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The *PNM2* mutation in the prion protein domain of *SUP35* has distinct effects on different variants of the [*PSI*$^+$] prion in yeast

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Abstract We have previously described different variants of the yeast prion [*PSI*$^+$] that can be obtained and maintained in the same genetic background. These [*PSI*$^+$] variants, which differ in the efficiency of nonsense suppression, mitotic stability and the efficiency of curing by GuHCl, may correspond to different [*PSI*$^+$] prion conformations of Sup35p or to different types of prion aggregates. Here we investigate the effects of overexpressing a mutant allele of *SUP35* and find different effects on weak and strong [*PSI*$^+$] variants: the suppressor phenotype of weak [*PSI*$^+$] factors is increased, whereas the suppressor effect of strong [*PSI*$^+$] factors is reduced. The *SUP35* mutation used was originally described as a "Psi no more" mutation (*PNM2*) because it caused loss of [*PSI*$^+$]. However, none of the [*PSI*$^+$] variants in the strains used in our study were cured by *PNM2*. Indeed, when overexpressed, *PNM2* induced the de novo appearance of both weak and strong [*PSI*$^+$] variants with approximately the same efficiency as the overexpressed wild-type *SUP35* allele. Our data suggest that the change in the region of oligopeptide repeats in the Sup35p N-terminus due to the *PNM2* mutation modifies, but does not impair, the function of the prion domain of Sup35p.

Key words [*PSI*$^+$] · *SUP35* · Prions · *PNM* mutant · Translation termination · Nonsense-suppression

Introduction

Prions are protein conformational variants capable of self-propagation. The concept and the term "prion" were introduced to describe a proteinaceous infectious agent, now presumed to be a conformational variant of the PrP protein and the cause of neurodegenerative diseases in humans (Creutzfeldt-Jakob Disease, Gerstmann-Shtraussler-Sheinker syndrome and fatal familial insomnia), sheep (scrapie), cattle (mad cow disease) and other mammals (Griffith 1967; Prusiner 1982; see also Caughey and Chesebro 1997; Horwich and Weissmann 1997; Wickner 1997 a; Prusiner et al. 1998 for reviews). The list of proteins capable of taking on self-propagating prion conformations was recently extended with the addition of two *Saccharomyces cerevisiae* proteins, Sup35p and Ure2p, and one *Podospora anserina* protein, Het-s (Wickner 1994; Coustou et al. 1997). The yeast non-Mendelian factor [*PIN*$^+$] was also proposed to be a prion, although the gene coding for Pinp is not known (Derkatch et al. 1997). Thus, the prion phenomenon is no longer limited to one mammalian protein. Moreover, data obtained by the investigators of [*Het-s**] suggest that the prion conformations of some proteins have distinct functions (Coustou et al. 1997; Wickner 1997 b).

A self-propagating prion conformation of Sup35p (Sup35^{PSI+}) was found in laboratory *S. cerevisiae* strains of different origin (Cox 1965; Liebman and All-Robyn 1984; Inge-Vechtomov et al. 1988) and is referred to as [*PSI*$^+$] (see Wickner et al. 1996; Tuite and Lindquist 1996; Lindquist 1997; Liebman and Derkatch 1999 for reviews). The appearance of Sup35p^{PSI+} can also be induced de novo in [*PIN*$^+$] derivatives of yeast strains following transient overexpression of the *SUP35* gene (Chernoff et al. 1993; Derkatch et al. 1996, 1997). The non-prion Sup35p isoform (Sup35^{psi-}) is presumed to function as the translation termination factor eRF3 (Stansfield et al. 1995; Zhouravleva et al. 1995; Frolova et al. 1996), whereas the prion Sup35p^{PSI+} conformation forms insoluble aggregates (Patino et al. 1996; Paushkin et al. 1996) and is impaired in release factor activity leading to an increase in translational nonsense-suppression in [*PSI*$^+$] strains (Cox 1965, 1971; Liebman and Sherman 1979; Ono et al. 1986; Tikhodeev et al. 1990). Different cellular factors, such as release factor eRF1 (Sup45p) and chaperone protein Hsp104 are involved in the biogenesis of Sup35p^{PSI+} and Sup35p^{psi-} conformations (Chernoff

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et al. 1995; Patino et al. 1996; Paushkin et al. 1996; Derkatch et al. 1998). However, purified Sup35p forms amyloid-like fibers in vitro and cell-free Sup35p aggregation can be seeded by pre-formed Sup35p fibers or $Sup35^{PSI+}$ aggregates (Glover et al. 1997; Paushkin et al. 1997 a).

Sup35p is a chimeric protein: its C-terminal part, starting at amino acid 254, exhibits co-linear homology to the EF-1α elongation factor (Kushnirov et al. 1988; Wilson and Culbertson 1988) and is sufficient to perform the essential (Ter-Avanesyan et al. 1993) Sup35p function. The N-terminal part of Sup35p is unique and not essential for viability, but is required for the maintenance of [*PSI*$^+$] (Ter-Avanesyan et al. 1993, 1994). Moreover, Sup35p fragments lacking the N-terminus do not join $Sup35p^{PSI+}$ aggregates (Paushkin et al. 1996). The N-terminal part of Sup35p is sufficient to induce the de novo appearance of [*PSI*$^+$] when overproduced in vivo (Derkatch et al. 1996) and, when purified, forms amyloid-like filaments in vitro (Glover et al. 1997; King et al. 1997). These data suggest the presence of a distinct prion domain within the Sup35p N-terminus that bears no homology to the mammalian prion protein but resembles PrP by its unusual amino-acid composition, the presence of oligopeptide repeats, and the predicted capability to form β-sheet-rich structures (Cox 1994; Tuite 1994; Kushnirov et al. 1995; Wickner et al. 1995; Glover et al. 1997; King et al. 1997).

All identified *SUP35* mutations causing [*PSI*$^+$] loss (Pnm or "psi no more" phenotype) or inhibiting [*PSI*$^+$]-associated nonsense-suppression (Asu or "antisuppressor" phenotype) have been located in two distinct regions of the Sup35p N-terminus. The first region, presumed to be crucial for [*PSI*$^+$] biogenesis, contains the oligopeptide repeats (residues 55–96). Doel et al. (1994) demonstrated that the dominant *PNM2* mutation, which caused the loss of [*PSI*$^+$] (Young and Cox 1971), is the result of a Gly→Asp substitution at amino-acid residue 58. Ter-Avanesyan et al. (1994) showed that deletion of amino acids 21–69, partially overlapping the region of repeats, causes a recessive Pnm and a dominant Asu phenotype. Not surprisingly, when overproduced, a Sup35p fragment bearing this deletion is unable to induce the de novo appearance of [*PSI*$^+$] (Derkatch et al. 1996). The second *SUP35* region in which dominant *PNM* and *ASU* mutations have been uncovered encodes the glutamine/asparagine-rich sequence at the extreme Sup35p N-terminus between amino-acid residues 8 and 24 (DePace et al. 1998). These mutants showed a diminished ability to be recruited into $Sup35p^{PSI+}$ aggregates, and *PNM* mutants generally caused a modest solubilization of such aggregates (*ibid*).

Sup35p has been proposed to exist in more than one prion conformation or to form more than one type of prion aggregate because [*PSI*$^+$] variants causing different levels of nonsense-suppression, and characterized by different mitotic stabilities, can be induced in the same yeast strain by overproduction of the identical Sup35 protein (Derkatch et al. 1996). Weak [*PSI*$^+$] variants cause weak nonsense suppression and are usually lost in approximately 3% of mitotic progeny, whereas strong [*PSI*$^+$] variants cause higher levels of nonsense suppression and are extremely stable in mitosis. Strong [*PSI*$^+$] variants are also lost less frequently on media containing guanidine hydrochloride (GuHCl) than weak [*PSI*$^+$] variants (*ibid*). The previously described non-Mendelian element [*ETA*$^+$] (Liebman and All-Robyn 1984) was recently shown to be a weak [*PSI*$^+$] variant (Zhou et al. 1999). The multiplicity of [*PSI*$^+$] variants is reminiscent of mammalian prion strains that cause different forms of transmissible spongiform encephalopathies and induce the formation of disease-specific prion proteins (PrP^{SC}) that are cleaved at different amino-terminal sites by proteinase K. Self-propagation of PrP^{SC} polymers with distinct three-dimensional structures was proposed to be the molecular basis of the distinction between mammalian prion strains (Bessen et al. 1995).

Here we show that neither strong nor weak [*PSI*$^+$] variants in our strain background are destabilized by overexpression of the *PNM2* allele of *SUP35*. This is in contrast to the efficient destabilization of [*PSI*$^+$] observed in another strain background, where the "Psi no more" phenotype of *PNM2* was originally described (Doel et al. 1994). We also show that a *PNM2* overdose has opposite effects on suppression efficiency in the presence of weak and strong [*PSI*$^+$] variants in an identical genetic background. Finally, we demonstrate that overexpression of the *PNM2* allele in isogenic [*psi*$^-$] derivatives induces the de novo appearance of both weak and strong [*PSI*$^+$] variants in a [*PIN*$^+$]-dependent manner.

Materials and methods

Strains. Two pairs of isogenic weak and strong [*PSI*$^+$] derivatives were obtained by overproducing the wild-type Sup35p or the Sup35p N-terminal fragment in a [*psi*$^-$][*PIN*$^+$] variant of 74-D694 (*MAT***a** *ade1-14*$_{UGA}$ *trp1-289 his3-200 ura3-52 leu2-3,112*). The *ade1-14* allele is efficiently suppressed in strong [*PSI*$^+$]-bearing derivatives whereas its suppression is poor in weak [*PSI*$^+$]-bearing derivatives. A [*psi*$^-$][*pin*$^-$] 74-D694 variant was obtained by incubating [psi$^-$][PIN$^+$] 74-D694 on YPD + GuHCl. 9ΔN-74-D694 and 12ΔN-74-D694 are independent mutants of [psi$^-$][PIN$^+$] 74-D694 which contain deletions of codons 2–254 of *SUP35* (Derkatch et al. 1997). An [*ETA*$^+$] derivative of SL1010-1A (*MAT*α *ade1-14*$_{UGA}$ *met8-1 leu2-1 his5-2 trp1-1 ura3-52*; Zhou et al. 1999) is a meiotic segregant from a cross of [psi$^-$][PIN$^+$] 74-D694 and [ETA$^+$] SL611-17 A (Liebman and All-Robyn 1984). An [*eta*$^-$][*PIN*$^+$] SL1010-1A derivative resulted from the spontaneous loss of [*ETA*$^+$] that occurs in about 1% of the mitotic progeny. [*PSI*$^+$] 783/4c (*MAT ade2-1*$_{UAA}$ *leu2-3,112 ura3-1 his3-11,15 SUQ5*; kindly provided by C. R. Nierras) was used as a control strain in experiments examining the effects of *PNM2* overexpression on [*PSI*$^+$] stability since this strain was originally used to demonstrate that *PNM2* overexpression causes the loss of [*PSI*$^+$] (Doel et al. 1994). In addition, two [*psi*$^-$] derivatives of strain 783/4c were obtained by overexpression of either *HSP104* or *PNM2* following the procedures described in Chernoff et al. (1995) and Doel et al. (1994), respectively, and three independent [*PSI*$^+$] derivatives were re-induced by overproduction of the Sup35p N-terminal fragment. Overexpression of *PNM2* caused the efficient loss of [*PSI*$^+$] in each of these four [*PSI*$^+$] variants (data not shown).

Plasmids. YEpSUP35 (also called pSTR7, Telckov et al. 1986) and YEpPNM2 (also called pSM128, Doel et al. 1994; kindly provided by C. R. Nierras and M. F. Tuite) are 2 μ *LEU2* plasmids bearing the complete *SUP35* wild-type gene and its *PNM2* allele, respectively. YEp13 (Broach and Hicks 1980), a standard 2 μ *LEU2* vector, was

used as a control in experiments involving YEpSUP35 and YEpPNM2. pGAL::SUP35 is a *CEN URA3* vector that contains the promoterless *SUP35* gene under the control of the inducible *GAL1* promoter (Derkatch et al. 1996). pRS316CG is a pRS316-based *URA3* vector containing the open reading frame of the *GFP* "super glow" allele under the control of the Cu^{2+}-inducible *CUP1* promoter (kindly provided by S. Lindquist). To construct the pPNM2nmGFP and pSUP35nmGFP plasmids, the promoterless *PNM2* and *SUP35* fragments encoding the N-terminal parts of the respective Sup35 proteins (amino acids 1–254, also called nm) were PCR-amplified from YEpPNM2 and pEMBL-SUP35 (Ter-Avanesyan et al. 1993) using primers 5′GCGGGATCCACAATGTCGGATTCAAACCA and 5′CCATCCGCGGCATATCGTTAACAACTTCGT. The amplification products were digested with *Bam*HI and *Sac*II and inserted into *Bam*HI- and *Sac*II-digested pRS316CG, downstream from *CUP1* and in-frame with *GFP*.

Methods and cultivation procedures. Standard yeast media and cultivation procedures were used (Sherman et al. 1986; Rose et al. 1990). Unless specifically mentioned yeast were grown on YPD. Transformants were grown on synthetic glucose media selective for plasmid maintenance (SD-Leu or SD-Ura). To evaluate the level of growth inhibition caused by overexpression of *PNM2* in [*PSI*$^+$] derivatives, transformants bearing YEpPNM2 or control plasmids were grown on SD-Leu and 10-fold serial dilutions were spotted on SD-Leu. The same cultures were used to determine plasmid stability. The stability of the 2 μm *LEU2* plasmids was estimated as the percentage of Leu$^+$ colony forming units in transformant cultures by colony purifying aliquots on YPD and replica plating the colonies to SD-Leu. At least three transformants with each plasmid were analyzed in each experiment, and at least 250 of the colonies formed were checked for the presence of *LEU2*. Suppression of the *ade1-14* and *ade2-1* nonsense mutations was estimated from growth at 20°C and/or 30°C on synthetic media lacking adenine and containing glucose or ethanol (2%) as a single carbon source (SD-Ade and SEt-Ade, respectively). Because, in addition to adenine auxotrophy, *ade1* and *ade2* mutations cause the accumulation of a red pigment, the color on YPD was also used to determine the efficiency of nonsense suppression. The better the growth on adenineless media and the lighter the color on YPD, the stronger the suppression. YPD medium containing 5 mM guanidine hydrochloride (YPD + GuHCl) was used to cure [*PSI*$^+$] (Tuite et al. 1981).

To test for [*PSI*$^+$] loss by overexpression of the *PNM2* allele, transformants bearing YEpPNM2 or the control YEp13 plasmid were patched on SD-Leu and, following growth on SD-Leu for approximately 14 cell generations, were colony purified on YPD where the proportion of red ([*psi*$^-$]) colonies was determined. Usually 500–700 colonies obtained from three transformants with each plasmid were analyzed. A higher proportion of red colonies in the progeny of YEpPNM2 transformants is indicative of [*PSI*$^+$] loss (Doel et al. 1994). A few red and pink or white colonies were picked up in each experiment and the lack or presence of [*PSI*$^+$], respectively, was confirmed following plasmid loss. Note that even though the presence of YEpPNM2 affects the suppression efficiency in [*PSI*$^+$] derivatives and induces the de novo appearance of [*PSI*$^+$] in [*psi*$^-$] derivatives (see Results), [*psi*$^-$] colonies are red and [*PSI*$^+$] colonies are white or pink in the presence of either YEpPNM2 or YEp13.

Tests for the de novo induction of [*PSI*$^+$] were performed as described previously (Chernoff et al. 1993; Derkatch et al. 1996). In qualitative tests involving pGAL::SUP35, transformants were patched and grown on SD-Ura, transferred to uracilless synthetic media containing 2% galactose as a single carbon source (SGal-Ura) and grown for 7 or 14 cell generations to induce the *GAL1* promoter, and then replica-plated to adenineless media where the *GAL1* promoter was repressed (SD-Ade and SEt-Ade). SD-Ura was used instead of SGal-Ura in the control experiment. Growth on adenineless medium following incubation on SGal-Ura but not on SD-Ura was indicative of the de novo appearance of [*PSI*$^+$]. In qualitative tests involving YEpPNM2, YEpSUP35 or YEp13, transformants were patched on SD-Leu and, following growth on SD-Leu for 7 or 14 cell generations, were replica-plated to adenineless media. The Ade$^+$ colonies that arose were presumed to be [*PSI*$^+$] if they retained suppression following plasmid loss and lost suppression following growth on YPD + GuHCl. The method of quantitative estimation of the frequency of the de novo induction of [*PSI*$^+$] is described in the legend to Table 1.

To test whether the *PNM2*-encoded protein (Pnm2p) is present in [*PSI*$^+$] aggregates, as normal Sup35p is, the pPNM2nmGFP construct, as well as control pSUP35nmGFP and pRS316CG plasmids, were introduced into [*PSI*$^+$] derivatives and fusion protein aggregates were visualized essentially as described previously (Patino et al. 1996; Zhou et al. 1999). Transformant cultures were grown to early log phase in SD-Ura. $CuSO_4$ was added to the final concentration of 5 μM to induce the *CUP1* promoter. The induction was for 1 h at 30°C.

Results

PNM2 is not always "Psi no more"

Because *PNM2* overexpression was previously shown to cause destabilization of [*PSI*$^+$] in the 783/4c background (Doel et al. 1994), we were surprised to find that *PNM2* overexpression did not destabilize [*PSI*$^+$] in our strains, 74-D694 and SL1010-1A. Four 74-D694 derivatives bearing weak or strong [*PSI*$^+$] variants and the SL1010-1A derivative bearing [*ETA*$^+$] were transformed with YEpPNM2 and YEp13. [*ETA*$^+$] was recently shown to be a weak [*PSI*$^+$] variant naturally occurring in some yeast strains (Liebman and All-Robyn 1984; Zhou et al. 1999). The stability of [*PSI*$^+$] was estimated in the transformants as described in Materials and methods. Neither the [*PSI*$^+$] variants nor [*ETA*$^+$] were destabilized by *PNM2* overexpression. On the contrary, the frequency of loss of weak [*PSI*$^+$] variants in the 74-D694 background was reduced from 3±0.7% to 0.4±0.4% in YEpPNM2 transformants (Fig. 1).

Although *PNM2* overexpression did not destabilize [*PSI*$^+$] in the strain derivatives used in this study, the effects of overexpressing *PNM2* differed from the effects of overexpressing wild-type *SUP35*. The major difference is that overexpression of wild-type *SUP35* is much more incompatible with strong [*PSI*$^+$] than is overexpression of *PNM2*. The incompatibility of strong [*PSI*$^+$] with excess *PNM2* was limited to the dramatic reduction of the YEpPNM2 plasmid stability relative to YEp13 (5–10% and 90%, respectively) and to a rather mild growth inhibition on plasmid selective, SD-Leu, medium (Fig. 2). In contrast, attempts to introduce the YEpSUP35 plasmid into derivatives bearing strong [*PSI*$^+$] resulted in a sharp reduction of transformation efficiency and a strong inhibition of growth. The rare transformants that arose appear to be due to plasmid integration or rearrangement. It was thus not possible to overexpress the wild-type *SUP35* allele in strong [*PSI*$^+$] derivatives. Another difference is that, unlike *PNM2*, overexpression of wild-type *SUP35* did not stabilize weak [*PSI*$^+$] variants. Indeed, more [*psi*$^-$] derivatives (red colonies) were observed in the progeny of YEpSUP35 compared to YEp13 transformants in one of the two 74-D694 derivatives bearing a weak [*PSI*$^+$] (see Fig. 1). This effect could be explained by either a destabilizing effect of wild-type *SUP35* overexpression on the propagation of some weak [*PSI*$^+$] variants or by selection for spontaneously occurring [*psi*$^-$] cells. [*ETA*$^+$] was not destabilized by overexpression of wild-type *SUP35*. Anal-

Fig. 1 *PNM2* overexpression stabilizes weak [*PSI*$^+$]. A 74-D694 derivative bearing weak [*PSI*$^+$] was transformed with multicopy plasmids containing *PNM2* or *SUP35*, or with control vector YEp13. Transformants were patched on SD-Leu medium and then subcloned on YPD (a representative plate is shown): YEp13 – top left, YEpPNM2 – top right, YEpSUP35 – bottom. The *color difference* reflects the difference in suppression efficiency. *Red sectors and colonies* indicate loss of [*PSI*$^+$]

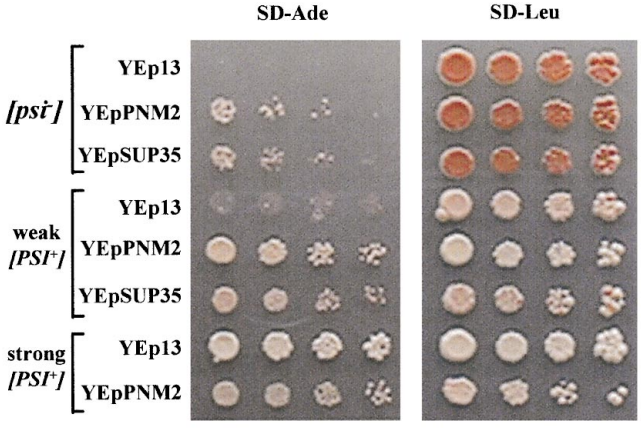

Fig. 2 Overexpression of the *PNM2* allele has different effects on weak and strong [*PSI*$^+$] variants. Spots of 10-fold serial dilutions show growth on SD-Ade or SD-Leu media of the indicated 74-D694 derivatives bearing YEp13 (Control), YEpPNM2 or YEpSUP35 multicopy plasmids. Growth on SD-Ade medium is due to the suppression of the *ade1-14* nonsense mutation. The SD-Leu medium is selective for plasmid maintenance

ysis of additional weak [*PSI*$^+$] variants in the 74-D694 background suggests that some of them are destabilized by *SUP35* overexpression and some are not.

Protein encoded by a *SUP35* fragment bearing the *PNM2* mutation fused to *GFP* can join pre-existing [*PSI*$^+$] aggregates

According to the model suggested by DePace et al. (1998), [*PSI*$^+$] is lost in strains bearing *PNM* mutations in *SUP35* because the protein encoded by the mutant alleles joins the [*PSI*$^+$] aggregates and prevents their further growth. Using this model, our finding of the lack of [*PSI*$^+$] destabilization by *PNM2* overexpression could be either due to the inability of the *PNM2*-encoded protein (Pnm2p) to join the [*PSI*$^+$] aggregates or to the inability of the Pnm2p that joins the [*PSI*$^+$] aggregates to block their further growth. To exclude the first possibility, 74-D694 derivatives bearing a weak or a strong [*PSI*$^+$] variant were transformed with pPNM2nmGFP, pSUP35nmGFP or pRS316CG. Following induction of the Gfp-fused proteins with $CuSO_4$, intense fluorescent foci were observed, respectively, in weak and strong [*PSI*$^+$] backgrounds in 10.27±1.65% and 60.2±11.38% of the cells in cultures of pPNM2nmGFP transformants and in 9.26±2.35% and 73.49±7.28% of the cells in cultures of pSUP35nmGFP transformants. In contrast, fluorescence was evenly distributed in cells bearing the pRS316CG vector indicating the lack of aggregation

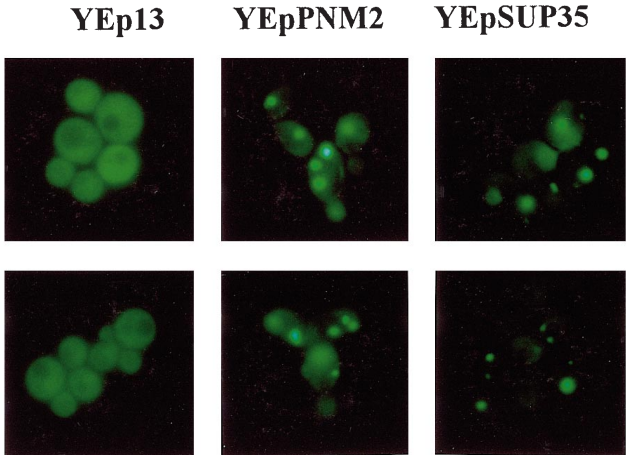

Fig. 3 Protein encoded by a *SUP35* fragment bearing the *PNM2* mutation fused to *GFP* joins [*PSI*$^+$] aggregates. Early log-phase cultures of 74-D694 derivatives bearing weak (top row) or strong (bottom row) [*PSI*$^+$] variants and the indicated plasmids were incubated in SD-Ura containing 5 μM $CuSO_4$ for 1 h at 30°C to induce the expression of the fusion construct

(Fig. 3). When pPNM2nmGFP and pSUP35nmGFP were introduced into a [*psi*$^-$] 74-D694 derivative, fluorescent foci were observed in less than 1% of the cells following *CUP1* induction. Thus, efficient aggregation of the Gfp-fusion proteins containing either of the Sup35 fragments depended upon pre-existing [*PSI*$^+$].

Overexpression of *PNM2* causes induction of both weak and strong [*PSI*$^+$] variants that can be maintained in the absence of *PNM2*

Overexpression of wild-type *SUP35* induces the de novo appearance of [*PSI*$^+$] (Chernoff et al. 1993) but only in the

Table 1 The efficiency of the de novo induction of [*PSI*$^+$] is not reduced in transformants with plasmids bearing the *PNM2* allele. To estimate the efficiency of the de novo induction of [*PSI*$^+$], transformants were patched on SD-Leu and, following growth for approximately 14 cell generations, were twice replica plated and colony purified on YPD. Plasmidless Leu$^-$ colonies were tested for the presence of [*PSI*$^+$], i.e., GuHCl-curable suppression of *ade-14*. Approximately ten Leu$^-$ colonies derived from each of 13 or 16 transformants with each plasmid were tested

Plasmid	Overexpressed *SUP35* allele	No. of transformants			No. of clones		
		Total	Giving rise to [*PSI*$^+$]		Total	Giving rise to [*PSI*$^+$]	
			Weak	Strong		Weak	Strong
YEpPNM2	*PNM2*	13	12	6	130	67	7
YEpSUP35	Wild type	13	13	7	126	58	9
YEp13	None	16	1	0	156	1	0

presence of the non-Mendelian [*PIN*$^+$] factor (Derkatch et al. 1997). We tested whether overexpression of the *PNM2* allele of *SUP35* can induce [*PSI*$^+$] and if this induction is dependent upon [*PIN*$^+$]. [*PIN*$^+$] and [*pin*$^-$] derivatives of [psi$^-$] 74-D694 were transformed with YEpSUP35 and YEpPNM2, as well as with the control YEp13 plasmid. The de novo appearance of [*PSI*$^+$] was detected in YEpPNM$_2$ and YEpSUP35-bearing transformants of 74-D694 [*PIN*$^+$] (Fig. 2) but not in [*pin*$^-$] derivatives (data not shown). This result indicates that overexpression of the *PNM2* allele, like overexpression of wild-type *SUP35*, causes the de novo appearance of [*PSI*$^+$] in a [*PIN*$^+$]-dependent manner. Because [*PSI*$^+$]-associated suppression was never lost following plasmid loss, we conclude that the [*PSI*$^+$] factors induced by *PNM2* overexpression can be efficiently maintained when only wild-type *SUP35* is present in the cell. The efficiency of [*PSI*$^+$] induction was not reduced in YEpPNM2 transformants relative to YEpSUP35 transformants, and the same array of weak and strong [*PSI*$^+$] derivatives was induced by both plasmids (Table 1). *PNM2* overexpression also caused the de novo appearance of [*PSI*$^+$] in an [*eta*$^-$][*PIN*$^+$] derivative of SL1010-1A and in a diploid resulting from a cross of [eta$^-$][PIN$^+$] SL1010-1A and [psi$^-$][PIN$^+$] 74-D694 (data not shown).

PNM2 is sufficient for the maintenance of [*PSI*$^+$] factors induced by the overexpression of wild-type *SUP35*.

To construct 74-D694 derivatives bearing the *PNM2* mutation at the *SUP35* locus, 9ΔN-74-D694 and 12ΔN-74-D694 strains bearing *SUP35* alleles lacking codons 2–254 were co-transformed with pGAL::SUP35 and a fragment of the yeast chromosomal DNA bearing the *PNM2* allele of *SUP35* (a *Xba*I fragment of YEpPNM2). Transformants were selected on SD-Ura that is selective for pGAL::SUP35 maintenance and replica-plated first to SGal-Ura where *GAL::SUP35* was induced and then to adenineless media where GAL::SUP35 was repressed to test for the de novo appearance of [*PSI*$^+$] (see Materials and methods). The presence of [*PSI*$^+$] cannot be detected on non-galactose adenineless media in 9ΔN-74-D694 and 12ΔN-74-D694 because the deletion of the region encoding the N-terminus of Sup35p makes these strains unable to maintain [*PSI*$^+$]. However, [*PSI*$^+$] could be detected on these media if [*PSI*$^+$] can be maintained in a strain exclusively expressing the *PNM2* allele of *SUP35* and if the *SUP35* deletion allele in 9ΔN-74-D694 and 12ΔN-74-D694 were gene-converted to the *PNM2* allele by the co-transformed *Xba*I fragment. (Note that pGAL::SUP35 bears a promoterless wild-type *SUP3*5 fragment that cannot gene convert the *SUP35* deletion allele in 9ΔN-74-D694 and 12ΔN-74-D694 to wild-type). Indeed, transformants in which [*PSI*$^+$] induction was easily detected appeared in both strains at a frequency typical of co-transformation (3 and 1 out of 56 and 56 Ura$^+$ transformants in 9ΔN-74-D694 and 12ΔN-74-D694, respectively). This indicates that [*PSI*$^+$] factors induced by wild-type *SUP35* overexpression can be maintained in yeast strains expressing exclusively *PNM2*.

PNM2 has different effects on weak and strong [*PSI*$^+$] variants.

Four 74-D694 derivatives bearing weak or strong [*PSI*$^+$] were transformed with YEpPNM2 or with the control YEp13 vector. Transformants bearing a weak [*PSI*$^+$] and carrying YEp13 exhibited the low level of suppression of *ade1-14* characteristic of weak [*PSI*$^+$] derivatives. However, YEpPNM2 transformants of weak [*PSI*$^+$] derivatives grew better on adenineless medium and had a whiter color than YEp13 transformants of this derivative, indicating that the level of suppression was significantly enhanced by overexpression of *PNM2* (Fig. 2). In 12 out of 12 colonies tested following the loss of the YEpPNM2 plasmid the suppressor phenotype returned to that characteristic of weak [*PSI*$^+$]. Thus, the increase in suppression (allosuppression) requires the presence of the *PNM2*-bearing plasmid and is not due to the high-frequency conversion of weak [*PSI*$^+$] variants into strong [*PSI*$^+$]. Likewise (data not shown), *PNM2* overexpression had an allosuppressor phenotype in an SL1010-1A derivative bearing [*ETA*$^+$]. The comparison of the effects of wild-type *SUP35* and *PNM2* overexpression in weak [*PSI*$^+$] backgrounds allowed for the conclusion that, like *PNM2*, wild-type *SUP35* causes allosuppression (see Fig. 2).

In contrast to the results described above for weak [*PSI*$^+$] variants, the suppressor phenotype associated with strong [*PSI*$^+$] variants was not increased in *PNM2*-bearing transformants (Fig. 2). However, further analysis of suppression efficiency was complicated by mild growth-inhibition. Therefore, YEpPNM2 and YEp13 transformants were colony purified on YPD where there was no selection for plasmid maintenance. The progeny of YEpPNM2 transformants formed pink and white colonies of equal size whereas colonies formed by the progeny of YEp13 transformants were of the same size but exclusively white. Es-

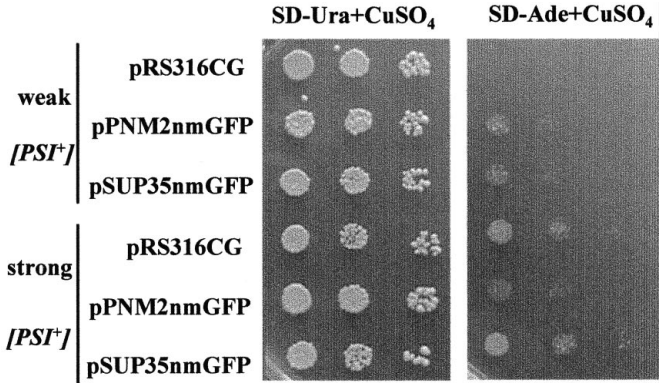

Fig. 4 Overexpression of the *PNM2* fragment encoding the prion domain is sufficient to cause allosuppression and antisuppression of weak and strong [*PSI*$^+$] variants, respectively. Spots of 10-fold serial dilutions show growth of the indicated 74-D694 derivatives bearing pRS316CG (Control), pPNM2nmGFP or pSUP35nmGFP plasmids on SD-Ade or SD-Ura media containing 0.25 μM $CuSO_4$. Growth on adenineless medium is due to the suppression of *ade1-14*. SD-Ura is selective for plasmid maintenance. $CuSO_4$ is required for the induction of the *CUP1* promoter

sentially all of the pink and all white colonies derived from YEpPNM2 transformants were Leu$^-$, indicative of plasmid loss, whereas 90% of the YEp13-derived colonies maintained the plasmid. The fact that the YEpPNM2-derived colonies were the same size as the YEp13-derived colonies was indicative of the lack of growth inhibition; this is not surprising since the YEpPNM2 plasmid was not present in all (usually) or most cells within a colony. However, half the colonies that arose from YEpPNM2 transformants but no longer carried the plasmid were pink, indicative of an antisuppressor phenotype. When transferred again to YPD, pink colonies grew up as white cultures indicative of strong [*PSI*$^+$]. This clearly shows that the *PNM2* overdose causes an antisuppressor effect that persists for several cell generations following plasmid loss (maybe as long as the protein is still present in the cells).

To test whether expression of the *PNM2* fragment encoding the prion domain, but not the translation termination domain, is sufficient to cause allosuppression and antisuppression in, respectively, weak and strong [*PSI*$^+$] backgrounds, 74-D694 derivatives bearing a weak or a strong [*PSI*$^+$] variant were transformed with pPNM2nmGFP or pRS316CG. Suppression was analyzed using 10-fold serial dilutions on SC-Ade medium containing 5 μM or 0.25 μM $CuSO_4$, where the *CUP1* promoter was induced. Indeed, relative to the pRS316CG transformants, the suppressor phenotype of pPNM2nmGFP transformants was increased in the weak [*PSI*$^+$] background and reduced in the strong [*PSI*$^+$] background. Both effects were clear even in the presence of only 0.25 μM of $CuSO_4$ when absolutely no growth inhibition was observed on the control SD-Ura plates (Fig. 4).

The effects of *PNM2* overdose on [*PSI*$^+$]-associated nonsense suppression were also tested in diploids resulting from crosses of various 74-D694 and SL1010-1A derivatives (Table 2). Suppression in diploids bearing the

Table 2 *PNM2* effects on the expression of weak and strong [*PSI*$^+$] derivatives in diploids

74-D694 parental derivative	SL1010-1A parental derivative	Suppression in diploid transformants with	
		YEpPNM2	YEp13 (control)
Weak [*PSI*$^+$]	[*psi*$^-$]	Moderate	Weak
[*psi*$^-$]	[*ETA*$^+$] (weak [*PSI*$^+$])	Moderate	Weak
Weak [*PSI*$^+$]	[*ETA*$^+$] (weak [*PSI*$^+$])	Moderate	Weak
Strong [*PSI*$^+$]	[*psi*$^-$]	Moderate to strong	Strong
Strong [*PSI*$^+$]	[*ETA*$^+$] (weak [*PSI*$^+$])	Moderate to strong	Strong
[*psi*$^-$]	[*psi*$^-$]	None[a]	None

[a] Induction of the de novo appearance of [*PSI*$^+$] under the conditions of *PNM2* overexpression is discussed the third section of the Results

control YEp13 vector was characteristic of the strongest [*PSI*$^+$] variant present in the parental strains. Analogously, overexpression of *PNM2* had an allosuppressor effect only in diploids bearing exclusively weak [*PSI*$^+$] variants. The presence of a strong [*PSI*$^+$] in either of the parents completely abolished allosuppression and caused antisuppression regardless of the presence of weak [*PSI*$^+$] in the other parent.

Discussion

We have previously described weak and strong variants of the yeast prion [*PSI*$^+$] that can be induced in the same genetic background by the overproduction of Sup35p (Derkatch et al. 1997). These [*PSI*$^+$] variants differ in the efficiency of nonsense suppression and in mitotic stability. It was proposed that, by analogy to mammalian prion "strains", different [*PSI*$^+$] variants correspond to different prion conformations of Sup35p or different types of aggregation of Sup35p^{PSI+} (Derkatch et al. 1996, 1997; Zhou et al. 1999). If this explanation is correct, certain mutant *SUP35* alleles could have different, even opposite, effects on the biogenesis of [*PSI*$^+$] variants as well as on phenotypes associated with different [*PSI*$^+$]s.

The *PNM2* allele of *SUP35* bears a missense mutation that changes one of the oligopeptide repeats that are presumed to be involved in [*PSI*$^+$] biogenesis (Cox 1994; Doel et al. 1994). Here we demonstrate that *PNM2* overexpression indeed has different effects on weak and strong [*PSI*$^+$] variants in the same strain background: nonsense suppression of weak [*PSI*$^+$] variants is increased, whereas suppression of strong [*PSI*$^+$] variants is reduced. This is true regardless of whether the [*PSI*$^+$] element examined is spontaneous or induced. Both antisuppressor and allosuppressor effects are transient, since the original level of suppression is restored when the *PNM2*-bearing plasmid is eliminated.

When two different [*PSI*$^+$] variants are combined in a diploid, the efficiency of the suppression phenotype in the presence of the control YEp13 vector as well as the *PNM2* overexpression effects are characteristic of the strongest [*PSI*$^+$] variant present in the parental strains. This demonstrates that strong [*PSI*$^+$] variants are dominant over weak [*PSI*$^+$] variants. This dominance may reflect the disappearance of weak [*PSI*$^+$] variants in diploids due to the more efficient propagation of strong [*PSI*$^+$].

The antisuppressor effect on strong [*PSI*$^+$], as opposed to the allosuppressor effect on weak [*PSI*$^+$], could be explained by the less-efficient conversion of Pnm2p^{psi-} than of Sup35p^{psi-} into the strong [*PSI*$^+$] shape or by the less-efficient incorporation of Pnm2p into the strong [*PSI*$^+$] aggregates, whereas Pnm2p^{psi-} prionization into weak [*PSI*$^+$] is slightly more efficient or comparable to that of Sup35p^{psi-}. Note, however, that we did not observe any difference in the proportion of cells with fluorescent foci in transformants with pPNM2nmGFP, or pSUP35nmGFP even though the method employed allowed us to see more efficient Sup35p aggregation in a strong vs. a weak [*PSI*$^+$] variant. Alternatively, incorporation of Pnm2p into weak or strong [*PSI*$^+$] aggregates could, respectively, increase or reduce the binding to the aggregates of other factors involved in translation termination, thus causing allosuppression or antisuppression. This explanation does not require Pnm2p and Sup35p to join [*PSI*$^+$] aggregates with different efficiency and is in agreement with the lower incompatibility of strong [*PSI*$^+$] with *PNM2* overexpression than with wild-type *SUP35* overexpression. Indeed, reduction in the binding of translation termination factors to the aggregates would simultaneously cause antisuppression and lower incompatibility by reducing the loss of termination activity. Sup45p could be one such factor because it was found in [*PSI*$^+$] aggregates (Paushkin et al. 1997 b; see, however, Patino et al. 1996) and because its overexpression rescues the lethality of Sup35p overexpression in a [*PSI*$^+$] background (Derkatch et al. 1998). Finally, incorporation of Pnm2p into weak and strong [*PSI*$^+$] aggregates could differentially affect their susceptibility to disaggregation (for example, by the Hsp104p chaperone) leading to changes in suppression and [*PSI*$^+$] stability. This hypothesis also explains our finding that *PNM2* overexpression stabilizes weak [*PSI*$^+$] variants.

It is interesting that the effect of *PNM2* overexpression on [*PSI*$^+$] stability in our strains was different from that originally observed by Doel et al. (1994). Although we reproduced the Doel et al. (1994) result when using their strain, 783/4c, we have never observed the efficient destabilization of any [*PSI*$^+$] (the "Psi no more" phenotype) upon *PNM2* overexpression in either the 74-D694 or SL1010-1A backgrounds. This observation explains the fact that DePace et al. (1998) failed to recover *PNM2* in their recent search for "Psi no more" mutants in 74-D694. Since we repeatedly observed the "Psi no more" effect of the overexpressed *PNM2* allele in the 783/4c background following curing and re-induction of [*PSI*$^+$] by overproduction of the Sup35p N-terminal fragment (data not shown), it appears that the difference in *PNM2* effects on [*PSI*$^+$] maintenance is strain-specific. The origin of this difference was not the subject of the current investigation; however, it should be mentioned that strain 783/4c differs from the strains used in our study by the presence of a tRNA suppressor *SUQ5*, and tRNA suppressors have different degrees of incompatibility with [*PSI*$^+$] and influence the expression of [*PSI*$^+$]-associated phenotypes (see Cox et al. 1988). Other strain differences could involve variability of Sup35p, Sup45p, Hsp104 or any other protein involved in [*PSI*$^+$] biogenesis.

It appears that Pnm2p can join pre-existing [*PSI*$^+$] aggregates without blocking further growth of the aggregates in our strains. Indeed, in both weak and strong [*PSI*$^+$] derivatives the fusion protein containing Gfp and the first 254 amino acids of Pnm2p was detected in aggregates that were previously suggested to be [*PSI*$^+$] (Patino et al. 1996). Moreover, Pnm2p is apparently sufficient for the maintenance of [*PSI*$^+$] factors induced by the overexpression of wild-type *SUP35*.

We also report that *PNM2* overexpression induces the de novo appearance of [*PSI*$^+$] and that the [*PSI*$^+$] factors induced do not require the presence of Pnm2p for their maintenance. We propose that the prion seeds in the *PNM2* overexpressing cells are formed by Pnm2p rather than by the low-abundant Sup35p encoded by the chromosomal wild-type *SUP35* allele. The possibility that overexpression of *PNM2* releases Sup35p from termination factors, and that only the released Sup35p forms [*PSI*$^+$] seeds, is unlikely because *PNM2* overexpression induces the de novo appearance of [*PSI*$^+$] as efficiently as the overexpressed wild-type *SUP35* allele. Indeed, it was previously shown that the efficiency of [*PSI*$^+$] induction correlates with the level of overproduction of Sup35p or Sup35p fragments capable of forming [*PSI*$^+$] seeds, and is not increased when Sup35p fragments lacking the [*PSI*$^+$] prion domain but capable of functioning in translational termination, and thus likely to be able to release Sup35p from termination complexes, are overproduced (Derkatch et al. 1996, 1998). The fact that *PNM2* overexpression results in the appearance of the same array of weak and strong [*PSI*$^+$] variants allows us to speculate that Pnm2p forms weak and strong [*PSI*$^+$] seeds with the same efficiency as the normal Sup35p, even though Pnm2p may join the pre-existing weak and strong aggregates with a different efficiency than the wild-type Sup35p. Interestingly, whether the [*PSI*$^+$] derivatives examined were originally induced by *PNM2* (data not shown) or a wild-type *SUP35* overdose, the weak and strong [*PSI*$^+$]s exhibited the same differential response to *PNM2* overexpression.

Our findings that *PNM2* overexpression can induce the de novo appearance of [*PSI*$^+$] re-opens the question of whether the region of nanopeptide repeats within the Sup35p N-terminus (amino acids 55–96) is crucial for [*PSI*$^+$] propagation. The arguments suggesting the importance of the repeats were based on data by Doel et al. (1994), who described the "Psi no more" effect of the *PNM2* allele (Gly58Asp), and on experiments involving plasmids bearing the *SUP35* allele deleted for the region encoding amino acids 21–69 (Ter-Avanesyan et al. 1994;

Derkatch et al. 1996). This deletion overlaps the first and part of the second repeat, but it also overlaps positions 24, 26 and 33 within the glutamine/asparagine-rich region where *PNM* mutations were localized by DePace et al. (1998). Our data indicate that the Gly58Asp change in the repeat region does not lead to a block of [*PSI*$^+$] propagation or the inability of the changed protein to join prion aggregates or form [*PSI*$^+$] seeds upon overproduction, even though it obviously affects the phenotypes of different [*PSI*$^+$] variants. This is in analogy with the mammalian prion PrP in which the region composed of five octapeptide repeats is obviously involved in scrapie biogenesis, but is not required for the formation of infectious prions. Indeed, the expansion of oligopeptide repeats is associated with familial prion diseases in humans (see Goldfarb et al. 1994) and with alteration of PrP aggregational properties and resistance to proteinase K (Lehmann and Harris 1996; Priola and Chesebro 1998). However, mice bearing a deletion of all but one of the repeats are fully susceptible to infection and produce normal titers of the scrapie agent (Fisher et al. 1996).

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