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Study of the mechanisms of influence of nonsense mutations in the *SUP35* gene on the [*PSI*$^{+}$] prion properties in the yeast *Saccharomyces cerevisiae*

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Introduction

Rationale of the study. Genetic control of protein synthesis in yeast *S. cerevisiae* has a long history of study. In the laboratory of physiological genetics of St. Petersburg State University, mutational analysis of key factors necessary for the final stage of this process — translation termination — was carried out. Disruption of the eRF1 and eRF3 factors in yeast can manifest itself as suppression, so the study of suppressor mutations in the *SUP45* and *SUP35* genes provides additional information about the processes occurring during translation termination. The question of the role of prions in the eukaryotic cell also remains relevant. The study is devoted to the compatibility of mutations in the vital *SUP35* gene and the [*PSI*$^+$] prion.

Extend of prior research on the topic. The Sup45 and Sup35 proteins function as translation termination factors eRF1 and eRF3 in *S. cerevisiae*, respectively, and work in complex. When one of them is deficient, or in the presence of mutations in the *SUP45* and *SUP35* genes, there is a decrease in the accuracy of translation termination, which is characterized by the appearance of a nonsense-suppressor phenotype in yeast cells. Cells containing the [*PSI*$^+$] prion have a similar phenotype, which suggested its role in the processes of translation termination regulation. Earlier in the work of D.A. Kiktev, synthetic lethality of the “strong” variant of the [*PSI*$^+$] prion and mutations in the *SUP45* gene was demonstrated. The cause of lethality was a simultaneous decrease in Sup35 protein in cells due to prionization and functional Sup45 protein due to *sup45* nonsense mutations. In this work, several *sup35* nonsense mutations were also investigated and it was shown that despite phenotypic similarity to *sup45*, the tested *sup35* mutations in the presence of the wild-type *SUP35* gene retain cell viability with “strong” and “weak” prion [*PSI*$^+$] variants.

Thus, the **aim of this work** was to study the effect of *sup35* nonsense mutations on the viability of *S. cerevisiae* cells with a strong prion variant [*PSI*$^+$].
To achieve this aim, it was necessary to complete the following **objectives**:

1. To test the viability of haploid and diploid yeast strains containing the strong [*PSI*$^+$] prion variant in combination with the *sup35* nonsense mutations;
2. Assess the effect of *sup35* nonsense mutations on the properties of the [*PSI*$^+$] prion.

Scientific novelty. In this study, we demonstrated that nonsense mutations in the *SUP35* gene can contribute to the appearance of a more stable and functionally active Sup35 protein and thereby increase the viability of *S. cerevisiae* cells. To analyze the compatibility of *sup35* nonsense mutations and the [*PSI*$^+$] prion in cells in more detail, we substituted plasmids carrying the wild-type *SUP35* gene for plasmids with mutant *sup35* alleles. For the first time, it was found that the compatibility of *sup35* nonsense mutations and the [*PSI*$^+$] prion in the absence of the wild-type *SUP35* gene depended on whether the mutant allele was present in the cells initially. The fulfillment of this condition depended on the method of combining mutations and prion — transformation of haploid or diploid [*psi*$^-$] cells carrying the *SUP35* gene on the plasmid with plasmids containing *sup35* or mating of [*psi*$^-$] and [*PSI*$^+$] strains carrying *sup35* and *SUP35* alleles, respectively. In addition, the work shows that the studied nonsense mutations in the *SUP35* gene can lead to changes in the [*PSI*$^+$] prion variant or to its loss.

Theoretical and practical significance. The results of the dissertation deepen the understanding of the mechanisms of the cell translational apparatus and its regulation and can be used in the courses " Particular genetics of yeast ", "Genetic control of translation" and "Prions" taught at the department of genetics and biotechnology, faculty of biology of St. Petersburg State University, as well as similar courses at other educational institutions.

Methods and materials of the research. The techniques traditionally used in yeast genetics, methods of molecular biology and microscopy were applied in the work. Baker's yeast *Saccharomyces cerevisiae* was used as a model organism.

Validity and approbation of the results. The main results of the dissertation were presented and discussed at **6** international conferences:

1. VI Congress of the Vavilov Society of Genetics and Breeders (VSGiB) and associated genetic symposia (Rostov-on-Don, Russia, 2014).
2. Protein misfolding in disease — Toxic aggregation-prone proteins in aging and age-related diseases: from structure to pathology and spreading (Roscoff, France, 2016).
3. The 43rd FEBS congress (Prague, Czech Republic, 2018).
4. VI Congress of Biochemists of Russia. The IX Russian symposium "Proteins and peptides" (Sochi, Russia, 2019).
5. International Congress "VII Congress of the Vavilov Society of Genetics and Breeders, dedicated to the 100th anniversary of the department of genetics, SPBU, and associated symposia" (St. Petersburg, Russia, 2019).
6. EMBO Workshop Protein quality control. From mechanisms to disease (Mallorca, Spain, 2019).

List of publications. The main results on the topic of the dissertation are presented in the following articles published in peer-reviewed publications included in the List of the Higher Attestation Commission (VAK) under the Ministry of education and science of the Russian Federation and equivalent foreign publications:

1. **Trubitsina, N. P.** From past to future: Suppressor mutations in yeast genes encoding translation termination factors / N. P. Trubitsina, O. M. Zemlyanko, S. E. Moskalenko, G. A. Zhouravleva // Biol Commun. — 2019. — Vol. 64(2). — P. 89-109.
2. **Trubitsina, N. P.** Nonsense mutations in the yeast *SUP35* gene affect the [*PSI*$^+$] prion propagation / N. P. Trubitsina, O. M. Zemlyanko, S. A. Bondarev, G. A. Zhouravleva // Int J Mol Sci. — 2020. — Vol. 21(5). — P. 1648.
3. Zhouravleva G. A. How big is the yeast prion universe? / G. A. Zhouravleva, S. A. Bondarev, **N. P. Trubitsina** // Int J Mol Sci. — 2023. — Vol. 24(14). — P. 11651.

Main scientific results. The dissertation presents the main scientific results of the research in the form of the publication of three scientific papers co-authored by the applicant.

1. In the review article “From past to future: Suppressor mutations in yeast genes encoding translation termination factors” [216], published in Biol. Commun. (Scopus), co-authored with O.M. Zemlyanko, S.E. Moskalenko, and G.A. Zhuravleva, summarized the literature data, as well as the results obtained in the laboratory of physiological genetics of St. Petersburg State University on suppressor mutations in the *SUP45* and *SUP35* genes.
2. The review article “How big is the yeast prion universe?” [245], published in Int. J. Mol. Sci. (Scopus, Web of Science Core Collection) co-authored with S.A. Bondarev, G.A. Zhuravleva provides a detailed review of all known yeast prions and considered various approaches used for their identification. The prospects for the discovery of new yeast prions are discussed.
3. One of the fundamental publications of the researcher is the article “Nonsense mutations in the yeast *SUP35* gene affect the [*PSI*$^+$] prion propagation” [216] published in co-authorship with O.M. Zemlyanko, S.A. Bondarev, and G.A. Zhuravleva in Int. J. Mol. Sci. (Scopus, Web of Science Core Collection). The main results of the research covered in the dissertation were published in this scientific work.

In all three scientific works, the applicant made a personal contribution in the form of developing a research design, collecting experimental material, statistical processing of the data obtained, preparing tables and figures, and writing the text.

The main thesis for defence.

1. When a transcript containing a premature stop codon in the *SUP35* gene is translated, nonsense suppression resulting from a reduction in Sup35 protein content can result in the substitution of an erroneous amino acid in place of the stop codon, resulting in the synthesis of a full-length Sup35 protein that is superior to the wild-type protein.

2. The viability of *S. cerevisiae* cells when they combine nonsense mutations in the *SUP35* gene and the [*PSI*$^+$] prion depends on whether they have previously undergone adaptation in the presence of only sup35 mutant alleles.
3. Nonsense mutations in the *SUP35* gene can lead to changes in the [*PSI*$^+$] prion properties or its loss due to the formation of truncated Sup35 proteins, which are included in pre-existing prion aggregates, as well as due to a decrease in the amount of full-length Sup35 protein in the cell.

Structure and the volume of the dissertation. The dissertation consists of an introduction, **4** chapters, conclusions and a list of references. The full volume of the dissertation is **108** pages with **25** figures and **5** tables. The bibliography contains **245** titles.

Chapter 1. Literature review. Translation termination and factors modifying its effectiveness in yeast *Saccharomyces cerevisiae*

1.1 General information about the translation process in eukaryotes

Translation or synthesis of a polypeptide chain on an mRNA template is a matrix process that in eukaryotes includes four stages: initiation, elongation, termination, and recycling. During initiation, the ribosomal complex is assembled, which consists of the large and small ribosomal subunits, mRNA, initiator methionyl-tRNA, and translation initiation factors (eIFs) (Figure 1). Three sites are identified in the collected 80S ribosome: A (aminoacyl) — binds incoming aminoacyl-tRNA (aa-tRNA), P (peptidyl — peptidyl transferase or catalytic center of the ribosome, accepts peptidyl-tRNA during elongation and E (exit) — site for deacylated tRNA. At the end of translation initiation, the anticodon of the initiator methionyl-tRNA occupying the P-site of the ribosome is located at the start codon, AUG. During translation elongation, the A-site of the ribosome receives aa-tRNAs. If the aa-tRNA anticodon and mRNA codon coincide, the ribosome, with the participation of elongation factors (eEFs), catalyzes the attachment of an amino acid residue (a.a.r.) to the growing polypeptide and shifts to the next mRNA codon, freeing the A site for the next aa-tRNA. At the end of the open reading frame (ORF), a stop codon enters the ribosome, and the synthesized polypeptide is separated from peptidyl-tRNA with the participation of translation termination factors (ERFs). At the final stage, the ribosome is recycled. A key participant in recycling — a protein factor from the ABCE1 family (ATP-binding cassette subfamily E member 1), promotes the dissociation of ribosomal subunits from mRNA and the release of deacylated tRNA (see review [191]).

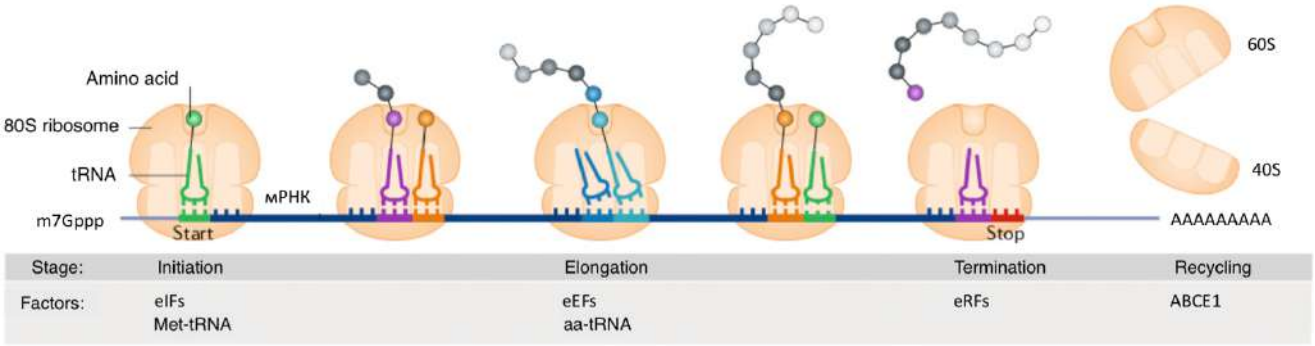

Figure 1. Schematic diagram of the translation process in eukaryotes. The diagram shows the translation stages: initiation, elongation, termination, and recycling. At the bottom of the diagram, the factors involved in these stages are indicated (modified from [191]).

Later in the review, we will discuss in more detail the main proteins involved in translation termination in eukaryotes and the factors influencing its efficiency.

1.2 Termination of translation in eukaryotes

Translation termination in eukaryotes requires the protein factor eRF1, which recognizes all three stop codons: UAA, UAG, and UGA [78]. The eRF1 factor binds to the ribosome in a complex with the translational GTPase eRF3 (Figure 2) [76, 203, 242]. Interaction of eRF1 and eRF3 is possible only if eRF3 is associated with GTP [121]. In the GTP-bound form, it holds eRF1 in an inactive conformation, which prevents interaction of the M-domain of eRF1 with the catalytic center of the ribosome [91, 195]. After eRF1 recognizes the stop codon, eRF3 hydrolyzes GTP and leaves the ribosome, releasing the M-domain of eRF1 for placement in the catalytic center of the ribosome [91]. Activation of the GTPase activity of eRF3 requires complex coupling with poly(A)-binding proteins (PABPs) located at the 3'end of the mRNA [220] (Figure 2). The GGQ motif of eRF1 stimulates hydrolysis of the peptidyl-tRNA ester bond in the catalytic center of the ribosome, thereby releasing the resulting polypeptide from the ribosome [79]. It should be noted that the eRF1 factor is capable of performing translation termination with low efficiency even in the absence of eRF3, as it was demonstrated [6, 71].

Delivery of eRF1 to the ribosome is accomplished by the Dbp5/DDX19 helicase. If its function is impaired, the eRF1 factor interacts prematurely with eRF3, resulting in

impaired translation termination. Then near-cognate tRNAs enter the ribosome's A-site and recognize the stop codon as significant [13, 86, 156].

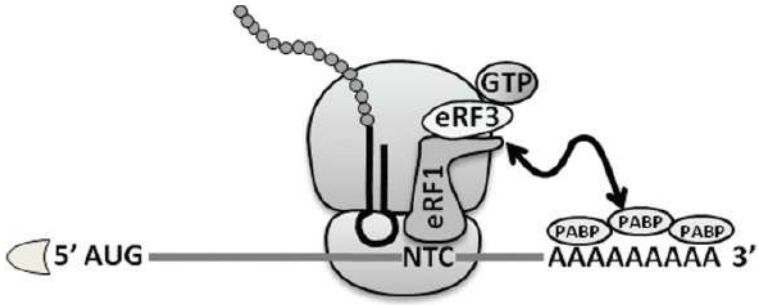

Figure 2. Scheme of translation termination in eukaryotes. NTC — normal termination codon; PABP — poly(A)-binding protein; eRF1 and eRF3 — translation termination factors (modified from [48]).

After translation termination, eRF1 remains bound to the ribosome and is released during recycling by Rli1 ATPase from the ABCE1 family [111, 177, 197]. For a clearer understanding of the process of translation termination in eukaryotes, it is necessary to have an idea of its main participants — eRF1 and eRF3, as well as factors affecting the efficiency of this process.

1.2.1 Translation termination factor eRF1

The amino acid sequence of eRF1 is highly conserved [78], although some organisms have several eRF1 paralogs, which may differ in the specificity of recognition of different stop codons [8, 34, 116, 117, 139]. In the yeast *S. cerevisiae*, the Sup45 protein is encoded by the vital *SUP45* gene [25]. Sup45 consists of three domains: N, M, and C (Figure 3), which perform various functions necessary for the translation termination process. Data from X-ray diffraction analysis of human and *Schizosaccharomyces pombe* eRF1 factors showed that eRF1 resembles a tRNA molecule in its three-dimensional structure [35, 200]. Structural data confirmed the universality of the molecular mimicry hypothesis proposed for prokaryotic translation termination factors [100].

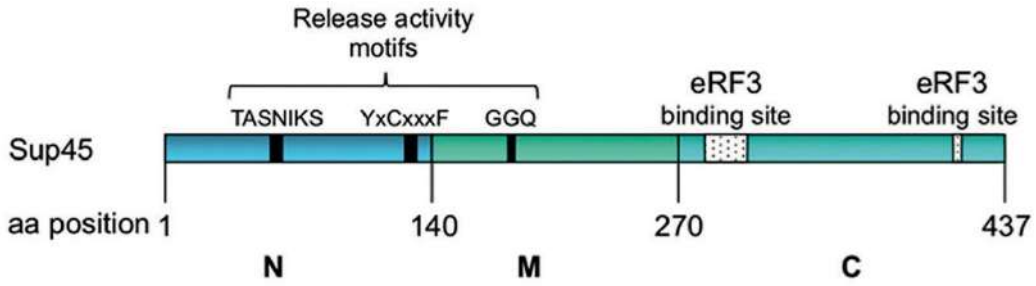

Figure 3. Sup45 protein scheme. N, M, C — Sup45 domains. The numbers below show their boundaries in the protein's amino acid sequence. The motifs necessary for Sup45 functioning during translation termination and eRF3 binding sites are noted.

The structure similar to tRNA allows the eRF1 factor to effectively compete for binding to the ribosome A-site and perform its functions. Sequences located within the N-domain are responsible for recognizing stop codons: the structural NIKS motif (Asn-Ile-Lys-Ser) and several other conservative elements, including the GTS (Gly–Thr–Ser) and YxCxxxF motifs [35, 71, 77, 183, 183, 201]. Using cryo-electron microscopy and catalytically inactive eRF1, it was shown that glutamic acid at position 55 and tyrosine at position 125 of eRF1 contribute to the recognition of purines and pyrimidines at the second and third codon positions [26]. Thus, they play a crucial role in the decoding of stop codons by the eRF1 factor. Hydrolysis of peptidyl-tRNA is performed by the GGQ motif (Gly–Gly–Gln) in the central M-domaine RF1, which, after decoding the stop codon, is located in the peptidyl transferase center of the ribosome [79, 192]. Like tRNA [187], eRF1 works in tandem with a GTPase, in this case the eRF3 protein [79]. The C-terminal region of eRF1 containing the amino acid sequence GFGGIG (G/A)XLRY is responsible for binding the eRF1 factor to eRF3 [69, 155].

1.2.2 Translation termination factor eRF3

The eRF3 factors of most eukaryotes, with the exception of some protozoa, have a three-domain structure (see review [98]). In *S. cerevisiae*, the eRF3 factor is encoded by the *SUP35* gene [203, 242], which is vital [115, 128, 237]. The Sup35 protein of *S. cerevisiae* is similar in its spatial organization and function in translation termination to eukaryotic and prokaryotic elongation factors eEF1A and EFTu respectively [124]. The need for the presence of eRF3 during translation termination is due to its GTPase activity,

by which eRF1 is stimulated. In addition, it has been shown that eRF3 can be involved in dissociation of eRF1 from the ribosome after the release of the synthesized polypeptide, which is especially critical when the concentration of eRF1 in the cell is reduced [70].

The ORC of the *SUP35* gene consists of 2055 base pairs (bp) and encodes the Sup35 protein with a length of 685 amino acid residues (a.a.r.). The amino acid sequence contains three methionine residues at positions 1, 124, and 254 (ATG codons), which can be divided into three domains — N, M and C (Figure 4) [128]. Using DNA hybridization of the *SUP35* gene with total RNA isolated from yeast cells, two transcripts were identified: the main transcript (2.3 kilobase (kb)) corresponding to the entire *SUP35* gene, and the minor transcript (1.4 kb), which corresponds to the 3'-terminal site of the gene encoding the C-domain of the Sup35 protein [206].

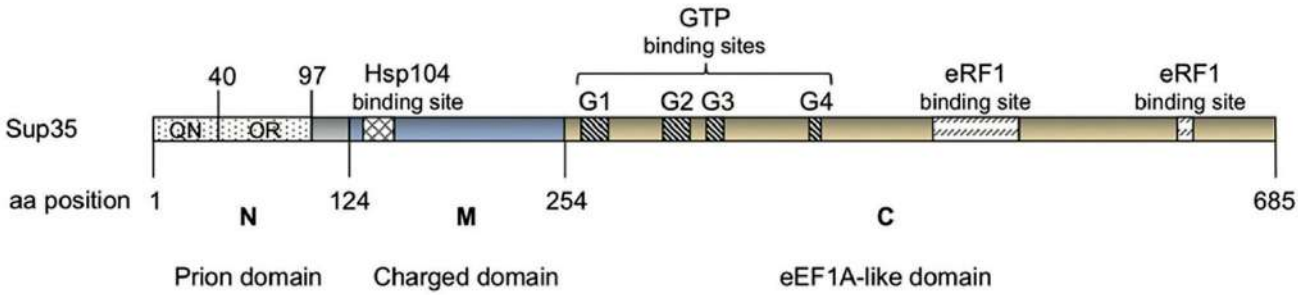

Figure 4. Sup35 protein scheme. N, M, C — Sup35 domains. The numbers below show their boundaries in the protein's amino acid sequence. Subdomains in the N-domain were identified: QN — a region rich in glutamine and asparagine, OR — a region of oligopeptide repeats. The binding sites of the Hsp104 chaperone, sites necessary for the binding of GTP and factor eRF1, are also noted.

Deletion analysis revealed that it is the C-domain that is essential for maintaining *S. cerevisiae* cell viability. In addition, it is proved that it is necessary and sufficient for efficient translation termination [213]. The Sup35 C-domain contains GTP binding sites [94, 127] and regions involved in interaction with the eRF1 factor [65, 101, 155, 174].

It has been shown that the N-domain of yeast Sup35 is not essential for cell life [213]. It is responsible for the possibility of Sup35 prionization with the formation of the [*PSI*$^{+}$] determinant [53, 173, 212]. It is known that the N-domain in the Sup35 NM region specifically interacts with the poly(A)-binding protein (Pab1/PABP) [42] and the

RNA-binding protein Pub1 (its human homologue Tia1), which is involved in the formation of stress granules [222].

Two subdomains can be conventionally distinguished in the N-domain: a Q- and N-rich region located within a.a.r. 1-40, and an oligopeptide repeat region (a.a.r. 41-97) (OR), which contains five complete PQGGYQ(Q)QYN repeats of nine amino acids and one incomplete repeat containing four PQGG amino acids [128]. The N-domain of eRF3 family factors is highly variable, with its amino acid sequence differing even among closely related species. In a number of fungal species close to *S. cerevisiae*, the N-terminus of the eRF3 protein has a structure similar to that of Saccharomyces: it is enriched with glutamine, asparagine, tyrosine, and glycine residues, and also contains ORs of different composition. At the same time, Q/N-rich regions and OR regions show the lowest degree of variability compared to other regions of the N-domain (see review [243]). Using chimeric constructs containing the C-domain of Sup35 of *S. cerevisiae* and N-domain of orthologous proteins, the prionisation of Sup35 of *Pichia methanolica*, *Candida albicans*, *Candida maltosa*, *Kluyveromyces lactis*, *Debaryomyces hansenii*, *Yarrowia lipolytica* and *Zygosaccharomyces rouxii* in *S. cerevisiae* cells was shown [92, 129, 161, 186].

The sequence of the M-domain is enriched in charged a.a.r. It serves as a linker between the N- and C-domains of the Sup35 protein and has a binding site with the Hsp104 chaperone (a.a.r. 129-148), which is necessary for maintaining the [*PSI*$^+$] prion [92].

In addition, data supporting the involvement of the Sup35 protein in phase separation in the cell in response to stress have recently been published. Acidic a.a.r. in the M-domain has been shown to be important for the formation of temporary condensates by the Sup35 protein in response to starvation and pH changes, because replacing these acidic a.a.r. with polar a.a.r. reduces the ability of Sup35 to respond to stress. It is assumed that the M-domain serves as a sensor, and N-domain apparently enhances the ability of Sup35 to enter condensates [74]. Some analogy can be drawn between protein condensates containing non-prion aggregates of the Sup35 protein and

stress granules, as well as P-bodies, since despite their different organization, they are temporary non-membrane formations that arise in the cell in response to stress.

1.2.2.1 Proteins that Sup35 interacts with

Sup35 interacts not only with Sup45, but also with many other proteins involved in controlling the efficiency of translation termination and other various cellular metabolic processes, such as transport of mRNA from the nucleus to the cytoplasm (Dbp5/DDX19 and Gle1), ribosome recycling (Rli1/ABCE1) (see above), mRNA degradation (Upf proteins) and translation initiation (Pab1/PABP) [42, 95].

The Upf1, Upf2, and Upf3 are key factors controlling NMD [46, 137, 138]. NMD is a process that destroys mRNA containing premature termination codons (PTC). Such codons may appear as a result of nonsense mutations, inaccurate or inefficient pre-mRNA splicing, or incorrect RNA editing. Upf1 is the main effector of NMD, and the factors Upf2 and Upf3 regulate its function [33]. They interact with each other, as well as with the ribosome, other NMD proteins, and translation termination factors. Upf1 binds to eRF1 and eRF3 and is involved in the process of translation termination and ribosome release at PTC [193]. Factors eRF1 and eRF3 bind to Upf1 and inhibit its ATPase activity [47]. Upf2 and Upf3 proteins interact only with eRF3 [229]. They compete with Upf1 for binding to eRF3 [29]. NMD factors, like eRF1, interact with the C-domain of eRF3. Upf1 binds to its first half (a.a.r. 254-465), while Upf2 and Upf3 interact with the second half of eRF3 (a.a.r. 465-685) ([229], see review [244]).

PABP proteins bind to the poly(A)-“tail” of mRNA and are one of the main participants in the regulation of the mRNA life cycle. According to their localization in the cell, they are divided into nuclear and cytoplasmic. Yeast has only one gene that encodes cytoplasmic PABP (Pab1 of *S. cerevisiae*), while several genes have been identified in mammalian cells [148]. Yeast eRF3 proteins directly interact with the L- and C-domains of Pab1 via NM domains [42, 181]. It is assumed that the eRF3-PABP association promotes efficient translation termination [42], binds it to various mRNA degradation pathways [80] and protects mRNA from NMD [110]. The association between PABP and eRF3 has been shown to maintain basal levels of nonsense repression

and negatively regulate translation termination [181]. Overproduction of the Pab1 protein increases the efficiency of translation termination in yeast [42], and deletion of the *PABPC1* gene in human cells leads to the reading of PTC [102]. It is noted that Pab1 can affect the interaction of eRF1 with eRF3, the GTPase activity of eRF3, and the effect of the Pab1-eRF3 complex on post-termination processes, such as recycling, is not excluded [12, 181].

In addition to protein synthesis, the involvement of eRF1 and eRF3 factors in various cellular processes is indicated by the presence of pleiotropic effects of mutations in the *SUP45* and *SUP35* genes, such as sensitivity to low and elevated temperature, increased osmotic pressure, and complete or partial inability to respiration (see review [98]). In addition, such mutants are characterized by hypersensitivity to benomyl, an agent that depolymerizes microtubules, suggesting that translation factors are involved in the control of microtubule-mediated cellular processes, such as chromosome segregation during cell division [21, 214].

One of the possible functions of the Sup35 protein is its interaction with the actin apparatus of the cell. The C-terminal domain of Sup35 protein has a structural similarity with the elongation factor eEFIA [68, 124, 128, 160]. Since eFFIA is involved in the organization of the cytoskeleton [68], the Sup35 protein may also be involved in this process. In addition, when the *SUP35* gene is repressed, cells show increased size and disruption of the actin cytoskeleton [224]. There is also evidence of the interaction of Sup35 with such actin cytoskeleton proteins as Sla1 [10] and Mlc1 [223].

1.3 Translation termination efficiency

The process of protein translation termination is not performed with 100% accuracy. There are natural mechanisms that can affect its effectiveness through specific regulation. One such mechanism is the reading of terminating codons as sense or nonsense suppression (Figure 5). In this case, the ribosome reads the stop codon as sense and continues protein synthesis until the next stop codon in the same reading frame. At the same time, nonsense suppression can be not only a decoding error, but also a means of producing various protein isoforms. Three types of stop codon decoding can be

distinguished: unprogrammed, programmed, and induced (see review [170]). Reading of any physiological stop codon or PTC can occur at the basal level in the absence of any additional effectors. Unprogrammed nonsense suppression is possible if mRNA with PTC is not eliminated by NMD [133]. This type of read is a rare event: just over 80% of nonsense mutations have a read level of 0.1% or less [72, 73, 179].

Programmed reading of stop codons as sense targets specific mRNAs [62, 75, 146] and represents a mechanism for expanding the proteome to allow the synthesis of specific protein isoforms. About 5% of yeast genes undergo programmed nonsense suppression [120].

Induced decoding of stop codons is mediated by specific molecules. When the ribosome reaches a stop codon, the presence of such molecules favours the recruitment of closely related tRNA instead of translation termination factors. In early studies, aminoglycosides such as paromomycin were shown to facilitate this type of readthrough process in bacteria and yeast [199]. Such molecules could potentially be used to treat pathologies associated with nonsense mutations.

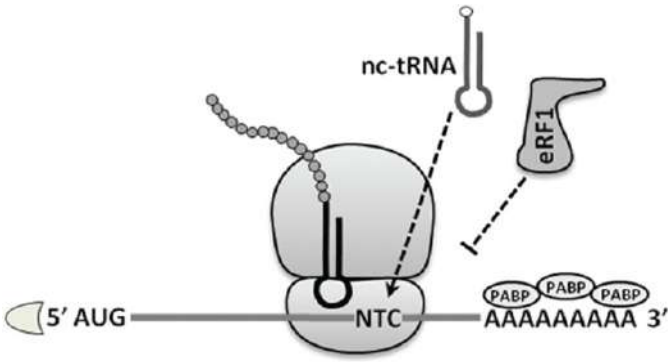

Figure 5. The principle of stop codon decoding (nonsense suppression). The "reading" of the stop codon occurs according to the principle of ambiguous matching. The suppressor tRNA, successfully competing with the eRF1 factor, interacts with the stop codon in violation of the canonical rules of nucleotide pairing. SC — stop codon; PABP — poly(A)-binding protein; eRF1 and eRF3 — translation termination factors (modified from [48]).

Various factors can affect the efficiency of reading a stop codon as significant in all three cases, including the type of stop codon and the surrounding nucleotide sequence [15, 96].

Despite the fact that eRF1, unlike prokaryotic factors RF1/RF2, recognises all three stop codons, the completion of protein synthesis at these codons occurs with different

efficiency. Experiments on mammalian cells have shown that the UGA codon has the greatest potential to be read by the suppressor tRNA as a significant one, while suppression of the UAG codon is less frequent, and UAA is the most reliable stop codon in terms of translation termination [15, 96, 145, 149]. However, these properties can be altered by the nucleotide context of the stop codon [19, 28, 153]. The nucleotide immediately following the stop codon has the strongest influence (+4, provided that the first nucleotide of the stop codon is labelled as +1) [153, 175, 200, 210]. It appears that a purine at this position, present in approximately 90% of the most highly expressed human genes, promotes more efficient translation termination [211], whereas pyrimidine promotes nonsense repression [27, 153, 210]. This has led to the theory that a four-nucleotide sequence is the signal for termination [27, 211]. Cryo-electron microscopy of the 80S ribosomal complex with eRF1 showed that during translation termination, nucleotides +4 and +5 dock with rRNA base pairs G626 and C1698, respectively, thus stabilizing the complex during stop codon recognition [26, 150, 195]. It has also been shown that some nucleotides downstream of the stop codon can contribute to nonsense suppression. For example, positions +6 to +9 are essential for the regulation of the terminal complex [45].

The nucleotide composition on the 5’-side from the stop codon also has an effect on translation termination efficiency, but appears to be less significant compared to the 3’-context [136]. Experiments in yeast have shown that the presence of adenine at positions -1 and -2 relative to the stop codon, stimulates nonsense repression of UAG and also probably the other stop codons. This is consistent with the observation that adenines on the 5'-side of the stop codon are evolutionarily conserved in genes regulated by nonsense repression [19, 215].

Proteins, RNAs, small molecules and the cell environment also affect the frequency of stop codon decoding (for review see [170]). For example, disruption of Dbp5/DDX19 protein function decreases the efficiency of translation termination (see above) [13, 86, 156].

Disruption of the function of eRF1 and eRF3 factors can also manifest as a nonsense-suppression effect. Complete inactivation (disruption) of *SUP45* or *SUP35*

genes of *S. cerevisiae* has a lethal effect [25, 93, 115, 128, 237]. Nevertheless, a number of mutations in these genes have been obtained, and their study has a long history. The foundation was established by studies in our laboratory, where recessive mutations *sup45* and *sup35*, initially named as s1 and s2, respectively, were obtained for the first time [2]. The study of the nature of *sup45* and *sup35* mutations showed that a significant part of them are missense mutations, which are located in the functional domains of the factors eRF1 and eRF3 ([4, 23, 159], see review [216]).

Unravelling the mechanisms of translation termination regulation is directly related to the study of suppressor mutations in *SUP45* or *SUP35* genes. The effects resulting from partial inactivation of the *SUP35* gene or prionization of the *SUP35* protein will be discussed in detail below.

1.3.1 Nonsense and missense mutations in the *SUP35* gene

Previously, it was assumed that nonsense mutants in the *SUP45* and *SUP35* genes are not viable in the absence of additional nonsense suppressors ([204], see review [216]). Nevertheless, our laboratory first obtained 5 nonsense mutations in the *SUP45* (*sup45-n*) gene, and then 11 nonsense mutations in the *SUP35* (*sup35-n*) gene (their selection was carried out simultaneously with the *sup45-m* and *sup35-m* missense mutations), which result in a PTC in the reading frame of the coding sequence [30, 159] (Figure 6).

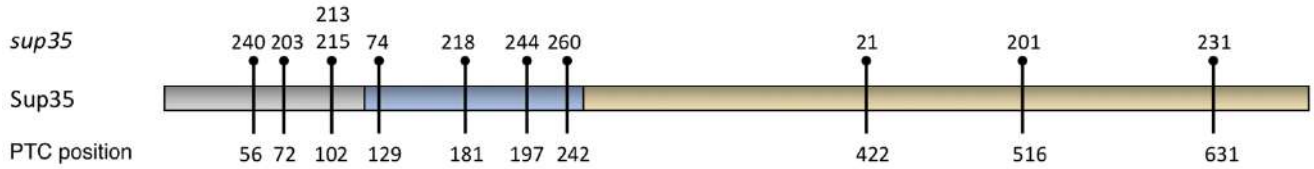

Figure 6. Nonsense-mutations in the *SUP35* gene obtained in our laboratory. The figure shows a schematic of the Sup35 protein. The numbers at the top indicate *sup35* mutations. PTC corresponds to the length of the shortened Sup35-n proteins resulting from translation termination at the corresponding nonsense mutation (modified from [30]).

Cells with nonsense mutations in the *SUP45* and *SUP35* genes are characterized by the presence of shortened proteins (N-terminal fragments) Sup45-n and Sup35-n, respectively. In the absence of the wild-type gene, they retain their viability by

suppressing nonsense mutations, resulting in the synthesis of the full-size Sup45 or Sup35 protein. This process is subject to self-regulation, since the Sup45 and Sup35 proteins are translation termination factors. Cells with the *sup45-n* and *sup35-n* mutations contain a reduced amount of the full-length Sup45 and Sup35 protein, respectively, compared to the wild type, for example, the *sup35-244* mutation leads to a decrease in the Sup35 level to 0.5 % [30]. The minimum amount of eRF3 or eRF1 sufficient to maintain cell viability is not known for certain. It was shown that decreasing the level of eRF3-C or eRF1 to 10 % reduced cell viability by a factor of 10-fold [224]. Thus, despite the fact that deletion of the *SUP45* and *SUP35* genes is lethal for the cell, a small amount of eRF1 and eRF3 proteins are sufficient to maintain viability.

Most of the resulting nonsense mutations are located in a “weak” nucleotide context, that is, a context that makes it easier to read the stop codon. Seven *sup35-n* nonsense mutations are localized in the first third of the *SUP35* gene encoding the eRF3 NM-domain (a.a.r.1-253). This uneven distribution of nonsense mutations can be explained by the increased content of codons, where a single substitution can lead to the appearance of a stop codon. Indeed, the *SUP35* gene region encoding the NM-domain contains 48 % of these codons, compared to 33 % in the region encoding the C-domain of eRF3 and 34 % in the entire yeast genome. There were also no *sup35-n* mutations leading to the appearance of the UGA stop codon, which can be explained by the reduced content of “potential” UGA codons (codons whose single mutations can lead to the appearance of the UGA codon). Indeed, the *SUP35* gene sequence contains 10 % of such codons, compared to 39 % and 51 % for potential UAG and UAA codons, respectively. These data correspond to the number of isolated TAG (4 mutations) and TAA (5 mutations) mutations [1]. It should be noted that in the screening of Ade+ revertants [23], 12 *sup35-n* mutations were isolated, of which six were TAG mutations, and the rest were evenly distributed between TAA and TGA mutations. The variations in the ratios of *sup35-n* mutation types can be explained by the differences in the methods of mutant selection.

Seven missense mutations in the *SUP35* gene were also characterized in our laboratory (see review [216]). They were selected by simultaneous reversion of *ade1-14*

(TGA) and *his7-1* (TAA) mutations. All missense mutations isolated in different strains are localized in the C-domain of eRF3 (a.a.r. 254-685), supporting the numerous data on the role of this domain in translation termination. The suppressor phenotype of isolated missense mutations cannot be explained by a reduced amount of eRF3, since it does not change, or by the inability of mutant eRF3 to interact with eRF1. The latter was proved by co-immunoprecipitation [30]. It is possible that these mutations affect the interaction of eRF3 with other proteins or the ribosome.

All missense mutations obtained in our laboratory result in amino acid substitutions conserved among eRF3 of *S. cerevisiae* and *S. pombe* (except for the *sup35-233* mutation). This allowed us to locate the corresponding amino acid substitutions in the tertiary structure of the C-terminal domain of eRF3 of *S. pombe* and clarify its functional significance [124, 216].

It should be noted that about half of the *sup35* mutations described in the literature are localized either in or near the *SUP35* sites encoding the GTPase domains of eRF3. Interestingly, the phenotypic expression of mutations located in the C-terminal domain of eRF3 may depend on the presence of the N-terminal domain of eRF3 [226]. Possibly, the interaction of this site with the mutant C-terminal domain reduces the GTPase activity of eRF3, which leads to an increase in suppression efficiency [1].

The nature of the suppression was investigated in details in two mutants in the GTPase domain of yeast eRF3 [183]. Initially, the authors obtained six mutations using site-specific mutagenesis. Only two of them, which resulted in substitutions of H348Q and R419G in the Sup35 protein, were able to maintain the viability of yeast cells, although their presence caused a decrease in the growth rate. Factors eRF3-H348Q and eRF3-R419G had a reduced efficiency of GTP hydrolysis, but led to a significant impairment of translation termination only at the UGA stop codon (the level of nonsense suppression increased 3.7-16.8-fold, depending on the nucleotide context). This was not the case for the UAG or UAA codons, but in the case of the tetranucleotides UAGC and UAAC, the level of suppression increased 3.2-4.4-fold compared with wild-type eRF3. These results indicated that GTPase activity of eRF3 is required to link recognition of eRF1 translation termination signals to efficient polypeptide chain release [183].

1.3.2 The [*PSI*$^+$] prion

Significant progress in the study of translation in eukaryotes has been achieved by the discovery of a hereditary determinant of protein nature [*PSI*$^+$], which is a prionized factor eRF3 (see review [234]). The phenomenon of prionization raises a lot of questions due to the ambiguous nature of prions. To date, it is not known for certain what function they perform in the vital activity of different organisms. A number of prion diseases, primarily neurodegenerative diseases, have been described in humans and animals (see review [41]). At the same time, in lower eukaryotes, yeast, prions can potentially be functional, for example, to protect the cell from stressful environmental conditions (see review [245]). Despite all the differences, prions of different organisms are united by their structural similarity and the ability to spread by copying the protein matrix. Much progress in understanding the nature of human prion and amyloid diseases has been made by studying the prions of the yeast *S. cerevisiae*. Saccharomycetes have the largest number of such proteins — more than 10. The study of prions is important not only from the point of view of finding approaches for the treatment of prion diseases, but also for solving the fundamental problem of determining the mechanisms of regulation of protein synthesis. The prion [*PSI*$^+$] can act as a regulatory element that ensures the ambiguity of translation termination, which has an adaptive significance in the process of evolution. Phenotypic manifestation of the prion [*PSI*$^+$] is related to its structural features, which depend on its variant (prion strain), as well as on the genotypic background of the yeast cell and the environment.

1.3.2.1 Occurrence of the [*PSI*$^+$] prion

The [*PSI*$^+$] prion, along with the factor [*URE3*], is one of the most well-known and studied yeast prions. The first mention of it was in 1965 in the work of Brian Cox [43], and in 1994 Reed Wickner suggested its prion nature [232]. Aggregates of the [*PSI*$^+$] factor consist of the prionized Sup35 protein, and the N-terminal domain is necessary and sufficient for its maintenance. Deletion of the N-domain leads to prion elimination [212]. The Sup35 region critical for prion maintenance includes the first 64

a.a.r. of this protein. This fragment includes a region rich in a.a.r. Q/N and the first OR [143, 169]. The Q/N site (a.a.r. 1-40) has been shown to be mainly responsible for the transition of the soluble form of Sup35 to the prion form [50], and the OR region (a.a.r. 41-97) plays an important role in the stable maintenance of the [*PSI*$^+$] prion in the cell [169]. Deletion of the region from 22 to 69 a.a.r. in Sup35 interferes with both the induction of the [*PSI*$^+$] prion and its interaction with chaperones [20]. Deletion of the M-domain reduces the stability of the [*PSI*$^+$] prion during cell divisions [144].

The main phenotypic manifestation of the [*PSI*$^+$] factor is a decrease in translation accuracy (see review [44]), similar to the mutations in the *SUP35* — the structural gene of the [*PSI*$^+$]. Spontaneous transition of [*psi*$^-$] to [*PSI*$^+$] is a rare phenomenon that occurs with a frequency of approximately 5.8×10^{-7} [61]. Various stressful environmental conditions are known to increase prion production in yeast. Spontaneous generation of the [*PSI*$^+$] factor increases after prolonged incubation of yeast cells at low temperature [39], heat stress [158], osmotic or oxidative stress [60, 84] and under endoplasmic reticulum stress (impaired proteostasis) [89, 166, 219]. A necessary condition for the appearance of the [*PSI*$^+$] prion in the cell is the presence of the [*PIN*$^+$] factor (from [*PSI*$^+$] inducibility) [54]. However, [*PIN*$^+$] is not required to maintain [*PSI*$^+$] [56]. [*PIN*$^+$] satisfies all the criteria of yeast prions and is characterized by the transition of Rnq1 to the aggregated state [54]. The biological function of this phenomenon is still unknown. There are two points of view on how the prion [*PIN*$^+$] stimulates *de novo* appearance of the [*PSI*$^+$] prion [56]. According to the first one, the soluble form of Rnq1 inhibits *de novo* formation of [*PSI*$^+$] factor. When Rnq1 transitions to an insoluble state with the occurrence of [*PIN*$^+$] factor, the effect of inhibition is mitigated. The second theory, which is the most widely accepted to date, states that Rnq1 aggregates serve as a matrix for forming the prion form of Sup35, which has been experimentally confirmed [53].

1.3.2.2 Structure of the [*PSI*$^+$] aggregates

Yeast [*PSI*$^+$] cells accumulate prionized Sup35 in the form of high-molecular aggregates [173]. Sup35 amyloid aggregates have a fibrillar morphology, where each

fibril consists of several protofibrils twisted together [97]. Protein molecules in protofibrils are arranged in stacks that form a so-called cross-β structure, in which the β-chains are oriented perpendicular to the protofibril axis and are located at a certain distance from each other (Figure 7). The distance between the β-chains in the β-sheet is 4.7-4.8 Å; each protofibril consists of at least two β-sheets located at a distance of ~10 Å. These repeating elements, located at fixed distances from each other (4.7-4.8 and 10 Å, respectively), form a regular structure that provides a characteristic pattern of electron or X-ray diffraction with two meridional and two equatorial reflections that correspond to the gaps between β-chains and β-sheets ([85], see review [216]).

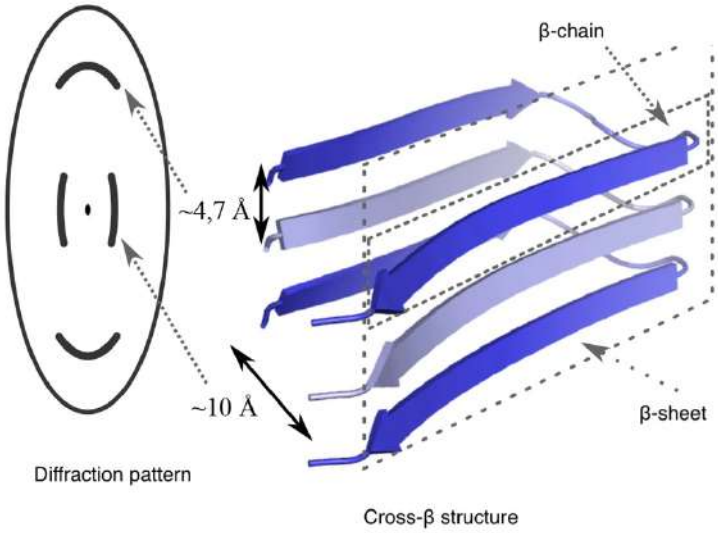

Figure 7. Protofibril structure. Distances between β-chains and β-sheets in amyloid aggregates with a cross-β structure and corresponding diffraction pattern are shown. Protein molecules alternating in the fibril are indicated by different colors (modified from [151]).

There are three fundamental models that describe different variants of the cross-β structure, where the orientation of β-chains in β-sheets can be "parallel in register", "parallel out-of-register", spiral (β-solenoid) and antiparallel. The parallel type in the register is probably the most common pattern for amyloids, in which the β-chains of neighboring protomers are located in parallel, and the corresponding residues from neighboring protomers are located close to each other (in the register) (see review [126]).

Various models have been proposed for the structural characterization of [*PSI*$^+$] prion aggregates, one of the most popular being the superpleated β-structure model [107], in which the β-chains are oriented parallel in the register. A sufficient amount of data has

been accumulated to support this model (see review [141]) (Figure 8). This model implies the stacking of Sup35 N-domains in a parallel superpleated structure, in which the β-layers are located perpendicular to the fibril axis. This structure is stabilized by hydrogen bonds acting between the polar a.a.r. of individual monomers parallel to the fibril axis.

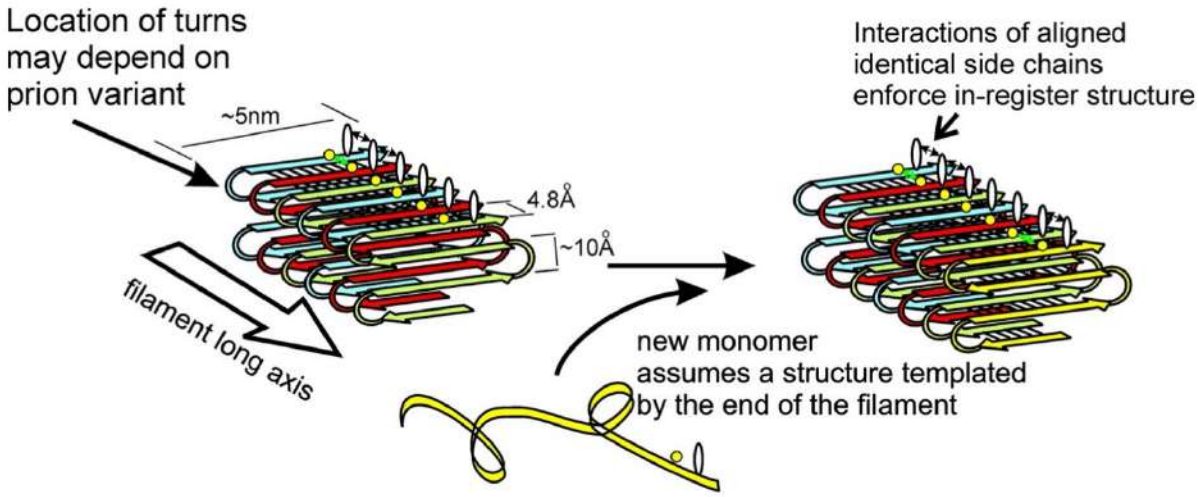

Figure 8. Model of the parallel superpleated β-structure of the [*PSI*$^+$] fibrils. The figure shows the moment when Sup35 monomer is attached to the growing fibril (modified from [236]).

It is still unclear which parts of the Sup35 N-domain are crucial for fibril formation. In a recent study, regions of various prion variants [*PSI*$^+$] resistant to protein kinase A (PKA) were characterized, which could presumably represent the amyloid core [51]. In this case, Sup35 aggregates were obtained from yeast cells rather than bacterial cells (which is commonly used for recombinant protein production). With this approach, the uniformity of the selected Sup35 fibrils can be guaranteed (see review [126]). The detected PKA-resistant regions were not located randomly: in all variants of [*PSI*$^+$], they were enclosed within the sequences of a.a.r. 1-72, 73-124, 125-153, and 154-221, which in this study were designated as amyloid core 1, 2, 3, and 4 [51]. Interestingly, the boundary between regions 2 and 3 coincided well with the boundary of domains N and M. Core 1 was the largest of these structures and best protected from PKA, and it also mainly determined the nonsense-suppressor phenotype of the [*PSI*$^+$] prion [51]. The authors concluded that region 2-32 is a key structural element of all the [*PSI*$^+$] variants studied in this paper. It was noted that the region between core 1 and core 2, which was described as “weak” and completely protected from PKA, is mainly

formed by OR (a.a.r.41-97) [51]. However, the data obtained in this study were poorly compatible with the model of a superpleated β-structure. Further studies are needed to obtain an accurate model of the structure of prion aggregates.

1.3.2.3 Prion variants

As in mammalian prions, the yeast prion protein sequence can be the basis for a wide range of inherited, clearly distinguishable so-called “prion variants” or “prion strains” [23, 53, 189]. Strain variations of several yeast prions have been reported, including [*PIN*$^+$], [*URE3*], and [*PSI*$^+$] [57, 232].

The first [*PSI*$^+$] variant that became known was the [*ETA*$^+$] factor, which was considered as “other forms of the same element” before the nature of [*PSI*$^+$] was revealed [140, 241]. Later, by overproduction of Sup35 protein in yeast, “strong”, “medium” and “weak” variants of the [*PSI*$^+$] prion were produced, which differed in the severity of the nonsense-suppressor phenotype reflecting the level of deficiency of functional Sup35 protein [53, 221]. A weak “indeterminate” variant [*PSI*$^+$], which could form both strong and weak variants, was also identified [196]. This variant was similar to the weak variant described previously [123], which could spontaneously turn into a strong one. In many laboratories, variants of the [*PSI*$^+$] prion [*PSI*$^+$] were similarly isolated. In the King’s lab, three stable variants of [*PSI*$^+$] were isolated, called VH (strong), VK, and VL (weak) [118]. VH and VK were subsequently reproduced by transformation of yeast cells with Sup35(1-61)-GFP fibrils obtained *in vitro* at incubation temperatures of 4 °C and 22 °C, respectively [119]. A similar experiment was done in Tanaka's lab [207], where it was later shown that environmental conditions and *sup35* mutations affect the stacking of conformational precursors, which eventually transformed into separate amyloid “seeds” [167, 168]. It has been shown, strong strain [*PSI*$^+$] can be present in the cell as a “cloud” of variants, dynamic structures that mutually transform, thus causing fluctuations in the composition of the cloud. In this case, the strength of the prion does not change, indicating that the range of fluctuations is limited, so a weak variant of the prion cannot manifest [11].

[*PSI*$^{+}$] variants can vary considerably in their stability — the frequency with which they are lost in a series of cell divisions. There is some correlation between the strength of the variant and its stability — the stronger the prion variant, the more stable it is. This is explained by the observed shorter fibril sizes of the strong [*PSI*$^{+}$] and their longer length in the weak variant. It is believed that with a short size, a larger number of filament ends are available, which contributes to the capture of a larger proportion of Sup35 monomers and, thus, to the manifestation of a strong suppressor phenotype [88, 108, 208]. Such differences between prion strains are thought to be mediated by the variable disaggregase action of the chaperone Hsp104 [208].

1.3.2.4 Factors affecting prion [*PSI*$^{+}$] maintenance

The Hsp104 protein is a disaggregase that acts on denatured proteins in conjunction with Hsp70 and Hsp40 [83]. It belongs to the family AAA ATPases (ATPases Associated with diverse cellular Activities) and is a hexamer. Hsp104 is thought to cleave prion aggregates into oligomers, thus ensuring their stable inheritance, provided a certain level of chaperone production and polymer growth rate [132, 173]. This process is carried out by dragging the fibril through the channel of the Hsp104 protein, during which monomers are separated from the center of the fibril, and it is divided into two parts [157, 163]. Normal levels of the Hsp104 chaperone have been shown to cure most [*PSI*$^{+}$] variants. In strains with the mutation *hsp104*^{T160M}, the prion is formed 10 times more often than at the basal spontaneous level (see review [236]).

When Hsp104 is inhibited, prion filaments continue to grow, but do not separate into parts to form new polymers, which leads to the loss of the prion in a series of cell divisions [164, 173]. Deletion of the HSP104 gene leads to the same consequences. This effect is partially compensated by the overproduction of the Hsp70-Ssa chaperone, which restores prion fragmentation and propagation when it works together with Hsp104 [113]. When Hsp104 is overproduced, cleavage proceeds with higher efficiency and a large number of oligomers are formed, which disrupts the inheritance of the prion [*PSI*$^{+}$] [37].

Guanidine hydrochloride (GdnHCl) is a universal anti-prion agent that eliminates all known yeast prions in millimolar concentrations (see review [141]). GdnHCl cures the

[*PSI*$^+$] prion only in dividing cells by blocking prion proliferation [64]. However, it has been confirmed that GdnHCl does not stop Sup35 polymerization in the [*PSI*$^+$] strain and does not destroy aggregates [162]. These data are consistent with the fact that GdnHCl specifically suppresses Hsp104 activity [103].

The subfamily of Hsp70 chaperones, Ssb, is represented by two non-vital, structurally similar proteins Ssb1 and Ssb2. Ssb1 is associated with translating ribosomes through a heterodimeric ribosome-associated complex (RAC), which consists of the proteins Zuo1 (homolog of Hsp40) and Ssz1 (homolog of Hsp70). Ssb1 provides folding of the resulting polypeptide chain. Unlike proteins of the other Hsp70 subfamily, Ssa, Ssb overproduction stimulates the elimination of the [*PSI*$^+$] prion under conditions of an excess of Hsp104 and prevents the formation of some variants of the [*PSI*$^+$] factor. Ssb protein deficiency stimulates *de novo* [*PSI*$^+$] prion formation and prevents [*PSI*$^+$] elimination under conditions of Hsp104 excess. *In vitro*, Ssb protein inhibits amyloid formation by the NM region of Sup35 protein. This effect is further enhanced in the presence of Zuo1/Ssz1 proteins [113].

The mechanism of the anti-prion effect in Hsp104 overproduction is still controversial to date, so it is actively being studied. From one point of view, Hsp104 has a specific binding site in the Sup35 M-domain [92] and therefore an excess of Hsp104 blocks the access of Hsp70 chaperones to filaments, thereby preventing their cleavage [238]. Other studies show that overproduction of Hsp104 results in asymmetric segregation of prion seeds (or propagons) in the cell, leading to frequent prion loss [164]. It is also suggested that overproduction of Hsp104 begins to remove monomers from the ends of fibrils in a process that has been called trimming (as opposed to the standard splitting of fibrils in the middle) [171, 240].

1.3.2.5 Mutations in the *SUP35* gene affecting the [*PSI*$^+$] prion maintenance

The N-domain of the Sup35 protein is necessary and sufficient for prion [*PSI*$^+$] maintenance [131, 212]. In order to identify the functional sites required for prion [*PSI*$^+$] maintenance, a number of studies were conducted using deletion and mutation analysis of the *SUP35* gene region corresponding to the N-domain of the Sup35 protein.

A number of mutations, termed *PNM* (from "[*PSI*$^+$] no more"), have been identified in the *SUP35* gene that affect the maintenance of the [*PSI*$^+$] factor. One of the first mutations mapped was *PNM2-1* (G58D) [59]. A detailed study of *PNM2-1*-induced elimination of [*PSI*$^+$] factor showed that mutants were not defective in [*PSI*$^+$] prion aggregation or induction [169]. Earlier studies indicated that Sup35 in *PNM2-1* mutants was capable of forming [*PSI*$^+$] *in vivo* [55, 118, 122]. Indeed, in [*psi*$^-$] [*PIN*$^+$] yeast strains, overproduction of Sup35-GFP protein with a G58D substitution in the presence of a wild-type copy of the *SUP35* gene resulted in the appearance of fluorescent foci. At the same time, a reduced efficiency of prion [*PSI*$^+$] transmission into daughter cells was observed [169]. The reason for the elimination of the [*PSI*$^+$] factor in the case of *PNM2-1*, in addition to the disruption of its inheritance, is potentially the increased fragmentation of prion aggregates by Sup35 chaperones, leading to their resolubilization [58, 176, 225].

Currently, many mutations in the *SUP35* gene have been described that lead to a change or elimination of the prion [*PSI*$^+$]. At the same time, they can have specific manifestations in different [*PSI*$^+$] prion strains [55, 118, 142]. Most of these mutations are found in the *SUP35* gene region, which encode charged a.a.r. Q or N within the N-domain [50]. In recent work, nonsense mutations in the N-domain coding region of the *SUP35* gene were found that can increase the frequency of [*PSI*$^+$] *de novo* occurrence by four orders of magnitude, bringing it close to the frequency of [*PSI*$^+$] occurrence in the case of Sup35 or Sup35NM protein overproduction [52]. It cannot be excluded that *sup35* mutations leading to substitutions in the functional region of the Sup35 protein can also affect prion stability [*PSI*$^+$]. Thus, it was shown that substitutions T314A or T314D in the C-domain of Sup35 lead to prion loss [104].

Previously, in our laboratory double *sup35KK* mutations localized in a region of the *SUP35* gene that encodes the N-domain of the Sup35 protein were obtained [18]. The study of these mutations was to facilitate with the search for regions of the Sup35 N-domain critical for fibril formation and maintenance of the [*PSI*$^+$] phenotype. The mutant proteins contained amino acid substitutions in one of the ORs of Sup35. According to the model, the constructed mutations should change the stability of the [*PSI*$^+$] prion only if they occur in the region that forms the superpleated

β-structure. Substitutions *sup35-M1* (Y46K/Q47K) and *sup35-M2* (Q61K/Q62K) resulted in loss of the prion, whereas *sup35-M3* (Q70K/Q71K), *sup35-M4* (Q80K/Q81K) and *sup35-M5* (Q89K/Q90K) were able to maintain the [*PSI*$^+$] prion. These results indicate that the part of the N-domain that is most critical for the formation of a parallel superpleated β-structure is between 63-69 a.a.r. [18]. Later, another mutation named *sup35-M0* (Q33K/A34K) was obtained in our laboratory and added to the study [49]. It is located in an additional OR that was detected using the T-REKS program [18]. In the presence of a new mutation, the [*PSI*$^+$] prion could form with very low efficiency and was able to support only weak variants. In an *in vivo* system, the Sup35-M0 protein could coaggregate with the wild-type Sup35 protein [49]. Presumably, the *sup35KK* mutations investigated in our laboratory affect the properties of the [*PSI*$^+$] factor by changing the structure of prion aggregates.

Electron microscopy showed that amyloid fibrils formed by Sup35NM protein with *sup35KK* substitutions and wild-type Sup35NM had significant differences in overall morphology. Moreover, *sup35-M5* had the least effect on the size and clustering of Sup35NM amyloid fibrils. Investigation of the secondary structure using circular dichroism spectroscopy showed that the fibrils formed from wild-type Sup35NM, as well as from Sup35-M4 and Sup35-M5, have a significant content of ordered β-structure. The observed variations in the content of the β-folded structure suggested differences in the length of the amino acid sequence forming the backbone of amyloid fibrils [204].

1.3.2.6 Viability of *S. cerevisiae* cells in the presence of [*PSI*$^+$]

The question of whether the prion [*PSI*$^+$] has a positive or negative effect on *S. cerevisiae* cells cannot be answered unequivocally this day. Studies by different groups have shown that prions are rarely found in yeast in natural populations. No natural strains of [*PSI*$^+$] have yet been identified, but two “wild” strains of [*PIN*$^+$] have been found [38, 180]. At the same time, it was shown that the Saccharomycetes clade evolutionarily shows an increase in the number of Q/N-rich proteins compared not only with all other clades in the fungal kingdom [7], but also among the entire living world [188].

Earlier works claimed that the [*PSI*$^+$] factor does not affect the growth rate of yeast strains (see [44] for a review). Studies conducted on seven yeast strains using 150 different culturing conditions showed that in 50% of the cases [*PSI*$^+$] indeed had no effect on growth, while in the rest, both inhibition and stimulation of growth in the presence of [*PSI*$^+$] were observed [218]. In some cases, the [*PSI*$^+$] factor led to changes in colony morphology [218] and sensitivity of cells to paromomycin at 20 ºC [42].

In another report, some stress conditions slightly increased the frequency of the [*PSI*$^+$] occurrence in a strain with an altered prion domain of Sup35 [219], but the other two groups were unable to reproduce these results [109, 231]. Moreover, [*PSI*$^+$] reduced cell viability in most stressful conditions [219], indicating that induction of [*PSI*$^+$] was not an adaptive response.

Prions arise spontaneously in the cell, and [*PSI*$^+$] is often lethal or toxic to cells. The idea that yeast becomes [*PSI*$^+$] under stress is still untenable — it is necessary to show that any variant of [*PSI*$^+$] reproducibly increases the survival rate of yeast cells (see review [233]). Nevertheless, there is evidence that non-prone aggregation of Sup35 protein — the formation of temporary protein droplets — fulfils an adaptive role and increases the chances of yeast to survive under difficult conditions. This function of the Sup35 protein is performed by the N- and M-domains, which form a kind of two-component functional unit, in which the M-domain, rich in charged amino acids, acts as a stress sensor. The NM-domain has been shown to form protein droplets *in vitro* reversibly and in direct dependence on pH. Although the amino acid sequence of the M-domain can vary considerably among different representatives of yeasts that diverged over 400 million years ago, the charge distribution is conserved — this refers to the ability of Sup35 to form a condensed fraction by pH-dependent phase separation, but not a prion. In this context, Sup35 prionization in yeast is a sporadically occurring phenomenon [74].

1.4 Conclusion

In the present work, we investigate *sup35* mutant alleles that directly or indirectly affect the properties of prion [*PSI*$^+$]. Over the long history of studying mutations in *SUP35* and *SUP45* genes at the Department of Genetics and Biotechnology of

St. Petersburg State University, a considerable amount of data on their phenotypic manifestation has been accumulated. However, all possible mechanisms by which these mutations are manifested in the cell remain unexplored. In the present work we investigate a unique system in which two strong suppressors are simultaneously present in the cell, the presence of which cause changes in the Sup35 protein — these are nonsense mutations in the *SUP35* gene and the [*PSI*$^+$] prion. At the same time, we face the challenge of distinguishing between the observed phenotypic effects of the mutation or the prion, and furthermore the effects that are observed in its combination. Interestingly, in this case, the cell is able to maintain viability, but it uses different mechanisms to do so, which we have attempted to understand in this study.

At present, it is not yet possible to answer the question of whether prions in yeast are functional, or whether this is a random conformational error. At the same time, their discovery, study, and search for new prions in yeast helps to study human prion and amyloid diseases. The active accumulation of new data on the properties of prions will help to answer this question sooner or later.

Thus, the aim of this work was to study the effect of *sup35* nonsense mutations on the viability of *S. cerevisiae* cells with a strong prion variant [*PSI*$^+$].

To achieve this aim, it was necessary to complete the following objectives:

7. To test the viability of haploid and diploid yeast strains containing the strong [*PSI*$^+$] prion variant in combination with the *sup35* nonsense mutations;
8. Assess the effect of *sup35* nonsense mutations on the properties of the [*PSI*$^+$] prion.

Chapter 2. Materials and methods

2.1 Strains

Isogenic strains of S. cerevisiae (see Table 1) and their derivatives containing nonsense-mutant alleles *sup35-21*, *sup35-74*, *sup35-218*, and *sup35-240* (see Table 3 for mutation descriptions) were used.

Table 1 — Strains of *S. cerevisiae* used in the work

Strain	Genotype	Source
7A-D832	*MAT*α *ade1-14 his7-1 leu2-3,112 lys2-739 trp1-289 ura3-52 SUP35::TRP1* [pYCM-U2] [*psi*$^-$] [*PIN*$^+$]	Provided by A. S. Borchsenius
10-7A-D832	*MAT*α *ade1-14 his7-1 leu2-3,112 lys2-739 trp1-289 ura3-52 SUP35::TRP1* [pYCM-U2] [*PSI*$^+$]^S [*PIN*$^+$]	
a8-7A-D832	*MAT*a *ade1-14 his7-1 leu2-3,112 lys2-739 trp1-289 ura3-52 SUP35::TRP1* [pRSU2] [*psi*$^-$] [*PIN*$^+$]	
OT56	*MAT*a *ade1-14 trp1-289 his3-200 leu2-3,112 ura3-52* [*PSI*$^+$]^S[*PIN*$^+$]	[54, 165]
OT55	*MAT*a *ade1-14 trp1-289 his3-200 leu2-3,112 ura3-52* [*PSI*$^+$]^W [*pin*$^-$]	
1-OT56	*MAT*a *ade1-14 trp1-289 his3-200 leu2-3,112 ura3-52* [*psi*$^-$] [*PIN*$^+$]	[152]
D1691	*MAT*a/α *ade1-14/ade1-14 his7-1/his7-1 leu2-3,112/leu2-3,112 lys2-739/lys2-739 trp1-289/trp1-289 ura3-52/ura3-52 SUP35::TRP1/SUP35::TRP1*[pRSU1] [pRSU2] [*psi*$^-$] [*PIN*$^+$]	This work, obtained by mating strains a8-7A-D832 and 7A-D832
D1692	*MAT*a/α *ade1-14/ade1-14 his7-1/his7-1 leu2-3,112/leu2-3,112 lys2-739/lys2-739 trp1-289/trp1-289 ura3-52/ura3-52 SUP35::TRP1/SUP35::TRP1*[pRSU1] [pRSU2] [*PSI*$^+$]^S[*PIN*$^+$]	This work, obtained by mating strains a8-7A-D832 and 10-7A-D832

Standard designations for mutations associated with auxotrophy for specific amino acids and nitrogenous bases were used in the table. The alleles *ade1-14* and *lys2-739* contain the premature stop codon UGA and lead to auxotrophy for adenine and lysine, respectively; the nonsense mutation *his7-1* (UAA) leads to auxotrophy for histidine; the nonsense mutation *trp1-289* (UAG) leads to auxotrophy for tryptophan. Missense mutations *ura3-52*, and *leu2-2,112* lead to auxotrophy on uracil and leucine, respectively. Originally, strains 7A-D832 and 10-7A-D832 harbored the plasmid pYCM-U2-SUP35 and strain a8-7A-D832 harbored the plasmid pASB2-SUP35, which compensated for the effect of *SUP35* (*sup35::TRP1*) gene disruption. These plasmids were replaced by pRSU2 and pRSU1 series plasmids, respectively. [*PSI*$^+$]^S is a strong variant of the [*PSI*$^+$] prion. [*PSI*$^+$]^W — weak variant of the [*PSI*$^+$] prion.

The bacterial strain *E. coli* DH5α (*supE44* Δ*lacU169* (*φ80 lacZ*Δ*M15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*) was used for plasmid production [90].

2.2 Plasmids

The plasmids used in this work are listed in Table *2*. Plasmids pRSU1 and pRSU2 containing the wild-type *SUP35* allele under its own promoter [227] were derived from centromeric vectors pRS315 and pRS316 [198] carrying yeast selective markers *LEU2* and *URA3*, respectively.

Table 2 — Plasmids used in the study

Plasmid	Brief description*	Source
pRSU1 (pRS315-SUP35)	*CEN*, *amp^R*, *LEU2, SUP35*	[227]
pRSU2 (pRS316-SUP35)	*CEN*, *amp^R*, *URA3*, *SUP35*	
pRSU3 (pRS415-SUP35)	*2μ*, *amp^R*, *LEU2*, *SUP35*	
pRSU1-21	*CEN*, *amp^R*, *LEU2*, *sup35-n*	[30]
pRSU1-74		
pRSU1-218		
pRSU1-240		
pRSU1-Q56Y	*CEN*, *amp^R*, *LEU2*, *sup35*	This work
pRSU1-Q56K		
pRS315	*CEN*, *amp^R*, *LEU2*	[198]
pRS316	*CEN*, *amp^R*, *URA3*	
pRS316-pCUP-GFP	*CEN*, *amp^R*, *URA3*, *GFP*	[82] original name of the plasmid is pRS316CG
pRS316-pCUP-SUP35NM-GFP	*CEN*, *amp^R*, *URA3*, *SUP35-GFP*	[194] original name of the plasmid is pmCUP-NMGFP
pRS316-pCUP-sup35-240-GFP	*CEN*, *amp^R*, *URA3*, *sup35-240-GFP*	This work

* The following characteristics are indicated for each construct: copy number in yeast cells (CEN — centromeric, 2μ — multicopy), bacterial selective marker, yeast selective marker, insertion gene. Hereafter in this work, we will denote the presence of a plasmid (e.g., pRSU1) in the following form for brevity: [*SUP35 LEU2*].

A series of pRSU1 plasmids with *sup35-n* alleles was constructed by S.V. Shabelskaya [30] by replacing the *SUP35* fragment of the pRSU1 plasmid with similar fragments containing *sup35-n* mutations.

The plasmid pRS316-pCUP-sup35-240-GFP was derived from pRS316-pCUP-SUP35NM-GFP: a SacII restriction site was added after the 166th nucleotide residue in the *SUP35NM* sequence by site-directed mutagenesis with primers 240_SacII-F and 240_SacII-R (Table 4). The resulting construct was restricted by SacII at two sites (the new one within the *SUP35NM* sequence and the original one between *SUP35NM* and GFP). The long fragment was ligated to itself.

Plasmids pRSU1-Q56Y and pRSU1-Q56K with mutations in the *SUP35* gene that result in Q56K and Q56Y substitutions in the Sup35 protein were constructed by site-directed mutagenesis with complementary primers Q56K-f and Q56K-r or Q56Y-f and Q56Y-r, respectively (Table 4).

Table 3 — Characteristics of nonsense mutant *SUP35* alleles, studied in the work

Name of the allele	Nucleotide position	Substitution	Amino acid position	Substitution
sup35-21	1264	CAA → TAA	422	Gln → (TAA)
sup35-74	388	CAA → TAA	129	Gln → (TAA)
sup35-218	541	GAA → TAA	181	Glu → (TAA)
sup35-240	166	CAA → TAA	56	Gln → (TAA)

* Triplets containing nucleotide substitutions are shown; mutant nucleotides are underlined.

2.3 Media and cultivation methods

A complete LB medium and a selective LBa medium containing the antibiotic ampicillin at a concentration of 100 μg/mL were used for bacteria cultivation [185]. Bacterial transformation was performed using competent cells [99]. Complete YPD media, synthetic SC media, selective media that do not contain individual components of the SC medium, and minimal growth media were used to work with yeast cultures [106]. The 1/4 YPD medium was used to visualize the suppressor phenotype [64]. To eliminate plasmids with the *URA3* selective marker from yeast cells, medium with 5-fluorotoric acid (5-FOA, Sigma) at a concentration of 1 g/L was used [106]. To induce the *CUP1* promoter, $CuSO_4$ was added to the growth medium to a final concentration of 50 μM [194]. To assess the stability of Sup35 protein, 100 μg/mL of cycloheximide, which inhibits translation, was added to the medium. The viability of some *sup35* mutants was evaluated on medium with the addition of paromomycin

(0.25 to 1 mg/mL), which also affects the accuracy of translation [98]. Bacterial cultures were grown at 37 °C, yeast — at 26 °C (in case of heat-sensitive strains with *sup35-n* mutations) and 30 °C. When cells were cultured in a liquid medium, the speed on a thermostatic shaker was 300 rpm for bacteria and 200 rpm for yeast.

2.4 Yeast genetics methods

Standard methods of working with yeast cell cultures were used in this work [106]. Yeast cells were transformed using lithium acetate and ballast DNA [81].

The viability of yeast cells was visualized by seeding with tenfold dilutions. For this purpose, the cells were collected from a solid medium and resuspended in sterile distilled water. The optical density of the obtained suspensions was measured at a wavelength of transmitted light of 600 nm (OD_{600}). Cell suspensions were equalized in density by adding the required amount of water. The obtained samples were diluted 10 times, repeating dilutions 4 times and seeded on solid medium in drops of 7.5 μl each.

Direct plasmid substitution (shuffling) [17] was performed as follows: a yeast strain carrying the pRSU2 plasmid was transformed with plasmids of the pRSU1 series. Next, loss of the pRSU2 plasmid was carried out selectively or non-selectively in [*URA3*] [*LEU2*] strains. In the first case, [*LEU2*] strains were selected after the growth of transformants on 5-FOA medium. Nonselective plasmid loss was performed in YEPD medium. The grown colonies were printed on media without uracil (SC-U) and leucine (SC-L), and then Ura-Leu+ cells were selected. For reverse plasmid substitution (reshuffling) [17], the [*LEU2*] strain obtained during shuffling was transformed with plasmid pRSU2. Next, a non-selective loss of plasmids was performed and Ura+Leu- cells were selected.

[PSI^+] prion induction in yeast was performed by overproduction of Sup35 protein using a plasmid carrying the *SUP35* gene under the control of the *CUP1* promoter. A yeast colony carrying such a plasmid was seeded from a Petri dish into a liquid selective medium and grown overnight. The next day, the optical density of the culture was measured and diluted with fresh medium to OD_{600}= 0.1. The cells were grown for 2-4 hours and then induction was performed. To eliminate the [PSI^+] prion, the initial yeast

strains were grown on YPD medium containing GdnHCl at a concentration of 4 μM. After that, the clones were cloned on YPD medium and the phenotype of the obtained clones was analyzed.

2.4.1 Protein transformation of yeast

Protein transformation was performed according to previously published protocols [207, 209, 235] with modifications. For the preparation of spheroplasts, yeast cells were grown in liquid YPD medium until the optical density of the culture OD_{600}= 0.3 was reached, then they were sedimented and sequentially washed with sterile water, buffer (1 M sorbitol, 25 M EDTA, 50 mM dithiotreitol), 1 M sorbitol and buffer SCE (1 M sorbitol, 1 mM EDTA, 10 mM sodium citrate, pH 5.8). The cells were then spheroplasted in SCE buffer with 50 μl of zymoliase (120 u/ml) for 30 min at 30 °C. Spheroplasts were sequentially washed with 1 M sorbitol solution and STC buffer (1 M sorbitol, 10 mM $CaCl_2$, 10 mM Tris, pH 7.5). The cells were then resuspended in 1 ml of STC buffer. A 100 μl portion of the spheroplast suspension was mixed with 2 μl of ballast DNA (5 mg/ml), 500 ng of pRS316 plasmid, and 5 μl of a solution of cell extracts with prion particles or Sup35NM fibrils obtained *in vitro*. The mixture was incubated for 30 min at 25 °C. Then 900 μl of PEG buffer (20 % (w/v) PEG 8000, 10 mM $CaCl_2$, 10 mM Tris, pH 7.5) was added and incubated for another 30 min. The cells were sedimented, resuspended in 150 μl of SOS buffer (1 M sobritol, 7 mM $CaCl_2$, 0.25 % yeast extract), and incubated for 30 min at 30 °C. The transformants were seeded on a selective medium (SC-Ura, 1 M sorbitol, 2 % agar) and covered with a top layer of agar (SC-Ura, 1 M sorbitol, 3% agar).

2.5 Methods of working with DNA

Isolation of plasmid DNA from bacterial cells, isolation of DNA from agarose gel, purification of PCR products, as well as restriction and ligation were performed using appropriate commercial kits and enzymes according to the manufacturer's protocols (Thermo Fisher Scientific).

2.5.1 Agarose gel DNA electrophoresis

DNA fragments were separated by gel electrophoresis in 1.0 % (w/v) agarose gel based on 0.5x tris-borate buffer (TBE) according to the standard procedure [185]. After electrophoresis, the gel was incubated in a solution of ethidium bromide and placed on the working surface of the transilluminator. The fluorescence of ethidium bromide bound to DNA was registered using a digital video camera.

2.5.2 PCR

Amplification of the *SUP35* gene sequence was performed by PCR according to the standard protocol on a T100 apparatus (BioRad). 50 ng of plasmid DNA per 10 µl of prepared mixture was used as a template. The final concentrations of the components in the reaction were as follows: primers (Evrogen) — 0.25 µM each, Taq polymerase (SibEnzyme) — 0.05 u/µl, deoxyribonucleotides (Fermentas) — 0.2 mM. The reaction was carried out in a buffer for Taq polymerase (SibEnzyme).

Table 4 — Sequences of primers used during plasmid construction

Name of the primer	Plasmid construction	Sequence (5 ' → 3')
240_SacII-F*	pRS316-pCUP-sup35-240-GFP	CCAAGGTTATTCTGGGTACCCGCGGG GTTATCAACAGTAC
240_SacII-R*		GTACTGTTGATAGCCACCCCGCGGGT ACCCAGAATAACCTTGG
Q56Y-f**	pRSU1-Q56Y	CCAAAATTACCAAGGTTATTCTGGGT ACTACCAAGGTGGCTATCAACAGTAC AATCCCG
Q56Y-r**		CGGGATTGTACTGTTGATAGCCACCT TGGTAGTACCCAGAATAACCTTGGTA ATTTTGG
Q56K-f**	pRSU1-Q56K	CCAAAATTACCAAGGTTATTCTGGGT ACAAGCAAGGTGGCTATCAACAGTAC AATCCCG
Q56K-r**		CGGGATTGTACTGTTGATAGCCACCT TGCTTGTACCCAGAATAACCTTGGTA ATTTTGG

* The SacII restriction site is highlighted
** Nucleotide substitutions are underlined

Amplification of the pRS316-pCUP-SUP35NM-GFP long fragment during the construction of pRS316-pCUP-sup35-240-GFP (Table 4) was performed using high-fidelity high-processing polymerase AccuPrime^{TM}Pfx (Invitrogen). Site-directed mutagenesis to obtain plasmids pRSU1-Q56Y and pRSU1-Q56K was also performed using Pfx on the pRSU1 template. After PCR, the product was restricted with Dpn1 to get rid of the original matrix and transfected with it into *E.coli* DH5α.

Validation of the obtained plasmids was performed by PCR from bacterial colonies. One colony was resuspended in 10 µl of water, incubated at 100 °C for 2 min, and centrifuged for 5 min at 14,000 rpm. For PCR, 2-5 µl of the resulting mixture was taken.

2.5.3 Sequencing

Sequencing of plasmid DNA fragments containing the coding sequence of the *SUP35* gene with the studied mutations was performed in the Research resource center for molecular and cell technologies of Saint Petersburg State University using the primers listed in Table 5.

Table 5 — Sequences of primers used for *SUP35* gene sequencing

Title	Sequence (5 ' → 3’)
CFPstart-r	GTGCCCATTAACATCACCATC
Sup35 BamHI	AAGGATCCATGTCGGATTCAAACCAAGG
116	CATTGGCATTGTTGGTATTATGTGTTG
sup3	GAGCATTGATGTGACGGT
112	CTTGCTCACCACACATAGCCATATCAAC
113	GGAGGAGAAACCAGTCCAGACTGAAG

2.6 Methods of working with proteins

2.6.1 Isolation and separation of proteins under denaturing conditions

Protein isolation from yeast and bacteria under denaturing conditions was performed according to published protocols [129, 239]. Electrophoresis of denatured

proteins in 15 % (v/v) polyacrylamide gel in the presence of SDS (SDS-PAGE) was performed according to the standard procedure [134]. After electrophoresis, proteins were transferred to a PVDF Hybond-P membrane (Amersham) using a Trans-Blot Turbo apparatus (Bio-Rad).

2.6.2 Isolation and separation of proteins under semi-denaturing conditions

To isolate proteins from yeast under semi-denaturing conditions (SDD-AGE), we used a modified technique based on previously published works [87, 125]. A 10-50 ml cell culture was grown until an optical density of OD_{600} = 0.6-0.8 was achieved. All manipulations with the samples were performed at 4 °C or in ice. The cells were sedimented for 5 min at 5,000 rpm in up to 50 ml test tube. The supernatant was removed, the cells were washed in 1 ml of water and sedimented under the same conditions. The cell pellet was resuspended in 500 µl of water, transferred to Eppendorf, and centrifuged for 1 min at 14,000 rpm. The supernatant was removed, and the cell pellet was resuspended in an equal volume of lysis buffer (100 mM Tris-HCl, pH = 7.5, 50 mM NaCl, 10 mM β-mercaptoethanol, 2 mM PMSF, 20 µl of a cocktail of protease inhibitors (Sigma) per 1 ml of buffer). Pure eppendorfs were filled with glass beads (Sigma) the volume of which was approximately equal to the volume of the cell suspension was added. Cells were transferred to the beads and lysis was performed on a FastPrep24 homogenizer (M. P. Biomedicals) for 20 seconds at a rate of “6.0”, followed by incubation of the cells at 4 °C for 2 minutes. The cycle was repeated three times until the cells were completely destroyed. Lysates were selected and transferred to new eppendorfs. The beads were washed once with a lysis buffer, the volume of which was equal to or half the volume of lysate, and transferred to a test tube in lysate. The samples were precipitated for 10 min at a speed of 2,000 rpm and the upper fraction was transferred to new eppendorfs.

Total amount of protein in samples was measured using the method of M. Bradford [22], adding the required amount of lysing buffer. Samples for electrophoresis contained 30-50 µg of total protein and 4x application buffer (2x TAE,

20% (v/v) glycerol, 8% SDS (w/v), bromphenol blue) was added. Before applying to the gel, the samples were incubated for 5 min at 25 °C.

Electrophoresis was performed using 1.5 % (w/v) agarose gel based on TAE buffer (0.04 M tris acetate, 0.002 EDTA) with 0.1 % (w/v) SDS. When preparing the gel, SDS was added after complete dissolution of agarose. If the agarose precipitated, the solution was heated until it dissolved, and boiling of the solution was avoided. Electrophoresis was performed in TAE buffer with 0.1 % (w/v) SDS, which was pre-cooled together with the electrophoresis chamber and gel in ice. The voltage applied to the camera during electrophoresis was calculated from the ratio of 3 V per 1 cm of gel.

Transfer of proteins from the agarose gel to the PVDF Hybond-P membrane (Amersham) was performed by capillary transfer in TBS buffer (100 mM NaCl, 25 mM Tris-HCl, pH = 7.6) [87].

2.6.3 Electrophoresis of proteins in polyacrylamide gel with preboiling

To check the presence of Sup35 protein aggregates in yeast, the SDS-PAGE method with preboiling was used [130]. Protein was isolated using the SDD-AGE protocol. Before applying to the gel, the sample was divided into 2 parts, one of which was incubated at 100 °C, the other at 25 °C, and applied to a 10 % polyacrylamide gel. After the dye level reached the middle of the gel, the electrophoresis was stopped, the gels were taken out, the wells were sealed and the gels were boiled for 10 min in a container with water. After that, the setup was reassembled and electrophoresis was completed.

2.6.4 Detection of proteins by hybridization with antibodies (Western blotting)

The membrane was incubated for 1 h in TTBS buffer (0.8 % (w/v) NaCl, 0.02 M Tris-HCl, 0.1 % (v/v) Tween-20 (pH 7.6)) with the addition of 5 % (w/v) skimmed milk powder. This and subsequent procedures were carried out under conditions of constant stirring of the solution. The membrane was then placed for 1 h in a solution with primary antibodies. At the next stage, the membrane was washed of excess

antibodies with TTBS buffer 2 times for 10 min. After that, a membrane with AntiRabbit secondary antibodies from the ECL Plus Western Blotting Reagent Pack (Amersham) was incubated (dilution in 5 % (w/v) milk 1:25000). Then the excess antibodies were washed off again, as described above. Detection of protein-antibody complexes was performed using the ECL Plus Western Blotting Detection System (Amerhsam) kit and the GeneGnom 5 imaging system (Syngene Bio Imagine). Then, if necessary, the membrane was stained in a Coomassie solution (0.1 % Coumassie R 250, 50 % methyl alcohol, 50 % water). Polyclonal antibody SE42901 [30] (dilution in 5% (w/v) milk 1:1000) was used to detect Sup35 protein. Anti-Tag(CGY)FP antibody (Evrogen, #AB121) was used to detect GFP protein.

2.7 Microscopy techniques

For the preparation of temporary slides, yeast cultures were grown in a liquid selective medium to a logarithmic growth stage (OD_{600} = 0.2-0.4). Next, expression of the reporter construct was induced for 2 h after adding 50 μM $CuSO_4$ to the culture (1 ml of the culture before induction was selected as a control). After that, the cells were precipitated, the supernatant was drained, leaving 5-10 μl, the cells were resuspended in the remaining medium, and the suspension was applied to a slide. A Zeiss Axio Scope.A1 fluorescence microscope with Zeiss Axiocam ERc s5 and Filter set 38 (excitation maximum: 470 nm; emission maximum: 525 nm) was used to visualize Sup35-GFP.

2.8 Data analysis

Quantitative assessment of Western blotting was performed using the ImageJ software [190]. The Wilcoxon rank sum test and the Mann-Whitney U-test were used to evaluate the differences. The Fischer exact test was used for statistical processing of protein transformation results [63]. The differences were considered statistically significant at $p < 0.05$. All calculations were performed using the R programming language [178].

The UGENE program (https://ugene.net/) was used for sequence alignment. Selection of primers and *in silico* plasmid construction were performed using SnapGene Viewer (https://www.snapgene.com/) and UGENE programs.

Chapter 3. Results

3.1 Effects of nonsense mutations in a gene *SUP35* on the viability of yeast cells

To study the effect of nonsense mutations in the *SUP35* gene and prion [*PSI*$^+$] on the viability of *S. cerevisiae*, we selected four *sup35-n* mutations [217], which were obtained in the work of S.V. Shabelskaya [30] (Figure 9). These mutations are located in different regions of *SUP35* and lead to the synthesis of N-terminal Sup35 fragments (Sup35-n) of different sizes (Figure 9, Table 3).

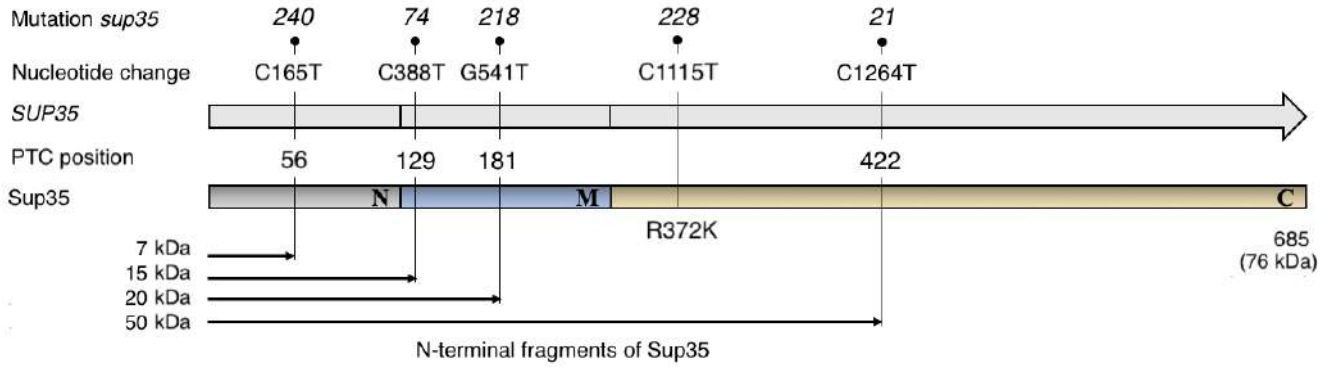

Figure 9. Localization of nonsense mutations in the *SUP35* gene. The schematic shows the *SUP35* gene and the Sup35 protein with the N-, M-, and C-domains labeled. Nonsense mutations *sup35-n* are indicated by numbers above, positions of mutations in the *SUP35* gene (nucleotide substitution is indicated) and positions of PTC resulting from mutations are indicated below. Arrows show the relative sizes of shortened Sup35-n proteins corresponding to mutant alleles; their approximate molecular masses are given on the left.

3.1.1 Characteristics of *sup35-n* nonsense mutations

At the first stage of our work, we characterized the phenotypic manifestation of the studied *sup35-n* alleles on a new genotypic background in comparison with the strains used previously [30]. We obtained a8-7A-D832 [*LEU2*] strain with *sup35-n* mutations by plasmid shuffling from the original a8-7A-D832 [*URA3 SUP35*] strain on 5-FOA medium. Cells carrying only mutant *sup35-n* alleles could suppress all marker nonsense mutations — *ade1-14*, *his7-1*, and *lys2-739* (Figure 10A).

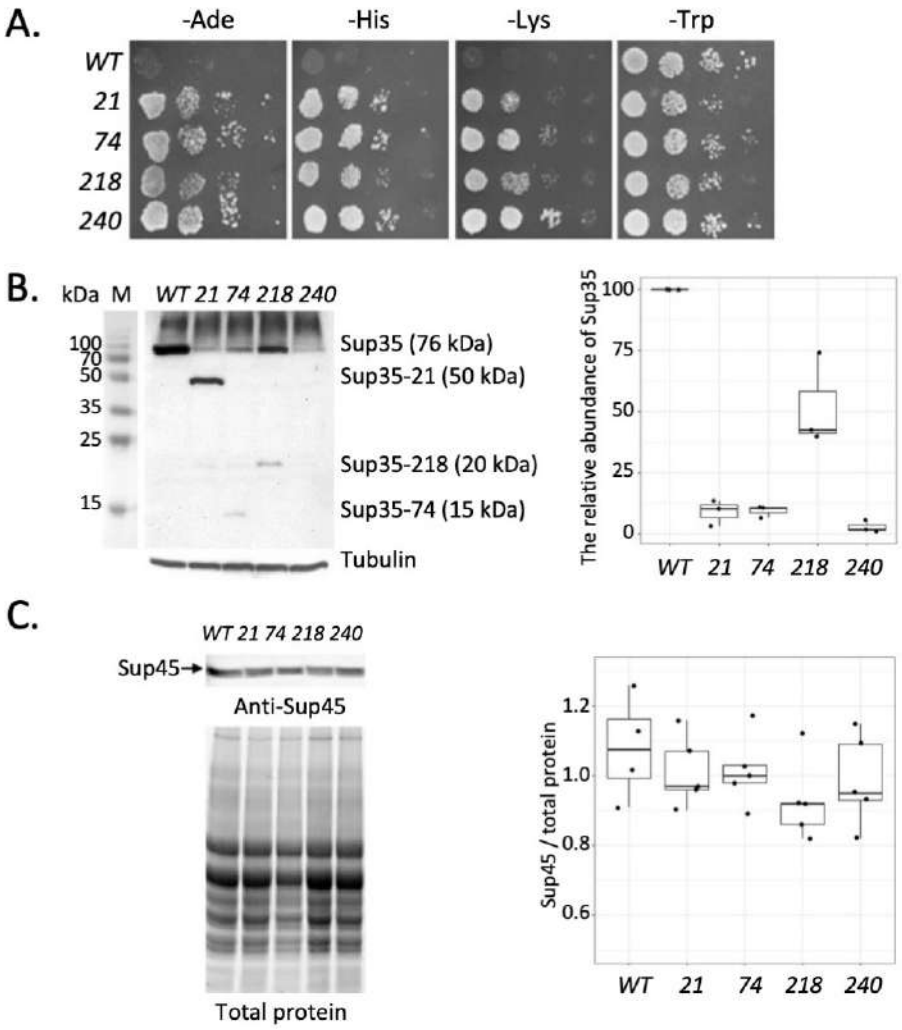

Figure 10. Characterization of *sup35-n* nonsense mutations. A. Mutations *sup35-n* result in omnipotent nonsense suppression. A series of ten-fold [*psi*$^-$] [*sup35-n*] cell dilutions are presented on media for detecting nonsense suppression after five days of incubation. Growth on a medium without tryptophan (–Trp) was used as a control. The numbers to the left of the figure indicate *sup35-n* mutations. **B.** Mutations *sup35-n* result in reduced amounts of full-length Sup35 protein. Cell lysates of the strains shown in panel A were analyzed by Western blot with antibodies to Sup35 protein and tubulin as loading control. The numbers above the lanes indicate the *sup35-n* mutation. M is a protein molecular weight marker in kDa. Fragments corresponding to the full-length and truncated Sup35 are marked by the arrows. Tubulin was used as a loading control. The plot shows the amount of Sup35 in [*sup35-n*] mutants relative to the wild type strain. Amount of Sup35 in the wild-type (*WT*) is taken as 100 %. **C.** Sup45 protein level in [*sup35-n*] mutants it doesn't differ from the wild type. The amount of Sup45 protein was normalized to the total protein, values for which were obtained by processing gels after electrophoresis in the gel documentation system (ChemiDoc XRS+ System, Bio-Rad). The Mann-Whitney U-test was used as a statistical criterion and showed that there was no statistically significant difference ($p < 0.05$) in the amount of Sup45 protein in wild type and [*sup35-n*] mutants. Each dot in the plot of panels B and C corresponds to one sample (independent isolation) of protein; the line marks the median level.

Using the SDS-PAGE method, we estimated the amount of full-length Sup35 protein and its Sup35- fragments in cells of a8-7A-D832 [*LEU2 sup35-n*] strain. All mutants were characterized by a decrease in the Sup35 protein level (Figure 10B). At

the same time, its smallest amount was detected in the strain in the presence of the *sup35-240* allele, which corresponds to the previous data [30]. In the case of cells carrying the *sup35-21*, *sup35-74* or *sup35-218* mutations, Sup35-n proteins were detected and their size corresponded to the expected size of 50, 15 and 20 kDa, respectively. The Sup35-240 protein could not be detected. This can be explained by the fact that the antibodies used to detect Sup35 do not recognize the epitope located in the N-terminal fragment of the Sup35. Another reason could be increased degradation of the Sup35-240 protein, since it is known that the NM-domain of the Sup35 protein is a substrate for proteasome degradation [105]. Nevertheless, we assume that the Sup35-240 protein is present in the corresponding cells, since both the presence of the *sup35-240* mutation and overproduction of Sup35-240 protein fused with GFP leads to the [*PSI*$^+$] prion elimination (see below).

The Sup45 and Sup35 proteins form a complex during translation termination. To test whether the Sup45 protein level changes in the presence of *sup35-n* mutations, we performed SDS-PAGE with [*sup35-n*] mutant lysates and Western blotting with the corresponding antibodies. The amount of Sup45 protein in cells with *sup35-n* mutations did not differ from the wild type (Figure 10C).

3.1.2 Mutations *sup35-n* can lead to the appearance of a more stable and functionally active Sup35 protein in yeast cells

The [*sup35-240*] cells exhibited the lowest amount of full-length Sup35 compared to the other [*sup35-n*] mutants, while they did not differ from the other mutants or the wild type in viability (Figure 10A). We assumed that the amino acid residue that is incorporated in the protein sequence during nonsense suppression of the *sup35-240* mutation improves the functional properties of Sup35. To test this hypothesis, we analyzed the effects of substituting glutamine (Q) for lysine (K) or tyrosine (Y) at the 56th position of the Sup35 a.a. sequence. It was previously shown that the yeast cell uses a.a.r. of Q, K, Y in the case of nonsense suppression at the UAA codon [16]. The

experiment, which allowed us to evaluate the stability and functionality of Sup35 protein with Q56K and Q56Y substitutions, was carried out together with S.A. Bondarev.

According to the literature data, Sup35 is a stable protein, but we did not find its exact half-life. There is evidence that it lasts 2 hours [105] or even about 33 hours [40]. Therefore, we performed prolonged (up to 20 hours) incubation with cycloheximide. The rate of degradation of the Sup35-Q56Y protein was similar to that of wild-type Sup35, whereas the Sup35-Q56K protein was more stable (Figure 11A).

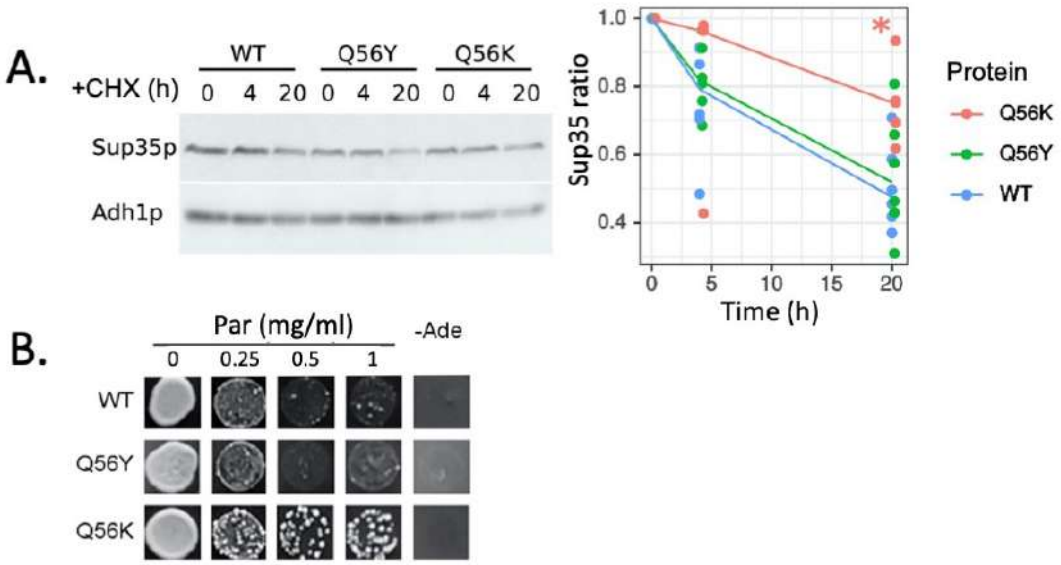

Figure 11. Nonsense suppression of *sup35-n* mutations may result in a more stable and functional full-length Sup35 protein. A. Increased stability of Sup35 in the case of *sup35-240* mutation. Steady-state level of Sup35 was estimated in 7A-D832 strain bearing pRSU1 plasmid with either wild-type *SUP35* (WT) or one of the mutant *sup35-Q56Y* (Q56Y) or *sup35-Q56K* (Q56K) alleles after incubation in YPD medium containing 100 μg/ml of cycloheximide (CHX) during 0, 4 or 20 hours. The amount of Sup35 in each probe at point 0 was taken as 1.0 and the relative Sup35 amount in other points was calculated. **B.** In the presence of Sup35-Q56K protein, yeast cells are less sensitive to paromomycin. Growth of the same transformants as at panel A was assessed on the YPD medium containing different concentrations of paromomycin (Par) after 5 days of incubation at 30 °C. Drops of yeast suspension of the same density were used. Three independent transformants with each plasmid were tested, and representative results are shown.

One of the pleiotropic effects of mutations *sup35* is a decrease in the rate of cell growth *S. cerevisiae* on a medium with paromomycin [98], an aminogligoside antibiotic that binds to rRNA and affects translation accuracy, as well as phenotypically suppresses nonsense mutations in yeast. Cells containing the Sup35-Q56Y and Sup35-Q56K proteins were tested for sensitivity to paromomycin. This experiment was performed in collaboration with O.M. Zemlyanko. In the presence of Sup35 and Sup35-Q56Y proteins, yeast were able to grow on medium with paromomycin at a concentration of up to

0.25 mg/mL. In the case of Sup35-Q56K protein, the cells survived at higher concentrations of antibiotic — up to 1 mg/mL (Figure 11B).

Thus, the full-length Sup35 protein, which is synthesized when the PTC is "read" as significant, may function better than the wild-type protein. This effect may occur in the case of the other *sup35-n* mutants. It is also important to note that the *sup35-Q56K* and *sup35-Q56Y* mutations themselves do not have a suppressor phenotype (Figure 11B).

3.1.3 Short Sup35-n proteins are stable and persist for a long time after blocking total protein synthesis in the cell

We evaluated the stability of Sup35-n proteins in strains 7A-D832 [*SUP35 LEU2*] and [*sup35-n LEU2*] after cell treatment with cycloheximide. The Sup35-21 and Sup35-218 proteins are stable, and can be detected 4 hours after blocking total protein synthesis in the cell (Sup35-21) and even 20 hours later (Sup35-218) (Figure 12A).

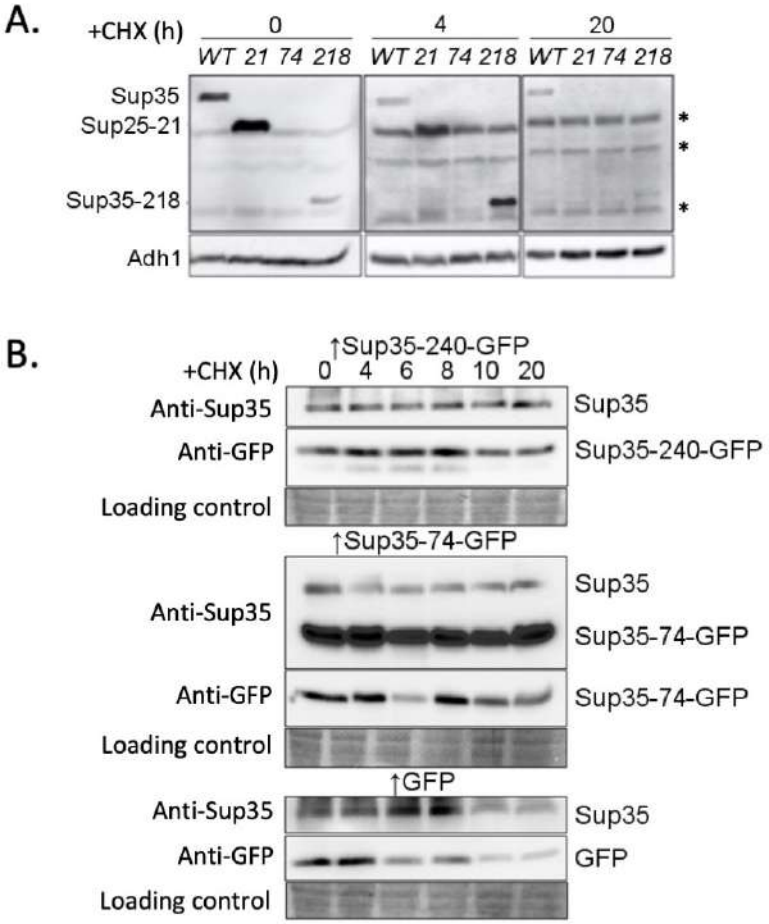

Figure 12. Stability of Sup35-n proteins after blocking total protein synthesis in the cell. A. Sup35-21 and Sup35-218 proteins are stable and persist for a long time after treatment with

cycloheximide. The results of Western blotting with antibodies to the Sup35 protein from lysates of strains 7A-D832 carrying the pRSU1 plasmid, either with the *SUP35* gene or with one of the *sup35-n* mutant alleles, after incubation in YPD medium containing 100 μg/ml of cycloheximide (CHX) for 0, 4 and 20 hours, are presented. The strains shown in Figure 10A were used. In the case of the *sup35-74* mutation, the short protein could not be detected. The Adh1 protein was used as a loading control. The symbol (*) to the right side of the figure marks additional protein bands (besides Sup35) that result from nonspecific interactions of the antibodies used with other proteins. To improve the visibility of the Sup35 protein bands, we increased the contrast of the images at 4 and 20 h, which also made the nonspecific bands much brighter. **B.** The chimeric Sup35-240-GFP and Sup35-74-GFP proteins persist for a long time after total protein synthesis is blocked in the cell, and their stability does not depend on the presence of the GFP label. To induce the *CUP1* promoter, which regulates the synthesis of the studied constructs with GFP, cells were incubated in a selective medium containing 50 μM $CuSO_4$ for 2 h. The amount of Sup35 protein or its N-terminal fragments fused to GFP was determined in strain 7A-D832 carrying the pRSU1 plasmid with the *SUP35* gene and one of the plasmids with the reporter constructs: RS316-pCUP-SUP35-240-GFP (Sup35-240-GFP) or pRS316-pCUP-SUP35-74-GFP (Sup35-74-GFP), after incubation in SC-Leu-Ura medium with cycloheximide at a concentration of 100 μg/mL for 0-20 h. The construct expressed from plasmid pRS316-pCUP-GFP (GFP) was used as a control, and Coomassie membrane staining after Western blotting was used as loading control.

The Sup35-74 protein was not detected in this experiment. For this reason, and to test the stability of the Sup35-240 protein, we repeated the cycloheximide experiment for chimeric constructs in which these Sup35 fragments were fused with the GFP reporter protein. The Sup35-240-GFP protein, which was detected only with anti-GFP antibodies, shows stability similar to the wild-type Sup35 protein (Figure 12B, top panel). The Sup35-74-GFP protein is recognized by both anti-GFP and anti-Sup35 antibodies, and is stable after incubation in the presence of cycloheximide for 20 hours (Figure 12B, middle panel). The stability of chimeric proteins cannot be explained by the presence of the GFP label, since the GFP protein itself is less stable than both constructs (Figure 12B, bottom panel). The results obtained are consistent with published data, where the half-life of the GFP protein was estimated to be approximately 7 hours [162].

3.1.4 Dominant manifestation of the *sup35-n* mutations

In order to describe the manifestation of *sup35-n* mutations in diploid cells, we mated strains a8-7A-D832 [*psi*$^-$] [*sup35-n*] with the isogenic strain 7A-D832 [*psi*$^-$] [*SUP35*]. The obtained diploid cells had a nonsense-suppressor phenotype, which confirms the presence of mutant *sup35-n* alleles (Figure 13A). The strains did not differ

significantly in Sup35 (Figure 13B) and Sup45 (Figure 13C) protein levels. Notably, we did not detect Sup35-n proteins in the presence of the two alleles of the *SUP35* gene, whereas after loss of the pRSU2 [*URA3*] plasmid with the wild-type *SUP35* gene, they were detected in strains with *sup35-21*, *sup35-218* and *sup35-74* mutations (Figure 13B).

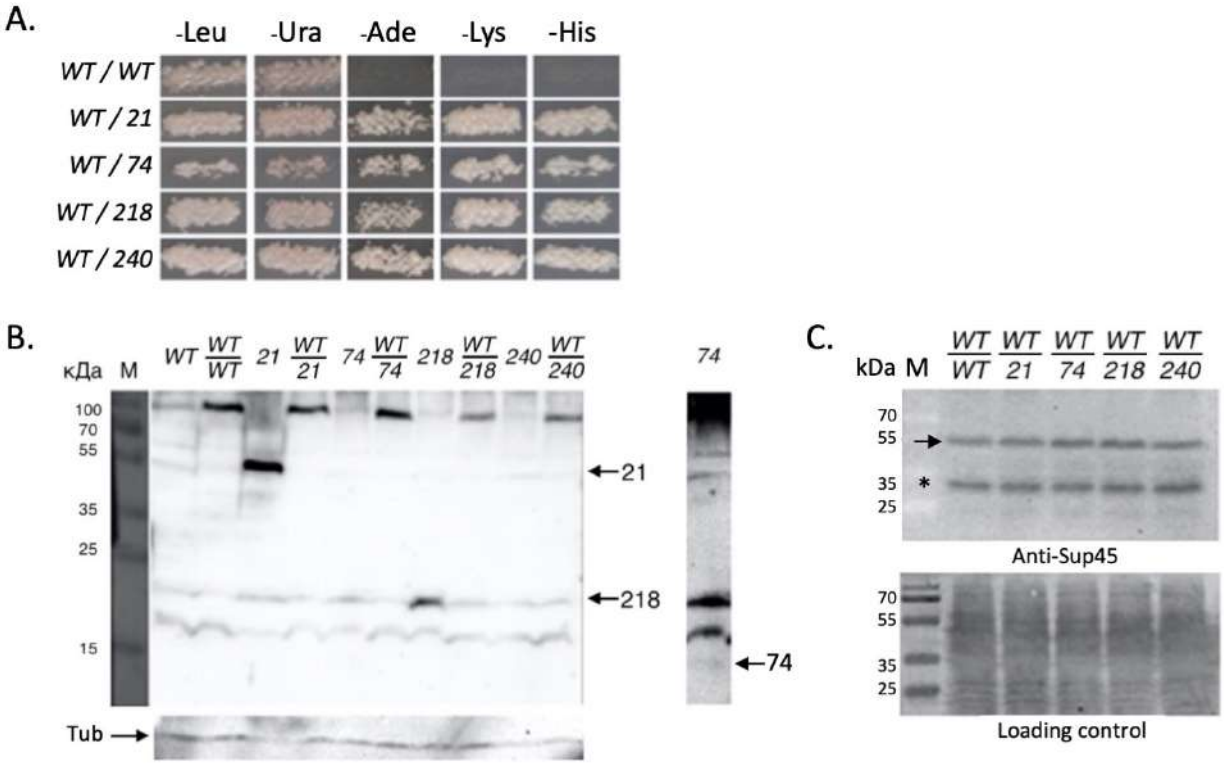

Figure 13. Dominant manifestation of *sup35-n* mutations. A. The [*psi*$^-$] [*SUP35*/*sup35-n*] diploid strains possess a nonsense-suppressor phenotype. Four independent diploid strains were tested, and representative results were presented after 4 days of incubation at 26 °C. The captions on the left indicate the combination of the *SUP35* wild type allele (*WT*) with *sup35-n* mutations. **B.** The presence of Sup35-n proteins, which are synthesized in the presence of *sup35-n* mutations in the cell, depends on the presence of an additional [*URA3 SUP35*] plasmid. Lysates of diploid [*psi*$^-$] strains containing a single [*sup35-n*] or [*SUP35*] plasmid or [*SUP35*/*sup35-n*] plasmids were analyzed by Western blotting with anti-Sup35 antibodies. M is a marker of the protein's molecular weight. Fragments corresponding to the short Sup35 protein are marked with arrows. Tubulin (Tub) was used as loading control. A separate band is shown for the short Sup35-74 protein after contrast enhancement (taken from the gel shown on the left). **C.** The diploid strains shown in panel A do not differ in the amount of Sup45 protein. Lysates from diploid [*psi*$^-$] strains were used for Western blotting with anti-Sup45 antibodies. The arrow shows bands of Sup45 protein. The symbol (*) indicates additional protein bands (besides Sup45), which result from non-specific interaction of the antibodies used with other proteins. Coomassie membrane staining was used as loading control.

3.2 Effect of nonsense mutations in the *SUP35* gene on the [*PSI*$^+$] prion maintenance

3.2.1 General scheme of the experiment

Previously, it was shown that nonsense mutations in the *SUP45* gene lead to synthetic lethality in combination with a strong [*PSI*$^+$] prion variant, even in the presence of the wild-type *SUP45* allele in the yeast *S.cerevisiae* [114]. Our objective was to identify the effects of mutations in the *SUP35* gene on cell viability [*PSI*$^+$]. We used two experimental approaches to combine *sup35-n* mutations and [*PSI*$^+$] prion in yeast: (1) plasmid shuffling in haploid and diploid strains (Figure 14A) and (2) mating of haploid strains (Figure 14B).

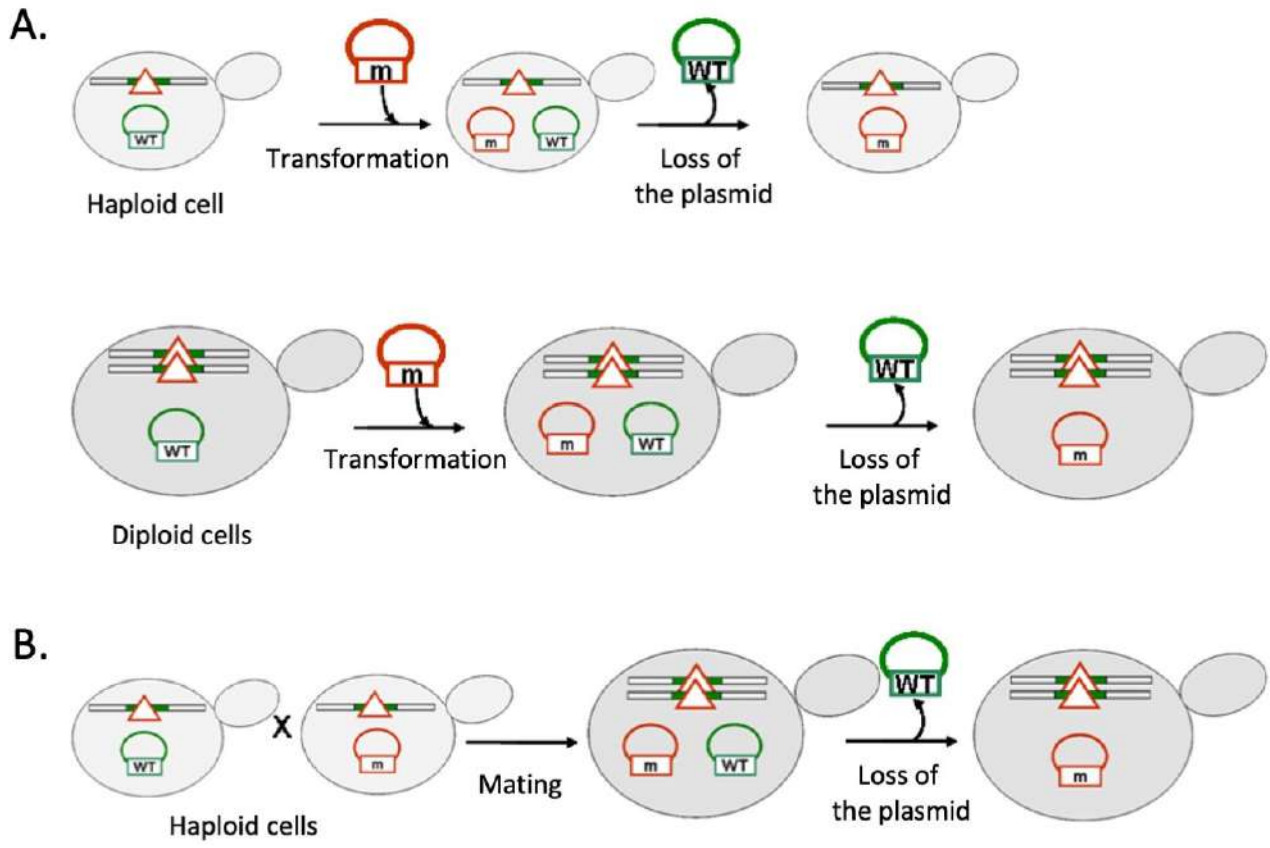

Figure 14. Experimental approaches used to combine *sup35-n* mutations with the [*PSI*$^+$] prion. **A.** Haploid [*PSI*$^+$] strains containing the wild-type *SUP35* gene on the pRSU2 [*URA3*] plasmid, were transformed with pRSU1 [*LEU2*] plasmids carrying *sup35-n* alleles. The resulting transformants were replica plated on 5-FOA medium to select clones that had lost the [*SUP35 URA3*] plasmid (upper panel). The same procedure was performed for diploid [*PSI*$^+$] strains (lower panel). **B.** A haploid [*PSI*$^+$] strain carrying the wild-type *SUP35* gene on the pRSU2 [*URA3*] plasmid was mated with derivatives of the [*psi*$^-$] strain carrying *sup35-n* alleles on the pRSU1 [*LEU2*] plasmid. The resulting diploid cells were replica plated on 5-FOA medium to select clones that had lost the [*SUP35 URA3*] plasmid. The [*psi*$^-$]

strain was used as a control in all cases. The *SUP35* allele (WT) is labeled in green, while the *sup35-n* (m) mutant alleles are labeled in red. A red triangle indicates a deletion of the *SUP35* gene on a chromosome.

In the first case, haploid or diploid [*PSI*$^+$] cells that contain a "strong" prion variant (Figure 15) acquired *sup35-n* mutations instead of the wild-type *SUP35* gene through transformation followed by loss of the original plasmid on 5-FOA medium (Figure 14A). In the second case, the haploid strain [*PSI*$^+$] [*SUP35*] was mated with isogenic haploid [*psi*$^-$] [*sup35-n*] strains. This was also followed by loss of plasmid carrying the wild-type *SUP35* gene on 5-FOA medium (Figure 14B). The isogenic [*psi*$^-$] strain was used as a control for all cases (Figure 15).

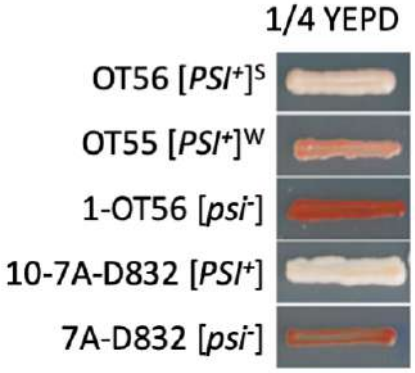

Figure 15. Strain 10-7A-D832 contains a "strong" variant of the [*PSI*$^+$] prion. For comparison, the growth of OT56 or OT55 isogenic strains carrying "strong" ([*PSI*$^+$]^S) and "weak" ([*PSI*$^+$]^W) prion variants, respectively [165], and growth of the strains used in this study on 1/4 YPD medium after 4 days of incubation at 30 °C are shown.

3.2.2 Prion [*PSI*$^+$] compatible with mutations *sup35-n* in the presence of an allele *SUP35* wild type in haploid and diploid strains

In order to test whether the studied *sup35-n* mutations can maintain the viability of haploid [*PSI*$^+$] cells, we transformed isogenic strains 7A-D832 [*psi*$^-$] and 10-7A-D832 [*PSI*$^+$] with pRSU1 [*sup35-n*] plasmids. It is important to note that the transformation of the [*PSI*$^+$] was less effective, in addition, the transformants had a reduced growth rate compared to the [*psi*$^-$] strain (Figure 16A). The growth inhibition of the strain that contains two alleles of the wild-type *SUP35* gene (*WT*/*WT* in Figure 16A) may be due to

prion toxicity caused by the increased amount of Sup35 protein in the cell and its sequestration in [*PSI*$^+$] aggregates (reviewed in [141]). In the case of *sup35-n* mutations, however, there is no significant increase in the total amount of Sup35 protein in the cells. Notably, transformants with *sup35-240* were characterized by an increased growth rate compared to the wild type or other mutants.

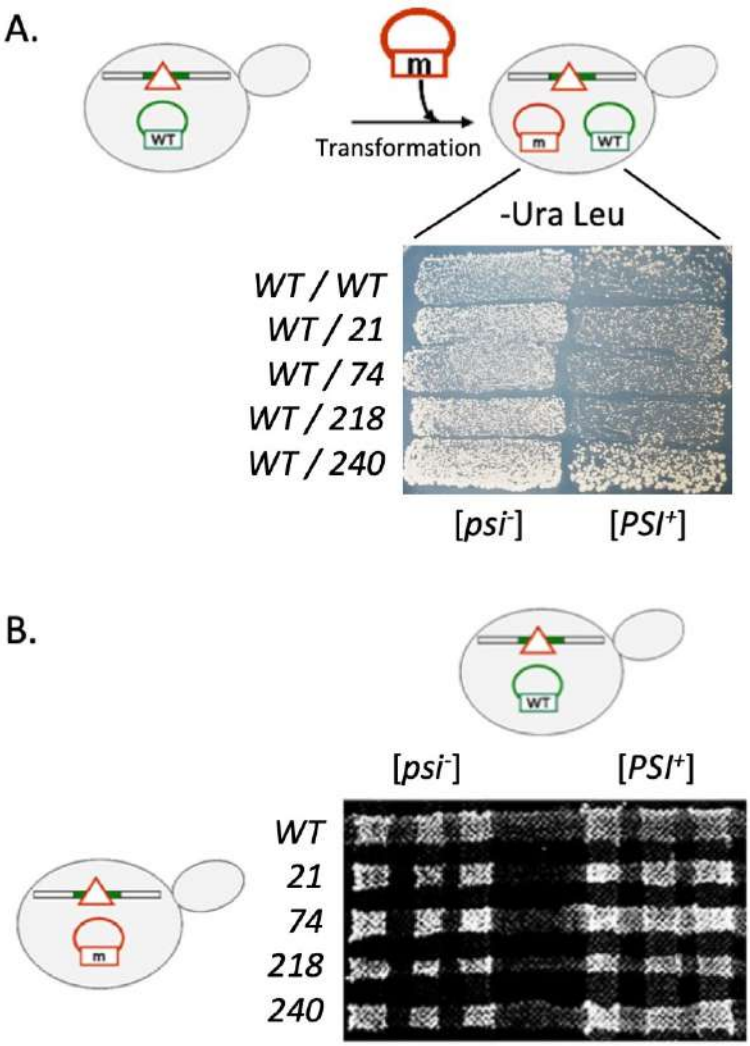

Figure 16. The [*PSI*$^+$] prion is compatible with *sup35-n* mutations in the presence of the wild-type *SUP35* allele. A. Haploid [*PSI*$^+$] [*SUP35*] cells are viable in the presence of *sup35-n* mutations. Strains 7A-D832 [*psi*$^-$] and 10-7A-D832 [*PSI*$^+$] carrying pRSU2 [*URA3*] plasmid with the wild-type *SUP35* gene were transformed with pRSU1 [*LEU2*] plasmids bearing mutant *sup35-n* alleles. In each case, the same number of cells and DNA were taken for transformation. After a standard transformation procedure, an equal amount of cell suspension was plated on media selective for both plasmids. The growth of transformants is shown after 7 days of incubation at 26 °C. **B.** Diploid [*PSI*$^+$] [*SUP35*] cells are viable in the presence of *sup35-n* mutations. The result of crossing haploid strains 7A-D832 [*psi*$^-$] [*SUP35*] and 10-7A-D832 [*PSI*$^+$] [*SUP35*] (vertical lines) with a8-7A-D832 [*psi*$^-$] [*sup35-n*] strains (horizontal lines) is shown. Photos of the plates were taken after five days of incubation at 26 °C.

To test whether *sup35-n* mutations maintain the viability of diploid [*PSI*$^+$] cells, we mated strains 10-7A-D832 [*PSI*$^+$] and 7A-D832 [*psi*$^-$] containing the *SUP35* gene with strains a8-7A-D832 [*psi*$^-$] [*sup35-n*]. Precisely this method of incompatibility analysis was used previously to detect the synthetic lethality of *sup45* mutations and the [*PSI*$^+$] prion [114]. In all cases, viable diploid [*PSI*$^+$] [*SUP35*] [*sup35-n*] strains were obtained (Figure 16B). Thus, in the system used, our results demonstrated the absence of synthetic lethality of *sup35-n* and prion [*PSI*$^+$] mutations in the presence of the wild-type *SUP35* allele.

3.2.3 Haploid and diploid strains retain prion aggregates [*PSI*$^+$] in the presence of *sup35-n* mutations and the wild type *SUP35* gene

Analysis of the suppressor phenotype in yeast strains containing the *ade1-14* nonsense mutation in their genome is often used to test for the presence of the [*PSI*$^+$] prion (for review see [141]). Because *sup35-n* mutants have the same suppressor phenotype as the [*PSI*$^+$] prion, we assessed the presence of *sup35* aggregates using SDD-AGE.

In *sup35-n* mutant cells, along with the full-length Sup35 protein, its shortened Sup35-n variant is present. We hypothesized that if the prion is retained in mutants, [*PSI*$^+$] aggregates may differ from wild-type Sup35 aggregates due to the incorporation of Sup35-n in both haploid and diploid [*PSI*$^+$] strains.

As a result of the analysis, Sup35 aggregates were detected in all [*PSI*$^+$] [*SUP35*/*sup35-n*] haploid cells, while they were absent in the control [*psi*$^-$] strain (Figure **17**A). In addition, the size of Sup35 aggregates in mutant cells did not differ from the wild type. Thus, in the presence of the wild-type *SUP35* gene*, sup35-n* mutations do not affect the properties of the [*PSI*$^+$] prion in haploid cells.

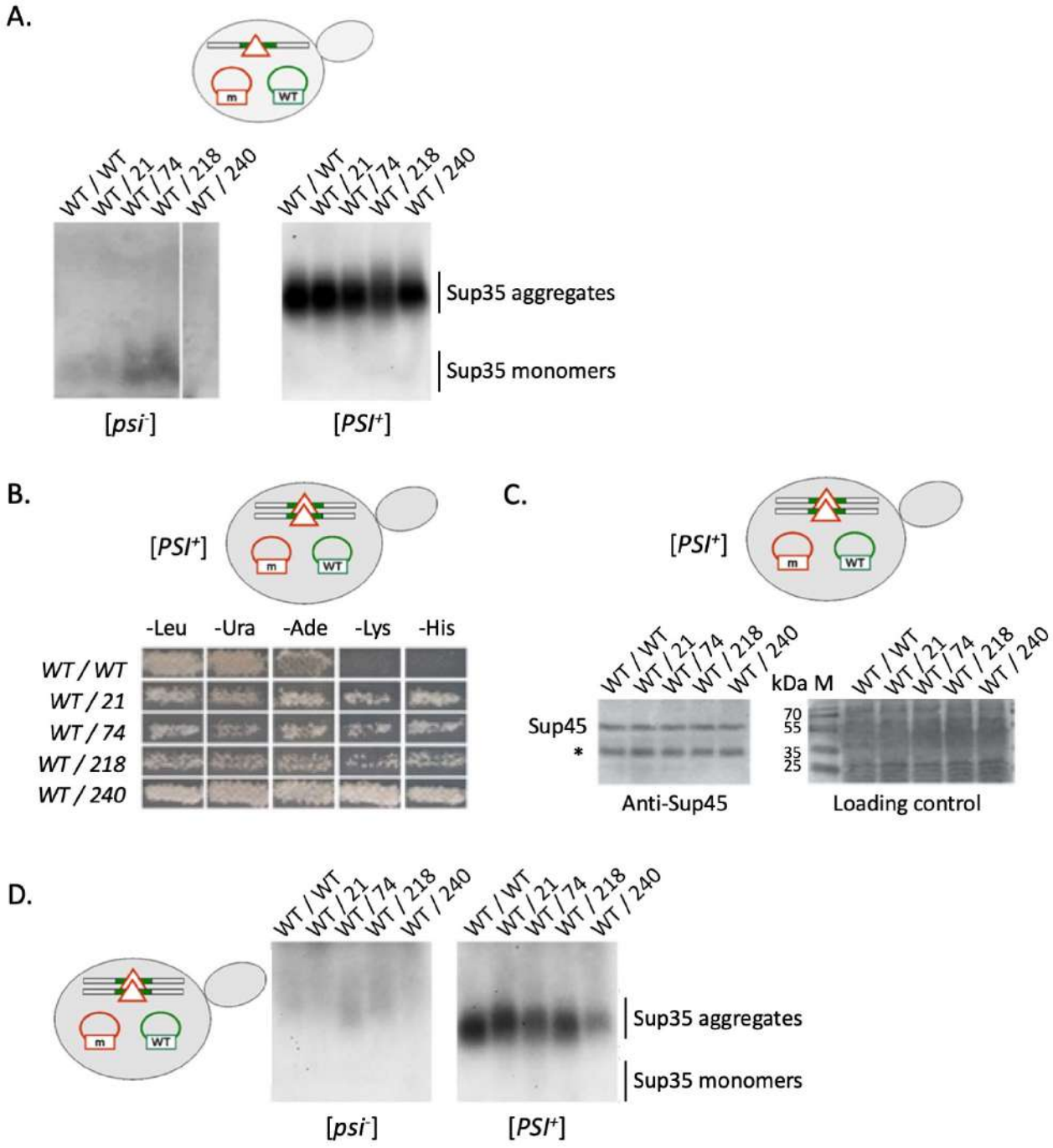

Figure 17. Haploid and diploid [*PSI*$^+$] strains retain Sup35 protein aggregates in the presence of *sup35-n* mutations and wild-type *SUP35* gene. A. Nonsense mutations *sup35-n* do not affect the size of [*PSI*$^+$] aggregates in the presence of the wild type *SUP35* allele in haploid strains. Yeast cell lysates from transformants containing the plasmid with the *SUP35* gene in combination with the plasmid carrying the indicated mutant allele were characterized by SDD-AGE followed by Western blotting with anti-Sup35 antibodies. **B.** [*SUP35*/*sup35-n*] [*PSI*$^+$] diploids are able to grow on media selective for nonsense suppression after 5 days of incubation at 26 °C. Media without leucine (-Leu) or uracil (-Ura) were used to control the presence of both plasmids. Growth on media without adenine (-Ade), histidine (-His), and leucine (-Lys) indicates the presence of strong suppressors in the strains. Ten independent transformants were tested in each case, representative results are shown. **C.** Diploid [*PSI*$^+$] strains containing two plasmids have the equal Sup45 amount. The same transformants shown in panel B were tested by Western blotting with anti-Sup45p antibodies. The symbol (*) marks additional complementary protein bands (besides Sup45) that result from nonspecific interactions of the antibodies used with other proteins. Coomassie staining of the same gel was used as a loading control. **D.** Diploid

[*PSI*$^+$] strains in the presence of *sup35-n* mutations and the wild-type *SUP35* gene allele are characterized by the presence of Sup35 protein aggregates. Lysates from diploid cells shown in panel B were characterized by SDD-AGE followed by Western blotting with antibodies to Sup35 protein.

In diploid [*PSI*$^+$] [*sup35-n*] strains, we monitored the nonsense-suppressor phenotype (Figure **17**B) and Sup45 protein levels before checking for Sup35 aggregates, which, as in previous similar experiments, did not differ from wild type in the mutants (Figure **17**C). According to SDD-AGE results, the prion was conserved in all [*PSI*$^+$] [*sup35-n*] mutants (Figure **17**D). Some heterogeneity in the size of Sup35 aggregates as well as the amount of protein incorporated into them could be noted. In addition, [*PSI*$^+$] [*sup35-240*] cells had fewer aggregates than the other mutants. We repeated SDD-AGE with lysates of newly obtained [*PSI*$^+$] [*sup35-n*] diploid strains several times, but did not find a significant difference in the content of aggregated Sup35 between them.

3.2.4 Nonsense mutations *sup35-21*, *-74* and *-218* lead to lethality of haploid [*PSI*$^+$] strains

As shown above, we tested the phenotype and presence of Sup35 aggregates in haploid and diploid strains [*PSI*$^+$] [*sup35*/*SUP35*] and found no differences between them and wild-type strains [*PSI*$^+$] [*SUP35*/*SUP35*]. We hypothesized that the effects of the mutations might be masked in the presence of the *SUP35* allele, so the next task was to obtain [*PSI*$^+$] mutants carrying only the *sup35-n* alleles.

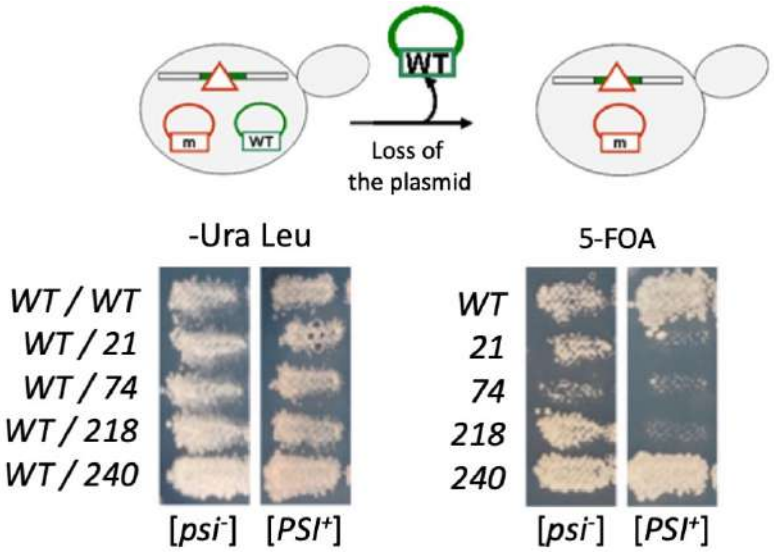

Figure 18. The combination of the [*PSI*$^+$] prion with *sup35-21*, *sup35-74*, or *sup35-218* mutations results in haploid cell lethality. Strains [*psi*$^-$] 7A-D832 and [*PSI*$^+$] 10-7A-D832 [*SUP35*/*sup35-n*], were replica plated on 5-FOA medium to select cells that retained only the [*LEU2 sup35-n*] plasmid. Only in the case of the *sup35-240* mutation, viable [*PSI*$^+$] cells were selected after five days of incubation at 26 ºC.

In haploid strains [*SUP35*/*sup35-n*], loss of plasmid [*SUP35 URA3*] on 5-FOA medium was performed. We observed incompatibility of the *sup35-21*, *sup35-74*, and *sup35-218* mutations and the prion [*PSI*$^+$] — such cells were not viable (Figure 18). Control transformants [*psi*$^-$] [*sup35-n*] were able to grow on 5-FOA medium, but with different efficiency. Strains with *sup35-21* and *sup35-74* mutations had the worst growth on the medium with 5-FOA.

3.2.5 The *sup35-240* mutation leads to the [*PSI*$^+$] prion loss in haploid strains in the absence of the wild type *SUP35* gene

In viable [*PSI*$^+$] cells collected from the *sup35-240* haploid strain (Figure 18), we tested for the nonsense suppressor phenotype and the presence of Sup35 aggregates. The selected cells were able to suppress the nonsense mutations *ade1-14*, *his7-1* and *lys2-739* (Figure 19A), indicating that the *sup35-240* allele is conserved after loss of the plasmid carrying the *SUP35* gene. The presence of the mutation was also confirmed by SDS-PAGE with [*PSI*$^+$] [*sup35-240*] haploid cells lysates (Figure 19B). The mutant strain contained a reduced amount of the full-length Sup35 protein compared to wild-type

cells. According to SDD-AGE results, haploid [*PSI*$^+$] [*sup35-240*] strains lack Sup35 aggregates (Figure 19C). Thus, these strains retained the mutation and lost the prion.

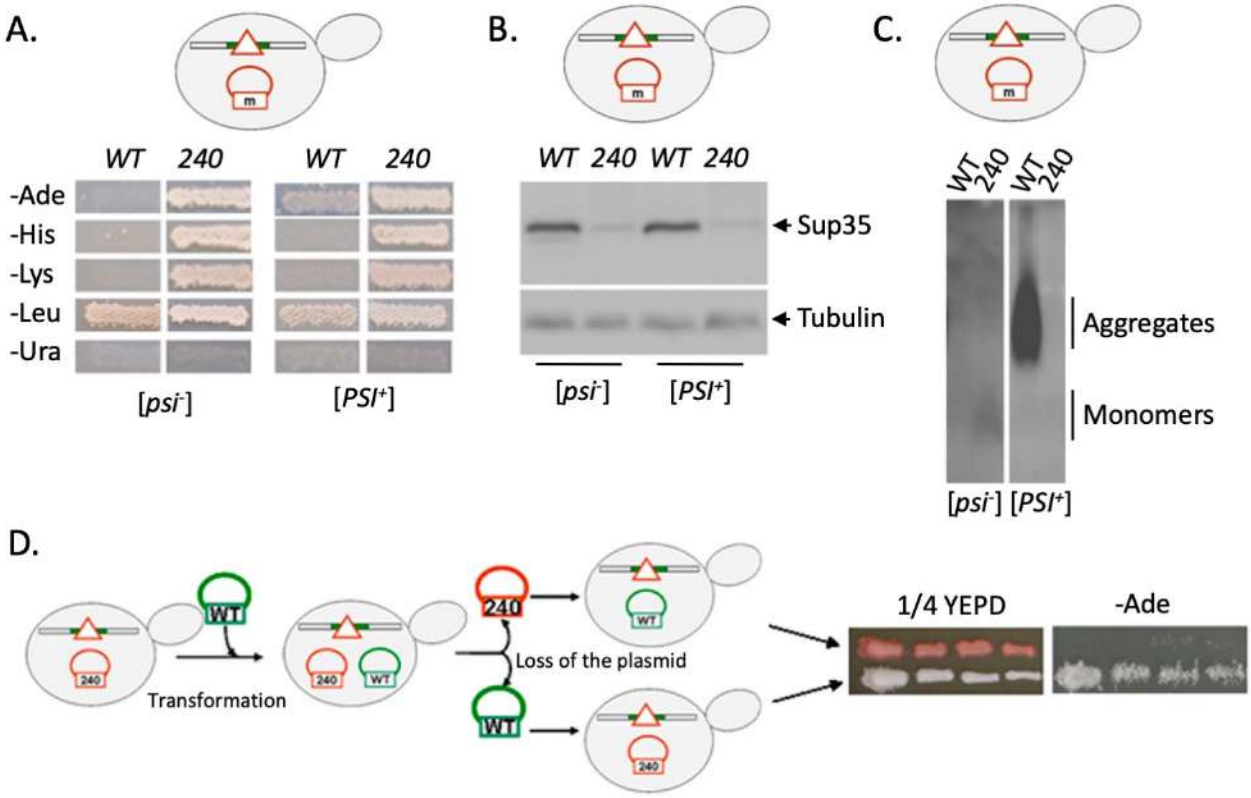

Figure 19. The *sup35-240* mutation leads to the [*PSI*$^+$] prion loss. A. The *sup35-240* mutation is retained in haploid [*PSI*$^+$] and [*psi*$^-$] strains after loss of the plasmid carrying the wild-type *SUP35* gene. Cells selected from 5-FOA medium (Figure 18) were plated onto media without adenine (-Ade), histidine (-His), and lysine (-Lys) to test for a nonsense-suppressor phenotype, and onto media without leucine (-Leu) and uracil (-Ura) to confirm for the presence of plasmid [*LEU2*] and absence of plasmid [*URA3*], respectively. Growth of strains for five days at 26 ºC is presented. **B, C.** The presence of the *sup35-240* mutation in haploid [*PSI*$^+$] and [*psi*$^-$] strains is confirmed by a decrease in the amount of Sup35 protein in cells (B) and results in the loss of Sup35 aggregates (C). Lysates from cells shown in Figure 18 were used for Western blotting with antibodies to Sup35 protein. Hybridization with antibodies to tubulin (Tub) was used as a loading control. Both proteins are indicated by arrows. **D.** The *sup35-240* mutation results in loss of prion in haploid [*PSI*$^+$] strains. Haploid [*PSI*$^+$] strains selected from 5-FOA medium (Figure 18) were transformed with a plasmid carrying the wild-type *SUP35* gene, whereupon spontaneous loss of [*sup35-n LEU2*] or [*SUP35 URA3*] plasmids was performed. Growth of cells bearing only one plasmid on 1/4 YPD or adenine-lacking medium (-Ade) after 5 days of incubation at 30 °C is shown.

To test the hypothesis that the *sup35-240* mutation leads to the [*PSI*$^+$] prion loss, we performed an experiment in which the [*sup35-240 LEU2*] cells were re-transformed with the [*SUP35 URA3*] plasmid (Figure 19D). This was followed by spontaneous loss of plasmids on complete YPD medium. Cells that retained the [*sup35-240 LEU2*] plasmid

had a nonsense-suppressor phenotype, while cells with the [*SUP35 URA3*] plasmid were characterized by its absence (Figure 19D).

Thus, in haploid [*PSI*$^+$] strains, plasmids carrying the mutant alleles *sup35-21*, *sup35-74*, and *sup35-218* are unable to replace the plasmid with the wild-type *SUP35* gene, which leads to the lethality of the corresponding cells on 5-FOA medium. In [*PSI*$^+$] cells, substitution for a plasmid with the *sup35-240* mutation results in prion loss.

3.2.6 Mutation incompatibility *sup35-n* and prion [*PSI*$^+$] in diploid cells depends on the method of obtaining such cells

We hypothesized that haploid and diploid yeast strains may differ in their compatibility with the prion [*PSI*$^+$]. To test this assumption, we used diploid strains that differed in the way they were obtained: transformation of diploid strains or mating of the corresponding haploid strains (see Figure 14).

In the first case, diploid strains [*PSI*$^+$] and [*psi*$^-$] bearing the [*SUP35 URA3*] plasmid were transformed with [*sup35-n LEU2*] plasmids. Viable [*PSI*$^+$] [*SUP35*/*sup35-n*] cells were obtained. We confirmed the nonsense-suppressor phenotype and the presence of [*PSI*$^+$] factor in the transformants by SDD-AGE followed by Western blotting with anti-Sup35 antibodies (data not shown). Diploid transformants [*SUP35*/*sup35-n*] were replica plated on a 5-FOA medium. Only in the case of the *sup35-240* mutation, the [*PSI*$^+$] [*sup35-n*] strain was obtained. The other mutations upon loss of the [*SUP35 URA3*] plasmid could not maintain the viability of diploid [*PSI*$^+$] transformants (Figure 20A). It should be noted that the efficiency of plasmid loss with the wild-type *SUP35* gene was also reduced in [*psi*$^-$] cells with *sup35-21* and *sup35-74* mutations (Figure 20A), which corresponds to the results obtained on haploid strains (see Figure 18).

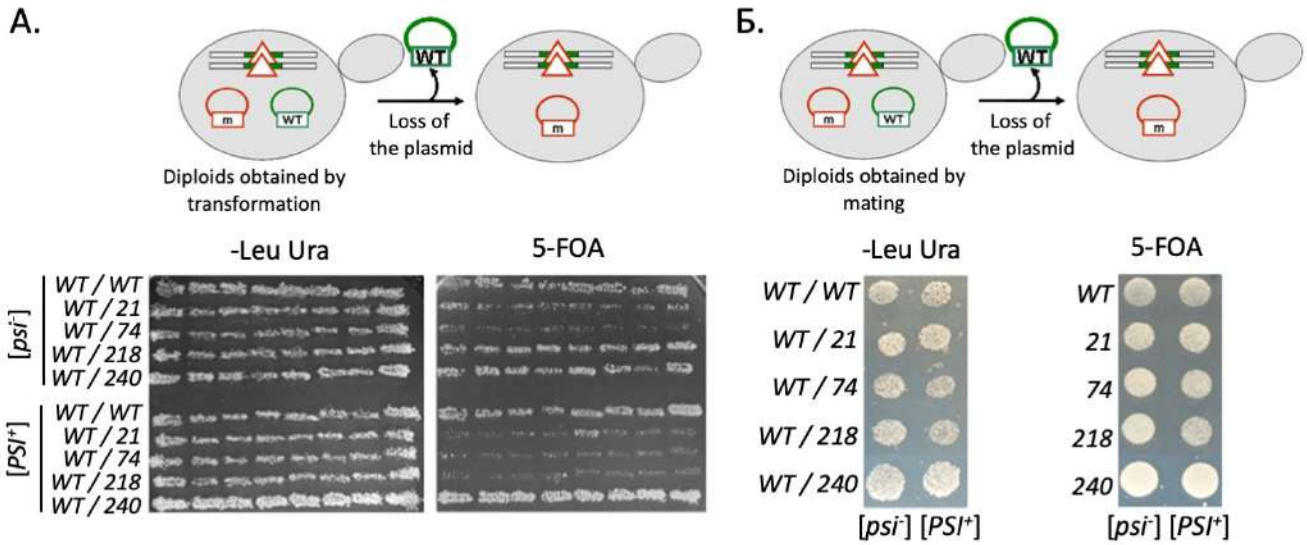

Figure 20. Lethality of the [*PSI*$^+$] diploids depends on the technique used to obtain [*SUP35*/*sup35-n*] cells. A. Cells bearing *sup35-n* mutations are lethal in combination with the [*PSI*$^+$] prion in diploid cells obtained by transformation. Diploid [*PSI*$^+$] and [*psi*$^-$] cells containing a plasmid with the wild-type *SUP35* gene were transformed with plasmids with *sup35-n* mutations. After growth of the resulting transformants on medium selective for both plasmids (left panel), cells were printed on 5-FOA medium for subsequent selection of clones that had lost the plasmid with the *SUP35* gene (right panel). Growth of eight independent transformants after seven days of incubation at 26 ºC is shown. **B.** Cells bearing *sup35-n* mutations are viable in combination with the prion [*PSI*$^+$] in diploid cells obtained by the mating of [*PSI*$^+$] [*SUP35*] strain with [*psi*$^-$] [*sup35-n*] strains. Left panel represents the growth of diploids on the medium selective for both plasmids, right panel shows that all cells are able to lose [*URA SUP35*] plasmid on 5-FOA media after 7 days of incubation at 26 °C. Eight independent transformants were tested in each case, representative results are shown.

In the second case, diploid strains were obtained by mating haploid [*psi*$^-$] [*sup35-n*] mutants with the [*PSI*$^+$] [*SUP35*] strain. All [*PSI*$^+$] [*SUP35*/*sup35-n*] mutant diploid strains obtained by mating are able to lose the plasmid with the wild-type *SUP35* gene (Figure 20B). The data suggest that the incompatibility between *sup35-n* mutations and [*PSI*$^+$] prion depends rather on the method of obtaining [*PSI*$^+$] [*SUP35*/*sup35-n*] cells than on the ploidy of the strains used, as it might initially appear. The [*psi*$^-$] [*sup35-n*] strains had the ability to adapt to mutations in the absence of prion and thereby increase the viability of diploid [*PSI*$^+$] [*sup35-n*] cells.

3.2.7 The *sup35-n* mutations change the properties of the prion [*PSI*$^+$] in diploid strains

Diploid strains [*PSI*$^+$] [*sup35-n*] obtained by crossing proved to be viable. We checked whether the mutation and prion are actually retained in such cells.

Diploid [*PSI*$^+$] [*sup35-n*] strains retain a nonsense-suppressor phenotype (Figure **21**A), confirming the presence of *sup35-n* mutations after loss of the plasmid carrying the wild-type *SUP35* gene.

As a result of SDD-AGE, we found either the absence or reduction of Sup35 aggregates in diploid strains [*PSI*$^+$] [*sup35-n*] (Figure **21**B, left panel). When we repeated this experiment with the newly obtained diploid mutants [*sup35-21*], [*sup35-74*], and [*sup35-218*], we observed heterogeneity in the size and number of Sup35 aggregates (Figure **21**C). At the same time, the [*sup35-240*] mutant had no Sup35 aggregates in all experiments. We hypothesize that Sup35-n proteins, when integrated into Sup35 aggregates, change the properties of the [*PSI*$^+$] factor. Also, the reduced amount of full-length Sup35 protein probably has an additional restriction on the maintenance of aggregates in the cell. In addition, we do not exclude the presence of possible additional inherited modifiers (see below) that affect the genetic background of cells and thus the phenotype. According to this, we can observe the presented variability.

To confirm the loss of the [*PSI*$^+$] prion or its maintenance in diploid cells, we performed an experiment in which we replaced plasmids with *sup35-n* mutations with a plasmid with the wild-type *SUP35* gene. Diploid cells [*sup35-n*] were transformed with [*SUP35 URA3*] plasmids. Lysates of the obtained diploid transformants [*SUP35*/*sup35-n*] were used for SDD-AGE. We hypothesized that with the return of the wild-type *SUP35* gene copy and, consequently, the appearance of sufficient amounts of full-length Sup35 protein in the cell, the aggregates might acquire their former characteristics (see Figure 17D). Nevertheless, we again detected either the absence or change in the properties of [*PSI*$^+$] aggregates, as was shown for diploid [*sup35-n*] strains (Figure 21B, middle panel). Next, we performed spontaneous loss of [*sup35-n LEU2*] plasmids in the resulted transformants and obtained diploid [*SUP35*] strains. All but one were characterized by the absence of Sup35 aggregates and a nonsense-suppressor phenotype (Figure 21B, right panel). Cells derived from the original diploid [*sup35-74 LEU2*] strain, had pink color of colonies on 1/4 YPD medium, which corresponded to the trace amounts of Sup35 protein aggregates detected by SDD-AGE (Figure 21B).

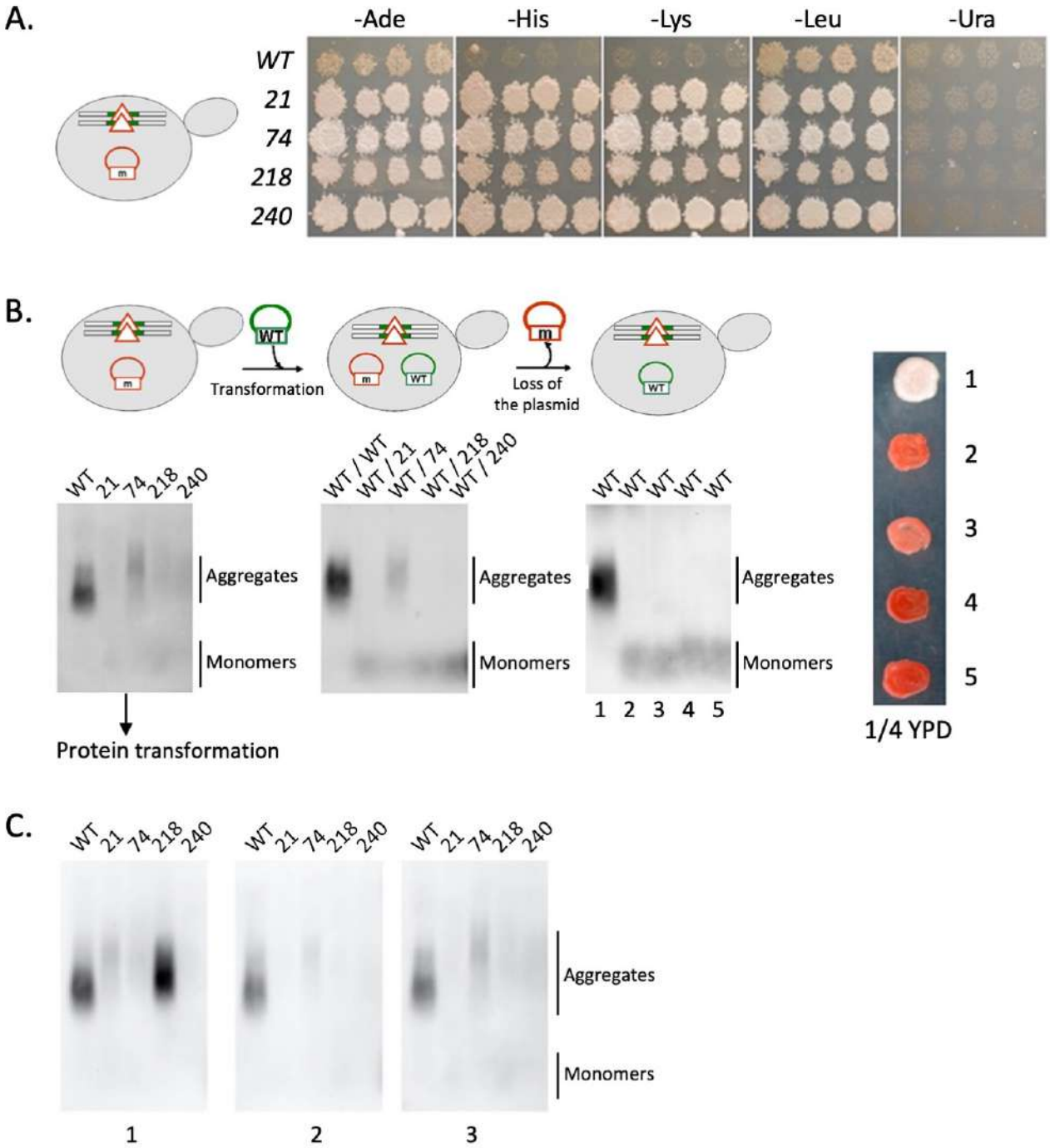

Figure 21. The *sup35-n* mutations alter the properties of the [*PSI*$^+$] prion in diploid strains. A. Nonsense-suppressor phenotype of [*PSI*$^+$] [*sup35-n*] diploid strains. Yeast cells selected in [*PSI*$^+$] background, as shown in Figure 20B, were plated on selective media to test nonsense suppression, as well on media lacking leucine (-Leu) or uracil (-Ura) to confirm the presence of [*sup35-n LEU2*] and the absence of [*SUP35 URA3*] plasmid, respectively. Four independent clones in each case are shown after 5 days of incubation at 26 °C. **B.** The presence of *sup35-n* mutations leads to modification of [*PSI*$^+$] aggregates properties in diploid strains. Results of SDD-AGE followed by immunoblotting with anti-Sup35 antibodies for lysates of diploid strains carrying different plasmids are presented. Left, cells selected on Figure 20B contain only plasmid with mutation. Middle, these cells were transformed by [*SUP35 URA3*] plasmid. Right, spontaneous loss of [*sup35-n LEU2*] plasmid was performed. The right panel represents the growth of transformants bearing only [*SUP35 URA3*] plasmid after loss of mutant plasmid on 1/4 YPD medium after 5 days of incubation at 26 °C. Numbers correspond to the lanes of the last SDD-AGE gel. **C.** [*PSI*$^+$] aggregates change their properties in the presence of *sup35-n* mutations in diploid strains. Cells selected as shown in Figure 20B were used for preparation of lysates followed by SDD-AGE. Three independent sets (1-3) of clones were analyzed.

Based on these results, we hypothesized that destabilization of the [*PSI*$^+$] factor with subsequent loss of Sup35 aggregates occurs in the presence of *sup35-n* mutations. We also do not exclude the possibility that *sup35-n* mutations may alter the [*PSI*$^+$] prion variant.

3.2.8 Sup35 protein aggregates in [*sup35-n*] mutants have reduced infectivity compared to the wild type

The change in the size of Sup35 aggregates (Figure 21B, left panel) and the absence of a prion phenotype after loss of [*sup35-n URA3*] plasmids (Figure 21B, right panel), led us to hypothesize that *sup35-n* mutations may lead to a change of [*PSI*$^+$] prion variant. To test this hypothesis, we isolated Sup35 aggregates from diploid [*PSI*$^+$] [*sup35-n*] strains and used them for protein transformation of [*psi*$^-$] cells. 1-OT56 and 2-OT56 strains were used as recipients, instead of 7A-D832, which had a low transformation efficiency. For the positive control, transformation was performed with Sup35NM fibrils, which were obtained *in vitro* by Maksyutenko E.M. The negative control was transformation with vector pRS316 or lysates of diploid [*psi*$^-$] strain D1691. The resulting transformants were phenotyped on 1/4 YPD medium (Figure 22A). Although transformation by lysates was performed with low efficiency, we were able to select suppressive clones. The nonsense-suppressor phenotype of all white and pink colonies was curable on the medium with the addition of GdnHCl (Figure 22B), which proved the presence of [*PSI*$^+$] prion in such cells.

We found that aggregates of the Sup35 protein of diploid [*PSI*$^+$] [*sup35-n*] strains have a reduced infectivity compared to the wild type [*PSI*$^+$] [*SUP35*] (Figure 22A). This observation is consistent with our initial assumption that the mutations studied alter the prion variant. The high heterogeneity of control transformant phenotypes may indicate that the diploid [*PSI*$^+$] strain D1692 contains a “cloud of prion variants” [11]. From this point of view, we can hypothesize that the presence of *sup35-n* mutations does not directly alter the variant factor [*PSI*$^+$], but leads to the selection of specific variants from

the existing cloud. However, this mechanism can also be considered as a change in the prion variant.

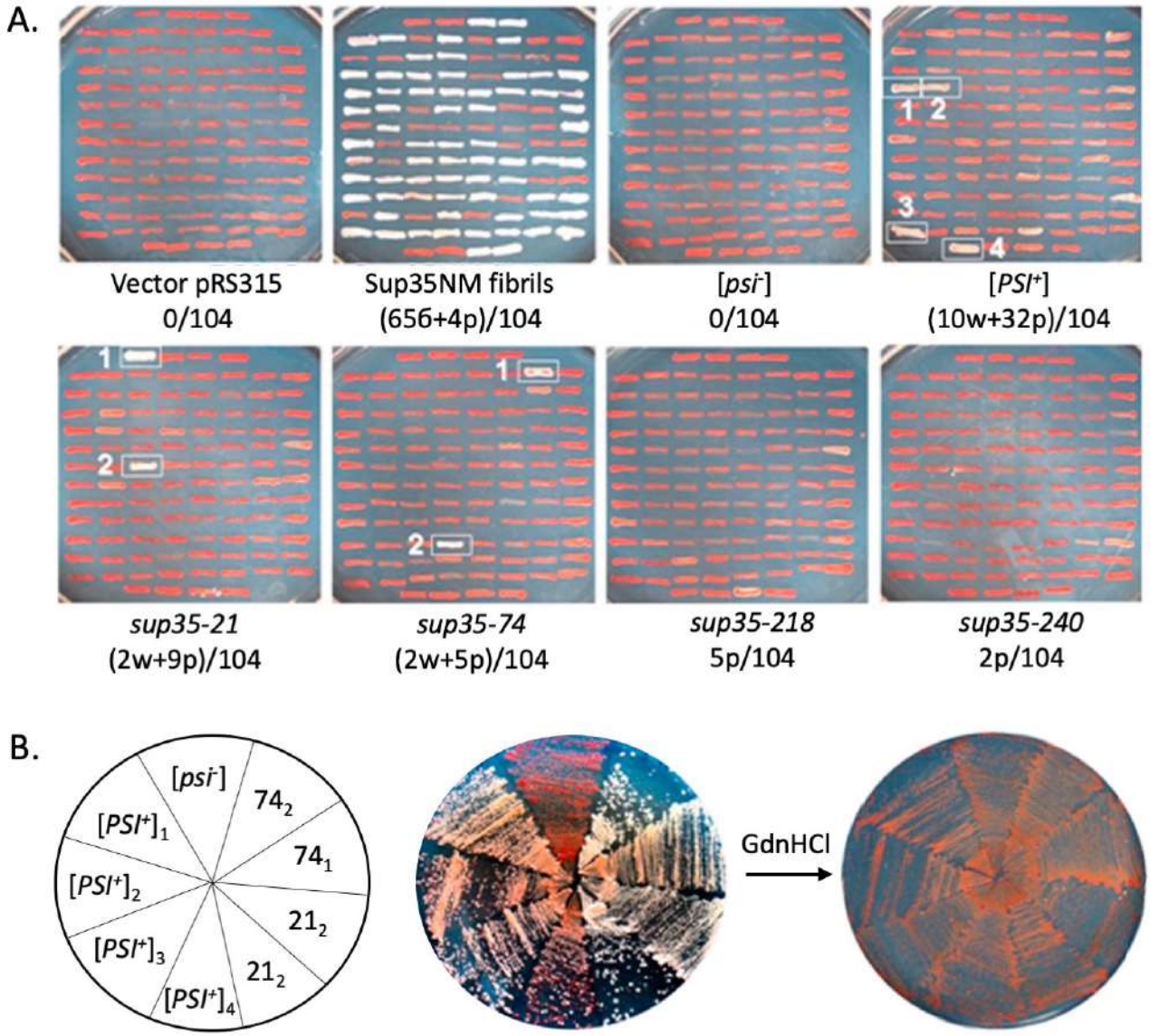

Figure 22. Sup35 protein aggregates from diploid [*PSI*$^+$] [*sup35-n*] strains have reduced infectivity compared with wild-type [*PSI*$^+$] [*SUP35*]. A. Infectivity of whole cell lysates of diploid strains D1691 [*psi*$^-$], D1692 [*PSI*$^+$] bearing *SUP35* or *sup35-n* mutant plasmid (selected as shown on Figure 20B), was determined by transformation into 1-OT56 [*psi*$^-$] [*PIN*$^+$]. Control transformation was performed with either vector pRS316 alone, or with fibrils of Sup35NM. Transformants were selected on SC-Ura media, and then replica plated on 1/4 YPD. In each case, growth of 104 independent transformants after 5 days of incubation at 30 °C is shown. The number of white (w) or pink (p) clones is shown below. **B.** Strains exhibiting a nonsense-suppressor phenotype after protein transformation acquired the [*PSI*$^+$] prion. The white yeast colonies shown in panel A were subcloned on 1/4 YPD medium followed by growth on GdnHCl medium. After three days of colony growth at 30 ºC the second passage on fresh GdnHCl medium was performed. After the third passage on GdnHCl medium, colonies were subcloned on 1/4 YPD medium to test the suppressor phenotype. The schematic diagram on the left shows the positions of the strains on the plates.

Thus, diploid [*PSI*$^+$] cells obtained by mating and carrying the mutant allele *sup35-21*, *sup35-74*, or *sup35-218* are able to maintain the prion. At the same time, it changes its properties, which is manifested in an increase in the size of Sup35 protein aggregates and their loss after replacement of the [*LEU2 sup35-n*] plasmid with a plasmid carrying the wild-type *SUP35* gene. Diploid cells containing the *sup35-240* mutation lose the [*PSI*$^+$] prion.

3.3 Mutation *sup35-240* prevents [*PSI*$^+$] prion propagation

3.3.1 The short protein Sup35-240 is included in the [*PSI*$^+$] prion aggregates

To determine the cause of the [*PSI*$^+$] prion loss in the presence of the *sup35-240* mutation, we conducted a series of experiments using the chimeric protein construct Sup35-240-GFP. The first task was to determine whether the Sup35-240-GFP incorporated into Sup35 aggregates in haploid [*PSI*$^+$] strains. The Sup35NM-GFP construct and the GFP protein were used as controls. We found that during overproduction, the Sup35-240-GFP protein decorates pre-existing [*PSI*$^+$] aggregates (Figure 23A). The fluorescent foci in the case of Sup35NM-GFP and Sup35-240-GFP constructs had different morphology. Cells overproducing Sup35-NM-GFP were characterized by the presence of large fluorescent foci, whereas many small foci were observed in cells with Sup35-240-GFP.

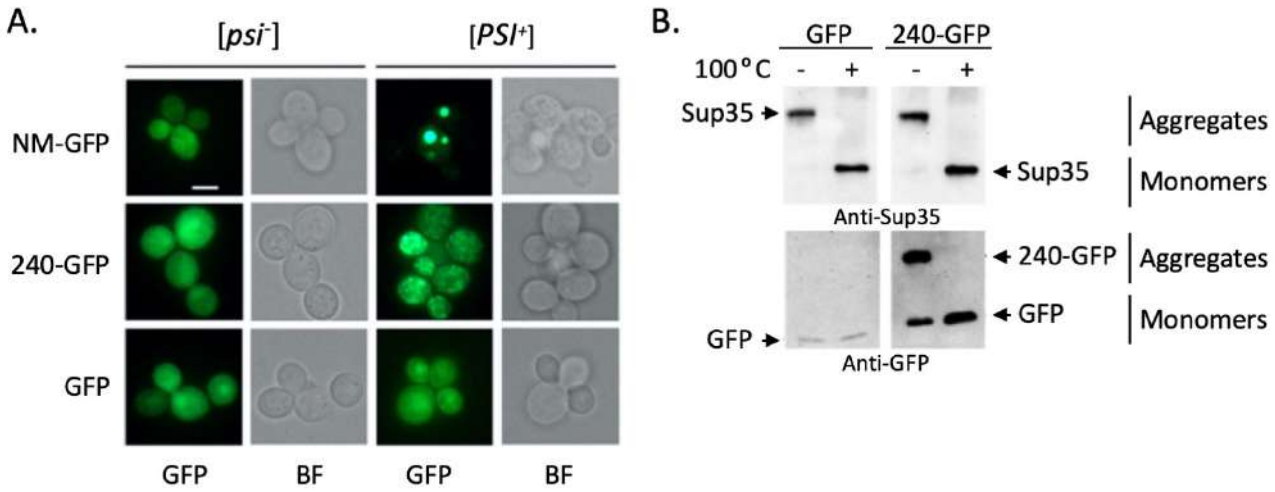

Figure 23. Sup35-240 protein is incorporated into [*PSI*$^+$] prion aggregates and leads to its destabilization. A. Strains 7A-D832 [*psi*$^-$] or 10-7A-D832 [*PSI*$^+$] were transformed with plasmids

pRS316-pCUP-GFP, pRS316-pCUP-SUP35NM-GFP and pRS316-pCUP-sup35-240-GFP. Logarithmic cultures of the obtained transformants were incubated with 50 μM $CuSO_4$ for two hours. Cells were visualized using fluorescence microscopy. Representative groups of cells are shown. Scale bar corresponds to 5 μm. BF — bright field. **B.** [*PSI*$^+$] cells in the presence of a vector with the *sup35-240-GFP* construct contain mainly aggregated Sup35 protein. Lysates of [*PSI*$^+$] and [*psi*$^-$] yeast cells shown in panel A were used to perform SDS-PAGE with preboiling. Immunoblotting was carried out with anti-Sup35 (upper panel) and anti-GFP (lower panel) antibodies. M — protein molecular weight marker (kDa) applied at the beginning of electrophoresis (M) and after boiling the gel (M*).

Next, we tested whether not only the colocalization of the Sup35-240-GFP protein is possible, but also its coaggregation with the full-length Sup35 protein in the [*PSI*$^+$] strain. SDS-PAGE with pre-boiling [130] was used for this task. In the presence of both control vector with GFP and plasmid with the Sup35-240-GFP construct, [*PSI*$^+$] cells contained a predominantly aggregated form of Sup35 protein (Figure 23B, top panel). Anti-Sup35 antibodies do not recognize the Sup35-240 protein, but using anti-GFP antibodies, we have shown that in [*PSI*$^+$] cells, the Sup35-240-GFP protein was predominantly present in the aggregated form (Figure 23B, bottom panel). Thus, the Sup35-240-GFP protein is probably included in the [*PSI*$^+$] prion aggregates formed by the full-length Sup35 protein, leading to the interruption of fibril growth (for more details, see Chapter 4. Discussion, 4.4). In this case, it may function as an anti-prion agent, causing the loss of [*PSI*$^+$] aggregates.

3.3.2 An increase in the Sup35-240 protein content in cells leads to the [*PSI*$^+$] prion destabilization

Since the Sup35-240-GFP protein is incorporated into Sup35 aggregates, we tested whether the properties of the [*PSI*$^+$] prion are altered when this protein is overproduced in cells. We compared the phenotypic characteristics of cells producing Sup35-240-GFP or GFP proteins for a long time. The [*PSI*$^+$] [*sup35-240-GFP*] cells were characterized by reduced nonsense-suppression, having pink colony formation on 1/4 YPD medium compared with control [*PSI*$^+$] cells (Figure 24). Thus, the presence of Sup35-240 protein leads to the destabilization of the [*PSI*$^+$] prion.

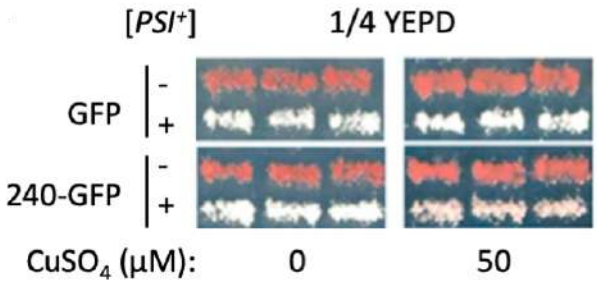

Figure 24. An increase in Sup35-240 protein destabilizes the [*PSI*$^+$] prion. The Sup35-240 protein destabilizes the [*PSI*$^+$] factor by incorporating into Sup35 protein aggregates. The transformants shown in **Figure 23**A were grown on plates in the presence of 50 μM $CuSO_4$ for three days and then replica plated on 1/4 YPD medium to test the efficiency of nonsense suppression. Six independent transformants were tested in each case. Representative results after five days of incubation at 30 ºC are shown.

3.3.3 Additional copy of the gene *SUP35* reduces the destabilizing effect of *sup35-240* mutation on the [*PSI*$^+$] prion

Previously, we did not observe any changes in [*PSI*$^+$] manifestation in diploid cells carrying simultaneously plasmids with *SUP35* and *sup35-240* alleles. To analyze this discrepancy, we performed an experiment in which we transformed the [*PSI*$^+$] strain (OT56) with a plasmid carrying the *sup35-240-GFP* construct in combination with the pRS315 vector or the pRSU1 [*SUP35*] centromeric plasmid. The combination of pRS316-pCUP1-GFP and pRS315 vectors was used as a negative control, and the pRSU1 plasmid combined with the pRS316 pCUP1-GFP vector was used as a positive control (Figure 25A). It should be noted that the studied effects were almost identical (possibly due to the presence of traces of copper in the medium). An additional copy of the *SUP35* gene protects the [*PSI*$^+$] prion from destabilization caused by temporary production of the Sup35-240-GFP protein. Thus, the effect of the *sup35-240* mutation depends on the ratio of the full-length and shortened Sup35 protein, which explains the preservation of [*PSI*$^+$] prion stability in diploid strains.

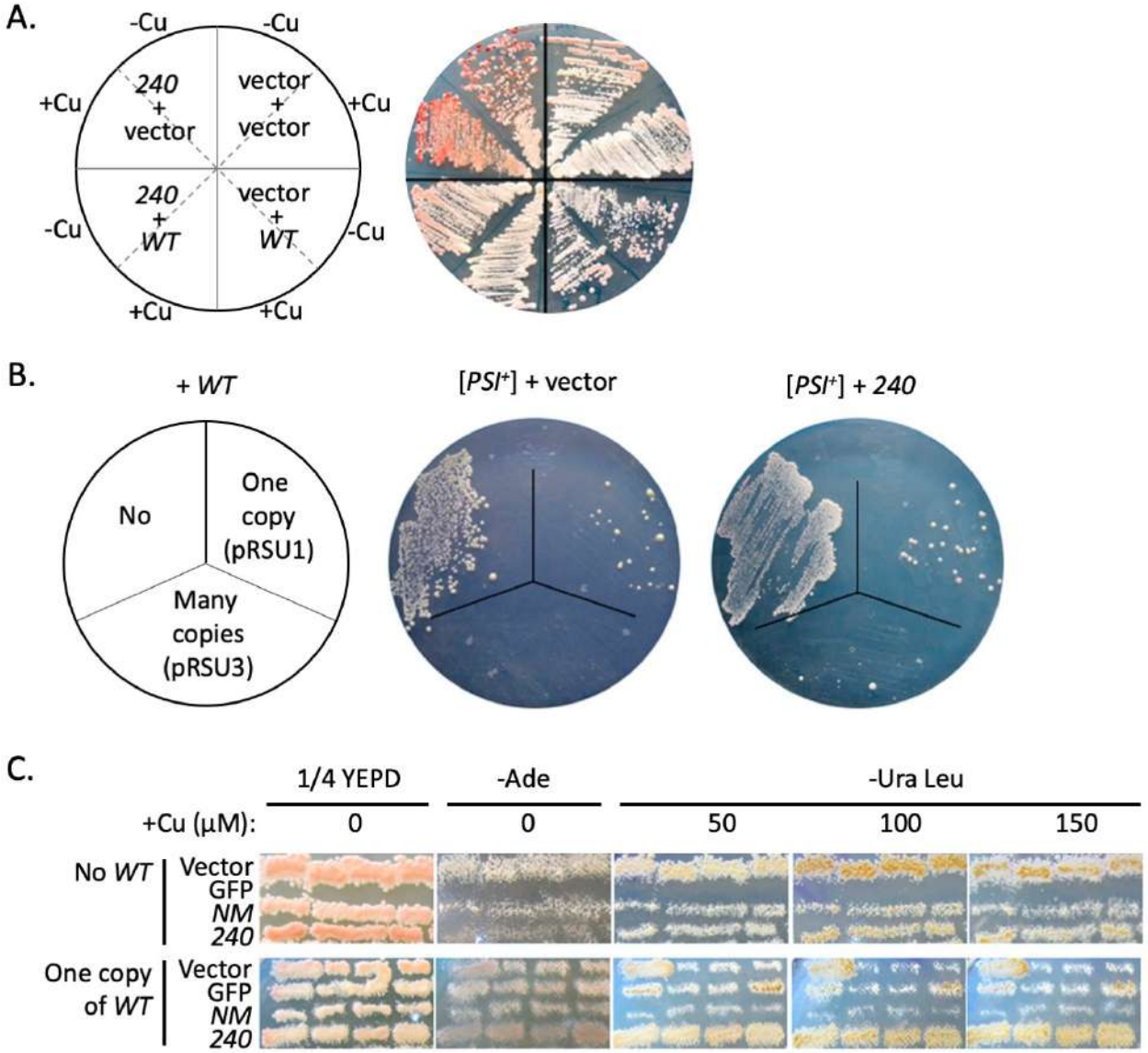

Figure 25. An additional copy of the *SUP35* gene protects the [*PSI*$^+$] prion from destabilization caused by temporary production of the Sup35-240-GFP protein. The [*PSI*$^+$] OT56 strain was transformed with a combination of the following vectors: pRS316-pCUP-GFP and pRS315 (vector + vector); pRS316-pCUP-GFP and pRSU1 (vector + *WT*); pRS316-pCUP-sup35-240-GFP and pRS315 (*240* + vector); pRS316-pCUP-sup35-240-GFP and pRSU1 (*240* + *WT*). Transformants were grown for 6 days on selective media under non-induced (– Cu) or induced (+ Cu) conditions (50 µM $CuSO_4$ was used), then transferred to 1/4 YPD medium and incubated for 5 days at 30 ºC. The schematic diagram on the right indicates the location of strains on the plate. **B.** Sup35-240 protein eliminates the toxicity associated with [*PSI*$^+$] prion. The OT56 ([*PSI*$^+$]) strain transformed with pRS316 or pRS316-pCUP-sup35-240-GFP vector was re-transformed with pRS315 (control), pRSU1 (one copy of *SUP35*) or pRSU3 (multicopy plasmid containing *SUP35*). Transformants were selected on SC–Leu Ura. In the case of combination of pRS316 with pRSU3 no transformants were selected, however in the presence of Sup35-240 ten transformants were recovered. The schematic diagram on the right indicates the location of strains on the plate. **C.** In [*PSI*$^+$] strains, in the presence of an additional copy of the *SUP35* gene, the effect of prion toxicity can be observed in all cases except the one where the plasmid pCUP-sup35-240-GFP is present. Strain OT56 ([*PSI*$^+$]) was transformed with the following plasmids: pRS315 (No *WT*); pRSU1 (One copy of *WT*). These plasmids were combined with either pRS316 (vector), pRS316-pCUP-GFP (GFP), pRS316-pCUP-NM-GFP (*NM*), or pRS316-pCUP-sup35-240-GFP (*240*). Transformants were grown for 6 days at 30 ºC.

It was previously shown that overexpression of the *SUP35* gene in [*PSI*$^+$] cells leads to toxicity [36]. It is noteworthy that this effect is evident even in the presence of an additional copy of the *SUP35* gene on the centromeric plasmid. In the presence of the Sup35-240 protein, the toxic phenotype completely disappeared (Figure 25B). When the [*PSI*$^+$] strain carrying the pRS316 vector was transformed with a multicopy plasmid carrying the *SUP35* gene, the transformants were not selected, possibly due to the effect of prion toxicity. Nevertheless, 10 transformants were selected in the presence of the pRS316-pCUP1-Sup35-240-GFP vector (Figure 25C). Thus, the Sup35-240 protein destabilizes the prion [*PSI*$^+$] even in the presence of full-length Sup in different yeast strains, but the effect depends on the amount of Sup35 protein.

Chapter 4. Discussion

Disruption of the eRF1 and eRF3 factors in yeast can manifest as suppression; therefore, the study of suppressor mutations in the *SUP45* and *SUP35* genes provides additional information about the processes occurring during translation termination. The *sup45-n* and *sup35-n* missense and nonsense mutations have been characterized in our laboratory. The *sup45-n* and *sup35-n* mutations studied are in a weak nucleotide context that facilitates stop codon reading and does not result in lethality in the absence of suppressor tRNAs ([30, 159], see review [216]). The subject of this study was *sup35-n* nonsense mutations, which we tested for compatibility with the [*PSI*$^+$] factor [217].

4.1 Consequences of nonsense mutations in a vital *SUP35* gene

First of all, we characterized the manifestation of mutations on the genotypic background used. As a result of nonsense suppression, [*sup35-n*] mutants synthesize full-length Sup35 protein, which provides them viability in the absence of the wild-type *SUP35* gene. However, some of the mutants studied contain very low amounts of the full-length Sup35 protein compared to the wild type: 1 % for *sup35-240*, 3 % for *sup35-21* and 10 % for *sup35-74* (Figure 10B). The *sup35-244* mutation in another study resulted in the greatest decrease in the Sup35 abundance, which was only 0.5 % [30]. According to this characteristic, [*sup35-n*] mutants remind [*PSI*$^+$] strains, in which the abundance of soluble full-length Sup35 protein is reduced due to its incorporation into [*PSI*$^+$] prion aggregates [172, 173]. According to various authors, [*PSI*$^+$] cells contain 0.5-2 % of the soluble Sup35 protein compared to [*psi*$^-$] cells [164, 241], which is comparable to what is observed in the case of [*sup35-n*] mutants. However, cells with *sup35-n* differ from [*PSI*$^+$], since along with full-length Sup35, they synthesize short proteins. It is possible that the maintenance of viability in mutants containing very low amounts of Sup45 or Sup35 is due to the fact that proteins with amino acid substitutions resulting from PTC nonsense-suppression are able to provide translation termination more efficiently than wild-type proteins. This assumption was confirmed in our work for the *sup35-240* mutation. We analyzed the effects of substituting Q for K or Y in the 56th position of the Sup35 amino asid sequence. It was previously shown that it is the a.c.o. of Q, K or Y that

is utilized by the yeast cell in the case of nonsense suppression of the UAA stop codon [16]. The degradation rate of Sup35-Q56Y protein was the same as that of wild-type Sup35, while Sup35-Q56K protein was more stable (Figure 11A). In addition, in the presence of Sup35-Q56K protein, cells survived at higher concentrations of paromomycin, up to 1 mg/mL (Figure 11B). It is noteworthy that such substitutions did not lead to stabilization of the Sup45 protein in [*sup45-n*] mutants. In contrast, in the case of the *sup45-102* and *sup45-105* mutations, the Sup45-Y102Q and Sup45-E105Y proteins, respectively, were less stable than Sup45, and the stability of the Sup45-E105Q protein did not differ from the wild-type protein. These results allowed to explain the reason why in the presence of the *sup45-102* mutation, cells were characterized by the lowest levels of full-length Sup45 protein with the most efficient nonsense suppression (20-30 %) among all [*sup45-n*] mutants. The [*sup45-105*] mutant showed the lowest efficiency of nonsense suppression (5-8 %), but the amount of Sup45 was at an average level [112].

In [*sup35-n*] mutants, short Sup35-n proteins are formed along with the full-length Sup35 protein. The question arises about their potential role in the cell. In the presence of the *sup35-240*, *-74*, and *-218* mutation, Sup35-n proteins consist of 56, 129, and 181 a.a.r., respectively, and do not include a C-domain. The Sup35-21 protein (422 a.a.r.) includes only part of the C-domain (part of the GTPase sites is missing and no eRF1 binding site) (Figure 6). Even small deletions of the Sup45 and Sup35 C-domains are known to impair their functions and cell viability [65, 69, 213]. Thus, Sup35-n proteins cannot exhibit GTPase activity and participate in translation termination. At the same time, Sup35-n (possibly, except for Sup35-240) should retain the ability to bind to the ribosome, as it has been shown for various Sup35 fragments that include at least a whole N- or C-domain [213].

The N-domain of Sup35 (Sup35N) is responsible for the ability to prionize Sup35 to form the [*PSI*$^+$] determinant [53, 173, 212], therefore, Sup35-n proteins may participate in its maintenance or potentially even contribute to *de novo* prion formation. [*PSI*$^+$] has a very low rate of spontaneous formation (estimated as 6×10^{-7}) in cells with endogenous Sup35 production [135]. However, the frequency of [*PSI*$^+$] occurrence may increase

under various stresses and in old cells [202, 219, 228]. Also, temporary overproduction of only the prion domain (or part of it) promotes more efficient formation of Sup35 aggregates than overproduction of the full-length protein [24, 53, 169, 174], so potentially the presence of short Sup35-n proteins may increase the rate of spontaneous [*PSI*$^+$] formation. Indeed, according to literature data, production of short Sup35 proteins in the presence of wild-type protein (both proteins were obtained without overproduction, i.e. under their own promoter) in some cases leads to an increase in the frequency of *de novo* [*PSI*$^+$] formation by several orders of magnitude. The maximum frequency of spontaneous [*PSI*+] formation was observed in cells with fragment 1-112 of the Sup35 protein — 8×10^{-2} [52], which was comparable to the frequency of [*PSI*$^+$] *de novo* occurrence upon overexpression of the *SUP35* gene from a multicopy plasmid — 10^2 [184] and exceeded this value for an additional copy of the *SUP35* gene by about 6000-fold. Fragment 1-49 in this study did not lead to the [*PSI*$^+$] induction; fragments 1-64, 1-123, and 1-252 were approximately 15, 241, and 4 times more effective than the presence of an additional copy of the *SUP35* gene [52].

Two subdomains, a Q/N-rich region (a.a.r. 1-40) and an OR region (a.a.r. 41-97), were previously localized in Sup35N [128] and the critical role of the Q/N site and the first two ORs (a.a.r. 1-64) in prion propagation was shown [50, 118, 169]. Sup35 protein containing only this site aggregates and induces [*PSI*$^+$] at a level comparable to Sup35N [118, 169]. Sup35 with a partial deletion of the second OR (a.a.r. 1-57) can still aggregate and induce [*PSI*$^+$] but with reduced efficiency, and a protein completely lacking the second OR (a.a.r. 1-49) does not induce [*PSI*$^+$] *de novo* but can decorate pre-existing Sup35p aggregates [169], which is consistent with the results of the work described above [52].

Based on the assumptions above, in cells in the presence of the wild-type *SUP35* gene, the Sup35-240 protein (56 a.a.r.) potentially aggregates and induces [*PSI*$^+$], but with reduced efficiency compared to Sup35N (123 a.a.r.). The Sup35-74 protein forms Sup35 aggregates and would be most effective in inducing spontaneous [*PSI*$^+$], but cells may not be able to maintain it stably during divisions because Sup35-74 (like Sup35-240) lacks part of the M-domain with the Hsp104 chaperone binding site (a.a.r. 129-148) [92].

The Sup35-218 protein (181 a.a.r.), like Sup35-21 (422 a.a.r.) includes a site for Hsp104, and potentially [*PSI*$^+$] aggregates are stably inherited, but given the results of the above-mentioned work, the frequency of prion formation would be approximately the same as that observed in the presence of an extra copy of the wild-type *SUP35* gene in the cell [52].

4.2 Compatibility of *sup35-n* mutations and [*PSI*$^+$] prion

In this work, we analyzed the effects of combining *sup35-n* mutations with [*PSI*$^+$] prion in haploid and diploid strains of *S. cerevisiae*. We showed that such effects depend on the presence of a wild-type copy of the *SUP35* gene, the method of yeast clone selection, and the position of the nonsense mutation.

In contrast to *sup45-n*, *sup35-n* mutations do not lead to synthetic lethality of cells with the strong prion variant [*PSI*$^+$] in the presence of the wild-type *SUP35* gene. In our experiments, both diploid and haploid [*PSI*$^+$] [*SUP35*/*sup35-n*] strains were viable. The lethality of [*PSI*$^+$] [*SUP45*/*sup45-n*] mutants was caused by a simultaneous decrease in the activity of Sup45 (due to mutation) and Sup35 (due to prionization) proteins. Lethality was shown to be dose-dependent for both the *SUP45* and *SUP35* genes [114]. Haploid and diploid mutants of [*SUP35*/*sup35-n*], despite reduced Sup35 level, have not different amounts of Sup45 compared to wild type ([30] and Figure 10C), possibly contributing to the maintenance of viability. However, loss of the plasmid carrying the wild-type *SUP35* gene in these strains results in significantly reduced Sup35 protein levels and consequently different outcomes.

The viability of [*PSI*$^+$] [*sup35-n*] strains in our experiments depended on the genetic method used to generate them. Transformation of haploid or diploid [*PSI*$^+$] [*SUP35 URA3*] cells by a plasmid carrying the [*sup35-n LEU2*], followed by selective loss of the [*SUP35 URA3*] on 5-FOA medium (Figure 14A) leads to cell death. With nonselective plasmid loss in [*PSI*$^+$] [*SUP35*/*sup35-n*] mutants on complete YEPD medium, we could select [*LEU2*] cells that either retained the *sup35-n* mutation but lost the prion or (which occurred in rare cases) maintained the prion but instead of the *sup35-n* mutation retained the wild-type *SUP35* gene as a result of recombination between the

[*sup35-n LEU2*] and [*SUP35 URA3*] plasmids (data not shown). The exception was the *sup35-240* mutation, which maintained the viability of the original [*PSI*$^+$] strains by causing prion elimination (see below). This implies that in the absence of the *SUP35* gene, *sup35-n* mutations are incompatible with [*PSI*$^+$] in both haploid and diploid cells obtained by transformation (Figure 18 and Figure 20). Thus, in our study, the lethality of [*PSI*$^+$] strains manifested with a significant decrease in only one translation termination factor, eRF3, while eRF1 had normal cellular level. However, diploid strains with a similar combination of *sup35-n* and prion resulting from mating (Figure 14B) can lose wild-type *SUP35*, retaining only the mutant allele (see below).

On the one hand, cell lethality in the combination of *sup35-n* and [*PSI*$^+$] is predictable, since the amount of full-length Sup35 protein is significantly reduced in [*sup35-n*] mutants compared to the wild type (Figure 10), with a portion of functional Sup35 escaping into prion aggregates. Thus, the cause of [*PSI*$^+$] [*sup35-n*] cell lethality is an excessive reduction of functional Sup35 protein (less than 0.5 %, compared to 100 % in the wild type) (see above).

On the other hand, the prion might be expected to exhibit allosuppressor activity toward *sup35-n* mutations. As a result, the strength of suppression would increase and the cell would produce more functional Sup35 protein sufficient to maintain cell viability. However, we did not observe this effect and assumed that the strong variant of the prion [*PSI*$^+$], which we used in our work (Figure 15), is toxic to [*sup35-n*] cells, or becomes more toxic in the presence of short Sup35-n proteins. According to literature data, Sup35-74, Sup35-218, or Sup35-21 proteins are more efficiently incorporated into Sup35 aggregates (see above) and can potentially alter the [*PSI*$^+$] variant. Remarkably, more than half of the prion variants studied previously in screening were lethal or extremely toxic to cells [154].

In addition, the *sup35-2* nonsense mutation was previously shown to be incompatible with the [*ETA*$^+$] factor (weak variant [*PSI*$^+$]) [140, 241]. The *sup35-2* mutation results in the replacement of the UUG codon with the UAG stop codon, so a shortened Sup35 protein consisting of 109 a.a.r. (along with a reduced amount of full-length Sup35) is synthesized [241]. When mating the yeast lines [*ETA*$^+$] [*SUP35*] and

[*eta*$^-$] [*sup35-2*], 97 % of meiotic segregants [*sup35-2*] were not-viable. However, genetic analysis showed that only 70 % of viable offspring were [*ETA*$^+$]. This paradox, namely the high lethality of [*sup35-2*] mutants, is explained by the fact that the Sup35-109 protein is incorporated into [*ETA*$^+$] aggregates more efficiently than full-length Sup35 and contributes to their stability during cell divisions. As a result, the eRF3 factor becomes insufficient to maintain [*sup35-2*] cells viability [241].

In our case, it should be noted that if the *sup35-n* mutations change the [*PSI*$^+$], then we could expect a difference in viability between different [*sup35-n*], but we did not detect it — cells are 100 % lethal in the case of *sup35-21*, *-74*, and *-218* mutations. We also found no change in the size of in both haploid and diploid mutant strains [*PSI*$^+$] [*SUP35*/*sup35-n*] compared to the wild type (see below).

All diploid strains [*PSI*$^+$] [*sup35-n*] obtained by mating haploid [*PSI*$^+$] [*SUP35*] cells with [*psi*$^-$] [*sup35-n*] mutants and subsequent loss of [*SUP35 URA3*] plasmid [*3*] were viable. Initially, it was assumed that haploid [*psi*$^-$] [*sup35-n*] cells select additional mutations at other loci during growth, which improve the viability of such mutants. However, whole genome sequencing of [*psi*$^-$] [*sup45-n*] and [*psi*$^-$] [*sup35-n*] strains showed that the mutants did not have common single-nucleotide polymorphisms, insertions, or deletions. A systematic increase in the number of copies of plasmids carrying the mutant *sup45-n* or *sup35-n* alleles was found. In the case of [*sup35-n*] mutants, this ensures a sufficient level of the functional Sup35 protein in the cell, even in the presence of the [*PSI*$^+$] prion. Thus, adaptation of yeast cells to mutations in translation termination factor genes occurs by increasing the number of plasmids carrying mutant alleles or as a result of amplification of the number of copies of the translation termination factor gene [147]. The mutants also showed an increase in the expression of cyclin genes (at stages G1, S, and G2) regulated transcriptionally and a decrease in the efficiency of the anaphase-stimulating complex. Analysis of the literature data allowed to suggest that the increase in plasmid copy number probably occurs due to slowing down the cell cycle, defects, and delays in the synthetic phase, which allows intensive plasmid replication and accumulation of various proteins [3]. Presumably, it is due to this adaptation that diploid [*PSI*$^+$] [*SUP35*/*sup35-n*] strains obtained by mating, unlike haploid and diploid

[*PSI*$^{+}$] [*SUP35*/*sup35-n*] strains obtained by transformation, survive after the loss of a plasmid with the *SUP35* wild-type gene.

4.3 Mutations *sup35-n* change the properties of the [*PSI*$^{+}$] prion

All diploid [*sup35-n*] mutant strains (except [*sup35-240*]) retained [*PSI*$^{+}$] after loss of the *SUP35* gene, but some prion properties were altered (Figure 21B and Figure ***22***). By the size and number of Sup35 aggregates, we detected a variation even among clones with the same *sup35-n* mutation (Figure 21BC). We hypothesized that in [*PSI*$^{+}$] [*sup35-n*] mutant diploid strains, the prion is destabilized by the deficiency of full-length Sup35 protein, as well as by the incorporation of Sup35-n proteins into its aggregates.

To explain the change in the properties of [*PSI*$^{+}$] in the presence of *sup35-n* mutations, we assume at least two mechanisms. First, short Sup35-n proteins can be incorporated into prion aggregates with high efficiency, which in turn can lead to a change in their size. In addition to what has been said above about short Sup35-n proteins (see subsection 4.1), it has also been previously shown that various N-terminal Sup35 fragments ($Sup35_{1\text{-}133}$, $Sup35_{1\text{-}154}$ and $Sup35_{1\text{-}240}$) are capable of forming aggregates consisting of only such short Sup35 proteins [174]. However, it is not clear from these data whether the short Sup35 proteins can be incorporated into the [*PSI*$^{+}$] formed by the full-length protein, or whether they form aggregates independently. Aggregates of the $Sup35_{1\text{-}240}$ protein (designated Sup35NM in the cited work) are able to induce prionization of the full-length soluble Sup35 protein *in vitro* [173]. This suggests that this fragment is incorporated into [*PSI*$^{+}$] aggregates. In addition, it was shown that N-terminal Sup35 fragments can decorate pre-existing Sup35 aggregates in [*PSI*$^{+}$] strains [50]. However, in these experiments, short Sup35 proteins were fused to the M-GFP reporter protein, so this may potentially affect the interaction with Sup35.

The second mechanism suggests that incorporation of Sup35-n into [*PSI*$^{+}$] aggregates affects their fragmentation by cellular chaperones. Thus, the *sup35-n* mutation can lead to an increase in the size of aggregates and reduce the number of prion seeds in the cell. In turn, daughter cells will receive fewer propagons, which will lead to a weakening of the [*PSI*$^{+}$] factor. In the case of the *sup35-74* allele second mechanism is in

good agreement with the identification of the Hsp104 binding site in Sup35. The Sup35 protein site from 129 to 148 a.a.r. is important for the interaction of these two proteins and elimination [*PSI*$^+$] under conditions of Hsp104 overproduction. At the same time, the Sup35 protein, which lacks this region, is able to support the prion [92]. Similar *sup35-74* results obtained for the *sup35-218* mutation suggest the possible presence of an unknown chaperone site in the missing part of the M-domain of the Sup35-218 protein (a.a.r.182-256). However, this mechanism is not suitable for explaining the effect of the *sup35-21* mutation, since Sup35-21 includes the entire NM-domain of Sup 35.

Additional experiments are needed to verify the two mechanisms mentioned above. Thus, we have demonstrated that *sup35-n* nonsense mutations can affect the properties of [*PSI*$^+$], which in turn can lead to the formation of a new prion variant. The protein transformation experiment showed that the [*PSI*$^+$] variants maintained in diploid [*sup35-n*] mutants differ from the prion in the wild-type [*SUP35*] strain (Figure 22A). The detailed molecular mechanisms of this phenomenon require further studies.

Importantly, it was not possible to detect Sup35-n proteins in diploid [*SUP35*/*sup35-n*] cells (Figure 13B). Both factors, eRF1 and eRF3, are required for efficient translation termination [203], so it is possible that in cells carrying [*SUP35*/*sup35-n*] plasmids together with two genomic copies of *SUP45*, the amount of Sup35 is insufficient to show the antisuppressor effect (translation termination on the PTC resulting from the *sup35-n* nonsense mutation). In this case, the shortened Sup35 protein will not be produced by the cell (or its amount will be low and not detected in Western blots). However, the case may be more complicated. It was previously shown in Ian Stansfield's lab that transcriptional regulation of the *sup45-18* nonsense allele leads to increased expression of the full-length Sup45 protein [14]. The authors were unable to prove this hypothesis by Western blotting because their antibodies did not recognize the N-terminal part of Sup45, but their data indicate that the presence of the *sup45-n* allele may affect the amount of full-length Sup45. We can hypothesize the existence of a similar mechanism in the case of *sup35-n* mutations.

4.4 Allele *sup35-240* as a new *PNM* mutation

The *sup35-240* mutation is of most interest in this work because although it could compensate for the absence of the wild-type *SUP35* allele, prion loss occurred in [*PSI*$^+$] [*sup35-240*] strains. This resulted in the selection of only [*psi*$^-$] cells carrying the *sup35-240* allele. The minimal fragment of Sup35, which can be included in existing prion aggregates, consists of the first 49 a.a.r., and its slight elongation to the 57th residue increases the efficiency of this process [169]. In our work, using the same approach, we demonstrated that the short protein Sup35-240 (56 a.a.r.) decorates Sup35 aggregates (Figure 23A). Thus, it can be assumed that the inclusion of the Sup35-240 protein in prion aggregates leads to their destabilization, or to the formation of non-heritable Sup35 aggregates. Alternatively, single molecules of Sup35-240 can stick to aggregate ends, and thus interrupt fibril growth. Such a mechanism has been proposed to explain the interspecies barrier to prion transmission [5]. We find the first hypothesis more appropriate because Sup35-240 is incorporated into [*PSI*$^+$] aggregates with high efficiency (Figure 23). We previously hypothesized that in the [*PSI*$^+$] variant used in this study, the first 63-69 a.a.r. of the Sup35 protein are incorporated into the superpleated β-structure [18]. This means that Sup35-240 effectively incorporated into existing Sup35 aggregates, but it cannot properly copy their structure, which may lead to [*PSI*$^+$] destabilization. Unfortunately, we were unable to detect the Sup35-240 protein without GFP labeling in [*sup35-240*] strains using the available antibodies (Figure 10B). Nevertheless, we suppose that the truncated fragment is presented in the cells, because the effects of *sup35-240* and *sup35-240-GFP* construct for the corresponding protein are clearly detected and are the same (Figure 23).

The use of a short 61 a.a.r. chimeric Sup35 protein (Sup351-61-GFP) has been described in the literature to visualize pre-existing prion aggregates [118, 119] as well as to identifying types of prion strains [142]. It has been shown that prolonged overproduction of Sup351-61-GFP does not expel prion variants [VH-1] or [VK-1], but a small cure of [*PSI*$^+$] in cells carrying the [VL-1] variant has been observed [118, 119]. It has also been demonstrated that overproduction of Ure2p fragments, as well as its

fusion with GFP, cures the [*URE3*] prion [66, 67]. In addition, the conversion of normal human PrP protein to its prion form was inhibited by a peptide containing a conserved PrP sequence, both in the cell-free system [32] and in tissue culture cells [31].

Thus, we can conclude that the *sup35-240* mutation prevents the propagation of [PSI^+] and can be considered as a novel *PNM* mutation. Most Pnm proteins show a reduced ability to be incorporated into [PSI^+] aggregates *in vivo*, and the presence of some mutant proteins leads to solubilization of Sup35 aggregates [50].

4.5 Conclusion

The part of the *SUP35* gene in which the NM-domain of the Sup35 protein is encoded is saturated with potential stop codons (48% compared to 34% for the entire yeast genome) [216]. Consequently, the probability of spontaneous nonsense mutations in the corresponding region is high. Thus, we can assume that *sup35-n* mutations can be found in wild yeast strains. Apparently, the combination of *sup35-n* mutations with [PSI^+] prion is also possible, for example, by mating the corresponding haploid strains, or the appearance of a spontaneous mutation in diploid strains. However, in most cases [PSI^+] will be quickly excluded from the population. This fact is explained either by the lethality of the combination of *sup35-n* with prion (*sup35-21*, *sup35-74*, and *sup35-218*) or by the *PNM*-effect of the *sup35-n* mutation itself (*sup35-240*). If [PSI^+] is not eliminated for the above reasons, it will be weakened and eventually disappear from the population.

At least five spontaneous mutations in the *PRNP* gene (encodes the PrP protein) have been described, leading to the appearance of a stop codon instead of a sense codon [9]. All these mutations lead to the synthesis of the PrP protein shortened from the C-terminus, which lacks the GPI anchor binding to the cell surface. There are various experimental models for studying human prion diseases, among which mouse diseases are the most common. Most modern mouse models of prion diseases do not fully replicate the clinical, biochemical, and pathological features of human disease, although significant progress has been made in creating more accurate models [230]. However, the development of a successful model for studying the effects of nonsense mutations in the *PRNP* gene has not yet been reported. It is possible that yeast can serve as a successful

model for studying the effects of shortened prion protein sequences *in vivo*. In the future, such studies may offer a potential strategy for treating prion diseases in humans and animals.

Conclusions

1. When reading the stop codon resulting from the *sup35-240* mutation, full-length proteins may arise that differ in properties from the wild-type protein. The *sup35-240K* (Q56K) mutation leads to increased resistance of *S. cerevisiae* cells to paromomycin and stabilization of the Sup35-Q56K protein, while the *sup35-240Y* (Q56Y) mutation does not affect the properties of the Sup35 protein.
2. The method of obtaining diploid strains containing *sup35* nonsense mutations in combination with prion [*PSI*$^+$] (transformation or mating) determines their further viability.
3. Mutations *sup35-21*, *sup35-74*, and *sup35-218* are incompatible with the [*PSI*$^+$] prion in haploid and diploid strains obtained by transformation.
4. Mutations *sup35-21*, *sup35-74* and *sup35-218* change the properties of the [*PSI*$^+$] prion in diploid strains obtained by mating.
5. The nonsense mutation *sup35-240* leads to loss of the [*PSI*$^+$] prion and disappearance of its aggregates in haploid yeast strains, and therefore it can be considered as a new *PNM* ([*PSI*$^+$] No More) mutation.

Abbreviations

a.a. — amino acid (sequence)

a.a.r. — amino acid residue

bp — base pair

eEFs — eukaryotic elongation factors

eIFs — eukaryotic initiation factors

eRFs — eukaryotic termination factors

GdnHCl — guanidine hydrochloride

kb — kilobase

NMD — nonsense-mediated mRNA decay

OR — oligopeptide repeats

ORF — open reading frame

PKA — protein kinase A

PTC — premature termination codon

SDS — sodium dodecyl sulfate

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