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Effect of Charged Residues in the N-domain of Sup35 Protein on Prion [*PSI*$^+$] Stability and Propagation*

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Background: The prion domain (PrD) of Sup35p can aggregate to form the [*PSI*$^+$] prion.
Results: Introduction of charged lysine residues (*sup35*^{KK}) in the Sup35p PrD alters prion properties.
Conclusion: Some *sup35*^{KK} alleles lead to the formation of new prion variants.
Significance: Establishment of molecular interactions influencing [*PSI*$^+$] prion stability and maintenance is a step toward an understanding of prion folding.

Recent studies have shown that Sup35p prion fibrils probably have a parallel in-register β-structure. However, the part(s) of the N-domain critical for fibril formation and maintenance of the [*PSI*$^+$] phenotype remains unclear. Here we designed a set of five *SUP35* mutant alleles (*sup35*^{KK}) with lysine substitutions in each of five N-domain repeats, and investigated their effect on infectivity and ability of corresponding proteins to aggregate and coaggregate with wild type Sup35p in the [*PSI*$^+$] strain. Alleles *sup35-M1* (Y46K/Q47K) and *sup35-M2* (Q61K/Q62K) led to prion loss, whereas *sup35-M3* (Q70K/Q71K), *sup35-M4* (Q80K/Q81K), and *sup35-M5* (Q89K/Q90K) were able to maintain the [*PSI*$^+$] prion. This suggests that the critical part of the parallel in-register β-structure for the studied [*PSI*$^+$] prion variant lies in the first 63–69 residues. Our study also reveals an unexpected interplay between the wild type Sup35p and proteins expressed from the *sup35*^{KK} alleles during prionization. Both Sup35-M1p and Sup35-M2p coaggregated with Sup35p, but only *sup35-M2* led to prion loss in a dominant manner. We suggest that in the fibrils, Sup35p can bind to Sup35-M1p in the same conformation, whereas Sup35-M2p only allowed the Sup35p conformation that leads to the non-heritable fold. Mutations *sup35-M4* and *sup35-M5* influence the structure of the prion forming region to a lesser extent, and can lead to the formation of new prion variants.

The devastating symptoms of transmissible fatal spongiform encephalopathies or infectious prion diseases are caused by an aberrant conformational change of the normally innocuous cellular prion protein PrP, leading to its deposition in the form of amyloid fibrils (1). Apart from the mammalian PrP[3] protein, clear evidence for prions has only been found in fungal systems (2), with over 10 prions identified in *Saccharomyces cerevisiae* (3, 4). One of the most studied prions to date is [*PSI*$^+$], which arises as a consequence of prionization of yeast Sup35p (translation termination factor eRF3). In the non-prion state, Sup35p is essential for robust translation termination. Cells bearing [*PSI*$^+$] have a reduced pool of soluble Sup35p, which leads to increased read through of stop codons during translation (5, 6).

The Sup35 protein consists of three distinct functional domains (Fig. 1*A*). The C-proximal region has significant homology with the translation elongation factor eEF1-A, it is required and sufficient for translation termination and cell viability (7). The N-proximal region, or prion-forming domain, is uniformly rich in Gln and Asn residues and contains six oligonucleotide repeats (8). This region is not essential for viability or termination (9), but is required for [*PSI*$^+$] induction (10) and propagation (11). The charged middle (M) region is not required for viability or termination, but can influence [*PSI*$^+$] induction or propagation because of interaction with the protein disaggregase Hsp104p (12).

Over the past decade, substantial progress has been made toward elucidating the structural arrangements of the prion fibrils of Sup35p. Their overall molecular architecture represents an approximate 8-nm wide backbone fibril of the N-region, which is surrounded by a diffuse 65-nm wide cloud of globular C-domains connected to the fibril by the extended M-region (13). Two types of structural model of the N-region fibrils have been proposed, based on experimental data: a β-helical model (14, 15) and a superpleated β-structure model (16). Current data favor the superpleated β-structure model (17, 18). In this structure, each polypeptide chain zig-zags in a planar serpentine-fold, and successive chains are stacked in register, one on top of another (Fig. 1*B*). This arrangement generates an array of elongated parallel β-sheets, each composed of identical strands and aligned with the fibril axis. The strands form a so-

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[3] The abbreviations used are: PrP, prion protein; GdnHCl, guanidine hydrochloride; PNM, [*PSI*$^+$] no more mutation; 5-FOA, 5-fluoroorotic acid; SDD-AGE, semi-denaturing detergent-agarose gel electrophoresis.

called cross-β structure that runs perpendicular to the filament axis.

Despite progress in understanding the structural arrangement of the Sup35p fibrils, it is still unclear which part(s) of the N-domain of full-length protein is critical for fibril formation. Mutational analysis of the Sup35 protein (substitutions with proline, insertions of glycine, or deletions of the repeats) has shown that only the first half of the N-domain may be required for [*PSI*$^+$] phenotype and fibril formation (19–22). Region 38–56 amino acids appears to be essential for the incorporation of a polypeptide into a growing amyloid fibril (20). Similarly to mammalian PrP, Sup35p can adopt multiple conformational states of the aggregates or "strains," which differ in phenotypic or biochemical characteristics (10). Species barrier or interference between different prion strains have been reported for both mammalian and yeast prions (23–25). For most prion strains characterized to date, the first half of the N-domain is either involved in formation of the β-structure (22, 26, 27) or critical for the infectivity of amyloid fibrils (22). On the other hand, other studies suggested that in addition to this region, a downstream region (amino acids 102–107) can also initiate amyloid formation (26, 27). Furthermore, the solid-state NMR data suggest that almost the entire N-domain and even part of the M-domain have an in-register parallel β-structure (28).

Thus, a complete understanding of the relationship between the N-domain sequence and its ability to form fibrils and maintain the [*PSI*$^+$] phenotype is still lacking. To address this question, here we designed a set of alleles encoding Sup35p with substitutions in the N-domain and probed their effect on the propagation of [*PSI*$^+$].

EXPERIMENTAL PROCEDURES

Strains, Media, and Growth Condition—*S. cerevisiae* strain 7A-D832 (*Matα ade1–14 his7–1 leu2 lys2 trp1 ura3 sup35*Δ::*TRP1* [pYCH-U2-SUP35] [*psi*$^-$] [*PIN*$^+$] and its isogenic derivative bearing the strong variant of [*PSI*$^+$] 10-7A-D832 (both generous gifts of A. Borchsenius) were used in this study. Both strains contain the *sup35*Δ::*TRP1* knock-out on the chromosome, compensated by plasmids bearing the *SUP35* gene.

Yeast cultures were maintained on YPD (yeast extract/peptone/dextrose medium) or on synthetic complete minimal medium (SC) (29) at 30 °C. Solid media were prepared with the addition of agar (2.5%). YPD medium containing 4 mM guanidine hydrochloride (GdnHCl) was used to eliminate [*PSI*$^+$] (30). We utilized solid SC media with 5-fluoroorotic acid (1 mg/ml) for negative selection of plasmids bearing *URA3* (31). Nonsense suppression induced in [*PSI*$^+$] cells was detected by the ability to grow on SC medium lacking adenine (SC-Ade). Suppression was also scored by the color test on 1/4 YEPD (32) or MD 1/5Ade; this variant of MD medium (33) contained all the appropriate auxotrophic supplements and adenine at 4 mg/liters. [*PSI*$^+$] cells suppressing the *ade1–14* allele were white in color, whereas [*psi*$^-$] cells accumulated red pigment.

Plasmids—Plasmids bearing *sup35* mutations were constructed by subcloning the corresponding PCR products in centromeric plasmids pRSU1 (*LEU2 SUP35*) and pRSU2 (*URA3 SUP35*) (34), which express *SUP35* from its own promoter. Point mutations were generated using the fusion PCR approach (35), primer sequences are available upon request. The final PCR product was cloned into pRSU1 or pRSU2 digested using HindIII. To obtain pRSU1-PNM2 or pRSU2-PNM2 the HindIII fragment of pSM128 (36) was subcloned in the corresponding sites of pRSU1 or pRSU2. The centromeric *URA3* plasmid CEN-GAL-SUP35 contains the complete *S. cerevisiae SUP35* gene under control of the *GAL* promoter (10). We obtained derivatives of this vector bearing mutant alleles by site-directed mutagenesis using highly processive DNA polymerase (AccuPrime Pfx, Invitrogen). Primer sequences are available upon request. Products of amplifications were digested by DpnI and transformed in *Escherichia coli* competent cells. All mutations were verified by sequencing. Centromeric plasmid pRS315 encoded Sup35p fused with the 3-HA tag (37) was the generous gift of M.D. Ter-Avanesyan.

Genetic and Microbiological Procedures—Yeast transformation was performed by the lithium acetate protocol (38).

Plasmid Shuffle—Direct plasmid shuffle (from wild type to mutant allele) was performed as follows: the [*PSI*$^+$] *sup35*Δ strain with the *SUP35* gene on a *URA3* plasmid was transformed with *LEU2* plasmids bearing the wild type or mutant *SUP35* allele. Transformants, selected on a uracil- and leucine-omitted SC medium, were tested for suppression of the *ade1–14* mutation to determine the presence of [*PSI*$^+$]. Then transformants were streaked out on YPD media to allow for spontaneous loss of plasmids. Colonies were replica plated on SC-Leu or SC-Ura medium to identify which had lost one of the plasmids. The suppressor phenotype of strains obtained was analyzed on SC-Ade or 1/4 YEPD. Reverse shuffle (from mutant to wild type allele) was performed in a similar way.

[PSI$^+$] Loss and Transmission—[*PSI*$^+$] curing and transmission were scored according to the previously described procedure (37) with minor modifications. The [*PSI*$^+$] *sup35*Δ [*SUP35 URA3*] strain was transformed with a *LEU2* plasmid bearing the *SUP35* or its mutant allele. To estimate [*PSI*$^+$] curing, due to the presence of the mutated *sup35* variant, three transformants for each combination of *SUP35* and *sup35^{KK}* alleles (*sup35* double mutations with lysines substituted for two consecutive residues in the equivalent positions of Sup35p repeats) were passaged three times on a uracil-omitted medium, then suspended in water and plated on MD 1/5 Ade medium to obtain single colonies. The fraction of Ura$^+$ Leu$^-$ [*PSI*$^+$] colonies was estimated by a color test and replica plating to SC-Leu and SC-Ura.

To determine the efficiency of [*PSI*$^+$] transmission from Sup35p to mutant Sup35 proteins, 50 transformants for each combination of WT (wild type) *SUP35* and *sup35^{KK}* alleles were passaged on leucine-omitted medium with uracil to enable the cells to lose plasmid coding for wild type *SUP35*. To estimate the efficiency of [*PSI*$^+$] transmission, Ade$^+$ colonies were scored among Ura$^-$Leu$^+$ isolates.

[PSI$^+$] Induction—The plate assays for [*PSI*$^+$] induction were performed as described previously (10). Yeast transformants bearing plasmid CEN-GAL-SUP35, or CEN-GAL with mutant variants of *SUP35*, were grown on glucose-Ura medium and replica plated onto -Ura and -Ura/Gal media. After 3 to 4 days of incubation, each plate was replica plated onto glucose -Ade

medium. [*PSI*$^+$] induction was detected as heterogeneous growth on -Ade medium after 7 to 10 days of incubation.

Prion Loss in Exponentially Growing Cultures—For quantitative estimation of [*PSI*$^+$] elimination, the *sup35*Δ [*SUP35 LEU2*] strain was transformed by *URA3* plasmids bearing *sup35-M2*, *sup35-M3*, or *PNM2* mutations. Transformants were grown in SC-Leu-Ura liquid medium, which allowed selection for both plasmids. The culture was diluted at 12-h intervals with fresh medium to ensure exponential growth. Cell concentration was estimated by A_{600}. Equivalent amounts of cells were periodically plated on 5-FOA (5-fluoroorotic acid) solid medium to select for cells that lost plasmid with the *sup35*^{KK} allele. The fraction of [*PSI*$^+$] colonies was estimated by a color test after replica plating on MD 1/5 Ade medium. The kinetics of [*PSI*$^+$] elimination by GdnHCl treatment was estimated according to the previously published procedure (32). Each experiment was performed three times.

β-Gal Assay—The β-galactosidase reporter system was used for quantitative characterization of nonsense suppression (39). Vectors utilized in this assay were the generous gift of S. Peltz and W. Wang. The efficiency of suppression was calculated as the ratio of β-galactosidase activity in cells harboring *lacZ* with the premature termination codon to that in cells harboring *lacZ* (WT). Values for liquid β-galactosidase assay represent the mean of five independent assays. For statistical analysis the Mann-Whitney *U* test was performed (40).

Protein Analysis—Sup35p amounts in different strains were quantified using the Western blotting procedure with rabbit polyclonal anti-eRF3 antibodies (41). Monoclonal anti-tubulin antibodies (T6074, Sigma) were used for tubulin detection. Densitometry measurements were performed in ImageJ software. The Mann-Whitney *U* test (40) was performed for statistical analysis. SDD-PAGE with additional boiling (42) was performed to detect Sup35–3HAp and Sup35p in the aggregated and soluble fractions. For the analysis of Sup35p amyloid aggregates, semidenaturing detergent-agarose gel electrophoresis (SDD-AGE) (43, 44) was used.

RESULTS

Rationale Behind the Design of Mutations in the N-region of Sup35p—We used the T-REKS program for identification of tandem repeats in sequences (45) and detected eight repeats (R0–R7) between residues 28 and 118, instead of the previously identified six repeats spanning the region of amino acids 41–97 (8) (Fig. 1*A*). To select a set of mutations, we assumed that the Sup35p fibrils have the parallel and in-register stack of β-serpentines. Taking into consideration the repetitive sequence pattern and the fact that proline and charged side chains are preferentially located in the loops, we derived a β-serpentine spanning the entire N-domain with putative β-strands and bends (Fig. 1*B*). The interior of the fibril may accommodate both apolar residues and uncharged polar Asn and Gln engaged in H-bonded ladders, as well as Ser, Thr, and Cys, interacting with each other and/or with the polypeptide backbone (46, 47). In contrast, charged residues (Lys, Arg, Glu, or Asp) that are not involved in the salt bridges can destabilize a parallel in-register stacking of β-strands because of electrostatic repulsion of the juxtaposed charged residues of the same sign that are placed

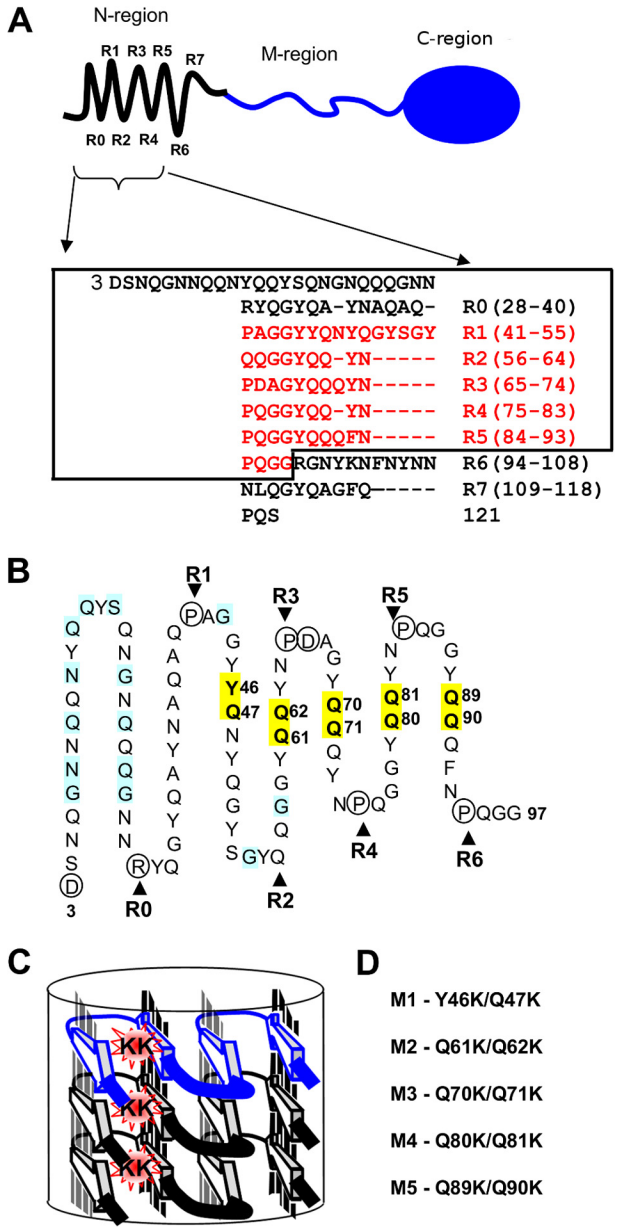

FIGURE 1. **Localization of mutated Sup35 residues regarding the structural model of the N-terminal domain.** *A, top*, schematic representation of Sup35p domain organization: the N-terminal region (1–123 amino acids) is shown with an array of eight repeats (R0–R7), discovered with the T-REKS program, the M-region (124–245 amino acids), C-terminal region (246–685 amino acids) has a globular-fold of the GTP-binding domain essential for robust translation termination. *Bottom*, new assignment of the Sup35p tandem repeats (T-REKS), which includes a previous one (8) colored in *red*. *B*, sequence of the N-terminal domain (the *rectangle* within the *panel A*) arranged in a serpentine with linear regions and bends, corresponding to putative β-strands and loop regions, respectively. The *circled* residues tend to localize to the loop regions (proline and charged residues). Positions where substitutions with charged residues lead to the PNM phenotype in previous reports (19, 36, 48) are highlighted in *blue*. Residues highlighted in *yellow* are mutated to lysines in this study. *C*, a model for the super-pleated β-structure that demonstrates how the introduction of charged residues in the β-strand region destabilizes the fibril by the electrostatic repulsion of same-sign charges along the fibril axis. *D*, *sup35* mutations studied in this work (numbering of the mutations corresponds to the repeat number shown above).

one over another along the fibril (Fig. 1*C*). This repulsion is particularly strong if such a charged axial ladder is located inside the fibril. Therefore, the substitution of uncharged resi-

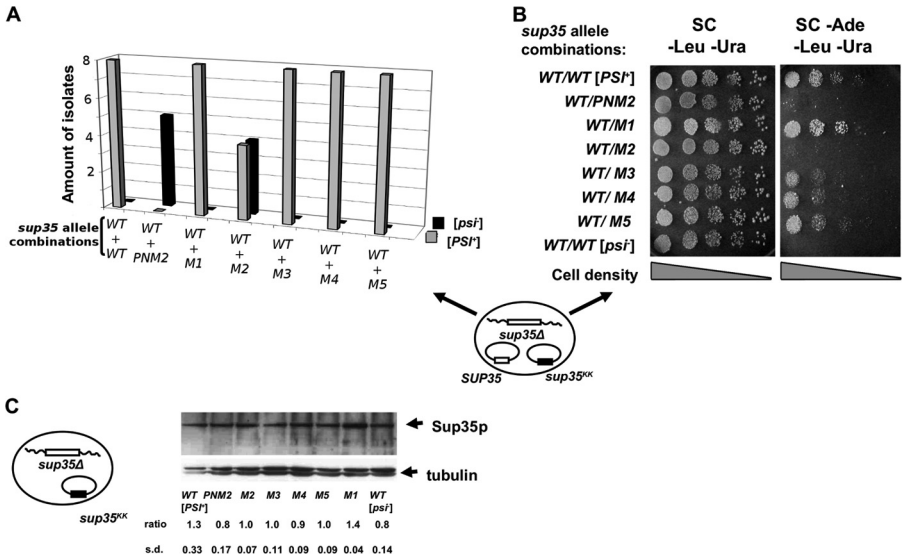

FIGURE 2. ***sup35-M2* and *PNM2* eliminate [*PSI*$^+$] in the presence of wild type *SUP35*.** *A,* transformants of yeast strain 10-7A-D832 (*sup35*Δ [*PSI*$^+$]) contain two plasmids: pYCH-U2 (*URA3*) with a wild type copy of *SUP35*, and pRSU1 (*LEU2*) with wild type or mutant alleles of *SUP35* (schematic representation of plasmid combination shown at bottom). Eight transformants were tested for each combination (except for *PNM2* where 5 transformants were tested). In the case of *sup35-M2* the [*PSI*$^+$]-dependent suppression of *ade1–14* was eliminated in 4 of 8 transformants. *B,* five serial dilutions of the indicated transformants were spotted onto SC medium selective for both plasmids with or without adenine, the tested *sup35* allele combinations are shown on the *left*. A typical Ade$^-$ transformant of *sup35-M2* is shown. Yeast strain 7A-D832 (*sup35*Δ [*psi*$^-$]) bearing two plasmids with a wild type copy of *SUP35* was used as a negative control. *C*, 10-7A-D832 (*sup35*Δ) derivatives bearing pRSU1-SUP35 or pRSU1-sup35^{KK} plasmids were analyzed by Western blotting with an anti-eRF3 antibody. The *captions below* the lanes indicate the *sup35^{KK}* allele. The "ratio" represents the relative abundance of Sup35p in each mutant compared with the amount of tubulin.

dues for charged ones in the region that forms the parallel and in-register β-structure could inhibit fibril formation and may affect the [*PSI*$^+$] phenotype. In the alternative β-solenoid model, the inserted charged residues will be further away from each other and the repulsive effect should be, in general, negligibly small. Importantly, the inhibitory effects of the other already tested mutations, such as deletions, insertions, or substitutions for proline residues (21, 22), are not specific to the parallel and in-register β-structures and can also be explained by the β-solenoid arrangements. Thus, the substitution of uncharged residues for charged residues appears to be the most suitable type of mutation for the establishment of boundaries of the parallel and in-register β-structures.

Previous reports have shown that many known *SUP35* mutants with the PNM ([*PSI*$^+$] no more) phenotype have neutral residues substituted for charged residues in the region upstream of the tandem repeats and within the first R1 and R2 repeats of Sup35p (Fig. 1*B*) (19, 36, 48). The parallel superpleated β-structure provides a straightforward explanation for this effect (Fig. 1*C*). Our goal was to test whether the effect of charged residues is also observed downstream of the R2 repeat. For this purpose, we constructed five double mutations with lysines substituted for two consecutive residues in the equivalent positions of Sup35p repeats R1–R5 (Fig. 1*D*). Two charged residues in each mutant were introduced to ensure the destabilization effect on the assumed parallel β-structure. Five mutant alleles (collectively termed the *sup35^{KK}* alleles) were constructed as follows, with the numbering (M1–M5) corresponding to the mutated repeat (R1–R5): *sup35-M1* (Y46K/Q47K), *sup35-M2* (Q61K/Q62K), *sup35-M3* (Q70K/Q71K), *sup35-M4* (Q80K/Q81K), and *sup35-M5* (Q89K/Q90K) (Fig. 1). A well characterized *PNM2* mutation (G58D) (36, 49, 50) was used as a control for prion destabilization.

The Effect of sup35^{KK} Alleles on [PSI$^+$] Prion in the Presence of Wild Type SUP35—First, we sought to determine the effect of *sup35^{KK}* alleles on the [*PSI*$^+$] prion in the presence of wild type *SUP35*. The 10-7A-D832 (*sup35*Δ [*PSI*$^+$]) strain that ectopically expresses wild type Sup35p (*URA3* plasmid) was transformed with *LEU2* plasmids containing either wild type *SUP35* or one of the five *sup35^{KK}* alleles. A *LEU2* plasmid expressing the *PNM2* allele was used as a control for prion destabilization. Leu$^+$,Ura$^+$ transformants were plated on media lacking adenine to test for suppression of *ade1–14* (UGA), which occurs if [*PSI*$^+$] is present. Eight transformants for each combination of plasmids were tested (except for *PNM2*, for which five transformants were studied) (Fig. 2*A*). Substitutions within the R2 repeat (*sup35-M2*) in half of the tested transformants, as well as the *PNM2* mutation (in all transformants tested) abolished growth on adenine-deficient medium, indicating elimination of [*PSI*$^+$] in these isolates (Fig. 2*B*). Thus, in some transformants the *sup35-M2* allele can cause an effective [*PSI*$^+$] loss in a dominant manner. In contrast, other *sup35^{KK}* alleles (*sup35-M1*, *sup35-M3*, *sup35-M4*, and *sup35-M5*) failed to eliminate the nonsense suppression phenotype in the presence of wild type *SUP35*.

To determine whether *sup35^{KK}* alleles themselves have a nonsense suppression phenotype, we transformed an isogenic [*psi*$^-$] strain (7A-D832) with plasmids bearing mutant alleles. Transformants failed to grow on media lacking adenine (data not shown). In addition, we measured steady-state levels of Sup35p in cells ectopically expressing the *sup35^{KK}* alleles. No significant variation in Sup35p levels in the [*psi*$^-$] strain was observed (Fig. 2*C*).

Effects of sup35^{KK} Alleles on the [PSI$^+$] Phenotype in the Homozygous State—To check [*PSI*$^+$] propagation in the presence of mutant alleles alone, we selected clones that lost the

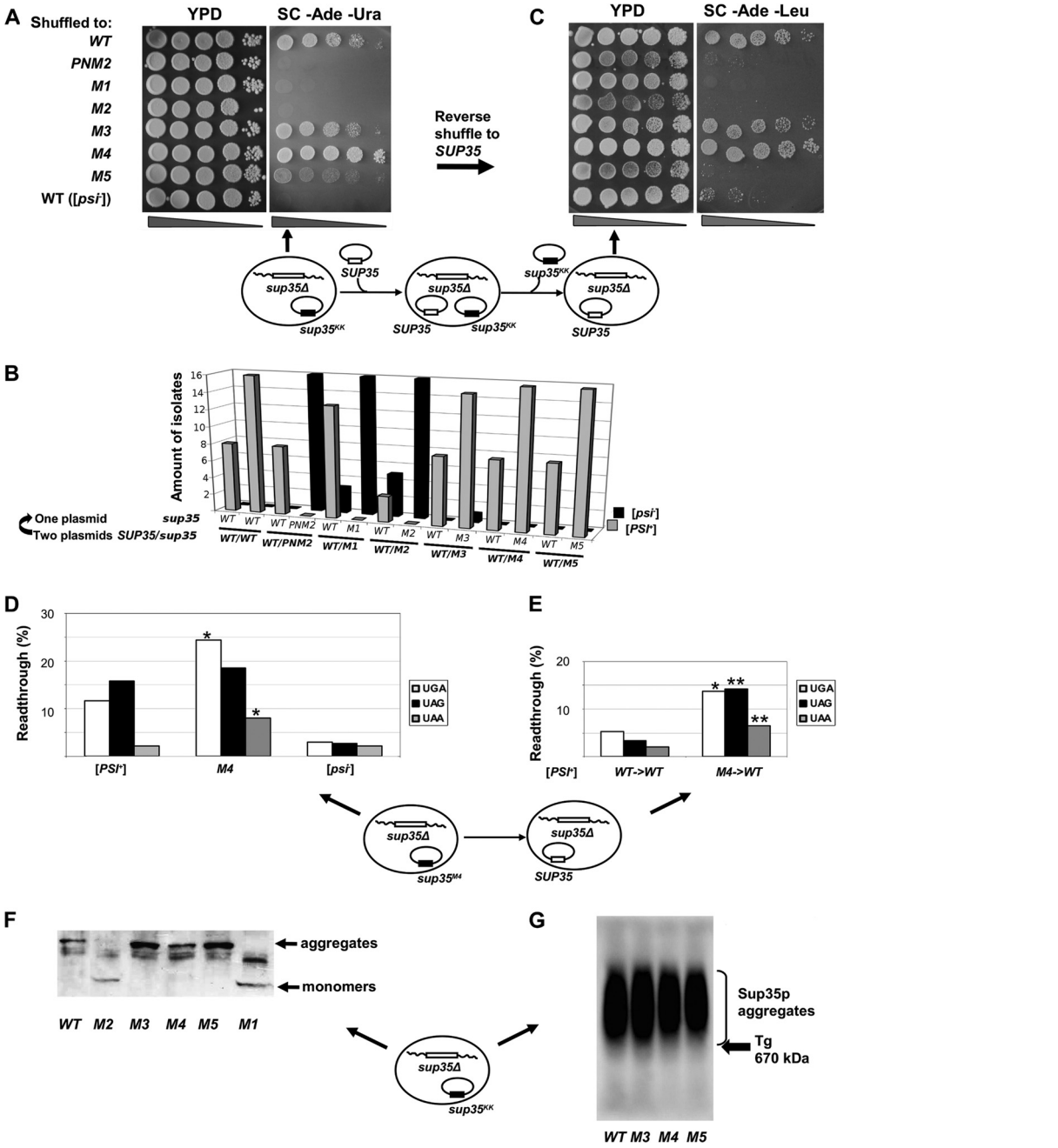

FIGURE 3. ***sup35^{KK}* alleles have the opposite effect on [*PSI*$^+$] phenotype in the absence of wild type *SUP35*.** *A*, five serial dilutions of 10-7A-D832 (*sup35*Δ) derivatives containing the pRSU1 plasmid with wild type or mutant alleles of *SUP35*. *B*, amount of [*PSI*$^+$] and [*psi*$^-$] isolates after the loss of one of the plasmids. *C*, isolates shown on *panel A* were retransformed with pRSU2 (*SUP35* (WT)) with subsequent loss of the pRSU1 plasmid, one representative clone from 16 tested in each case is shown. In both *panels A* and *C* one representative transformant was spotted onto YPD and SC-Ade. Derivatives of 7A-D832 (*sup35*Δ [*psi*$^-$]) containing the corresponding plasmids with a wild type copy of *SUP35* were used as a negative control in *panels A–C*. Schematic representation of the plasmid shuffling assay is shown under *panels A* and *C*. *Arrows* indicate where the corresponding phenotype is demonstrated. *D* and *E*, read through efficiency in [*PSI*$^+$] 10-7A-D832 derivatives bearing *sup35-M4* mutation after direct (*C*) and reverse (*D*) shuffle measured by *in vivo* β-galactosidase activity assay. The efficiency of suppression was calculated as a ratio of β-galactosidase activity in cells harboring *lacZ* with premature termination codon to *lacZ* (*WT*) control. The results are from at least three separate experiments. Significant differences from wild type [*PSI*$^+$] are shown by *one* or *two asterisks* ($p < 0.05$ and $p < 0.01$, respectively). *F*, SDS-PAGE with additional boiling of [*PSI*$^+$] aggregates in 10-7A-D832 (*sup35*Δ) derivatives containing the pRSU1 plasmid with wild type *SUP35* or *sup35^{KK}*. The *captions below* the lanes indicate the *sup35* allele. A schematic representation of the plasmid combination is shown near the panel. *G*, SDD-AGE analysis of Sup35p aggregates in transformants shown in *panel A*. The *arrow* marks the position of thyroglobulin (*Tg*, 670 kDa) in gel revealed by Coomassie staining.

LEU2 plasmid encoding wild type Sup35p. The nonsense suppression phenotype of the selected clones was tested on medium lacking adenine. We found that *PNM2*, *sup35-M1*, and *sup35-M2* have a strong inhibitory effect on [*PSI*$^+$] propagation. Each of these alleles was able to eliminate growth of the [*PSI*$^+$] strain on adenine-deficient medium upon loss of wild type (Fig. 3*A*) in all colonies tested (Fig. 3*B*). A strong effect of *sup35-M1* and *sup35-M2* mutations was also evident for clones that retained the plasmid with the wild type *SUP35* gene, as some of them also lost [*PSI*$^+$] (Fig. 3*B*). Loss of [*PSI*$^+$] was irreversible for *sup35-M1*, *sup35-M2*, and *PNM2* alleles, as the growth of mutant strains on adenine-deficient medium was not

restored after shuffling back the plasmid encoding wild type Sup35p (Fig. 3*C*).

Interestingly, the *sup35-M4* allele alone led to an increase in nonsense suppression efficiency compared with the original [*PSI*$^+$] strain. This was observed by the presence of white colonies in 1/4 YEPD medium (data not shown) and better growth on adenine-deficient medium compared with wild type *SUP35* (Fig. 3*A*). Read through efficiency for UGA and UAA stop codons in strains bearing the *sup35-M4* mutation was also significantly higher ($p < 0.05$) than that of the [*PSI*$^+$] strain with wild type *SUP35*, as measured with the β-galactosidase assay. No difference was observed for the UAG codon (Fig. 3*D*). This strong [*PSI*$^+$] phenotype was maintained after reverse shuffle (from *sup35-M4* to *SUP35*) that was confirmed by better growth on adenine-deficient medium as compared with wild type *SUP35* (Fig. 3*B*) and statistically significant read through efficiency for UGA and UAG stop codons (Fig. 3*E*). Suppression of the *ade1–14* mutation in the presence of *sup35-M3* was indistinguishable from the original [*PSI*$^+$] strain (Fig. 3*A*), and was not changed after reverse shuffle (Fig. 3*C*). We did not find any significant difference in read through efficiency between the native [*PSI*$^+$] strain and the [*PSI*$^+$] variants supported by the *sup35-M3* and *sup35-M5* alleles (data not shown). These data suggest that the [*PSI*$^+$] variant supported by the *sup35-M4* allele is stronger than the initial [*PSI*$^+$].

The *sup35-M5* allele alone decreases the nonsense suppression efficiency, as observed from weak growth on adenine-deficient medium (Fig. 3*A*). Replacement of *sup35-M5* by wild type *SUP35* led to failure of growth on adenine-deficient medium (Fig. 3*C*). Among 17 independent isolates of the [*PSI*$^+$]^{M5} (prion variants maintained by *sup35-M5*) strain tested after reverse shuffle, none retained the nonsense suppression phenotype, indicating the loss of [*PSI*$^+$] due to the inability of wild type *SUP35* to support the [*PSI*$^+$]^{M5} prion isolate (data not shown).

We next sought to characterize [*PSI*$^+$] aggregates in the presence of *sup35*^{KK} alleles using a modified version of SDS-PAGE with additional boiling (42) and SDD-AGE (43, 44). Using the SDD-AGE approach, differences in the size of the aggregates among prion strain variants can be detected as changes in electrophoretic mobility in agarose gels. Prion aggregates from a strong [*PSI*$^+$] strain migrate further into the gel than aggregates from a weak [*PSI*$^+$] strain (43). Using both techniques, we found that the presence of *sup35-M1* or *sup35-M2* in the homozygous state led to a complete absence of prion aggregates, with Sup35p present only in the soluble fraction (Fig. 3*F* and data not shown). This finding is consistent with the lack of nonsense suppression phenotype in these strains, and provides additional support for the conclusion that *sup35-M1* and *sup35-M2* in the homozygous state lead to the elimination of [*PSI*$^+$].

Mutant Sup35-M3, Sup35-M4, and Sup35-M5 proteins from corresponding *ade1–14* suppressing cells were found in aggregates (Fig. 3*G*). There was no significant difference in the sizes of the Sup35-M3p and wild type Sup35p aggregates (Fig. 3*G*), whereas both the Sup35-M4p and Sup35-M5p aggregates migrated more slowly through the gel than [*PSI*$^+$] aggregates from *SUP35WT* cells (Fig. 3*G*). In the case of *sup35-M5*, this observation is consistent with weak growth on the adenine-deficient medium (Fig. 3*A*), suggesting the presence of a "weak" [*PSI*$^+$] variant in this strain. However, in regard to *sup35-M4*, this is inconsistent with the strong suppressor phenotype of this mutation (Fig. 3, *A* and *D*).

In summary, the absence of wild type *SUP35*, *sup35-M1*, and *sup35-M2* mutations led to the elimination of both the nonsense suppression phenotype and Sup35p aggregates in yeast cells, indicating a complete cure of the [*PSI*$^+$] prion. We did not observe any significant influence of *sup35-M3* on [*PSI*$^+$]-mediated phenotypes, whereas *sup35-M4* increased the strength of nonsense suppression and formed larger aggregates. [*PSI*$^+$] prion isolates in the presence of *sup35-M5* were characterized by decreased strength of nonsense suppression of *ade1–14* and by aggregates of higher molecular weight that could not be supported by wild type *SUP35* upon loss of *sup35-M5*.

The Effects of sup35^{KK} *Alleles on Prion Transmission from [PSI*$^+$*]*^{WT} *to [PSI*$^+$*]*^{mut} *and [PSI*$^+$*]*^{WT} *Loss*—The inability of strains bearing *sup35-M1* and *sup35-M2* alleles in the homozygous state to grow on medium without adenine may reflect the inability of the mutant protein to adopt prion conformation. To study this phenomenon, we employed methods previously used in the characterization of the species barrier for transmission of [*PSI*$^+$] (37). We used the cells that ectopically express wild type *SUP35* and *sup35*^{KK} alleles from two independent vectors (Fig. 2*A*). We induced spontaneous loss of one of the two vectors by growing the cells in a medium selective for only one of the two plasmids. The nonsense suppressor phenotype observed upon loss of the wild type plasmid allowed us to estimate the transfer of the prion state from the wild type Sup35p to mutant protein. Concomitantly, loss of the plasmid with the *sup35*^{KK} allele and a subsequent assessment of the percentage of [*PSI*$^+$] cells allowed us to estimate the effect of the mutant allele on the stability of native [*PSI*$^+$].

The prion state was transmitted with 100% efficiency from the wild type to mutant proteins Sup35-M3, Sup35-M4, and Sup35-M5 (Fig. 4*A*). *PNM2* was characterized by a low capacity for prionization, because only 7.5% of the clones maintained the [*PSI*$^+$] phenotype. Transfer of the [*PSI*$^+$] state for the mutant proteins Sup35-M1 and Sup35-M2 was not observed, in accordance with the data shown in Fig. 3*A*.

The transient presence of *sup35-M2* caused destabilization of the native [*PSI*$^+$] (Fig. 4*B*), and the *PNM2* allele showed similar results, although to a lesser extent. The presence of *sup35-M1*, *sup35-M3*, *sup35-M4*, and *sup35-M5* alleles did not affect the stability of the original [*PSI*$^+$].

To test the influence of *sup35-M1* and *sup35-M2* alleles on *de novo* [*PSI*$^+$] formation we overproduced wild type *SUP35* and its mutant variants in [*psi*$^-$] background (Fig. 4*C*). The *de novo* appearance of [*PSI*$^+$] was detected in all transformants except for transformants with a control empty vector, indicating the potential ability of the *sup35-M1* and *sup35-M2* alleles to form prions.

To determine whether the mutant Sup35-M1 and Sup35-M2 proteins are found in aggregates in the presence of wild type Sup35p, we used SDS-PAGE with additional boiling (42). For these experiments we use the derivative of the 10-7A-D832 [*PSI*$^+$] strain bearing *SUP35-HA* on a centromeric vector (37).

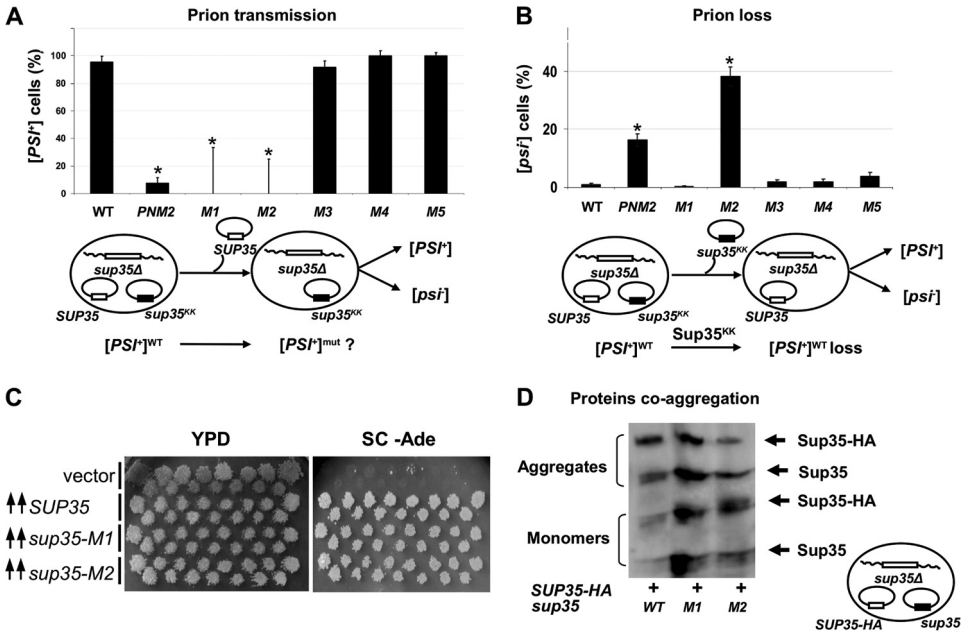

FIGURE 4. **Influence of *sup35^{KK}* alleles on [*PSI*$^+$] transmission, loss, induction, and protein coaggregation.** *A*, [*PSI*$^+$] transmission from the wild type to the indicated *sup35* allele. A fraction of cells that retained prions after loss of the wild type allele is shown on the graph. *B*, [*PSI*$^+$] loss induced by transient expression of the *sup35^{KK}* alleles. The fraction of cells that have lost prions after loss of the *sup35^{KK}* allele is shown. Significant differences from control variant (WT) in *A* and *B* are shown by *asterisks* ($p < 0.05$). *C*, transient overexpression of *sup35-M1* or *sup35-M2* leads to [*PSI*$^+$] induction. The appearance of prions was detected by growth on the adenine-omitted medium. Transformants of the 7A-D832 strain (*SUP35* WT [*psi*$^-$]) were incubated on -Ura/Gal medium and replica plated onto glucose -Ade medium. Plates were photographed after 5 days of incubation. *D*, cell lysates of 10-7A-D832 derivatives containing wild type Sup35p-HA together with mutant Sup35p were separated using SDS-PAGE with additional boiling. Blotted proteins were probed with anti-eRF3 antibody, which revealed Sup35p-HA and Sup35p migrating in monomeric and aggregate fractions. Schematic representations of experiments and the plasmid combinations are shown *below* each panel.

This construction encodes wild type hemagglutinin-tagged Sup35p (Sup35WT-HAp), which allowed us to discriminate it from the mutant Sup35p via an increase in molecular weight. The strain was transformed with plasmids coding for Sup35-M1p or Sup35-M2p. Transformants were selected and analyzed for coaggregation of Sup35^{KK}p with wild type Sup35p. We observed coaggregation of both Sup35-M1p and Sup35-M2p with Sup35WT-HAp, whereas the amount of monomers in both alleles was higher compared with the control variant (*SUP35* in the homozygous state) (Fig. 4*D*).

Prion Loss in the Presence of sup35-M2 but Not sup35-M1 Depends on Generation Number—To obtain further insight into the molecular mechanisms of [*PSI*$^+$] loss in the presence of *sup35-M1* and *sup35-M2* alleles, we determined the kinetics of loss of [*PSI*$^+$] during cell division. GdnHCl treatment of the *SUP35* WT [*PSI*$^+$] strain was used for comparison. The [*PSI*$^+$] *sup35*Δ strain ectopically expressing wild type Sup35p was transformed with plasmids bearing either *sup35-M1* or *sup35-M2* alleles. Transformants were grown in liquid medium selective for both plasmids, and cultures were maintained in the logarithmic growth phase by regular dilution with fresh medium. Equivalent amounts of cells were regularly plated onto solid medium with 5-FOA to select for cells that lost the *URA3* plasmid carrying the *sup35^{KK}* mutant allele. The obtained colonies were screened for [*PSI*$^+$] by replica plating on MD 1/5 Ade medium. White, pink, and sectored colonies were scored as [*PSI*$^+$], whereas red colonies were scored as [*psi*$^-$]. This experiment allowed us to study the effect of the transient expression of selected *sup35^{KK}* alleles on [*PSI*$^+$] stability.

The *sup35-M1* allele did not lead to an increase in red colonies upon loss of mutant allele expression compared with wild type *SUP35* (Fig. 5*A*). This result is in agreement with our data showing that this allele does not lead to loss of [*PSI*$^+$] in the heterozygous condition (Fig. 2). In control experiments, [*PSI*$^+$] cells containing plasmids with wild type *SUP35* were white, and the isogenic [*psi*$^-$] cells remained red (data not shown).

Transient expression of *sup35-M2* led to destabilization of the [*PSI*$^+$], as evidenced by the scoring of 70% of cells as [*psi*$^-$] after 20 generations (Fig. 5*A*). However, after this observation and until the 50th generation, we did not detect significant prion loss. One of the possible explanations for this phenomenon is that *sup35-M2* may convert the original strong [*PSI*$^+$] strain into a weaker version, characterized by reduced mitotic stability. To test this hypothesis, we maintained plasmids encoding wild type and mutant *SUP35* in [*PSI*$^+$] *sup35*Δ cells for more than 50 generations. Cells from seven independent isolates obtained on 5-FOA to eliminate the *URA3* plasmid bearing the *sup35-M2* allele did not display any red [*psi*$^-$] colonies when subcloned on MD 1/5 Ade and did not differ in growth in Ade$^-$ medium from the wild type strain (Fig. 5*B*, *lower panel*). SDD-AGE analysis of Sup35p aggregates in these seven isolates revealed variation in their sizes (Fig. 5*B*, *top panel*). Thus, the *sup35-M2* allele led to destabilization of the [*PSI*$^+$], but did not cause complete elimination of [*PSI*$^+$].

DISCUSSION

We constructed five mutant alleles of the *SUP35* gene, each carrying a pair of positively charged lysine residues in equiva-

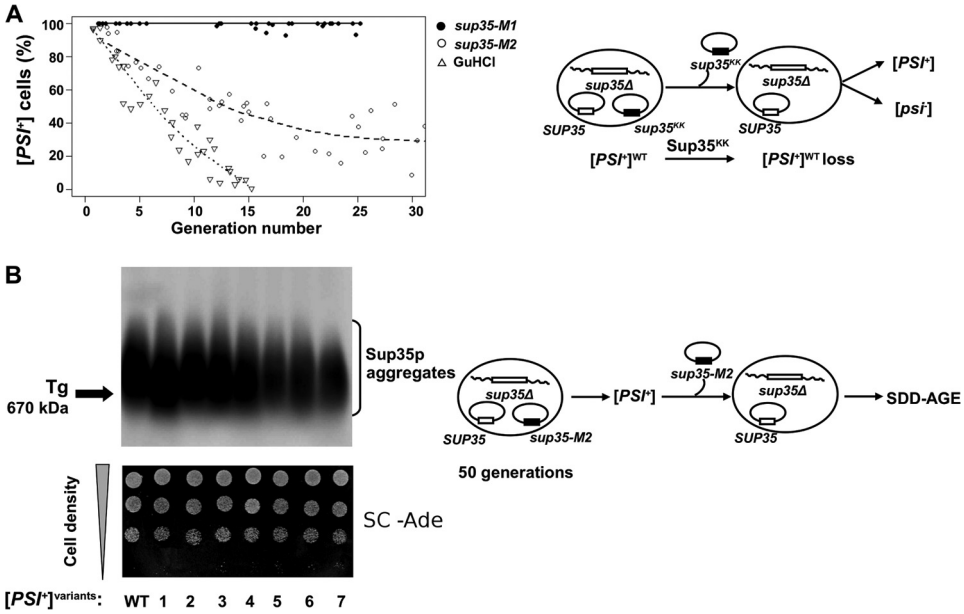

FIGURE 5. **Kinetics of [*PSI*$^+$] loss in the presence of *sup35-M1* and *sup35-M2* alleles.** *A,* transformants of 10-7A-D832 bearing two plasmids (with *SUP35WT* and *sup35*^{KK}) were grown in liquid medium selective for both plasmids. At regular time points, culture aliquots were taken and plated on 5-FOA solid medium (see "Experimental Procedures"). 5-FOA resistant colonies were tested for the presence of [*PSI*$^+$] by replica plating onto MD 1/5 Ade medium. The percentages of [*PSI*$^+$] cells have been plotted as a function of generation number passed from the beginning of the experiment, which was estimated from measurements of A_{600}. [*PSI*$^+$] elimination in the presence of 4 mM GdnHCl was used as control. *B,* SDD-AGE analysis of [*PSI*$^+$] aggregates in independent Ade$^+$ isolates that have lost *sup35-M2* after more than 50 generations of growth as in *panel A* (*top panel*). *WT,* wild type strain 10-7A-D832. The *arrow* marks the position of thyroglobulin (*Tg*, 670 kDa) in the gel as revealed by Coomassie staining. Ade$^+$ phenotype of the same isolates (*lower panel*). Ten serial dilutions of one representative clone were spotted onto SC medium without adenine. Schematic representation of the plasmid shuffling assay is shown to the *right* of each panel.

lent positions in the five repeats, to uniformly cover the entire repeat-containing N-domain up to the C-terminal end of the putative prion-forming region (Fig. 1). We demonstrated that the *sup35-M1* and *sup35-M*2 mutant alleles (amino acid positions 46–47 and 61–62) led to prion loss in direct shuffling experiments (Fig. 3*A*). The other alleles were able to maintain the [*PSI*$^+$] prion with 100% efficiency (Fig. 4*A*). Introduction of the charged residues into the parallel in-register superpleated structure would destabilize the prion fibrils, whereas the charged residues placed outside of the prion-forming region would have no effect. This suggests that the critical part of the parallel in-register β-structure for the studied [*PSI*$^+$] prion variant lies within the first 63–69 residues.

It is also noteworthy that both proteins Sup35-M1 and Sup35-M2 can induce prions when overproduced (Fig. 4*C*). This resembles the *PNM2* (G58D) allele, which can also induce the *de novo* appearance of [*PSI*$^+$] upon overexpression (50). Thus, the observed effects of corresponding alleles on [*PSI*$^+$] propagation is not associated with a global influence on prion folding but rather interferes with a specific prion variant. As the dominant-negative effect of the *sup35-M2* allele is similar to that of *PNM2* (Figs. 2, *A* and *B*, and 3, *A–C*), this property could be explained by the increased sensitivity of Sup35-M2p and Sup35p heteroaggregates to Hsp104p as shown for the *PNM2* allele coexpressed with wild type *SUP35* (51) (see below).

At the same time, our study reveals a more complex interplay between the Sup35p wild type and mutant proteins. Mutant proteins Sup35-M1 and Sup35-M2 both coaggregate with Sup35p but lead to a transmission barrier in accordance with a previous observation where it was shown that the ability to coaggregate does not exclude the existence of a cross-species barrier (52). Prion transmission from wild type *SUP35* to both *sup35-M1* and *sup35-M2* was not observed (Fig. 4*A*). We hypothesize that the mechanisms leading to this outcome are different for the two *sup35*^{KK} alleles. In the case of *sup35-M1*, in addition to the inability to transmit prions from wild type to *sup35-M1* allele, the transient expression of Sup35-M1p does not induce prion loss (Fig. 4*B*). Finally, Sup35-M1p was able to coaggregate with Sup35p (Fig. 4*D*). To explain these findings we hypothesized that the failure of prion transmission in the case of *sup35-M1* is caused by the inability of the mutant protein to form homoaggregates due to electrostatic repulsion in the parallel and in-register β-structure (Fig. 6). This hypothesis is in accordance with data that residues 47 and 48 lie within one of the three consensus sequences of amyloid stretches (53). The *sup35-M1* can maintain [*PSI*$^+$] in the presence of the wild type *SUP35* allele because Sup35-M1p molecules are required to be interspersed with Sup35p along the axis of the prion fibril so that positively charged lysine residues of Sup35-M1p are placed further away from each other. This arrangement does not significantly change prion conformation, as the loss of *sup35-M1* expression does not induce a loss of [*PSI*$^+$], suggesting that the conformation of the heteropolymer fibril is still compatible with maintenance by wild type Sup35p only.

The *sup35-M2* mutant allele also could not maintain [*PSI*$^+$] in the absence of wild type *SUP35*. However, in contrast to *sup35-M1*, it has a dominant-negative effect and eliminated [*PSI*$^+$] even in the presence of wild type *SUP35* in a large proportion of cells (Fig. 2). We show that prion elimination by Sup35-M2p depends on cell divisions (Fig. 5*A*). It is worth mentioning that prion elimination in the *SUP35*/*sup35-M2* heterozygote was not complete, and ~30% of cells maintained

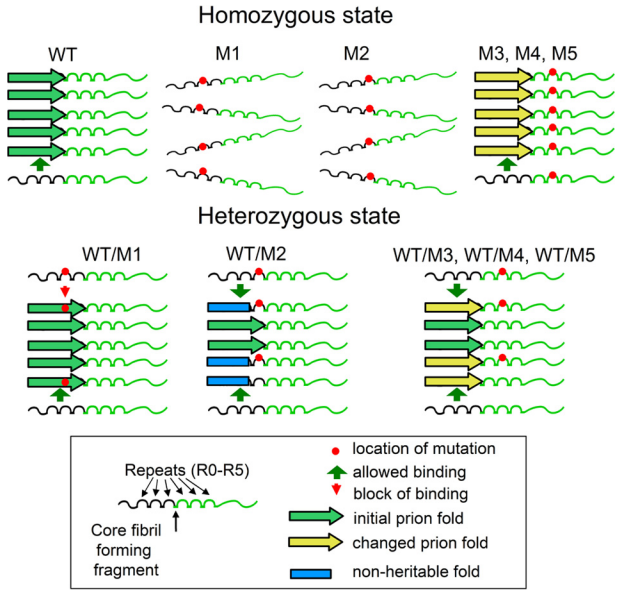

FIGURE 6. **The model describing interactions of mutant Sup35p with prion filaments.** Mutations *sup35-M1* and *sup35-M2* are located within the core region of the parallel and in-register superpleated β-structure. In the homozygous state, both Sup35-M1p and Sup35-M2p do not form fibrils. In the heterozygous state, Sup35-M1p and Sup35-M2p can coaggregate with Sup35p. Sup35-M1p does not affect the prion, whereas Sup35-M2p leads to conformational changes, formation of non-heritable fibril-folds, and hence loss of [*PSI*$^+$] in the majority of cells. We suggest that in the case of Sup35-M1p, homoaggregates are not permitted. Thus in the growing fibril Sup35p and Sup35 are perfectly interspersed, one following the other along the axis of the prion filament. We speculate that regions mutated in Sup35-M3p, Sup35-M4p, and Sup35-M5p lie outside of the superpleated β-structure of Sup35p in the conformation of the prion strain utilized in this study. Hence these alleles cannot eliminate [*PSI*$^+$] under the conditions tested.

prion. [*PSI*$^+$] maintained in these cells was stable in mitosis. Our data show evidence of heteroaggregation of Sup35-M2p and Sup35p (Fig. 4*D*). We speculate that only part of the prion-forming region of Sup35-M2p can bind aggregates of wild type protein and change β-structure arrangement (Fig. 6). The next protein molecule (mutant or wild-type) that joins the end of the fibril replicates the change made by Sup35-M2p in the β-structure. It is plausible that some aggregates (~70%) acquired a non-heritable fold that led to prion loss. Evidence for such a conformational change comes from the observation of aggregates of varying size in cells that retain [*PSI*$^+$] after the presence of *sup35-M2* (Fig. 5*B*). Similar reasoning has been previously used to explain the mechanism of the Sup35p prion interspecies barrier (37). A minor fraction of the fibrils could preserve the prion-fold, thus maintaining the ability to persist through generations of cells.

Alternatively, prion loss could be explained by the terminal blocking of fibril growth, called "growth poisoning," by mutant Sup35-M2p (37, 52, 53). Such a scenario implies a significant increase in the monomeric fraction of Sup35p; however, we observed only a mild increase in the soluble fraction when *SUP35* and *sup35-M2* were co-expressed in cells that managed to maintain [*PSI*$^+$] (Fig. 4*D*). Another potential mechanism for prion loss could be due to Sup35-M2p and Sup35p heteroaggregates being more susceptible to Hsp104p disaggregase activity. This explanation was proposed for *PNM2* mutation (51). Indeed, an optimal level of this chaperone activity is required to maintain [*PSI*$^+$] (12, 54). If Hsp104p fails to act on heteroaggregates, the kinetics of prion loss should match those obtained under GdnHCl treatment (55), eventually leading to complete prion-free cultures. In the case of the *SUP35*/*sup35-M2* heterozygote, 30% of cells contained prion stably maintained during cell divisions (Fig. 5*A*), thus rendering the hypothesis of reduced Hsp104p activity less plausible. Although we cannot rule out any of these scenarios, we favor our initial hypothesis, according to which Sup35-M2p induces a conformational switch in heteroaggregates (Fig. 6). Such a switch may lead, in most cases, to destabilization of prion structure and prion loss; however, some conformations can permit weak [*PSI*$^+$] characterized by long aggregates that are stably inherited from one generation to the other.

The mutations *sup35-M4* and *sup35-M5* in the N-region downstream of *sup35-M1* and *sup35-M2* have some effect, albeit to a lesser extent. Alleles *sup35-M4* and *sup35-M5* increased and decreased the nonsense suppressor phenotype of [*PSI*$^+$], respectively (Fig. 3*A*). Sup35p aggregates increased in size in the presence of *sup35-M4* and *sup35-M5* alleles (Fig. 3*G*). Mutant alleles also differed in the ability for reverse transmission of prion to the wild type allele. Indeed, the prion determinant was lost upon reverse shuffle of [*PSI*$^+$] cells from *sup35-M5* back to wild type (Fig. 3*C*). An analogous phenomenon was mentioned in the literature as asymmetry of cross-species prion transmission (52, 53). Hence, we conclude that the [*PSI*$^+$] strain maintained with *sup35-M5* differs substantially from the [*PSI*$^+$] strain maintained with the wild type *SUP35* allele. In our opinion, the observed asymmetry of the "cross-strain" barrier stems from the inability of the wild type Sup35p to efficiently adopt prion conformation of the Sup35-M5p fibril. Allele *sup35-M4* leads to irreversible alteration of prion properties (Fig. 4, *D* and *E*). Analogous results were obtained previously during investigation of the inter-species barrier and it is noteworthy that the region responsible for changes in [*PSI*$^+$] properties includes position of the *sup35-M4* mutation (53).

We suggest that although *sup35-M4* and *sup35-M5* mutations are located outside of the core fibril-forming region and can maintain [*PSI*$^+$], they may still cause rearrangement of the fibril structure. It is worth mentioning that the superpleated β-structure is more permissive to such polymorphisms compared with the other types of protein structures. This fold allows different locations and numbers of β-strands and turns within the same sequence. Structural variations of this kind offer a plausible explanation for the phenomenon of prion variants (22, 56). A similar hypothesis of the effect of amino acid substitutions in the PrP protein on prion fibril conformation was previously proposed by Chen and colleagues (57) in their *in silico* study. We propose that *sup35-M4* and *sup35-M5* lead to the formation of new prion variants. Analogous phenomena were previously described, for example, when the *SUP35* WT allele was combined with one from another species or a mutant one (53, 58) and for the PrP prion when it was transmitted between species (23). Based on an analytical model of prion strain variations (59), we speculate that [*PSI*$^+$]^{M5} has a decreased division rate. This assumption is in agreement with the increase in size of the weaker nonsense-suppression pheno-

type Sup35p aggregates. [PSI^+]^{M4} (prion variants maintained by *sup35-M4*) possesses a stronger nonsense-suppression phenotype compared with [PSI^+] in the 10-7A-D832 strain, although the prion aggregates are slightly increased in size. These data can be explained by the increased fibril growth rate.

Taken together, our results suggest that residues 46–47 (*sup35-M1*) and 61–62 (*sup35-M2*) are located within the parallel and in-register superpleated β-structure for the [PSI^+] variant studied in this work. These mutants do not form homoaggregates and lead to prion loss (Fig. 6). However, they influence propagation of the Sup35p aggregates in a different manner. The *sup35-M1* mutant does not affect the prion in the presence of *SUP35*, but *sup35-M2* leads to prion loss in a dominant manner and the formation of non-heritable aggregates. To explain these data, we suggest that in the fibrils, Sup35p can bind to Sup35-M1p in the same conformation, whereas Sup35-M2p allowed the Sup35p conformation that leads to non-heritable fibrils (Fig. 6). The smaller effect of the mutations on *sup35-M4* and *sup35-M5* can be explained by their location within the parallel in-register β-structure but outside of its critical core region. These mutations have some influence on protein structure and can lead to the formation of new prion variants.

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