling activity. The yeast Snf/Swi and RSC are subsets of this enzyme [3, 4]. Both types are multi-subunit protein complexes and similar in structure; sharing two identical subunits and at least four other homologous components. RSC is approximately ten times more abundant than Snf/Swi [4]. However, the *in vivo* functions of each complex seem to be distinct. Snf/Swi is not essential for viability, whereas loss of RSC is lethal. In addition, RSC is required for cell cycle progression through mitosis [5, 6], whereas Snf/Swi is not. While the involvement of Snf/Swi in transcriptional control has been extensively studied both genetically and biochemically, the precise role of RSC in transcription or in other chromatin-related processes *in vivo* has not yet been established.

Nps1p/Sth1p is an ATPase subunit of the RSC complex. This protein harbors conserved ATPase domain and the bromodomain at the center- and carboxy (C)-terminal portions, respectively, similar to the prototype ATPase of this family, Snf2p/Swi2p. Previous works by Akada *et al*. and by us showed that the overexpression of C-terminal 270 amino acid portion of Nps1p containing the bromodomain causes growth inhibition in the wild-type yeast cells, while neither that of the full-length Nps1p nor that of the ATPase domain produced any effect [7, Tsuchiya *et al*. unpublished result]. To examine the function of the C-terminal portion of Nps1p, we screened mutations of this region and isolated three mutations that show temperature sensitivity for growth at 38˚C. One of these mutations alters amino acids within the bromodomain and exhibits multiple phenotypes including DNA-damage sensitivity. The bromodomain is a structural domain of 110 amino acids, containing a central motif sequence of 60 amino acids which has been identified in over 40 proteins implicated in transcriptional control [8, 9]. Recent studies demonstrated that bromodomains bind acetylated lysine residues within the amino-terminal histone tails [10-12]. At the same time, other studies indicated the importance of this domain for protein-protein interactions in the transcription-related proteins [13-15], but the extent to which these interactions are dependent on acetylation of some of its partners' is obscure. The role of bromodomains in ATP-dependent nucleosome-remodeling complexes has been controversial because their deletion has widely different consequences. Loss of the bromodomain of the yeast Snf2p/Swi2p causes no discernible phenotype [16], and similar results are seen in its *Drosophila* and human homologs, *brahma* (brm) and hbrm, respectively [17, 18]. On the other hand, the second bromodomains of Rsc1p and Rsc2p, additional RSC subunits each contain two tandem bromodomains, are essential [19], and partial deletions of the bromodomain in Sth1p/Nps1p cause growth defect at high temperature [20]. In addition, detailed functions of bromodomains in these proteins, including their binding partners, are not yet understood.

Here we show that the bromodomain of Nps1p plays important role in the maintenance of the integrity of RSC and the deterioration of functional RSC cause enhanced sensitivity to

DNA-damaging conditions.

2. Materials and methods

2.1. Strains, media and plasmids

All *S. cerevisiae* strains were derived from W303-1A (*MATa ade2-1 leu2-3, 122 his3-11, 15 trp1-1 ura3-1 can 1-100*) or W303-1B (*MATα*) [21]. WET-1A (*MATa nps1Δ::HIS3*/ YCp50*NPS1*), WTH-1A (*MATa nps1-105*) and WHS-r2HA-1A (*MATa rsc2Δ::HIS3*/pRSC2.2XHA) were as described [22, 23]. WHK20-1A (*MATa nps1Δ1247*), WHK21-1A (*MATa nps1Δ1274*) and WHK22-1A (*MATa nps1Δ1338*) were constructed by introducing a stop codon (TAG) after C-3738, A-3819 and C-4011 by PCR, respectively. To construct *nps1Δ1247* and *nps1Δ1274*, PCR reactions were carried out with primers del-U (5'-gatttacaggcgaattctagagctcac-3') and del1247 (5'-gggaaaaatcccatgggactaggattt-3'), and del-U and del1274 (5'-agcttttcaaccatgggaattcatggatgggatgc-3'), respectively, and using pUC119 carrying the *Sac*I-*Nco*I fragment of *NPS1* as template. To construct *nps1Δ1338*, the PCR reaction was carried out with primers del1338 (5'-aatgaagagggatcctgaattcacgtttac-3') and pUCR (5'-tttcacacaggaaacagctatgac-3'), and using pUC119 carrying the *Bam*HI-*Cla*I fragment of *NPS1* as template. After verifying that the sequence was correct by sequencing, each PCR product was used for interchanging with the respective wild-type sequence on the plasmid pUC*NPS1* (carrying 5.6 kb *Eco*RI-*Cla*I fragment). Each mutant *nps1* gene was excised from respective plasmid by *Eco*RI and *Sal*I digestion and used to replace the *nps1Δ* locus as described previously [22]. The *SFH1HA* allele was constructed as follows. The 2.2 kb DNA fragment containing the *SFH1* open reading frame (ORF) was generated by PCR using primers SFHU (5'-ggaaaggatcccgttcaagtgcact-3') and SFHR (5'-aattagtcgacatagaaacaagatc-3') with genomic DNA as template. The amplified fragment was digested with *Bam*HI and *Hin*CII, and cloned into pUC119 (pUC*SFH1*). *sfh1Δ::HIS3* and *SFH1-3HA* were constructed by inserting the *Sma*I-*Hin*CII fragment containing the *HIS3* gene or the 82 bp *Nhe*I fragment containing the triad HA epitope sequence into the unique *Nco*I or *Xba*I site within the *SFH1*

ORF, respectively, on the plasmid pUC*SFH1*. The correct direction of the HA insertion was verified by sequencing. *SFH1-3HA* was excised by *Bam*HI and *Sph*I digestion and inserted into the respective sites in YEp13 (YEp*SFH1-3HA*). The diploid W303 (W303D) strain was transformed with the *Bam*HI-*Hin*CII fragment excised from pUC*sfh1Δ::HIS3* and then with YEp*SFH1-3HA*, and His^+ Leu^+ haploid progenies were selected after sporulation (MY40-1A). Disruption of genomic *SFH1* locus and expression of Sfh1-Hap of MY40-1A were verified by Southern- and Western-blot analyses. p314.RSC2-2XHA (*RSC2-HA TRP1 CEN6*) was a gifted from B. R. Cairns [19]. WMY44-1A (*MATa nps1-13 sfh1Δ*/YEp*SFH1-3HA*), WHK20sf-1A (*MATa nps1Δ1247 sfh1Δ*/YEp*SFH1-3HA*) and WHK13r2HA-1A (*nps1-13 rsc2Δ*/p314.RSC2-2XHA) were generated by crosses between WMY40-1A (*MATa*) and WHK13-1B (*MATα*), WHK20-1A and WMY40-1B (*MATα*), and WHS-r2HA-1A and WHK13-1B, respectively. A tetracycline-regulatable *NPS1* allele was constructed as follows. The *Xho*I-*Puv*II fragment containing $tetO^7$ and the minimal CMV promoter, and the *Sma*I-*Xba*I fragment containing the *NPS1* ORF and terminator sequences were excised form pTRE2 (Clontech) and pASZ11*NPS1*, respectively, and inserted into pRS316 (*CEN6 ARS114 URA3*) to construct YCpTRE*NPS1*. The tTA gene encoding a TetR-VP16 fusion protein was excised from pTet-Off (Clontech) by *Xho*I and *Pvu*II digestion, and inserted between the *Sal*I and *Sma*I sites within pRS425 to construct YEptTA. YCpTRE*NPS1* and YEptTA, or pASZ11*NPS1* and YEptTA were introduced into WET-1A by using the plasmid shuffling technique [24] to construct WHK30-1A or WHK30C-1A, respectively.

Preparation of rich (yeast extract-peptone-dextrose, YEPD) and synthetic complete (SC) media and standard genetic methods were as described [25].

2.2. Isolation of nps1 mutants

Introduction of mutations into the C-terminal portion of *NPS1* was carried out by PCR mutagenesis according to the method described by Fromant *et al.* [26] with primers A (5’-ttacaggcccaagatagagctcac-3’) and B (5’-ttcaatataatatcaatggccatg-3’) using genomic DNA as template. Amplified fragments were digested with *Sac*I and *Nco*I and used to replace the

same region of the wild-type gene on plasmid pASZ11*NPS1* (*ARS1 CEN4 TRP1 NPS1*). The resulting plasmids were introduced into WET-1A. Approximately 6000 transformants grown selectively for both plasmids were replicated to supplemented SC medium containing 5-fluoroorotic acid [25] to counterselect against the plasmid carrying the wild-type *NPS1* gene [24]. Transformants carrying only the mutagenized plasmid (3000) were screened for lethality at 38˚C. Twenty-six mutants were temperature sensitive for growth. Plasmid DNA was recovered from these colonies and the DNA sequence between *Sac*I and *Nco*I were determined. All of the mutagenized fragments contained 10 to 15 nucleotide changes. To determine the nucleotide changes responsible for temperature sensitivity, these *Sac*I-*Nco*I fragments were further digested with *Stu*I, *Pma*CI and *Pst*I, and the resulting *Sac*I-*Stu*I, *Stu*I-*Pma*CI, *Pma*CI-*Pst*I and *Pst*I-*Nco*I fragments were used for fragment interchanging with the respective wild-type sequences on pASZ11*NPS1*. After repeating the screening as described above, three temperature sensitive mutations were identified. The nucleotide changes (and predicted amino acid changes) of *nps1-13* (WHK13-1A), *nps1-17* (WHK17-1A) and *nps1-19* (WHK19-1A) were as follows; *nps1-13* contained A3768T (E1256D), T3797C (L1266S), T3806C (V1269A) and A3815T (H1272L), *nps1-17* contained A2864C (E955A), A2896G (T966A), A2998G (K1000E) and A3002G (E1001G), and *nps1-19* contained A2906G (D969G), T2993C (I998T) and T3008C (M1003T).

2.3. Immunoprecipitation and Western blotting

Cells were grown to mid-log phase in YEPD. Preparation of whole-cell extracts and immunoprecipitation were as described [27]. To 100 μl of the extract containing 400 μg of protein, 2 μg of anti-HA mouse monoclonal antibody (clone HA11, Babco) was added. Anti-Nps1p antibody and Western blotting were as described [5]. The amount of each protein band detected by immunoblotting was estimated by densitometry using Bio Image 60S (Bio Image).

3. Results and discussion

3.1. A novel temperature sensitive mutation of NPS1 maps to the conserved bromodomain

To characterize the function of C-terminal region of Nps1p, temperature-sensitive (ts) mutations were generated by PCR mutagenesis of a cloned *NPS1* fragment encoding alanine at 901 to methionine at 1298. Three independent ts mutations were identified in a screen of about 3000 transformants using the plasmid shuffling technique [24]. The integrated alleles segregated 2+:2- for growth at 38˚C in crosses to a wild-type strain. Two of the mutations (*nps1-17 and nps1-19*) were recessive and the other one (*nps1-13*) was semi-dominant for temperature sensitive growth. All mutations caused cells to arrest in G2/M of the cell cycle at the restrictive temperature and to be sensitive to thiabendazole (TBZ). These phenotypes are similar to our previously identified ATPase domain mutation, *nps1-105* [22]. To further characterize the phenotypes of each mutation, we tested the growth of these mutants on media containing caffeine, formamide or hydroxyurea (HU), which have been reported to cause growth inhibition in mutations of other RSC components [19], the DNA-damaging agent methyl methanesulfonate (MMS) and UV irradiation. As shown in Fig.1, *nps1-13*, but not other mutations, showed impaired growth under all conditions tested at 30˚C, the permissive temperature of this mutation. Other mutations, including *nps1-105*, also showed sensitivity to these reagents or UV irradiation at semi-permissive temperature, 35˚C (data not shown). Caffeine inhibits the kinase activity of ATM (ataxia telangiectasia mutated) and ATR (ataxia telangiectasia related), which encode protein kinases involved in the DNA damage checkpoint pathway [28, 29], and HU inhibits ribonucleotide reductase to block DNA replication. Replication block frequently causes a break of stalled forks, a process called replication fork collapse [30]. Therefore, *nps1* mutations, especially *nps1-13*, were thought to enhance sensitivity of the cells to DNA damage. The *nps1-13* mutation alters amino acids in the conserved bromodomain that extends from amino acids 1253 to 1359. The bromodomain consists of four α helices (termed helices Z, A, B and C from the amino terminal side, Fig. 2A) and long intervening loops between helices Z and A (ZA loop), and helices B and C (BC loop) [8, 10]. The amino acid substitutions of *nps1-13* are E1261D, L1266S, V1269A and H1272L locating within and near helix Z of the bromodomain. The other two mutations identified in this study alter amino acids between amino acids 955 and 1003, the region

franking the ATPase domain.

Du *et al*. reported that deletions of amino acids from 1274 to 1359 (corresponding to the region from helix A of the bromodomain to C terminus) and from 1338 to 1359 (corresponding to helix C of the bromodomain to C-terminus) show weak and strong temperature sensitivity, respectively [20]. Because phenotypes of complete deletion of the bromodomain have not yet been characterized, we constructed *nps1Δ1247*, which lacks all of the bromodomain, in addition to previously described two previously descibed deletion alleles, *sth1*/*nps1Δ1274* and *sth1*/*nps1Δ1238*, and accessed their growth under various conditions. As described by Du *et al*, *nps1Δ1274* and *nps1Δ1238* exhibited weak and strong temperature sensitivity, respectively (Fig. 2B). However, *nps1Δ1247* exhibited no discernible phenotype compared to the wild type. Interestingly, among these bromodomain deletion mutations, only *nps1Δ1238* exhibited sensitivity to TBZ and DNA-damaging conditions similar to *nps1-13*.

3.2. Bromodomain mutations of NPS1 perturb interaction between RSC components

A number of studies have shown the interaction between bromodomains and peptides derived from histone N-terminal tails, in a manner dependent on acetylation of lysine residues within the peptide [10-12]. Acetyl-lysine binding occurs through residues in the ZA loop and BC loop. *nps1-17* contained an additional amino acid substitution within the bromodomain, Y1290H, when it was originally isolated. This residue is absolutely conserved in bromodomains and has been demonstrated to be implicated in acetyl-lysine interaction [10-12]. However, the growth of the Y1290H mutation alone was indistinguishable to the wild type (data not shown).

Recent studies suggest the implication of bromodomains in the interaction with proteins other than histones [13-15]. We thought it was possible that the bromodomain of Nps1p contributes to the interaction with other components of the RSC complex. Because Sfh1p interacts directly with Nps1p [6], we generated strains expressing HA-tagged Sfh1p and

performed coimmunoprecipitation experiments. Although anti-HA antibodies could coimmunoprecipitate Nps1p in all the strains tested, the amount of Nps1p in the precipitates from *nps1Δ1247* and *nps1-13* cell lysates decreased to 69% and 18% of that from the wild-type cells, respectively (Fig. 3A). We also performed coimmunoprecipitation experiment using HA-tagged Rsc2p, a component of RSC [19], and obtained similar results (Fig. 3B). These results indicated that the bromodomain of Nps1p plays an important role in the interaction with other RSC components. The observation that the complete deletion of bromodomain (*nps1Δ1247*) caused moderate disturbance of Nps1p-Sfh1p interaction while the *nps1-13* mutation, which alters amino acids in helix Z, caused severe disturbance in Nps1p-Sfh1p interaction suggests that the bromodomain of Nps1p may not be a direct binding site for other RSC components. *nps1Δ1338*, which lacks the bromodomain helix C, and *nps1-13* exhibited similar phenotypes. Possibly these two mutations cause alterations of the ternary structure of the bromodomain and this may also result in the alteration of Nps1p total structure. Although the domain of Nps1p involved in the interaction with Sfh1p or other RSC components remained to be determined, we speculate that the bromodomain of Nps1p assists the binding by supporting the proper structure of the binding site. Whether the interaction between Nps1p and Rsc2p is direct or not is unclear [19]. A recent study on Snf5p, a component of Snf/Swi complex and a prototype paralog of Sfh1p, indicated the importance of this protein to the integrity of the complex [32]. Therefore, it is possible that the reduced interaction between Nps1p and Sfh1p results in the release of Rsc2p from the complex.

3.3.Reduced expression of Nps1p enhances sensitivity to DNA damage

The above observations that the *nps1Δ1247* mutation, which caused moderate reduction of the interaction with Sfh1p, showed no discernible growth defect, while the *nps1-13* mutation, which caused severe disturbance in the interaction, showed multiple growth defects, suggested that an abundance of Nps1p/RSC is required for the tolerance to TBZ or DNA-damaging agents, or for the growth at high temperatures. To test this possibility, we

constructed a strain that expresses *NPS1* under the control of a tetracycline (TC)-regulatable promoter (tet*NPS1*, *nps1Δ*/YCpTRE*NPS1* YEptTE). We first determined that Nps1p levels responded to the TC concentration. In the absence of TC, the level of Nps1p in tet*NPS1* was about 76% of that in the *NPS1* wild-type control strain (WHK30C-1A, *nps1Δ*/pASZ11*NPS1* YEptTE, referred to as wild type here after), rapidly decreased to 35% by the addition of 1 μg/ml of TC and then gradually decreased to 12% by the addition of 50 μg/ml TC (Fig. 4A). The addition of 100 μg/ml TC caused severe growth delay with the accumulation of G2/M cells in tet*NPS1* (data not shown). Nevertheless, the addition of up to 50 μg/ml TC did not affect the viability of both the tet*NPS1* and the wild type strains (Fig. 4C, data of the control strain are not shown).

Next, we examined the effect of TBZ, HU, MMS, UV irradiation and high temperature (37˚C) on the growth of tet*NPS1*. The addition of 120 μg/ml of TBZ to the medium or the incubation at 37˚C did not affect the growth of tet*NPS1* regardless of the presence of TC (Fig. 4B). On the other hand, the growth of tet*NPS1* was severely impaired by 200 mM HU, 0.005% MMS or 20 J/m^2 UV irradiation when 10 μg/ml TC was present. As shown in Fig. 4C, viability of the tet*NPS1* cells rapidly declined by the addition of HU or MMS, or by UV irradiation in concert with the increase of the TC concentration present in the medium; viability of the cells declined to 25% (HU) or 50% (MMS or UV) in the presence of 1 μg/ml of TC and to less than 1% (HU) or about 6% (MMS or UV) in the presence of 10 μg/ml of TC. Under the same conditions, viability of the wild type strain remained unchanged (data not shown). The *nps1-13* mutation, but not tet*NPS1*, showed TBZ- and temperature sensitivities indicating that the aberrant structure of the bromodomain in *nps1-13* may affect not only the interaction with other RSC components but also the catalytic activity of Nps1p. Overall, the decrease of the functional RSC in both tet*NPS1* and *nps1-13* enhanced the sensitivity to DNA-damaging conditions, suggesting that an abundance of RSC is important for the maintenance of viability under the DNA-damaging conditions. Taken together that *nps1* mutations other than *nps1-13* also enhance sensitivity to DNA-damaging conditions at semi-permissive temperature, RSC is likely to be involved in DNA-damage response pathway.

We observed the cell-cycle arrest occurred in *nps1-13* when treated with HU or MMS, suggesting that the mutation may not affect the DNA-damage checkpoint pathway (Koyama *et al.*, unpublished result). DNA damage is known to arise spontaneously during the proliferation process. Abundant RSC could facilitate the rapid response of the cells to overcome DNA damage by transcriptional activation or repression, and/or assisting the access of repair enzymes to damage sites. Further analysis on the functions of RSC in DNA-damage response is under progress in our laboratory.

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Figure legends

Fig. 1. Phenotypes of temperature sensitive *nps1* mutations. Wild type (WT, W303-1A), *nps1-105* (WHT-1A), *nps1-13* (WHK13-1A), *nps1-17* (WHK17-1A) and *nps1-19* (WHK19-1A) were grown to mid-log phase and spotted on YEPD plates containing various drugs indicated in the figure. Unless otherwise indicated, plates were incubated at 30˚C for 3 days.

Fig. 2. Phenotypes of *nps1* bromodomain mutants. (A) A schematic diagram of the Nps1 protein is shown. Regions of α-helix within the bromodomain were referred to the definition by Jeanmougen *et al*. [8]. (B) Wild type (WT, W303-1A), *nps1-105* (WHT-1A), *nps1-13* (WHK13-1A), *nps1Δ1247* (WHK20-1A), *nps1Δ1274* (WHK21-1A), and *nps1Δ1338* (WHK22-1A) were grown to mid-log phase and spotted on YEPD plates containing various drugs indicated in the figure. Unless otherwise indicated, plates were incubated at 30˚C for 3 days.

Fig. 3. *nps1-13* mutation perturbs interaction between the RSC components. Whole-cell extracts sere prepared from WMY40-1A [*NPS1* (WT) *sfh1Δ*/YEp*SFH1*HA], WMY44-1A (*nps1-13 sfh1Δ*/YEp*SFH1*HA), WHK20sf-1A (*nps1Δ1247 sfh1Δ*/YEp*SFH1*HA), WHS-r2HA-1A (*rsc2Δ*/pRSC2.2XHA) and WHK13r2HA-1A (*nps1-13 rsc2Δ*/pRSC2.2XHA) and utilized for immunoprecipitation experiments using anti-HA antibody. The immuno precipitation pellet derived from 400 μg of whole-cell extract was divided into two portions, separated through SDS-7.5% acrylamide gels and immunoblotted with anti-Nps1p or anti-HA antibody. In “input” lanes, 100 μg of a whole-cell extract from each strain was loaded. The numbers under each panel indicate fold changes of immunoprecipitated Nps1p versus Sfh1HAp or Rsc2Hap estimated by densitometry (± SD, n=3). The values of the wild type cells are referred to as 1. (A) WT: WMY40-1A; *nps1-13*: WMY44-1A; *nps1Δ1247*: WHK20sf-1A. (B) WT: WHS-r2HA-1A; *nps1-13*: WHK13r2HA-1A

Fig. 4. Phenotypes of the strain that expresses *NPS1* under the control of a tetracycline-regulatable promoter. (A) Nps1p levels in WHK30C-1A (*NPS1*, *nps1Δ*/pASZ11*NPS1* YEptTE) and WHK30-1A (tet*NPS1*, *nps1Δ*/YCpTRE*NPS1* YEptTE) are shown. Whole cell extracts were prepared from the cells grown for 12 h in YEPD medium containing various amounts of tetracycline (TC) indicated in the figure. The numbers under each lane indicate fold changes of Nps1p versus Cdc28p estimated by densitometry (± SD, n=3). The values of the WHK30C-1A cells in the absence of TC are referred to as 1. (B) WHK30C-1A, WHK30-1A and WHK13-1A grown to mid-log phase were spotted on YEPD plates containing various drugs indicated in the figure. Unless otherwise indicated, plates were incubated at 30˚C for 3 days. (C) Effect of HU (200 mM, closed circles with solid line), MMS (0.005%, closed diamonds with fine dotted line) and UV irradiation (20 J/m^2, closed rectangles with dotted line) on the viability of WHK30-1A. The cells (300~3000) were plated on YEPD plates containing various amount TC, TC and HU or MMS indicated in the figure. After 3-days incubation at 30˚C, colonies arose on each plate were counted. Viability (colony number/ total cell number) of WHK30C-1A grown in the absence of TC is referred to as 100. Open circles with solid line indicate the viability of WHK30-1A on the plates containing only TC. Average of three independent experiments with five plates per drug concentration is plotted.

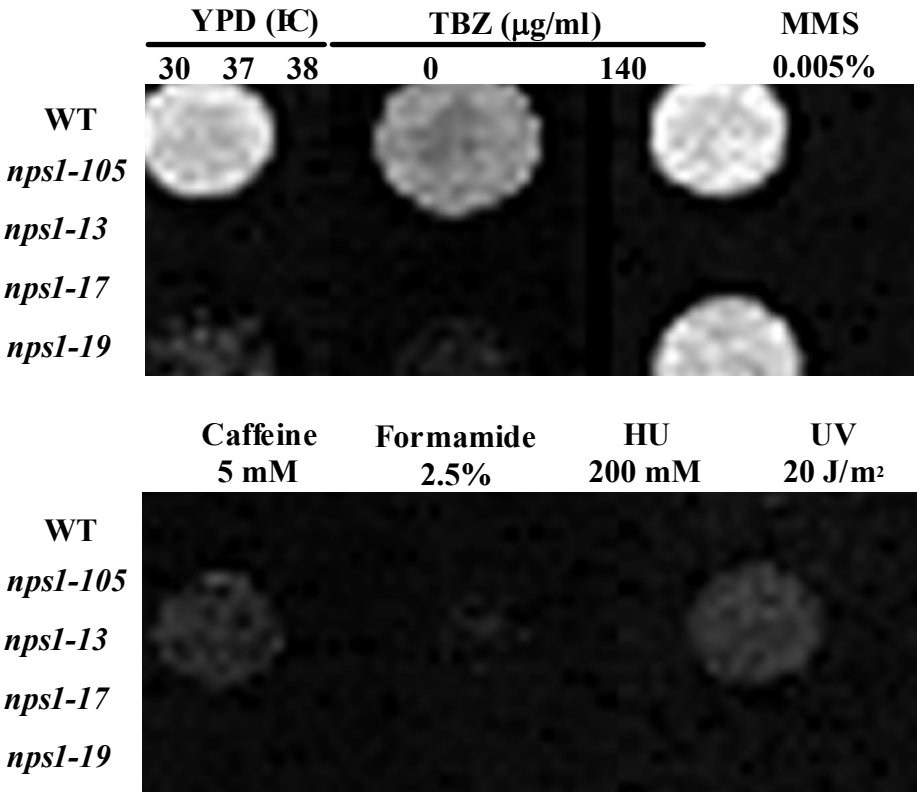

Fig. 1. Koyama *et al.*.

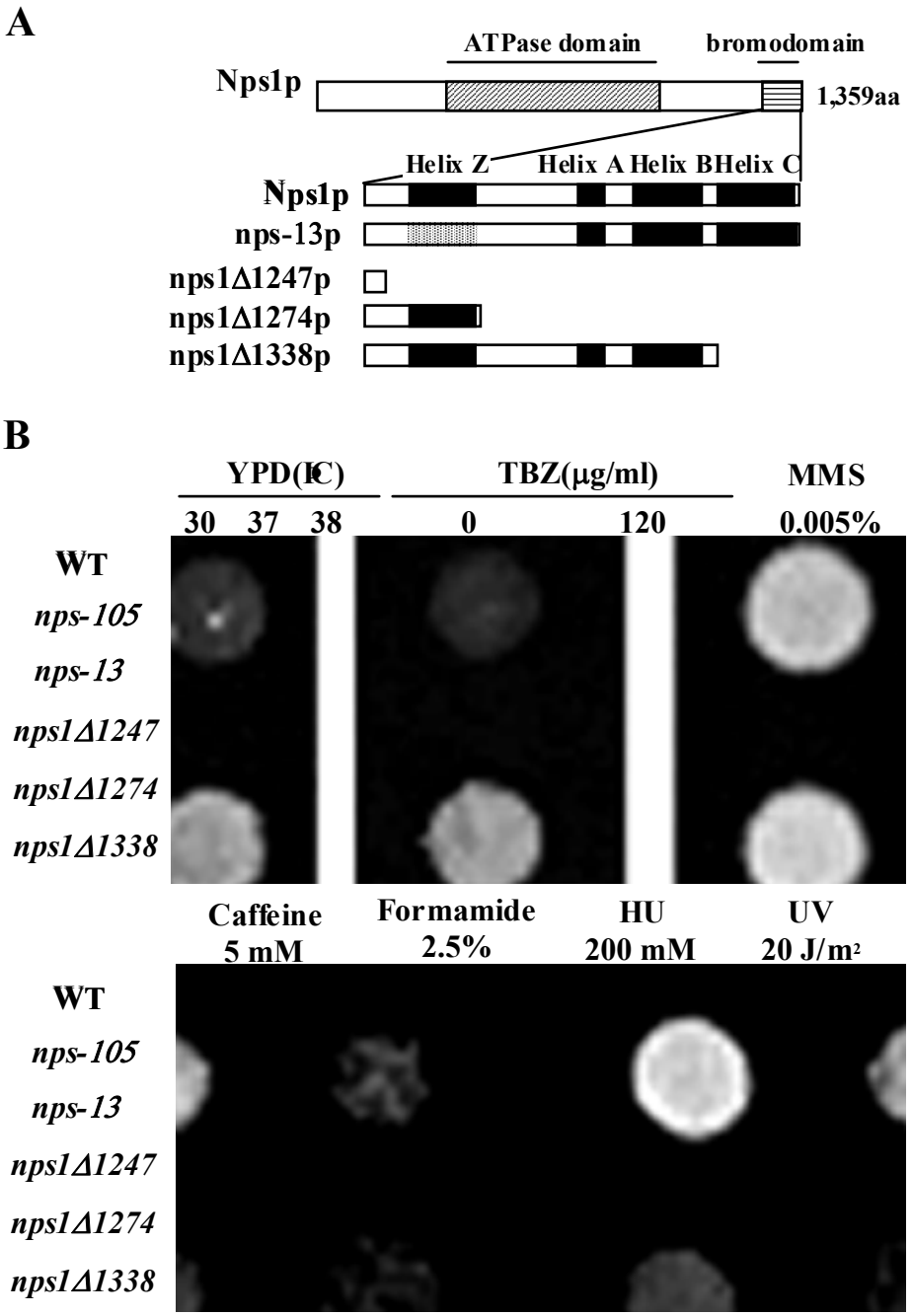

Fig. 2. Koyama *et al.*.

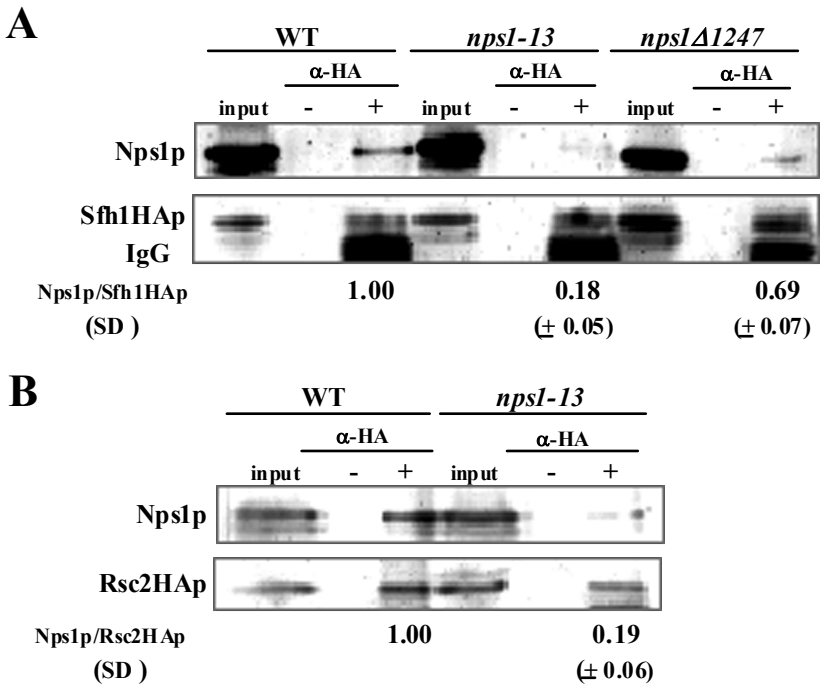

Fig. 3. Koyama *et.al.*

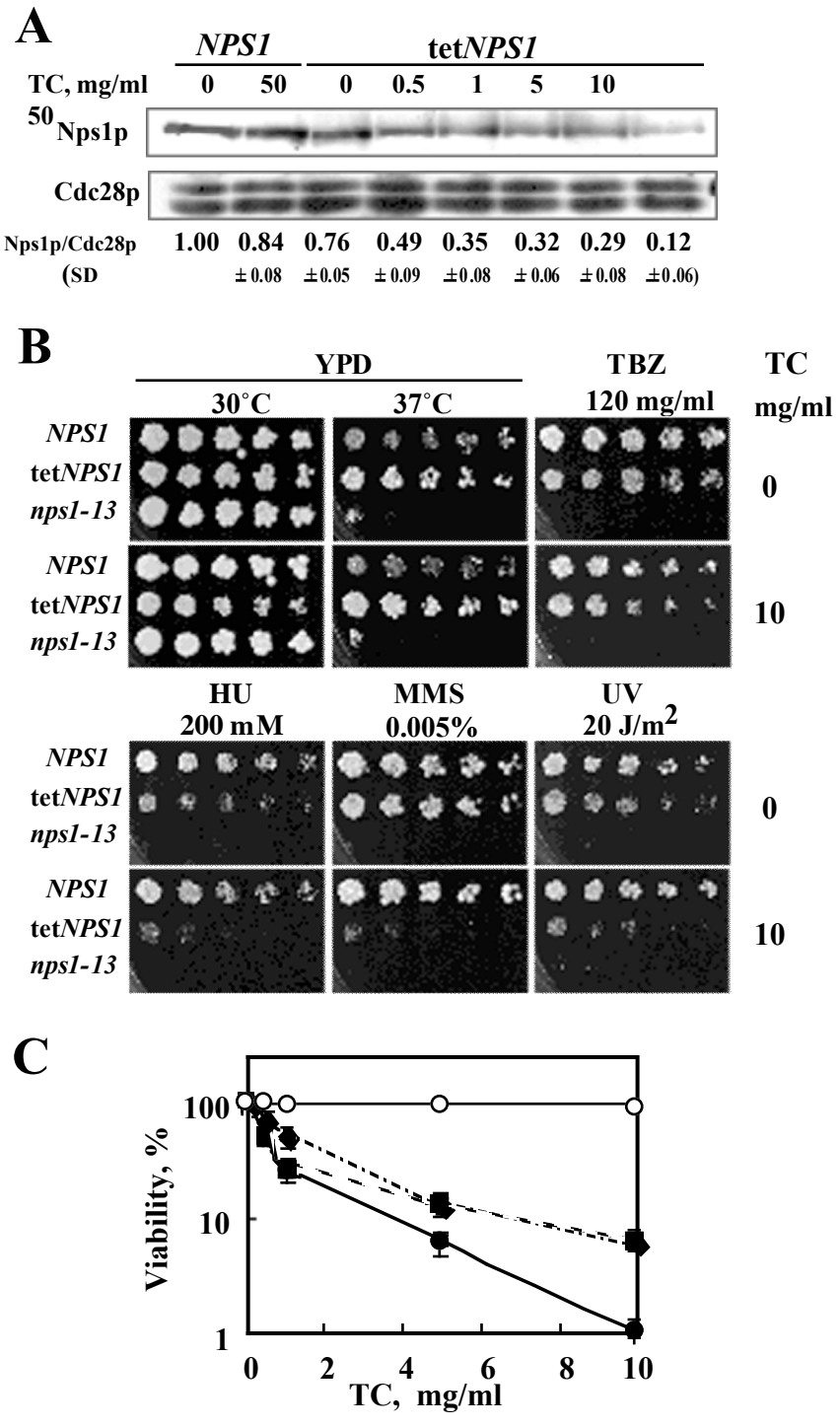

Fig. 4. Koyama *et al.*