June 14, 2013

Review

of the Dissertation of Anton Nizhnikov

submitted for the degree of Doctor of Philosophy in Biology

at the Saint-Petersburg State University

The dissertation consists of 24 pages of text, list of references and 5 published articles. The experimental results included in the published articles and described verbally in the text.

The main goal of the performed studies was to conduct the search for novel genetic and epigenetic nonsense suppressors in *Saccharomyces cerevisiae* yeast strain. The screens were performed in the strains defective for SUP35 (sensitized background). The results obtained by Mr Nizhnikov indicated that these strains exhibit decreased functional activity of eRF3 translation termination factor (Nizhnikov at al, 2013A). As a result of the screen nine novel genetic supressors and one epigenetic suppressor [*NSI+*] have been identified (Saifitdinova at al, 2010). The majority of the thesis work was related on exploring these initial results and on characterization the properties of these hits. Standard methods of yeast genetics have been used, and there is no doubt that Mr Nizhnikov acquired extensive knowledge and experience with genetic studies while performing the project.

In my opinion results characterization of epigenetic suppressor [*NSI+*] is the most interesting and provocative part of the work. It is demonstrated that the properties of [*NSI+*] are consistent with expected properties of yeast prions. [*NSI+*] posseses non-

chromosomal inheritance and cytoplasmic infectivity and spontaneously appears *de novo* (Saifitdinova at al, 2010). The infectivity by [*NSI*+] is high in protein transformation assay but it is only 11-14% in cytoduction assay (Saifitdinova at al, 2010). As discussed by Mr Nizhnikov, these properties are consistent with the nuclear protein, which is present in cytoplasm in relatively low abundance.

In the course of the work several attempts have been made to identify a gene encoding [*NSI*+]. Candidate approach was taken and it was shown that [NSI+] is stably maintained on the background of several previously identified yeast prions (Saifitdinova at al, 2010). This result suggested that [NSI+] is likely to be a novel prion factor . Overexpression screen was performed to identify genes that impact the phenotype of [NSI+] ((Nizhnikov at al, 2012B). Obtained results indicated that nonsense suppression in the [NSI+] is related to functional activity of eRF3 translation termination factor. This kind of result provide limited insight to the nature of [NSI+], as it is related to nonsense suppression per se and not to a specific suppressor. It appears that genetic methods have been largely exhausted at this point in search for [NSI+] –encoding gene. Biochemcial methods such as fractionation of yeast lysates and functional reconstitution of [NSI+] activity will be probably necessary to identify [NSI+] –encoding gene. Mr Nizhnikov mentions a possibility of biochemical approach briefly in the Future Directions section of the thesis, but no attempts at such studies have been undertaken by the candidate so far.

Second part of the thesis deals with novel genetic modulators of nonsense suppression. Mr Nizhnikov demonstrated that overexpression of these genes causes nonsense suppression in sensitized yeast strains with reduced activity of eRF3. Some of these genes encoded RNA-binding proteins (3 genes) and some genes encoded transcription factors (6 genes). Some mechanistic information was obtained regarding mechanisms involved in nonsense suppression by VTS1 RNA-binding protein. Potential mechanisms

involved in actions of other factors discussed in the thesis, but these have not been tested experimentally by Mr Nizhnikov.

Overall, significant amount of genetic studies have been performed to study function of novel nonsense supressors in yeast. Publication activity is very good and thesis is clearly written. Mr Nizhnikov appears to have a good grasp on the literature in the field and good understanding of genetic methods. My main criticism of the work is its exclusive reliance on genetic methods. Some of these problems, in particular identification of [NSI+] –encoding gene, may benefit from application of biochemical techniques. Mr Nizhnikov mentions this possibility in the Future Directions section of the thesis and I hope that he will persue this avenue of research more aggressively in the future.

Based on my careful reading of the thesis and evaluation of Mr Nizhnikov's published reports I conclude that this dissertation complies with the international standard for PhD dissertation in the field of genetics. I recommend that Mr Nizhnikov proceeds with thesis defence for PhD SPbSU degree in Biology.

Ilya Bezprozvanny, PhD, DSci
Professor of Physiology

Review
of the Dissertation of Anton Nizhnikov
submitted for the degree of Doctor of Philosophy in Biology
at the Saint-Petersburg State University

The dissertation is 110 pages long, consists of five chapters and includes an introduction, a brief description of the results and a discussion followed by a detailed literature list. The fifth chapter comprises copies of papers arising from this thesis, four of which are published and one which has been accepted for publication. All tables and figures of the dissertation are presented in the body of the published papers.

The dissertation, entitled "Novel genetic and epigenetic suppressors of nonsense mutations in *Saccharomyces cerevisiae*", examines the molecular mechanisms of nonsense suppression using baker's yeast as a model system.

One-third of inherited genetic disorders including cystic fibrosis, Duchenne muscular dystrophy, hemophilia, and several hereditary cancer syndromes are caused by nonsense mutations that create premature termination codons (PTC) in genes encoding fundamental, disease-associated proteins. Premature termination codons are potentially deleterious because the truncated proteins generated are often non-functional or exert dominant-negative effects. Nonsense suppression is a process that changes the effects of nonsense mutations by altering the reading of premature stop codons. Many efforts have been made to develop suppression therapy allowing read-through of a premature termination codon to rescue the production of a full-length protein. Efficiency of read-through inversely correlates with efficiency of translation-termination and is influenced by many cellular factors. To ensure progress in the development of suppression therapies for treating patients and discovery of pharmacological agents with nonsense suppression activities, a deeper understanding of the molecular basis of nonsense suppression is extremely important.

The main goal of this thesis is identification and characterization of novel genetic and epigenetic suppressors of nonsense mutations in *S. cerevisiae*. The aim of the thesis and the major questions addressed are both clearly stated at the end of Introduction. To accomplish this aim several new approaches were developed and tested. Using a dual-reporter assay to measure the nonsense suppression efficiency candidate demonstrates that strains expressing Sup35 with N-terminal domain deleted or modified (Aβ-

Sup35MC) reveal significantly increased read-through of termination codons. Such features make these strains a useful tool to increase sensitivity of screens to identify novel nonsense suppressors. The benefits of using these strains were successfully demonstrated in this work. Novel non-chromosomal determinant [*NSI*$^+$] (nonsense suppression inducer) causing nonsense suppression in strains bearing N-terminally deleted or modified variant of *SUP35* gene was identified. [*NSI*$^+$] enhances nonsense codon read-through and inhibits cells growth. In extensive large- scale screen for the genes whose overexpression affects suppression, 3 genes, *VTS1*, *NAB2* and *NAB3*, coding for the NQ-rich RNA-binding proteins, were identified as the novel nonsense suppressors. A targeting approach was successfully used to identify novel modulators of nonsense suppression among the genes encoding NQ-rich transcription factors that regulate the expression of proteins involved in translation termination. In total nine novel genetic suppressors and one epigenetic suppressor were identified and characterized in this dissertation.

The molecular features of [*NSI*$^+$] and *VTS1* were analyzed further. It has been demonstrated, that nonsense suppression in the [*NSI*$^+$] strains is modulated by translation termination factors, Sup35 and Sup45, and by Vts1 protein, involved in deadenylation- dependent mRNA degradation.
In the thesis, the candidate demonstrates that [*NSI*$^+$] exhibits characteristic of yeast prions, specifically, dominant non-Mendelian inheritance, reversible curability and transmissibility by protein transformation. [*NSI*$^+$] was not found to correspond to any of 8 tested previously identified prions. Based on the obtained data a conclusion was made that [*NSI*$^+$] is a novel prion determinant involved in the epigenetic control of nonsense suppression in *S.cerevisiae*.

Overexpression of Vts1 causes translational read-through and growth defects, which is similar to [*NSI*$^+$] induction. Like yeast prion proteins, Vts1 is enriched in NQ-residues and when overexpressed it forms cellular aggregates as detected by observing GFP tagged protein in live cells. Despite this, the prion form of Vts1 was not detected. It has been suggested earlier that conversion of soluble proteins into the prion form may play a protective role against protein degradation under unfavorable conditions and/or in the adaptation of organisms to changing environment. Considering this, the finding of this dissertation that several proven prion proteins and some proteins with NQ-rich prion –like domain are involved in nonsense suppression in the conditions when translation termination is altered is very

exiting and suggests that these proteins may be part of regulatory network of protein translation in response to the fluctuating physiological environment. Further analysis of Vts1 and other NQ-rich proteins involved in nonsense suppression will allow for discovery of a new significance of proteins with prion or prion-like properties in a highly tuned function of translation machinery.

The data obtained in this research form a strong base for the future work required to characterize the complete landscape of yeast nonsense suppressors. Finding that nonsense suppression by [NSI^{+}] was more prominent in strains with general defects of translation, such as cells growing in the presence of aminoglycoside antibiotics inhibiting translation, or cells expressing modified Sup35 with N-terminal deleted or replaced with Aβ sequence, allowed to develop a more sensitive screen for novel genetic suppressors of nonsense mutations as well as understand the specific role of NQ-rich prion forming proteins in nonsense suppression.

The candidate demonstrates great competence in applying the modern methods of molecular and cellular biology in addition to the advanced knowledge and skills of classical methods of yeast genetics. All methods used to get answers to raised scientific questions were adequate, properly applied and correctly executed.

Dissertation has a well-defined logical structure. The material is organized into the Introduction, Results and Discussion section. The candidate ensures that presented material is properly organized within each relevant chapter. There is only necessary minimal repetition or restatement of basic facts between the chapters. Dissertation has a narrative flow and easy and enjoyable to read.

The candidate demonstrates a deep theoretical knowledge and use of the literature in the field as well as excellent orientation in all scientific questions discussed in the thesis. Overall 90 bibliography sources are quoted in this thesis. References of the thesis are up-to-date and demonstrate clearly that candidate was aware of any new development in the field, and consistently incorporated it into his work.

The thesis presents the very interesting and valuable results of high quality. All necessary controls in experimental design are included. Results are

described in a lucid and scholarly manner. Statistic analysis for all quantitative data is provided.

The thesis evidently demonstrates that the candidate possesses all necessary practical skills, theoretical knowledge and high motivation for research work. The candidate's input into the achievement of the dissertation results is clearly reflected by his impressive list of publications. Examples include his accomplishments as a first co-author in one publication and a first author in four other publications.

Text of dissertation is written in English language. It is unnecessary to comment on papers already published in internationally accepted journals, which went through the peer review process. The rest of the dissertation is well composed, easy to understand and demonstrate a good knowledge of English language.

Work presented in this thesis have already been published and was thus previously revised and corrected through the pier review process, making it difficult to find additional shortcomings. However, a short list of comments is appropriate:

1. *Abbreviations*: It would be useful to include in the dissertation a list of defined abbreviations. Also, abbreviations used in the thesis should be spelt out fully when first used. *For example*: abbreviation "NSI" was spelt out after it was already used in the text several times.

2. *References*: A consistent approach should be used for formatting of references. In the thesis different references in chapter IV are presented in a different format. Also, should be brought to the candidate's consideration that when introducing the topic of yeast prions in his theses it would be appropriate to cite the most recent extensive review. An example of such review is "Prion in Yeast" by S. W. Liebman and Y. O. Chernoff, 2012. The candidate's choice of reference was "Alberti et al., 2009, with modifications" p5, is not as recent; further the words "with modification" cause some confusion when there is no further explanation from the author.

3. *Protein levels of Vts1*: Candidate has shown that presence of [NSI^{+}] leads to an increase in the amount of *VTS1* mRNA. Due to the differences in protein's half-life and factors affecting protein degradation machinery

increase in the amount of mRNA does not always correlate with the amount of protein present in the cell. Considering this, the question arises of the need for a comparison of the levels of Vts1 protein in [*NSI*$^{+}$] and [*nsi*$^{-}$] cells.

4. *Structural features of Vts1*: Vts1 protein possesses structural features of yeast prions and its overexpression mimics the effect of [*NSI*$^{+}$] prion on nonsense suppression. This suggests the possibility that Vts1 itself may convert into a prion form, although, it was not confirmed by the traditional tests for identification of prions. Considerations of the possibility that Vts1 forms an unstable prion form under specific environmental conditions could be useful, along with the applicable testing approach.

My overall conclusion is that the dissertation by Anton A. Nizhnikov complies with the international standard for PhD dissertations in Biology. Therefore, after successful defense of the PhD thesis, I recommend the award of a PhD degree to Anton A. Nizhnikov.

June 15, 2013

Tatiana Chernova

Tatiana Chernova, Ph.D.
Assistant Professor RT
Emory University School of Medicine
Department of Biochemistry
RRC, 1510 Clifton Rd., Room 4070
Atlanta, GA 30322
Tel: 404-727-0412
Fax: 404-727-3452
Email: tcherno@emory.edu

REVIEW

of the Dissertation of Anton Nizhnikov
submitted for the degree of Doctor of Philosophy in Biology
at the Saint-Petersburg State University

The dissertation consists of 110 of pages and includes 4 published articles, and 1 article accepted for publication. The articles include a total of 17 figures and 21 table.

The Dissertation summarizes the work aimed at searching for and characterizing genetic and epigenetic suppressors of nonsense mutations in yeast *Saccharomyces cerevisiae*. The goal is ambitious and important. Indeed, a large number of human heritable diseases and cancers are linked to nonsense mutations in various genes, and finding the ways to modulate the translational read-through of such premature stop codons may ultimately lead to major therapeutic advances. Yet, while nonsense suppression has been studied for over half a century, the cellular factors and mechanisms that fine-tune the overall level of translational fidelity in the cell are still unclear.

The laboratory of Prof. Inge-Vechtomov at Saint-Petersburg State University pioneered the studies of omnipotent nonsense suppression in yeast. The unique genetic collections and experimental systems developed in Inge-Vechtomov lab are now extensively and successfully used in the laboratory of Dr. Galkin at N.I. Vavilov Institue of General Genetics, where this Dissertation was done. The most significant modification of the experimental approach distinguishing this Dissertation from earlier studies in these research groups is that the search for novel nonsense suppressors is performed in strains with a genetically modified translation termination factor Sup35 (eRF3). The genetic modifications reduce the efficiency of Sup35 and create an “error-prone” translational environment highly sensitive to even the slightest variations in translational accuracy. Consequently, while earlier searches in unmodified genetic backgrounds allowed to identify the major components of translation termination machinery, such as translation termination factors, this study performed in “error-prone” cells revealed an array of cellular factors that modulate the fidelity of translation indirectly, e.g. through transcriptional regulation.

The study identifies both genetic and epigenetic factors affecting the suppression of nonsense mutations. [*NSI+*] was detected as a nonsense suppressor in cells where Sup35 is replaced by a construct where the A-beta$_{1-40}$ amyloidogenic sequence substitutes for the N-terminal domain of Sup35 (A-beta$_{1-40}$-Sup35MC). Thorough genetic analysis is used to confirm that [*NSI*$^{+}$] satisfies all genetic criteria for prions and has characteristic features of a Q/N-rich amyloid prion: dominant non-Mendelian inheritance, reversible curability, infectivity in cytoductions and protein transformations, and dependence on the Hsp104 chaperone. While the coding gene for [*NSI*$^{+}$] has not been identified in this study,

the possibility that this is a variant of a known yeast prion is excluded convincingly, and I concur with the conclusion that [*NSI*$^+$] is formed by a nuclear protein, probably involved in transcriptional regulation or mRNA turnover. The focus of subsequent work was on elucidating the mechanism of nonsense suppression in [*NSI*$^+$] cells. At this stage, the *ade1-14* reporter for nonsense suppression is complimented by a dual luciferase stop-codon read-through reporter and the strain with reduced transcription of the *SUP35* gene is used along with mutants lacking the N-terminal part of the Sup35 protein. These adjustments to the experimental system allow to establish that [*NSI*$^+$] acts through a general increase of nonsense codon read-through and that [*NSI*$^+$]-associated nonsense suppression is not limited to cells with a mutant translation termination factor. Together these findings underscore the importance of further investigation of [*NSI*$^+$].

Genetic factors affecting nonsense suppression were identified through two genomic overexpression screens, as well as through a candidate approach based on extensive data mining. Screening for genes that, upon overexpression in a strain with modified Sup35, cause nonsense suppression or increase the suppressor effect of [*NSI*$^+$] yielded 3 candidates, *NAB2, NAB3* and *VTS1*. All three proteins possess Q/N-rich prion-like domains and are engaged in mRNA turnover. Nab2 is a component of the mRNA nuclear export system and participates in mRNA poly-adenilation. Nab3 regulates termination of transcription from Pol II promoters. And Vts1 is a component of the mRNA degradation system coupled with Ccr4-Pop2-Not de-adenylation. The first important outcome of the screens is the confirmation that multiple proteins with prion domains contribute to the regulation of translational accuracy. This conclusion was further supported by the candidate-based approach where a subset of prion candidates was chosen based on the likelyhood of being implicated in transcriptional regulation of translation termination factors. This approach yielded 6 more weak nonsense suppressors, *ABF1*, *GLN3*, *FKH2, MCM1, MOT3*, and *REB1*, which is 50% of the candidates tested. While so far there is no clear understanding of how all these proteins affect nonsense suppression, and what is the role of Q/N-rich domains, no doubt that having identified all these proteins would greatly facilitate future research progress. As the first attempt to gain an insight into the mechanism of such the modulation of translational accuracy by Q/N-rich proteins, Vts1, which was selected both as a nonsense suppressor and as an allosuppressor of [*NSI*$^+$], was studied in more detail. It was found to be an almost perfect phenocopy of the [*NSI*$^+$] prion. Indeed, excess Vts1 leads to an increase in nonsense read-through, and the effect is not mediated by the prion domain of Sup35. Furthermore, the fact that [*NSI*$^+$] upturns the levels of Vts1, is indicative of an interaction between these proteins.

In summary, the main achievements of this research project are: (i) the discovery of a new prion, [*NSI*$^+$]; (ii) identification of an array of genetic and epigenetic factors that simultaneously possess Q/N-rich prion-like domains and affect the

accuracy of translation termination; (iii) gaining first insights into the mechanism of the modulation of translation by these factors.

The results of the study have been presented in 5 research publications. Anton Nizhnikov is an equally contributing first co-author in the first article describing the discovery of the [*NSI+*] prion, and the only first author in the other four articles. In my opinion, this shows convincingly that Anton is the major contributor to the project, and that he obtained most of the experimental evidence leading to the conclusions made in this Dissertation.

In my opinion these achievements are sufficient and actually exceed the requirements for the Ph.D. Dissertation study. Furthermore, I would like to stress that the data are not only extensive, but of very high quality. The technical level of experimentation is high – all required controls are present and statistical analysis of results is appropriate. Also, as can be seen from the previous paragraphs, Anton uses a variety of approaches, which allows him to broaden the search, to validate data and to look at the problem from different angles.

The core of the Dissertation are the research articles that are already published or accepted for publication. The articles are preceded by a general Introduction that reviews previous works and puts this study into a context of the current state of knowledge in the area. In the introduction Anton demonstrates good knowledge of relevant scientific literature, starting from the first reports on nonsense suppression to the most recent studies of cellular regulation networks that could modulate translation. The Dissertation also includes a Discussion that provides a global summary of findings presented in different articles and outlines the directions of future studies. While quite short, the Discussion is logical and well-structured. One can see how findings form several projects mutually validate each other and reveal a global phenomenon – the involvement of proteins with prion-like domains in the regulation of translational accuracy.

The Dissertation is written clearly and in proper English language. The only section, for which I would suggest language revisions, is the translation of the article recently accepted for publication in Russian Journal of Genetics.

As for the shortcomings of this work, they are either limited to the suggestions for minor textual revisions, or are already addressed in the future research plans. The major textual correction is on page 4 of the Introduction, where the codons and anticodons of $tRNA_{Gln}$ are mixed up the text. On a more global scale, in my opinion, the conclusion that the effect of the newly identified suppressors with Q/N-rich domains is not due to their prionization is a bit premature. Several of these proteins are known prions, and to make such a conclusion it would be advisable to make sure that the presence of these prions does not cause the same phenotypes as their overexpression.

In conclusion, I find this Dissertation fully complying with the international standard for PhD dissertations in biology and enthusiastically recommend granting the degree of Doctor of Philosophy in Biology at the Saint-Petersburg State University to Anton Nizhnikov

Irina L. Derkatch, June 16, 2013

Candidate of Biological Sciences
Adjunct Associate Research Scientist
Department of Neuroscience,
Columbia University
New York, USA

Review

of the Dissertation of Anton Nizhnikov

submitted for the degree of Doctor of Philosophy in Biology

at the Saint-Petersburg State University

The dissertation of Anton Nizhnikov consists of 110 pages, includes four published articles and one article accepted for publication. The main subject, to which the dissertation is dedicated, is the study of nonsense suppression and search for novel suppressors of nonsense mutations in *Saccharomyces cerevisiae*. This is very interesting and significant problem not only due to basic purposes, but also because a number of human inherited diseases are associated with premature nonsense codons. The analysis of nonsense suppression is the simplest way to detect factors that affect fidelity of translation termination, and it is available only for lower eukaryotes, such as *S. cerevisiae*, which is the most convenient organism to study this process. The work of A. Nizhnikov considerably expands the knowledge of suppressors of nonsense mutations describing nine novel genetic (*ABF1*, *GLN3*, *FKH2*, *MCM1*, *MOT3*, *NAB2*, *NAB3*, *REB1*, and *VTS1*) and one epigenetic suppressor, [*NSI*$^{+}$], which is likely to be a yeast prion.

The sufficiency and quality of the material used in this work is high enough. The experiments were performed on a large number of yeast strains and plasmids, most of which were obtained or constructed directly by the author. The combination of methods of classical genetics and molecular biology was successfully employed in this study. All these methods are adequate and used only in such cases, when it is really necessary. It is important that author tries to use quantitative methods like qPCR or dual-reporter assay to prove the effects found by genetic studies, such as influence on nonsense suppression or read-through of termination codons. Especially, I want to mention that dissertation involves a quite impressive amount of experimental data. The author performed two large-scale genomic screenings, analyzed its results (about 25000 clones) and constructed more than twenty plasmids. Nevertheless, this large dataset based on five scientific

papers is merged in the dissertation by one main idea of characterization of novel suppressors of nonsense mutations. Thus, in general, the logic of dissertation structure is easy to read and understand. In particular, it becomes clear from the "Brief Description of Results" section, which, though written on just few pages, but creates a holistic perception of the results of entire work.

The good validity of results presented in dissertation is supported by that these results were published in four peer-reviewed scientific papers (fifth peer-reviewed paper is accepted for publication). It is very important that dissertation contains no unpublished data. Also, A. Nizhnikov uses the statistical apparatus correctly and, in cases, if it is possible, takes the samples of sufficient size to be processed statistically.

Author demonstrated a good knowledge of the literature in the field of dissertation. The "Introduction" section includes a brief, but relatively complete review of suppressors of nonsense mutations identified to date in yeast. The author draws the reader's attention on the interesting phenomenon of epigenetic regulation of nonsense suppression in yeast by prions. Only minor correction to this section is that codon specific tRNA suppressors are usually denoted by three letters amino acid nomenclature, but author uses single letter designations. Also, a comparison of own data with the results obtained by other investigators is included in "Discussion" section. This comparison is quite detailed and critical, and includes hypotheses about the possible molecular mechanisms of nonsense suppression that underlie the effects of genes and factors identified in this work.

The results of dissertation make a considerable contribution to the area of study of nonsense suppression not only from the position of identification of novel suppressors, but also in the context of interactions of these suppressors, because in this study at least three suppressors (*SUP45*, *SUP35* and *VTS1*) were demonstrated to interact with [*NSI*$^{+}$] prion-like determinant. These findings suggest that the system of regulation of nonsense suppression (and termination of translation) contains a number of participants forming a set of genetic and epigenetic networks, the significance of which has yet to comprehend.

A. Nizhnikov made the main contribution in the results included in dissertation. He is the first author of all five papers (first paper, "[*NSI*$^+$]: a novel non-Mendelian suppressor determinant in *Saccharomyces cerevisiae*", has two first co-authors that made the equal contributions). Also, it is noteworthy that A. Nizhnikov is the corresponding author of the last paper, "The analysis of interactions of prion-like determinant [*NSI*$^+$] with *SUP35* and *VTS1* genes in *Saccharomyces cerevisiae*", accepted for publication in "Russian Journal of Genetics". This suggests that A. Nizhnikov has brought not only considerable experimental, but also significant intellectual contribution to this work.

The work is written in good English. Also, two articles originally published in Russian and included in dissertation, were translated by A. Nizhnikov himself.

The manuscript has some shortcomings. First, author did not include the list of abbreviations, which would be useful for better understanding. Second, when A. Nizhnikov describes epigenetic modulation of nonsense suppression in yeast, he does not discuss the role of [*PIN*$^+$] determinant, which, although indirectly, modulates nonsense suppression influencing the spontaneous reappearance of [*PSI*$^+$] *de novo*. Also, I suppose that the role of *SUP45* in the phenotypic manifestation of [*NSI*$^+$] is not completely studied, although the author speculates about this role in the "Discussion" section. It would be also interesting to have more comments on the role of Sup35 specific activity in [*NSI*$^+$] mediated nonsense-suppression. Nevertheless, all these comments are minor and do not affect the scientific value of the work.

On the basis of the above-mentioned criteria, the dissertation of Anton Nizhnikov submitted for the degree of Doctor of Philosophy in Biology at the Saint-Petersburg State University complies with the international standard for PhD dissertations in the corresponding field and.

2013-06-06

Professor Sergey G. Inge-Vechtomov,
Full Member of Russian Academy of Sciences,
Chairman,
Department of Genetics and Biotechnology, SPbSU

REVIEW
of the Dissertation of Anton Nizhnikov

"Novel genetic and epigenetic suppressors of nonsense mutations in *Saccharomyces cerevisiae* "
submitted for the degree of Doctor of Philosophy in Biology at the Saint-Petersburg State University

It has became evident during the last years that a key cellular processes, even in unicellular organisms, are controlled by factors operating at different regulatory levels. The yeast Saccharomyces cerevisiae provides a unique opportunity to identify many of these factors and to study their interaction thanks to detailed genetic and molecular characterization of yeast. Especially important is recently discovered participation of prions, genetic determinants of protein nature, in regulation of fundamental functions performed in the cell. At least several of them are involved in translation, among them is [NSI+], found in the group, to which Anton Nizhnikov belongs, and studied systematically in his dissertation. Besides, he revealed and characterized some genes influencing translation accuracy, specifically nonsense codons read through. So, the importance of investigation performed by A. Nizhnikov is obvious.

The dissertation consists of 110 pages and includes INTRODUCTION (5 pages), BRIEF DESCRIPTION OF THE RESULTS (4 PAGES) and DISCUSSION (13 PAGES). The list of references consists of 90 works. This part of dissertation includes neither tables no figures, because all illustrative materials are contained in the texts of 5 papers, which represent the rest part of the manuscript. 4 of them have been published; one paper is accepted for publication.

In INTRODUCTION the author carefully explains why study of nonsense suppression in yeast is the efficient instrument allowing characterization molecular mechanisms of translation termination. He lists different types of genetic and epigenetic nonsense suppressors and characterizes their molecular mechanisms. In conclusion, he briefly describes the non-chromosomal determinant [NSI+] and formulates questions that should be answered in the dissertation.

In the next chapter “BRIEF DESCRIPTION OF THE RESULTS”, A. Nizhnikov in a very laconic manner just lists main results obtained in his work: the data indicating a prion nature of [NSI+]; identification of *Vts1* as a new presumably prionogenic protein influencing translation termination and interacting with [NSI+]; characterization of [NSI+] interaction with Sup35p and its derivative Aβ-Sup35MC; finally, detection of 8 genes encoding transcriptional factors, some of which are potential prions, as genes affecting nonsense suppression. All these data are analyzed in details in the next part of thesis, DISCUSSION. This analysis makes possible to conclude that [NSI+] is a new yeast prion, since its features correspond to features demonstrated by typical yeast prions, but at the same time it is not identical to some one of them. The other important and well established conclusion is that nonsense suppression observed in [NSI+] strains depends on the functional activity of translation termination factor eRF3 (Sup35p). Less clear is interpretation of results indicating a dependence of [NSI+] manifestation on *SUP45* encoding the second translation termination factor, eRF1 (Sup45p). The author has shown that only one additional copy of wild type *SUP45* masks nonsense suppression caused by [NSI+]. The increase of Sup45p amount in this case is undetectable. Therefore A. Nizhnikov concludes that nonsense suppression in [NSI+] strains depends on functional inactivation of Sup45p, which takes place in the background of decreased Sup35p activity. Hence, my **first question is: *what could be the nature and possible mechanism of the proposed Sup45 functional inactivation?***

The second question is related to effects caused by a change in the level of *VTS1* expression. ***What is the explanation of similar effects of VTS1 overexpression and deletion?*** This similarity looks very unusual, since effects of overproduction and absence of the protein are opposite as a rule. Nevertheless, identification of a novel component of a system regulating translation fidelity should be considered as the interesting and significant result.

The same conclusion should be done concerning the data indicating involvement of additional 8 genes in regulation of nonsense suppression efficiency. Summarizing these data, it should be noted that nonsense suppression, known as classical genetic effect, is much more complex than it was believed earlier and is determined by numerous factors of different nature.

In the last section of DISCUSSION, A. Nizhnikov writes about perspectives of future studies with special attention to experimental approaches that would be helpful for identification of [NSI+] structural gene. The author explains why this task is difficult and compares different ways that might solve this problem.

All the data represented in this part of the thesis are supported by 5 papers, where A. Nizhnikov is either the first author, or contributed equally with one of co-authors.

As to methods used in this study, in addition to routine genetic and molecular methods, A. Nizhnikov applied a lot of contemporary methods, in particular, fluorescent microscopy, protein transformation, etc. This fact granted a high quality of the data obtained and their validity.

The author showed a well knowledge of the literature on the subject. I noticed only one inaccuracy. Sorry, that it is related to my papers. When A. Nizhnikov writes about genes influencing nonsense suppression, he reminds *PPZ2*, which has been found in the work of my group. But before *PPZ2* we identified two other genes, *HAL3* and *PPZ1* and published two papers describing their influence on suppression efficiency. For some reason these genes are not included by applicant in the list of genes functionally related to translation termination.

In conclusion, it should be noted that the structure of thesis is rational, it is written by rather good English and easy readable. It is no doubt that all results represented in dissertation were obtained by A. Nizhnikov or, in some cases, he immediately participates in the experiments performed. The work of A. Nizhnikov contributes essentially to the study of genetic and epigenetic factors controlling

translation termination in *Saccharomyces cerevisiae* and gives possibilities for future studies in this area.

The material of A. Nizhnikov's dissertation and its design correspond to international standards and are sufficient to apply for the degree of Doctor of Philosophy in Biology at the Saint-Petersburg State University.

June, 10. 2013.

/Professor, Doctor of Science, Lyudmila Mironova/

Review

of the dissertation of Anton A. Nizhnikov "Novel genetic and epigenetic suppressors of nonsense mutations in *Saccharomyces cerevisiae*", submitted for the degree of Doctor of Philosophy in Biology at the Saint-Petersburg State University

The mechanisms of gene expression have been studied for many years and are one of the central problems of the contemporary biology. Although the basic principles of these mechanisms, including mechanisms of protein biosynthesis, are well understood, many details of these processes are still unclear. Specifically, translation termination represents one of the less studied stages of protein biosynthesis in eukaryotes.

The investigation of translation termination mechanisms is not only of academic interest. At present more than 200 various heritable human diseases are attributed to nonsense mutations in different genes, which make the study of translation termination important for developing approaches to treating such diseases. It is obvious that the yeast *Saccharomyces cerevisiae* represents a very tractable model organism for the investigation of translation termination. This is because all the power of classic genetic analysis and novel DNA recombinant techniques which are easily applied in this unicellular eukaryotic microorganism. It is especially important that the efficient homologous recombination characteristic of *S. cerevisiae* enables functional characterization of genes, since this allows their easy isolation, *in vitro* modification and re-introduction into the genome. This, combined with the complete establishment of the entire genome structure, makes this organism an ideal experimental eukaryotic model, which allows obtaining results that can be extrapolated to higher eukaryotes. Thus, one can state that the work of A.A. Nizhnikov devoted to the study of translation control seems to be both timely and important.

The dissertation of A.A. Nizhnikov consists of 110 pages and includes the following chapters: Introduction, Brief description of the results, References and Papers arising from this thesis.

The introduction, which includes a brief review of the published data, contains all information required for the understanding of work. It starts from the definitions and brief description of translational suppression in yeast. Then, author describes elements of epigenetic (prion) control of this process and indicates that the work is devoted to the study of a novel non-chromosomally-inherited factor which controls suppression. At the end of the chapter author lists all the aims of the work.

I have only a couple of minor remarks to this chapter. First, it seems unusual to use single-letter amino acid code for tRNA designation (p. 3). The expression "cycloheximide

mutants crl are localized in genes" is incorrect (p. 4). Only mutations but not mutants can be localized in genes.

In the beginning of the chapter "Brief Description of the Results" the author presents a list of his papers. This list is rather impressive. A.A. Nizhnikov is a coauthor of four papers, two of which are published in an international peer-reviewed journal. Also, one more paper has been accepted for publication.

Though the results of the work are described very briefly, in 3 pages, this chapter allows understanding of the content of the work. Formally, this chapter can be subdivided into several sections (which was not done by the author).

In the first section the author describes the properties of the genetic determinant [*NSI*$^+$], which suggest its prion nature and show that this determinant is not related to known proteins with established prion properties. This makes clear that [*NSI*$^+$] is related to an unidentified yeast prion protein. The second section describes results of the search for the genes modulating phenotypic expressions of the [*NSI*$^+$] determinant.

Remarks to this chapter are of a technical nature. To me, the expression «causes the slow suppression of *ade1-14*" (p. 10) sounds strange. Suppression can be inefficient or weak, but not slow. Concerning aminoglycoside antibiotics (p.10), it is my opinion, that it is more important that such antibiotics induce translation misreading, rather than inhibit translation.

All the results obtained are discussed in the third, largest (13 pages) chapter. The chapter contains four sections, which facilitates understanding the significance of the results. The order of the discussion of results partially corresponds to the order of their description in the previous chapter.

In the first section the author discusses the results that suggest prion nature of the [*NSI*$^+$] determinant and shows that this determinant differs from all described earlier. It should be noted that author's argumentation is valid and his conclusions seem to be correct.

The second section discusses the results of studying effects of the release factor-mediated modulation of the suppressor effect of [*NSI*$^+$]. Again, the data are discussed correctly. Nonetheless, the reviewer has one question. The author reasonably states that the suppressor effect of [*NSI*$^+$] is manifested only at decreased functional activity of Sup35. It should be noted that one fact may contradict this interpretation. In fact, [*NSI*$^+$] is manifested in cells expressing Sup35MC instead of wild type Sup35. However, it is known that N-terminal truncation of Sup35 increases, rather than decreases, its activity in translation termination (Valouev et al., Cell Motil Cytoskelet, 2002; Volkov et al., FEMS Yeast Res, 2007). The author also obtained data indication inactivation of Sup45 by [*NSI*$^+$] however western-blotting did not reveal differences in

the level of Sup45 in [*NSI*$^{+}$] and [*nsi*$^{-}$] strains. I'd like to give advice to author to repeat western-blotting with serially diluted samples.

The third chapter is devoted to the *VTS1* gene, which was identified in this work. The author presents quite rational considerations on the role of this gene in suppression. It should be however mentioned that such suppressors are usually designated as multicopy suppressors, because they suppress mutations only in the multicopy state.

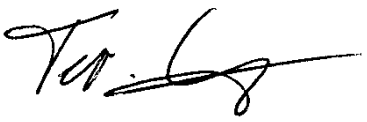
In the fourth section the author discusses the nature of the remaining multicopy suppressors identified in the work. Some of these genes encode RNA-binding proteins, while other encode transcription factors and, therefore, it is likely that they affect [*NSI*$^{+}$]-mediated suppression in a very indirect way. Notably, none of the identified proteins can physically interact with Sup35 and Sup45 release factors.

The fifth section is devoted to further perspectives of the work. All the plans seem to be reasonable and the reviewer would like to wish the author good luck in his future interesting studies.

The last chapter "References" contains 90 citations, all appropriate.

To summarize it should be stated that A.A. Nizhnikov has completed a well designed work and obtained interesting and important results, which are accurately described and discussed in his thesis. The content of the published papers corresponds well with the text of the dissertation. This leads this reviewer to conclude that the dissertation of Anton A. Nizhnikov "Novel genetic and epigenetic suppressors of nonsense mutations in *Saccharomyces cerevisiae*" satisfies all the requirements for such dissertations and the author deserves obtaining the degree of Doctor of Philosophy in Biology at the Saint-Petersburg State University.

Michael D. Ter-Avanesyan,
Professor, Corresponding Member of
The Russian Academy of Sciences,
Head of the Laboratory of
Molecular Genetics,
A.N. Bach Institute of Biochemistry
of the Russian Academy of Sciences



The signature of M. Ter-Avanesyan is valid.
A.F. Orlovsky, PhD, scientific secretary of INBI RAS June 5, 2013

Review

of the Dissertation of Anton Nizhnikov "Novel genetic and epigenetic suppressors of nonsense mutations in *Saccharomyces cerevisiae*" submitted for the degree of Doctor of Philosophy in Biology at the Saint-Petersburg State University

The dissertation of Anton Nizhnikov is dedicated to the problem of translational misreading induced in yeast by different mechanisms. The dissertation consists of 110 pages and includes four published articles and one article accepted for publication.

The problem of protein synthesis control is studied in the laboratory of physiological genetics of St. Petersburg University during last 50 years. Mutations in sup1 and sup2 genes of yeast *Saccharomyces cerevisiae* were obtained by S.G. Inge-Vechtomov at the middle of 60-s. Later analogous mutations in genes designated as *SUP35* и *SUP45* were independently described by others. Important progress in study of indispensable genes *SUP35* and *SUP45* has been achieved after cloning of corresponding genes from prokaryotic and eukaryotic organisms. It has been shown that *SUP45* and *SUP35* genes encoded translation termination factors eRF1 and eRF3, respectively. It was also found that aggregation of Sup35 protein leads to an appearance of [*PSI*$^{+}$] prion. To date yeast contain more prions than all other organisms taken together, all of them are formed by different proteins. One of them, [*NSI*$^{+}$], is characterized in the PhD thesis of A. Nizhnikov. Thus an actuality of his work does not raise any doubts.

In the **Introduction**, written on five pages, the author demonstrates a good knowledge of the scientific literature on the research topic. Nevertheless, some of his references are incorrect and some of them are missing (for example, Doel et al, 1994, one of the first papers where connection of [*PSI*$^{+}$] with the prion isoform of the translation termination factor eRF3 (Sup35) was shown).

In the next chapter, **Brief Descriptions of Results**, which takes four pages, author gives a short summary of results, most of which is already published. The chapter **Discussion** (13 pages) shows the author's understanding of the theoretical context of his work and the ability to make links between the review and his own researches. The work is very fully reported and discussed. The weakness of both parts is the completely absence of any illustrations including tables or/and figures. Partly it can be compensated by illustrations shown in accompanying publications, however a general scheme of research as well as a figure, summarizing all the results obtained by the author are missing.

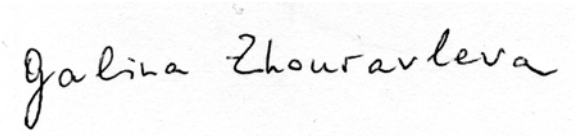
The last chapter, **References,** lists 90 works, most of them are written in English. As it was mentioned before, some of the important references for authors working in the field are missing, also some are strange, for example, what means "with modifications" (Alberti et al., 2009, with modifications, p.5)?

In conclusion, I can say that the thesis is very well written, the candidate shows that he can think critically about the data. The most of data are received by Anton Nizhnikov himself or with his active participation. During his research the

author used different methods, all of them are adequate to the task of research. The results are valid, most of them are already published in peer-reviewed international journals.

The work is important for understanding prionization phenomenon in yeast, in particular, and in mammals, in general.

Finally, I can conclude that the dissertation of Anton Nizhnikov "Novel genetic and epigenetic suppressors of nonsense mutations in *Saccharomyces cerevisiae*" complies with the international standard for PhD dissertations in the corresponding field.



Professor, Dr. of Sciences Galina Zhouravleva

June 13, 2013