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Novel Genetic and Epigenetic Suppressors of Nonsense Mutations in Saccharomyces Cerevisiae

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ABSTRACT

Nonsense suppression is a process that involves the inhibition of the phenotypic manifestation of nonsense mutations typically occurring due to a decrease in the translation termination fidelity. In the present study, we described nine novel genetic suppressors of nonsense mutations (*ABF1, GLN3, FKH2, MCM1, MOT3, NAB2, NAB3, REB1*, and *VTS1*) and characterized one epigenetic suppressor, [*NSI*+], which has the properties of a yeast prion. All of these suppressors were shown to affect nonsense suppression only in strains with decreased functional activity of eRF3. The obtained data expand the knowledge on the modulation of nonsense suppression in *S. cerevisiae* and suggest that the system of translation termination contains a number of precisely interacting genetic and epigenetics regulators.

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CONTENTS

LIST OF INCLUDED ARTICLES

PI   Nizhnikov A.A. et al. [*NSI*+] determinant has a pleiotropic phenotypic manifestation that is modulated by *SUP35*, *SUP45*, and *VTS1* genes. // Current Genetics. 2012B. Vol. 58. P. 35–47.

PII   Saifitdinova A.F.*, Nizhnikov A.A.*, Lada A.G., Rubel A.A., Magomedova Z.M., Ignatova V.V., Inge-Vechtomov S.G., Galkin A.P. [*NSI*⁺]: a novel non-Mendelian suppressor determinant in *Saccharomyces cerevisiae* // Current Genetics 2010. Vol. 56. P. 467–478 (*these authors contributed equally).

PIII   Nizhnikov A.A., Z.M. Magomedova, A.F. Saifitdinova, S.G. Inge-Vechtomov, A.P. Galkin. Identification of genes encoding potentially amyloidogenic proteins that take part in the regulation of nonsense suppression in yeast *Saccharomyces cerevisiae* // Russian Journal of Genetics: Applied Research. 2012A. Vol. 2. P. 399–405.

PIV   Nizhnikov A.A., Kondrashkina A.M., Antonets K.S., Galkin A.P. Overexpression of genes encoding asparagine-glutamine-rich transcriptional factors causes nonsense suppression in *Saccharomyces cerevisiae* // Ecological Genetics. 2013B. Vol. 11. P. 49–58.

PV   Nizhnikov A.A., Kondrashkina A.M., Galkin A.P. The analysis of interactions of prion-like determinant [*NSI*+] with *SUP35* and *VTS1* genes in *Saccharomyces cerevisiae.* // Russian Journal of Genetics. 2013A. (accepted for publication).

OTHER PUBLICATIONS

AI   Nizhnikov A.A., Magomedova Z.M., Kondrashkina A.M., Galkin A.P. [NSI⁺] determinant affects readthrough of termination codons by reducing the SUP45 mRNA amounts // Proceedings of the 4th EMBO meeting (2012, Nice, France), 2012, Vol.1, P.97.

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AIII   Nizhnikov A.A. [*NSI⁺*] — novel non-chromosomal factor in *Saccharomyces cerevisiae* // LAP Lambert, 2011, 78p (In Russian).

AIV   Nizhnikov A.A., Galkin A.P. Identification of novel prion-like determinant [*ABA*] in *Saccharomyces cerevisiae* // Proceedings of the V Congress of Vavilov Society of geneticists and breeders (June 21–27, 2009, Moscow, Russia), Vol.2 P.158 (In Russian).

AV   Nizhnikov A.A., Magomedova Z.M. Genomic screening for identification of structural gene of prion-like factor [*NSI⁺*] in yeast *Saccharomyces cerevisiae* // Proceedings of the XVII international conference of students and young scientists "Lomonosov" (2010, Moscow, Russia), Vol.1 P.89 (In Russian).

AVI   Nizhnikov A.A., Galkin A.P. Search for genes affecting phenotypic manifestation of [*NSI⁺*] prion-like factor in *Saccharomyces cerevisiae* // Biology – The Science of XXI Century: XIV International School-Conference of Young Scientists (April 19–23, 2010, Pushchino, Moscow), Book of abstracts, Vol.2 P.163 (In Russian).

AVII   Nizhnikov A.A., Galkin A.P. The demonstration of infectious properties of [NSI⁺] prion-like factor by protein transformation // Biology — The Science of XXI Century: XIV International School-Conference of Young Scientists (April 19–23, 2010, Pushchino, Moscow), Book of abstracts, Vol.2 P.248 (In Russian).

AVIII Nizhnikov A.A., Lada A.G., Saifitdinova A.F., Inge-Vechtomov S.G., Galkin A.P. [*PIN⁺*] factor affects the phenotypic manifestation of [*NSI⁺*] determinant in *Saccharomyces cerevisiae* // Biology – The Science of XXI Century: XV International School-Conference of Young Scientists (April 18–22, 2011, Pushchino, Moscow), Book of abstracts, Vol.1 P.22 (In Russian).

AIX   Nizhnikov A.A., Magomedova Z.M., Rubel A.A., Inge-Vechtomov S.G., Galkin A.P. The penenotypic manifestation of [*NSI*⁺] determinant is mediated by the *SUP35* and *SUP45* genes in *Saccharomyces cerevisiae* // Biology – The Science of XXI Century: XV International School-Conference of Young Scientists (April 18–22, 2011, Pushchino, Moscow), Book of abstracts, Vol.1 P.23 (In Russian).

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1   INTRODUCTION

Nonsense suppression is a process that involves the inhibition of the phenotypic manifestation of nonsense mutations. Typically, nonsense suppression occurs due to a decrease in the translation termination fidelity, which results in the read-through of premature termination codons. This process is important not only for basic purposes but also in biomedical studies because a large number of human heritable diseases and cancers are associated with nonsense mutations (Pichavant et al., 2011; Keeling et al., 2012; for review see: Loudon, 2010). According to some researchers, the proportion of this type of diseases among heritable diseases is approximately 10 percent (for a review, see Bidou et al., 2012). The analysis of nonsense suppression is a very simple and convenient tool for the identification of genes and factors that affect the translation termination fidelity. Unfortunately, the phenotypic detection of read-through by nonsense suppression is only available in lower eukaryotes, such as the baker's yeast *Saccharomyces cerevisiae* and not in higher eukaryotes. In these organisms, the translational read-through can be detected through a relatively complicated and expensive dual-reporter assay (for example, see Cardno et al., 2009). Thus, *S. cerevisiae* has all the prerequisites to become one of the most studied organisms in the field of the regulation of nonsense suppression.

The study of this phenomenon in yeast began in 1960th. All of the suppressors that were identified were divided into two groups: codon-specific and omnipotent. Codon-specific suppression, which is the suppression of only one of three termination codons (UAA — ochre, UAG — amber, and UGA — opal) is caused by mutations in the anticodons of different tRNAs. Some mutant tRNAs (E, K, L, Q, S, and Y) were shown to possess suppressor activity (for a review, see Sherman, 1982). The mutations and overexpression of some tRNA-encoding genes were subsequently shown to cause nonsense suppression. In particular, the overexpression of tRNA^{Gln} (CAG, CUG and UUG) causes amber suppression (Weiss and Friedberg, 1986; Weiss et al., 1987). This effect can be explained by the possibility of non-canonical base pairing of U in the first position of the codon with G in the third position of the anticodon, as first predicted by the "wobble hypothesis" proposed by F. Crick (Crick, 1966). A similar effect was also shown for tRNA^{Trp} (Kim et al., 1990), which is encoded by six genes in yeast. Two of these genes are relatively strong multicopy opal suppressors, whereas two of the remaining genes are weak suppressors,

and the other two do not affect suppression. This difference appears to be related to the different trans-regulatory elements of these genes (Ong et al., 1997).

In contrast to codon-specific suppression, omnipotent suppression in yeast is mediated by a set of protein-encoding genes. Thus, *SUP45* and *SUP35* genes, which encode the translation termination factors eRF1 (Sup45) and eRF3 (Sup35), respectively (Zhouravleva et al., 1995; Stanfield et al., 1995; Frolova et al., 1994), were identified as recessive omnipotent suppressors (these genes were initially denoted by different names, such as *s1* and *s2)* (Inge-Vechtomov, 1964; for a review, see Wakem and Sherman, 1990). Additionally, a set of dominant and semi-dominant omnipotent suppressors, including *SUP44* and *SUP46*, was identified. *SUP44* was shown to be the allele of *RPS4* (All-Robyn et al., 1990) and *SUP46–RPS13* (Vincent and Liebman, 1992; Ono et al., 1981). A group of cycloheximide-resistance mutants *crl* (McCusker and Haber, 1988), which are partly localized in genes encoding the yeast proteasome (Gerlinger et al., 1997), was demonstrated to cause the omnipotent suppression. Mutations in *TEF2* encoding the translation elongation factor also lead to omnipotent suppression (Sandbaken and Culbertson, 1988).

Allosuppressors are another group that enhances suppression in the presence of other suppressors. For example, mutant alleles of *UPF1, UPF2,* and *UPF3*, which encode components of the system that regulates the degradation of mRNAs containing premature termination codons (nonsense-mediated mRNA decay), may enhance nonsense suppression (Ono et al., 2005; Ono et al., 1986). Mutations in the *PPQ1* gene, which encodes a type I serine-threonine phosphatase-like protein, lead to allosuppression in the presence of mutant *SUP45* (Vincent et al., 1994). Additionally, *PPZ2* was shown to be involved in the regulation of nonsense suppression efficiency (Ivanov et al., 2010).

The most notable feature of nonsense suppression in yeast is that it is modulated not only by genetic but also by epigenetic suppressors that are prions. Prions are proteins that can exist in two or more conformations, of which at least one has infective properties (Alberti et al., 2009, with modifications). To date, at least eight yeast prions and a set of prion candidates have been identified (Wickner, 1994; Derkatch et al., 1997; Derkatch et al., 2001; Du et al., 2008; Patel et al., 2009; Alberti et al., 2009; Rogoza et al., 2010; Suzuki et al., 2012; Halfmann et al., 2012). Typically, yeast prions form amyloids with infectious properties. Amyloids are protein aggregates that are enriched with ordered β-sheets stabilized by intermolecular hydrogen bonds (for a review, see, for example, Toyama and Weissman, 2012). Amyloids of yeast prions exhibit infectious properties due to their fragmentation by chaperones, which reduce the size and amplify the number of aggregates, thereby allowing their efficient transmission into the daughter cells. If chaperones are inactivated (for example, by the addition of guanidine hydrochloride to the culture medium), the size of the aggregates increases, and these larger aggregates cannot be efficiently transmitted into the daughter cells, which results in the loss of the prion (for a review, see, for example, Reidy and Masison, 2011).

[*PSI*⁺] was one of the first yeast prions identified. Initially, it was described as a non-chromosomal allosuppressor of some mutant tRNAs (Cox, 1965). Furthermore, [*PSI*⁺] was later shown to be the prion isoform of the translation termination factor eRF3 (Sup35) (Wickner, 1994; Ter-Avanesyan et al., 1994; Chernoff et al., 1993; Paushkin et al., 1997A

and B; Paushkin et al., 1996), which acts as a recessive omnipotent nonsense suppressor. There are a number of nonsense alleles that are efficiently suppressed by [*PSI*⁺], the most well-known of which is *ade1–14*$_{UGA}$. The mechanism through which [*PSI*⁺] prion causes the suppression of *ade1–14*$_{UGA}$ is currently quite clear. Normally, *ade1–14*$_{UGA}$ blocks the translation of Ade1p, which is a component of adenine biosynthesis. This results in the absence of growth of [*psi*⁻] strains on selective medium lacking adenine. The emergence of [*PSI*⁺] causes the aggregation of Sup35p, and this leads to its functional inactivation, which enhances the frequency of reading the nonsense codons as sense codons during translation. This allows the synthesis of Ade1p and partially restores adenine biosynthesis, which ultimately results in the growth of [*PSI*⁺] strains on medium lacking adenine (for a review see, for example, Crow and Li, 2011).

The second prion involved in the regulation of nonsense suppression efficiency is [*ISP*⁺], which is a prion isoform of the transcriptional factor Sfp1p (Rogoza et al., 2010; Volkov et al., 2002). This prion is an epigenetic antisuppressor of a combination of mutations in the *SUP35* and *SUP45* genes. Its manifestation can be suppressed by mutations in *PPZ1* or by the overexpression of *HAL3*, both of which can act as allosuppressors (Aksenova et al., 2007).

Taken together, a large amount of data has been compiled on the study of nonsense suppression in *S. cerevisiae*. In addition to codon-specific tRNA suppressors, at least five functional groups of proteins (some factors of transcription and translation, nonsense-mediated mRNA decay proteins, components of proteasome, and serine-threonine phosphatases) are involved in this process. Nevertheless, it is obvious that these data are relatively incomplete and need to be expanded. We recently described a novel non-chromosomal determinant [*NSI*⁺] in *S. cerevisiae* (Saifitdinova et al., 2010). [*NSI*⁺] causes nonsense suppression in strains expressing different variants of Sup35 (Saifitdinova et al., 2010; Nizhnikov et al., 2012B) with decreased functional activity (Nizhnikov et al., 2013A), but does not affect nonsense suppression in strains producing full-length Sup35 (Saifitdinova et al., 2010; Nizhnikov et al., 2012B). Taking these data into consideration, we proposed that modifications of Sup35 that affect its functional activity create a specific genetic background that allows the identification of novel suppressors. This thesis is based on the experimental analysis of this hypothesis with a focus on both the characterization of [*NSI*⁺] and the search for genes that affect nonsense suppression on the abovementioned genetic background.

The aim of this thesis was the search for and the characterization of novel genetic and epigenetic suppressors of nonsense mutations in *S. cerevisiae*.

The following questions were addressed:

1. Does [*NSI*⁺] have the properties of a yeast prion?

2. Which genes affect the phenotypic manifestation of [*NSI*⁺]?

3. What are the molecular properties of nonsense suppression in [*NSI*⁺] strains? What are the possible functions of the structural gene of [*NSI*⁺]?

4. Does the decrease in the functional activity of Sup35 allow the identification of novel suppressors, and what are these suppressors?

2 BRIEF DESCRIPTION OF THE RESULTS

The results of this thesis were published in five papers:

1. Nizhnikov A. A. et al. [*NSI*+] determinant has a pleiotropic phenotypic manifestation that is modulated by *SUP35*, *SUP45*, and *VTS1* genes. // Current Genetics. 2012B. V. 58. P. 35–47.

2. Saifitdinova A. F.*, Nizhnikov A. A.*, Lada A. G., Rubel A. A., Magomedova Z. M., Ignatova V. V., Inge-Vechtomov S. G., Galkin A. P. [*NSI*+]: a novel non-Mendelian suppressor determinant in *Saccharomyces cerevisiae* // Current Genetics 2010. V. 56. P. 467–478 (*these authors contributed equally).

3. Nizhnikov A. A., Z. M. Magomedova, A. F. Saifitdinova, S. G. Inge-Vechtomov, A. P. Galkin. Identification of genes encoding potentially amyloidogenic proteins that take part in the regulation of nonsense suppression in yeast *Saccharomyces cerevisiae*. // Russian Journal of Genetics: Applied Research. 2012A. V. 2. P. 399–405.

4. Nizhnikov A. A., Kondrashkina A. M., Antonets K. S., Galkin A. P. Overexpression of genes encoding asparagine-glutamine-rich transcriptional factors causes nonsense suppression in *Saccharomyces cerevisiae*. // Ecological Genetics. 2013B. V. 11. P. 49–58.

5. Nizhnikov A. A., Kondrashkina A. M., Galkin A. P. The analysis of interactions of prion-like determinant [*NSI*+] with *SUP35* and *VTS1* genes in *Saccharomyces cerevisiae*. // Russian Journal of Genetics. 2013A. (accepted for publication).

The main reason for beginning this thesis was the identification of the novel non-chromosomal determinant [*NSI*+] in our laboratory. This determinant, in addition to [*PSI*+] and [*ISP*+], is involved in the epigenetic control of nonsense suppression. We demonstrated that [*NSI*+] causes nonsense suppression in strains bearing the N-terminally deleted (*SUP35MC*) or modified (*Aβ-SUP35MC*) variant of the *SUP35* gene but is not found in strains bearing an intact copy of *SUP35*. [*NSI*+] exhibits a dominant non-Mendelian inheritance and reversible curability and may be transmitted

by protein transformation. Similarly to yeast prions, this determinant can be cured by the deletion or mutational inactivation of Hsp104 but not by the overproduction of Hsp104. [*NSI*⁺] reappears *de novo* at a rate of approximately 5×10^{-6}. The efficiency of the protein transformation of [*NSI*⁺] is 5–7%. The non-Mendelian inheritance of [*NSI*⁺] was confirmed by tetrad analysis, in which the tetrads with four surviving spores exhibit the ratio of 4[*NSI*⁺]:0[*nsi*⁻]. We have also analyzed whether [*NSI*⁺] corresponds to the previously identified yeast prions. The data obtained show that [*NSI*⁺] is stably maintained on the background of deletions of *RNQ1, URE2, CYC8, MOT3,* or *NEW1*, which encode previously identified yeast prions, and is not induced by the overexpression of *SUP35* and *SWI1* (these genes are essential and cannot be deleted). Based on the data obtained, we proposed that [*NSI*⁺] is a novel prion factor involved in the epigenetic control of nonsense suppression (Saifitdinova et al., 2010).

We have shown that [*NSI*⁺] enhances nonsense codon read-through and inhibits vegetative growth in *S. cerevisiae*. Using a large-scale overexpression screen to identify genes that impact the phenotypic effects of [*NSI*⁺], we found that the *SUP35* and *SUP45* genes, which encode the translation termination factors eRF3 and eRF1, respectively, modulate nonsense suppression in the [*NSI*⁺] strains. Another gene that affects nonsense suppression in the [*NSI*⁺] strains was found to be *VTS1*. This gene encodes an NQ-enriched RNA-binding protein that affects nonsense-suppression in [*NSI*⁺] and [*nsi*⁻] strains. We demonstrated that *VTS1* overexpression, similarly to [*NSI*⁺] induction, causes translational read-through and growth defects in *S. cerevisiae*. The chimeric protein Vts1-GFP forms fluorescent aggregates under overexpression conditions. These aggregates are partially sedimentated by differential centrifugation. Additionally, we demonstrated that the deletion of *VTS1* causes the slow suppression of *ade-14* (Nizhnikov et al., 2012B).

The similarity between the phenotypic manifestations of [*NSI*⁺] and the overexpression of *VTS1* suggested that [*NSI*⁺] and *VTS1* might interact. We compared the relative amounts of *VTS1* in [*NSI*⁺] and [*nsi*⁻] strains and found that [*NSI*⁺] causes a statistically significant increase in the amounts of *VTS1* mRNA. This is an important observation that facilitates our understanding of the role of the [*NSI*⁺] structural gene (Nizhnikov et al., 2013A).

The interaction of [*NSI*⁺] with *SUP35* was also analyzed. We demonstrated that [*NSI*⁺] does not affect the amounts of *SUP35* mRNA but does cause suppression in strains expressing full-length *SUP35* at decreased levels. This finding suggests that nonsense suppression in the [*NSI*⁺] strains is modulated by the functional activity of Sup35 as eRF3. A comparison of the nonsense suppression efficiencies, which were determined through a dual-reporter assay, between full-length Sup35 and Aβ-Sup35MC demonstrated that the read-through of termination codons is significantly increased in the presence of Aβ-Sup35MC. Thus, Aβ-Sup35MC is a cryptic suppressor that enhances the phenotypic manifestation of [*NSI*⁺] or the overexpression of *VTS1*. Additionally, we demonstrated that [*NSI*⁺] causes nonsense suppression in strains producing full-length *SUP35* in media containing aminoglycoside antibiotics, which inhibit translation. This finding suggests that nonsense suppression in the [*NSI*⁺] strains is not specific to a

decrease in the functional activity of Sup35 (eRF3) and can be modulated by the general defects of translation (Nizhnikov et al., 2013A).

In this work, we conducted a search for novel genetic suppressors of nonsense mutations that may be identified only on a specific genetic background. We first performed a genomic screen for genes whose overexpression causes nonsense suppression in a strain producing the chimeric Aβ-Sup35MC protein and containing the deletion of the *SUP35* chromosomal copy. As a result of this screening, we found a set of fragments of the yeast genome whose multicopy expression affects nonsense suppression in this strain. A detailed analysis of the genes encoded by these fragments demonstrated that the overexpression of three genes, *NAB2, NAB3*, and *VTS1*, which encode potentially amyloidogenic proteins that are rich in asparagine and glutamine, causes nonsense suppression. In addition, the overexpression of *VTS1* causes omnipotent nonsense suppression, but the overexpression of *NAB2* and *NAB3* suppresses only the $ade1–14_{UGA}$ nonsense allele, which suggests that these genes might be codon-specific suppressors. The data obtained indicate that modifications of *SUP35* that affect the functional activity of eRF3 create a specific genetic content that allows the identification of novel suppressors of nonsense mutations that cannot be found in strains containing the intact *SUP35* (Nizhnikov et al., 2012A). This approach can be useful for the detection of novel functions of genes that influence the regulation of basic cellular processes.

Additionally, we used a targeted approach to find novel suppressors. We hypothesized that these suppressors could be found among genes that encode transcriptional factors that regulate the expression of translation termination factors. The effect of the overexpression of the *ABF1, CYC8, FKH2, GAL11, GLN3, INO4, MCM1, MOT3, PGD1, REB1*, and *SFP1* genes on nonsense suppression and vegetative growth in strains producing modified variants of Sup35 was analyzed. These genes encode transcriptional factors rich in asparagine and glutamine that are potential candidates for yeast prions. The overexpression of *ABF1*, *GLN3*, *FKH2, MCM1, MOT3*, and *REB1* was shown to affect nonsense suppression in *S. cerevisiae*. The overexpression of at least two genes, *ABF1* and *GLN3*, causes a statistically significant increase in the read-through of the UGA nonsense codon, which suggests that the fundamental processes of transcription and translation in living cells are, in some cases, interrelated.

3   DISCUSSION

3.1   [*NSI*⁺] determinant possesses the features of a yeast prion

We demonstrated that [*NSI*⁺] possesses non-chromosomal inheritance and cytoplasmic infectivity and spontaneously appears *de novo* (Saifitdinova et al., 2010). These properties are common to most of the amyloid yeast prions that have been identified to date. The cytoplasmic infectivity is an important feature of yeast prions, and it was demonstrated that [*NSI*⁺] (Saifitdinova et al., 2010) exhibits this property through a recently described protein transformation assay (Tanaka and Weissman, 2006). The frequency of [*NSI*⁺] transmission by protein transformation was found to be approximately equal to that found for the [*PIN*⁺] prion (Patel et al., 2007). In addition, [*NSI*⁺] is transmitted through cytoduction (another method for the demonstration of cytoplasmic infectivity) with an efficiency of approximately 11–14% (Saifitdinova et al., 2010), which is comparable with the frequencies found for the [*ISP*⁺], [*SWI*⁺], and [*OCT*⁺] determinants (Rogoza et al., 2010; Du et al., 2008; Patel et al., 2009) and significantly lower than the transmission frequencies of the [*PSI*⁺] prion determinant (approximately 100%; Cox, 1988). The differences observed are likely related to the nuclear localization of the structural proteins of [*ISP*⁺], [*SWI*⁺], and [*OCT*⁺] compared with the cytoplasmic localization of Sup35 and Ure2 (data from "*Saccharomyces Genome Database*", http://www.yeastgenome.org). Cytoduction leads to the fusion of the cytoplasms of both cells involved in this process and results in the elimination of the donor nucleus (Zaharov et al., 1969; Conde and Fink, 1976). It should be clarified that some fraction of the cellular proteins that possess the nuclear localization signal is always located in the cytoplasm because protein synthesis is conducted in the cytoplasm. Thus, prions, such as [*ISP*⁺], [*SWI*⁺], and [*OCT*⁺], can be transmitted through cytoduction at low frequencies if the prion conversion occurred before the corresponding proteins were transported from the cytoplasm to the nucleus. In the case of protein transformation, the recipient strain gains both the cytoplasmic and the nuclear proteins of the donor strain. Therefore, in this assay, the frequencies of transmission of both nuclear and cytoplasmic prions are expected to be approximately equal. The relatively low frequency of [*NSI*⁺] transmission through cytoduction and the

high frequency of [*NSI*⁺] transmission through protein transformation suggests that the structural protein of [*NSI*⁺] is generally localized in the nucleus.

A distinctive feature of yeast prions is their ability to spontaneously appear *de novo* after their elimination. We demonstrated that [*NSI*⁺] is capable of reappearing *de novo*, and this provides evidence of the prion nature of this determinant. Based on the results obtained (Saifitdinova et al., 2010), we may propose that [*PIN*⁺] affects the frequency of the spontaneous appearance of [*NSI*⁺]. Nevertheless, the absence of statistically significant differences between the data obtained with the [*nsi⁻*] [*pin⁻*] strain and the data obtained with the [*nsi⁻*] [*PIN*⁺] strain does not allow us to confirm this hypothesis. If [*PIN*⁺] really does increase the frequency of [*PIN*⁺], this information may aid the search for candidates for the role of the [*NSI*⁺] structural gene because this protein is expected to physically interact with the prion isoform of Rnq1. Taken together, the data show that [*NSI*⁺] is a prion in *S. cerevisiae*.

It was then important to understand whether [*NSI*⁺] was a previously identified yeast prion. The most reliable way to determine this is to obtain deletions of the structural genes of previously identified yeast prions in the [*NSI*⁺] strains because the deletion of the structural gene causes the elimination of the corresponding prion (for a review, see Wickner, 2007). We demonstrated that [*NSI*⁺] is stably maintained in the strains bearing deletions of *URE2*, *RNQ1*, *CYC8*, *MOT3*, and *SFP1* (Saifitdinova et al., 2010). Thus, the structural gene of [*NSI*⁺] is not found among these genes. Because *SUP35* and *SWI1* are essential genes, these genes cannot be deleted. In addition, the N-terminal domain of Sup35 is essential for the propagation of [*PSI*⁺] (Ter-Avanesyan et al., 1994), and [*NSI*⁺] is stable in the strains bearing the deletion of the sequence encoding *SUP35N*. Moreover, the overexpression of *SUP35* causes antisuppression in the [*NSI*⁺] strains. Based on these data, we may conclude that [*NSI*⁺] is unrelated to the prionization of Sup35. The [*SWI*⁺] prion is similar to [*NSI*⁺]: similarly to [*NSI*⁺], this prion inhibits galactose utilization (i.e., [*SWI*⁺] strains do not grow on MG medium). Nevertheless, there are differences between [*NSI*⁺] and [*SWI*⁺]. First, [*SWI*⁺] inhibits growth not only on media with galactose as the sole carbon source but also on media with raffinose (Du et al., 2008), whereas, the [*NSI*⁺] strains actively grow on raffinose medium. [*NSI*⁺] causes nonsense suppression, but [*SWI*⁺] modulates nonsense suppression via the induction of [*PSI*⁺] (Du et al., 2008). In addition, we have shown that the multicopy expression of *SWI1* does not cause the induction of [*NSI*⁺]. The novel prion [*MOD*⁺], similarly to [*SWI*⁺], facilitates the induction of [*PSI*⁺], and, in contrast to [*NSI*⁺], partially eliminates the overexpression of *HSP104* (Suzuki et al., 2012). Additionally, the overexpression of *MOD5* does not affect the frequency of [*NSI*⁺] induction (Nizhnikov et al., 2012A). Thus, [*NSI*⁺] is unrelated to the previously identified yeast prions and can be considered a novel prion determinant in *S. cerevisiae*.

3.2 Nonsense suppression in the [*NSI*⁺] strains is modulated by genes encoding release factors

The interrelation between nonsense suppression in the [*NSI*⁺] strains and the *SUP35* gene was one of the first questions studied in this work. [*NSI*⁺] was initially discovered in strains expressing the chimeric protein Aβ-Sup35MC. Furthermore, [*NSI*⁺] was demonstrated to cause nonsense suppression on the background of Sup35MC expression but not on the background of full-length Sup35 expression (Nizhnikov et al., 2012B; Saifitdinova et al., 2010). These findings suggested that the absence of the N-terminal domain of Sup35 is essential for nonsense suppression in the [*NSI*⁺] strains. This hypothesis was supported by the finding that Sup35N affects the degradation of mRNAs (Hosoda et al., 2003). Nevertheless, our data demonstrated that nonsense suppression in the [*NSI*⁺] strains is unrelated to the deletion of Sup35N and is caused by a decrease in the functional activity of Sup35 as eRF3. We demonstrated that [*NSI*⁺] causes nonsense suppression in a strain bearing the pNR-ΔABF1 plasmid, which causes a decreased level of expression of the full-length Sup35 (Nizhnikov et al., 2013A). Another confirmation was obtained from the analysis of nonsense suppression in the [*NSI*⁺] strains grown on media to which aminoglycoside antibiotics, which cause general defects in the translation mechanism, were added. We also demonstrated that [*NSI*⁺] causes strong suppression on this media in strains expressing the full-length Sup35 at the normal level (Nizhnikov et al., 2013A). Taken together, these data allow us to conclude that the deletion of Sup35N is not essential for nonsense suppression in the [*NSI*⁺] strains.

In fact, nonsense suppression in the [*NSI*⁺] strains depends on the functional activity of eRF3. This was clearly demonstrated in a relatively simple experiment, in which we estimated the dependence of nonsense suppression levels in the [*NSI*⁺] strains on the translational read-through in three isogenic [*nsi*⁻] strains through a dual-reporter assay (Nizhnikov et al., 2013A). We found the strongest suppression in the [*NSI*⁺] strain bearing the plasmid pNR-ΔABF1, which causes a decreased expression level of the full-length Sup35. The [*nsi*⁻] strain expressing this variant of Sup35 exhibited the highest level of read-through of the UGA nonsense codon. A relatively weaker suppression was observed in the isogenic [*NSI*⁺] strain expressing Aβ-Sup35MC, and the [*nsi*⁻] derivative of this strain exhibited a lower read-through of UGA. The third strain was the [*NSI*⁺] strain that expresses the full-length Sup35 at the physiological level. [*NSI*⁺] does not affect nonsense suppression in this strain, and its [*nsi*⁻] derivative demonstrated the lowest read-through of UGA (Nizhnikov et al., 2013A). Thus, we confirmed that nonsense suppression in the [*NSI*⁺] strains directly depends on the functional activity of eRF3.

The analysis of the influence of *SUP45* on nonsense suppression in the [*NSI*⁺] strain yielded other results. We found that only one additional copy of *SUP45* expressed from the centromeric plasmid masks nonsense suppression in the presence of [*NSI*⁺] (Nizhnikov et al., 2012B). The sequence of *SUP45* in the [*NSI*⁺] strains does not contain any nucleotide substitutions; thus, the observed effect cannot be explained by the mutational inactivation of Sup45. Based on this observation, we hypothesized that [*NSI*⁺] causes a decrease in the functional activity of eRF1 (Sup45), which can be

compensated by increasing the level of Sup45 expression. A comparison of the levels of Sup45 protein in the [*NSI*$^+$] and [*nsi*$^-$] strains demonstrated that these levels are identical (Nizhnikov et al., 2012B). Thus, the effects of *SUP45* on the nonsense suppression in the [*NSI*$^+$] strains appears to be unrelated to the possible influence of [*NSI*$^+$] on the amounts of Sup45 protein. Nevertheless, there might be hypothetical variants associated with a very fine tuning of the Sup45 amounts by [*NSI*$^+$] that are undetectable by the standard western-blot hybridization protocol.

Taken together, our data suggest that the nonsense suppression by [*NSI*$^+$] is modulated by *SUP45* likely due to the functional inactivation of eRF1 and can be detected only in those strains with a decreased functional activity of eRF3 or on a background with defects in the translation fidelity.

3.3 *VTS1*: novel omnipotent suppressor in *S. cerevisiae*

The novel omnipotent suppressor *VTS1* was identified in a search for genes whose overexpression affects nonsense suppression in the [*nsi*$^-$] strain expressing Aβ-Sup35MC (Nizhnikov et al., 2012A). The overexpression of *VTS1* is very similar to the phenotypic manifestation of [*NSI*$^+$] excluding the different growth dynamics on media containing glycerol as the sole carbon source (Nizhnikov et al., 2012B).

VTS1 is an omnipotent suppressor: its overexpression increases the read-through of at least two nonsense codons, UGA and UAG. The deletion of *VTS1* causes a weak suppression of *ade1–14*$_{UGA}$ in the [*nsi*$^-$] strain. Thus, *VTS1* is one of the genes involved in the regulation of the termination of translation. We hypothesized that a decrease in the translation termination fidelity, which could occur in the case of possible changes in the levels of *VTS1* expression under stress conditions, might have a potentially adaptive function. A similar example was shown for [*PSI*$^+$]: this prion causes not only omnipotent suppression but also frame-shift suppression that appears to enhance the efficiency of the programmed shift of the *OAZ1* open reading frame (Namy et al., 2008).

It is notable that the presence of [*NSI*$^+$] leads to an increase in the amounts of *VTS1* mRNA. Nevertheless, the nonsense suppression in the [*NSI*$^+$] strains is not related to changes in the *VTS1* expression because an increase in the amounts of *VTS1* mRNA caused by the presence of [*NSI*$^+$] is insufficient to affect suppression (Nizhnikov et al., 2013A). Thus, although [*NSI*$^+$] interacts with *VTS1*, the similarity of their phenotypic manifestations appears to be unrelated to their interactions but rather arises from their influence on the translational machinery.

With respect to this interaction, it is interesting to analyze how *VTS1* might affect nonsense suppression. *VTS1* encodes a protein involved in the posttranscriptional regulation of mRNAs containing specific hairpin SREs (SMAUG Recognition Elements) (Aviv et al., 2003). Initially, this gene was identified as the multicopy suppressor of *vti1–2* mutations that lead to defects in vacuolar transport (Dilcher et al., 2001). Vts1 was later shown to induce the degradation of SRE-containing RNAs via deadenylation mediated by the Ccr4p-Pop2p-Not complex (Rendl et al., 2008). This evolutionary conserved system, which is called Vts-mediated decay, is presented in *Drosophila melanogaster*

by the Vts1 homologue Smaug (Aviv et al., 2003). The components of the mRNA degradation system have been identified as suppressors of nonsense mutations. For example, the mutant alleles of *UPF1*, *UPF2*, and *UPF3*, which are the components of nonsense-mediated decay, were identified as weak recessive omnipotent allosuppressors of mutations in tRNA[Leu]-encoding gene (Ono et al., 2005). Thus, the increase in the degradation of Vts1-mediated decay-specific transcripts and the subsequent decrease in the amounts of the corresponding proteins is a possible mechanisms for the nonsense suppression induced by *VTS1* overexpression.

The analysis of the physical interactions of Vts1 was also informative. This protein interacts with at least 15 proteins ("*Saccharomyces Genome Database*", http://www.yeastgenome.org/). None of these proteins encode a component of the nonsense-mediated mRNA decay system. Vts1 has been shown to physically interact with Stm1 (Krogan et al., 2006), which mediates the interaction of the translation elongation factor eEF3 with the ribosome (Van Dyke et al., 2009). The changes in the expression of *STM1* cause a violation of translation, enhance the sensitivity of strains to the translational inhibitor anisomycin, and decrease the affinity of eEF3 to the ribosome (Van Dyke et al., 2009). An additional interaction of Vts1 with the translational machinery was recently described (Rendl et al., 2012). In this study, Vts1 was demonstrated to physically interact with Eap1, which is a negative regulator of the translation initiation factor eIF4E (Cosentino et al., 2000). Eap1 is essential for the efficient degradation of transcripts via Vts1-mediated decay and mediates the interaction of Vts1 with eIF4E, whose role is unclear (Rendl et al., 2012). Thus, Vts1 might affect suppression through its interaction with eIF4E. It is possible that the overexpression of *VTS1* strengthens the binding of Eap1 with eIF4E and that this may decrease the fidelity of translation termination.

Additionally, it is unclear how the overexpression of *VTS1* leads to defects in vegetative growth. Vts1 interacts with proteins encoded by *GAL80* and *SIN4* (Yu et al., 2008; Costanzo et al., 2010). *GAL80* encodes the transcriptional regulator of genes involved in galactose metabolism. Mutations in this gene blocks the growth of yeast strains on media containing galactose as the sole carbon source (Bhat and Iyer, 2009). Sin4 is a subunit of RNA-polymerase II and acts as a global transcriptional regulator. *SIN4* also represses growth on media containing galactose as the sole carbon source (Dudley et al., 2005). The influence of Vts1 on galactose metabolism is likely related to its interactions with *SIN4* or *GAL80*. Thus, the mechanism through which *VTS1* regulates the fidelity of translation termination and the connection of this effect with the observed defects in vegetative growth remain to be demonstrated.

3.4 The novel modulators of nonsense suppression in *S. cerevisiae*

A set of novel modulators of nonsense suppression, in addition to *VTS1*, was identified in this study. The genes that we identified through genetic screens were *ABF1*, *GLN3*, *FKH2, MCM1, MOT3, NAB2, NAB3,* and *REB1* (Nizhnikov et al., 2012A, Nizhnikov et al., 2012B; Nizhnikov et al., 2013A). We demonstrated that the overexpression of these genes causes nonsense suppression on the background of variants of eRF3 with

decreased functional activity (Aβ-Sup35MC was used) but does not affect nonsense suppression in strains that express full-length Sup35. These genes were subdivided into two groups: genes encoding RNA-binding proteins (*NAB2*, *NAB3* and *VTS1*; this group was discussed in the previous section) and genes encoding transcriptional factors (*ABF1*, *GLN3*, *FKH2*, *MCM1*, *MOT3*, and *REB1*). The mechanisms through which these genes affect nonsense suppression are unclear at this stage of our work, but we will discuss the probable mechanisms.

Nab2 is a component of the nuclear export system. This protein participates in the mRNA polyadenylation process and regulates the nuclear export of more than 2,000 different mRNAs (Fasken et al., 2008). Nab3 is also an RNA-binding protein that regulates the termination of transcription from RNA-polymerase II promoters (Conrad et al., 2000). It is difficult to hypothesize the molecular origin of nonsense suppression induced by the overexpression of *NAB2* and *NAB3* because these genes regulate a number of cellular processes. These genes have not been previously identified as suppressors of nonsense mutations. Nevertheless, there is one piece of evidence that may explain the influence of these genes on nonsense suppression. Nab2 and Nab3 were previously shown to physically interact with the poly(A)-binding protein Pab1 (Batisse et al., 2009; Gavin et al., 2006), which is part of the 3'-end mRNA processing complex and interacts with the translation factors eIF4-G (Kessler and Sachs, 1998) and eRF3 (Cosson et al., 2002). This finding suggests that Nab2 and Nab3 are at least physically associated with the translational machinery and thus may affect the fidelity of this process.

In the present study, we also demonstrated that the overexpression of the *ABF1*, *GLN3*, *FKH2*, *MCM1*, *MOT3*, and *REB1* genes, which encode transcriptional factors, cause nonsense suppression on the same genetic background as *NAB2* and *NAB3*. All of these genes, in addition to *VTS1*, can be considered allosuppressors because these genes affect nonsense suppression in a strain expressing Aβ-Sup35MC, which is the variant of eRF3 with decreased functional activity (Nizhnikov et al., 2013A). Although the influence of transcriptional factors on nonsense suppression is not surprising, it has only been previously identified with [*ISP*+], which is a prion isoform of the global transcriptional regulator Sfp1 (Rogoza et al., 2010). The simplest explanation of the effects of *ABF1*, *GLN3*, *FKH2*, *MCM1*, *MOT3*, and *REB1* on nonsense suppression is that these genes affect the transcription of *SUP35* or *SUP45*. Additionally, we cannot exclude the hypothesis that these genes could affect the expression of other as-yet-unidentified suppressors. *ABF1* is a transcriptional factor for *SUP35* and *SUP45* (Dagkessamanskaya et al., 1997). *REB1* regulates the transcription of *SUP45* (Venters and Pugh, 2009). *FKH2*, *MCM1*, and *MOT3* were identified as potential transcriptional factors for *SUP45* because the promoter region of this gene contains binding sites for these factors (Pic et al., 2000; Wynne and Treisman, 1992; Madison et al., 1998). *FKH2* and *MOT3* are also potential regulators of *SUP35* transcription (Pic et al., 2000; Madison et al., 1998). *GLN3* encodes a transcriptional factor that regulates nitrogen metabolism (Mitchell and Magasanik, 1984; Magasanik and Kaiser, 2002), interacts with Ure2 (Cox et al., 2000), and likely forms a prion (Alberti et al., 2009). Additionally, Gln3 regulates the expression of a set of genes that encode ribosomal proteins (Staschke et al., 2010) and *VTS1* (Staschke et al., 2010). These data may explain the effects of *GLN3* on nonsense suppression.

To summarize, in the present study, we performed a search for novel genetic and epigenetic suppressors in *Saccharomyces cerevisiae*. This search was very effective: we described nine novel genetic suppressors (*ABF1*, *GLN3*, *FKH2, MCM1, MOT3*, *NAB2*, *NAB3, REB1*, and *VTS1)* and characterized one epigenetic suppressor, [*NSI*+]. All of these genes affect nonsense suppression in strains expressing the chimeric protein Aβ-Sup35MC and bearing the deletion of the *SUP35* chromosomal copy but are not manifested in strains expressing the full-length Sup35. Initially, we hypothesized that this effect was due to the deletion of the sequence encoding Sup35N (Saifitdinova et al., 2010), but we later demonstrated that Aβ-Sup35MC exhibits decreased functional activity as eRF3 (Nizhnikov et al., 2013A). This finding clearly explains why all of these suppressors are not manifested in strains expressing intact *SUP35* and demonstrates that the use of strains with inhibited functional activity of the released factor is a very effective tool for the search for novel suppressors. In this work, we demonstrated the prion properties of the [*NSI*+] determinant: non-chromosomal inheritance, cytoplasmic infectivity, and spontaneous appearance *de novo* (Saifitdinova et al., 2010). This determinant was shown to cause translational read-through and to affect vegetative growth (Nizhnikov et al., 2012B). The nonsense suppression in the [*NSI*+] strains is mediated by the *SUP45* gene (Nizhnikov et al., 2012B) and was detected only in strains expressing variants of Sup35 with decreased functional activity or in strains grown on media to which aminoglycoside antibiotics, which inhibit translation, were added (Nizhnikov et al., 2013A). [*NSI*+] was demonstrated to affect the amount of *VTS1* mRNA. This finding suggests that the structural gene of [*NSI*+] encodes a transcriptional factor or an RNA-binding protein. We demonstrated that the overexpression of *VTS1* is a phenocopy of [*NSI*+]. Similarly to [*NSI*+], the overexpression of *VTS1* causes the read-through of termination codons and defects in the vegetative growth. The deletion of *VTS1* causes weak nonsense suppression (Nizhnikov et al., 2012B). Additionally, a set of novel genetic suppressors (*ABF1*, *GLN3*, *FKH2, MCM1, MOT3*, *NAB2*, *NAB3*, and *REB1)* was identified (Nizhnikov et al., 2012A; Nizhnikov et al., 2013B). These genes encode transcriptional factors and RNA-binding proteins. The obtained data expand the knowledge on the modulation of nonsense suppression in *S. cerevisiae* and suggest that the system of translation termination contains a number of precisely interacting genetic and epigenetics regulators.

3.5 Suggestions for further work

The results obtained in this study create a basis for further investigations in at least two fields. First, it is important to understand which molecular mechanisms are responsible for the nonsense suppression caused by the overexpression of the genes identified in this work. Although some probable mechanisms were proposed in the discussion, these assumptions need to be experimentally analyzed. In this respect, it is important not only to analyze the suppressors identified in this work but also to conduct other searches for novel suppressors of nonsense mutations because these findings will help explain the data. Although one of the promising approaches for the achievement of this aim was

used in this work, there are other potentially useful methods, such as the use of chemicals that inhibit translation in combination with mutations in *SUP35* and *SUP45*. In addition, although this work is laborious, it is almost always productive. For instance, the standard genetic screen includes approximately 2,000 yeast transformations and the analysis of approximately 20,000 clones. Nevertheless, this is the only effective method for the identification of novel suppressors of nonsense mutations among different functional groups of proteins; however, targeted approaches, such as the analysis of the effects of transcriptional factors on nonsense suppression performed in this study, can also be effective. In general, one of the main objectives of our future work is to characterize the complete landscape of yeast nonsense suppressors.

A second area of interest arising from this work is the identification of novel prions and amyloids. In this study, we demonstrated the prion properties of [*NSI*+], but the structural gene of this determinant remain to be elucidated. There are a number of strategies for the identification of prions in yeast, but none of these guarantee the identification. A genomic screen is the classical approach. There are two different types of screens for prion determinants: overexpression and deletion. The strategy of an overexpression screen is based on the assumption that the overexpression of a structural gene induces the frequency of appearance of the corresponding prion. Unfortunately, this approach works only in a specific genetic and epigenetic background. Thus, for example, the overexpression of *SUP35* does not cause the induction of [*PSI*+] in [*psi*-][*pin*-] strains (Derckatch et al., 2001). Another approach is the screening of a deletion library. This approach was successfully used for the identification of the [*ISP*+] prion (Rogoza et al., 2010). However, this finding was very fortutitous, and the researchers were lucky twice: first, because Sfp1, which is the structural gene of [*ISP*+], is non-essential for the viability of yeast cells, and second, because the phenotypic manifestation of *SFP1* deletion is equal to [*isp*-] instead of [*ISP*+]. Otherwise, the structural gene of [*ISP*+] would never have been found. Thus, the prospects of the identification of the [*NSI*+] structural gene through genetic strategies are relatively dim, particularly because the genetic background in which [*NSI*+] causes nonsense suppression leads to the identifications of dozens of candidates for the role of its determinant (Nizhnikov et al., 2012A) that are very difficult to analyze.

Based on the above arguments, the most promising approach for the identification of the [*NSI*+] determinant is the development of a biochemical method for the identification of prions and amyloids. We are currently working to improve the method that was originally proposed by the Laboratory of Prof. M. D. Ter-Avanesyan (Kushnirov et al., 2006). This method is based on the isolation and purification of amyloids that form detergent-resistant polymers. We have proposed modifications that result in a significant increase in the resolution of this method. In particular, we identified a number of amyloid proteins (Rnq1, PrP, and Aβ-GFP) and several proteins that are potentially new yeast amyloids. Further improvements to this approach might not only allow the identification of [*NSI*+] but also create the world's first method for the proteomic screening of amyloid proteins in various organisms, including human.

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