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Assessing the regulation dependencies of thioredoxin 1 transcript variants by means of interactomic dynamic-with-induction profiling approach

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Antioxidant protein thioredoxin 1 (TXN) has been shown to play a critical role in the regulation of several redox-sensitive transcription factors. As thioredoxin 1 is rather the important nuclear regulator of the gene expression than immediate antioxidant, complex regulation of TXN expression may be required for the cell in order to isolate very distinct protective and regulatory functions of TXN. Thus, we aimed at revealing whether 1) thioredoxin 1 transcripts are differentially expressed in NFE2L2 pathway-inductive conditions, 2) any of thioredoxin 1 transcripts resemble NFE2L2-induced genes in the expression dynamics or regulation character. Using repetitive sampling approach we herein demonstrate experimental evidences of the differential regulation of both constitutive and inducible expression of the TXN transcript variants, and plausible TXN transcript variant 1
expression control by NFE2L2 pathway.

Keywords: oxidative status, interactomics, thioredoxin 1, independent expression regulation of transcript variants

Introduction

Thioredoxin 1, a protein containing a dithiol-disulfide site [8], has been shown to play a critical role in the regulation of several redox-sensitive transcription factors, including AP1 and NF-kappaB, which are capable of regulating cellular survival, proliferation, differentiation and apoptosis [3]. These functionally distinct factors also possess protective functions in response to specific pro-oxidant shifts in the cellular oxidative status, including oxidative, electrophilic, nitrosative and endoplasmic reticulum stresses [2, 10]. Kim et al. (2003) [6] showed that thioredoxin 1 expression is redox-sensitive (tBHQ-responsive) and is up-regulated in the manner specific to the antioxidant response element-dependent genes. Antioxidant

response element — dependent protective cellular reactions are provided by a subfamily of nuclear erythroid factor 2 related factors, predominantly NFE2L2. NFE2L2 has recently been shown to pair with at least some AP1 components and to rely on APEX1 activity, thus TXN forms following feed-forward system [10]: NFE2L2 (possibly cooperating with AP1 factors) induces TXN expression via ARE [10], and TXN provides induction of AREdependent genes by facilitating APEX1-dependent NFE2L2 and AP1 DNA binding [1, 4]. NFE2L2-involving pathways are reach in feed-forward and feedback circuits enabling the fast induction and inactivation of the principle cellular antioxidant regulatory system [11]. NFE2L2 regulatory partners (AP1, BACH1, HDAC3 and others; for more information please refer to Oxidative Status Interactome Map [11]) are all pleiotropic and multi-systemic. As such, they all may bear regulatory features already shown for BACH1. BACH1 has 2 proteincoding transcript variants, and only one of them is regulated by the intronic AREsequence [5]. Thus, BACH1-NFE2L2 regulatory system represents a positivefeedback loop allowing for the fast inactivation of NFE2L2 transcriptional activity in justnormalafter-stress cellular conditions. At the same time this partial regulatory independence of BACH1 transcript variants severs the NFE2L2dependent and NFE2L2-independent BACH1-dependent cellular processes. As cellular functional roles of TXN are at least BACH1-fashion diverse, while no certain information on TXN transcript variants regulation is available yet, we proposed TXN to have the independent transcript variant expression regulation in terms of ARE control. Thus, we aimed at revealing the character of gene expression regulation of thioredoxin 1 transcript variants by means of inductiondependent dynamic correlation/concordance assay.

Materials and methods

Study design and participants

The present study was a part of the project aimed at assessing the dynamic properties of a set of ARE-dependent genes (NQO1, PRDX6, HMOX1, SRXN1, KEAP1, BACH1, FOSL1; publication in process) in young adults under the academic examination and term stress conditions. The study was approved by Southern federal university Bioethical committee. All participants signed informed consent. The participants of the study (N=8) were students of Southern federal university Biological faculty, of matching age (21±1 years old). The study was undertaken during the winter examination session 2013 and the spring term 2013. All the

participants filled the questionary allowing to trace the environmental and organismal factors affecting oxidative status pathways: health state and necessary family anamnesis; medication, vitamin supplementation; coffee, alcohol consumption and smooking; contact with pollutants and radiation. Weather conditions were accounted and were similar during all the study stages. The blood was collected 4 times in total from the same subjects: 2 times during the winter examination session (24 h apart, representing the dependent dynamic expression patterns) and, one month later, 2 times during the beginning of the spring term (approximately 1 month apart, representing the assumed-independent expression patterns). Three-day stress level was assessed by the participants themselves using the 11-point analogue scale (stress level 0 to 10) in the morning of each blood collection days. Preliminary data analysis revealed the median stress levels for the session stages to be 6.75 (25th—75th percentiles: 5.9—7.1) and 5.75 (4.9—6.75), respectively, and for the term stages 2.5 (1.0—3.0) and 2.0 (1.25—3.1). Intracondition (session or term) stress levels were similar, while inter-condition stress levels were significantly different ($p=0.011$).

Semiquantitative PCR

Blood was collected into the vacuum blood collection systems with EDTA from elbow vein between 9:45 and 10:15 in the morning on the every stage of the study. Total RNA was isolated within 20 minutes after the blood collection using the commercial kit ‘RNA extran’ (Syntol, Russia) and was immediately treated with DNAse I (Syntol, Russia). The RNA integrity was assessed using non-denaturing 1.5% agarose gel electrophoresis. Clear 18S and 28S bands were observed with no signs of RNA degradation. The RNA was reverse transcribed using ‘RT kit’(Syntol, Russia) with the template denaturation step and using oligo(dT) primer. Reverse transcription (with M-MLV enzyme) was performed during 1 hour incubation at 38 ^{0}C. PCR was performed with the use of reagents by Syntol (Russia) (Mg^{2+}, dNTP mix), NEB TaqPol and PCR Buffer, and authors’ design primers (available on request) for NFE2L2 (3 transcripts), KEAP1 (2 transcripts), NQO1 (3 transcripts), PRDX6, HMOX1, BACH1 (transcript variant #2), SRXN1, FOSL1, TXN, and TBP (2 transcripts) as reference gene. We herein discuss only the dynamic and the inducible properties of thioredoxin 1 gene, so no redundant data on the factors listed will be presented here (the respective publication is in process). Transcript variants of thioredoxin 1 were detected using the restriction analysis of

the amplified TXN fragments with the use of MnlI restriction enzyme yielding heterogenous restricted band of 170 bp for transcript variant 1 and nonrestricted 285 bp band of transcript variant 2. DNA ladder used was Quick- Load PCR Marker (NEB, USA). Densitometry was performed using ImageJ (NIH, USA).

Statistical analysis

All target products' band intensities were normalized to that of TBP. Data were analyzed using MedCalc 11.4.2 and Statistica 7. All data were analyzed using the appropriate non-parametric criteria: Friedman test for the dependent time-series analysis; Mann-Whitney test for the stress conditions comparison; Wilcoxon test for the intra-condition time-series analysis; Spearman's correlation analysis. Data on the figures are medians (mid-line) and 25th—75th percentiles (bars).

Results and discussion

In the present study thioredoxin 1 transcript variants demonstrated complex character of the dynamic and stress state-dependent gene expression.

Under the academic stress conditions, the group expression levels of TXN transcript variant 1 (TXN tv1) increased in 24 hours (p=0.0078) (Figure 1) - similarly to the expression dynamics of the canonical NFE2L2 targets. NFE2L2 expression itself did not show any significant changes (data not shown). TXN transcript variant 2 (TXN tv2) expression remained unchanged (though close to significance of increase, p=0.078).

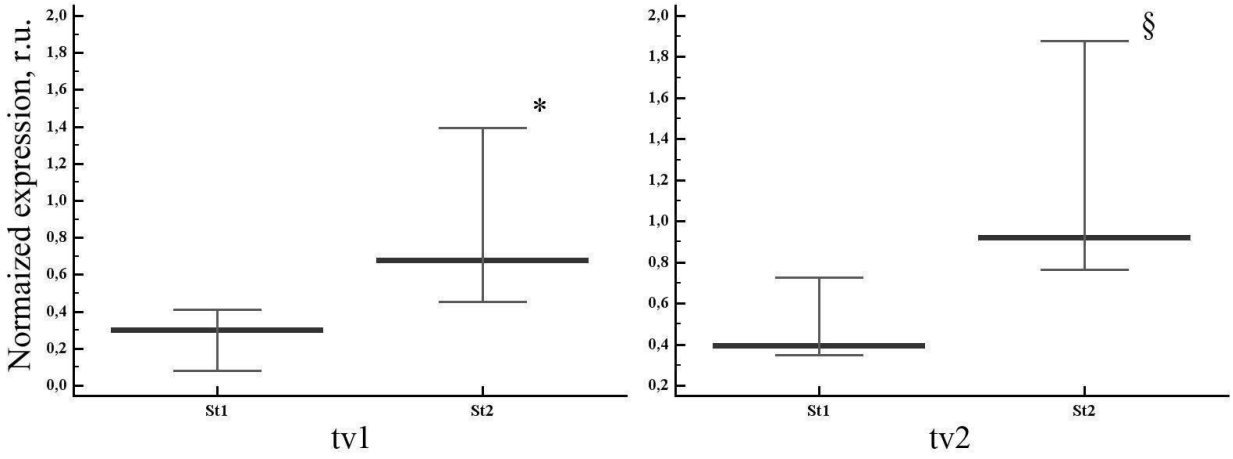

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*Figure 1 — RNA expression of TXN transcript variants 1 and 2 in the two-point 24h dynamics under the stressful conditions. St1 — day 0, the first blood collection stage; St2 — the day-after blood collection. * - p=0.0078 differences are significant between St1 and St2 for TXN tv1 expression; § — p=0.078 — differences are close to significance between St1 and St2 for TXN tv2 expression.*

Data presented in and underlying the Figure 1 do not allow to answer whether: 1) any of TXN transcripts is ARE-dependent, and 2) the increase in either transcript's expression is due to the interactomic pathway activation (e.g. NFE2L2 system stimulation) or to increase in putative transcription factor's RNA and(or) protein expression. However, contrary to similarities in the group trends of the TXN tv1 and tv2 expression, correlation analysis reveals no connection between the two transcripts suggesting them to be individually, though in the same trend, regulated. Moreover, under the academic exam stress conditions only transcript variant 1 expression correlated with the expression of the classic NFE2L2 targets (approvingly intercorrelated; data not shown).

According to set theory in gene expression regulation, all selected NFE2L2 targets have more than one regulator, while NFE2L2 is the only known common transcription factor of the inducible expression for KEAP1, NQO1, PRDX6, HMOX1, BACH1, SRXN1 and FOSL1. Consequently, concordant dynamic or inductive trends of interrelated in such a way factors would suggest for changes in the common variable's state, i.e. shifts in the NFE2L2 expression or pathway activation. As NFE2L2 expression was stable over a day during the session stages, we have concluded NFE2L2 pathway to be activated. Taking together data on the properties of the selected set of genes, the NFE2L2 pathway activation under the examination stress conditions, the correlation of the expression levels of TXN tv1 and FOSL1 (r=0.59, p=0.016), HMOX1 (r=0.859, p=0.0001), NQO1 (r=0.61,

0.012), we suggested TXN transcripts to be at least differentially regulated and,

even more, TXN tv1 to be NFE2L2-dependent and TXN tv2 to be NFE2L2independent.

Furthermore, TXN transcript variants further demonstrate clear differences in responsiveness to the socio-psychological stress: while TXN tv1 expression is decreased (p=0.0093) in the more stressful conditions, tv2 demonstrates no changes

between the session and the term (Figure 2). Surprisingly higher TXN tv1 expression under the mild stress conditions (term) may reflect the interference between stress-responsive pathways and other regulators of TXN tv1 expression, e.g. overflow-type inhibitory interactions between AP1 and NFE2L2 [9]. In fact, NFE2L2 expression, though generally stable in the dynamics over the whole period of the study, was also found to be slightly-but-significantly higher under mild stress conditions (data not shown). Nevertheless, the data presented on Figure 2 supports our mention on the differential regulation of the TXN transcripts' expression.

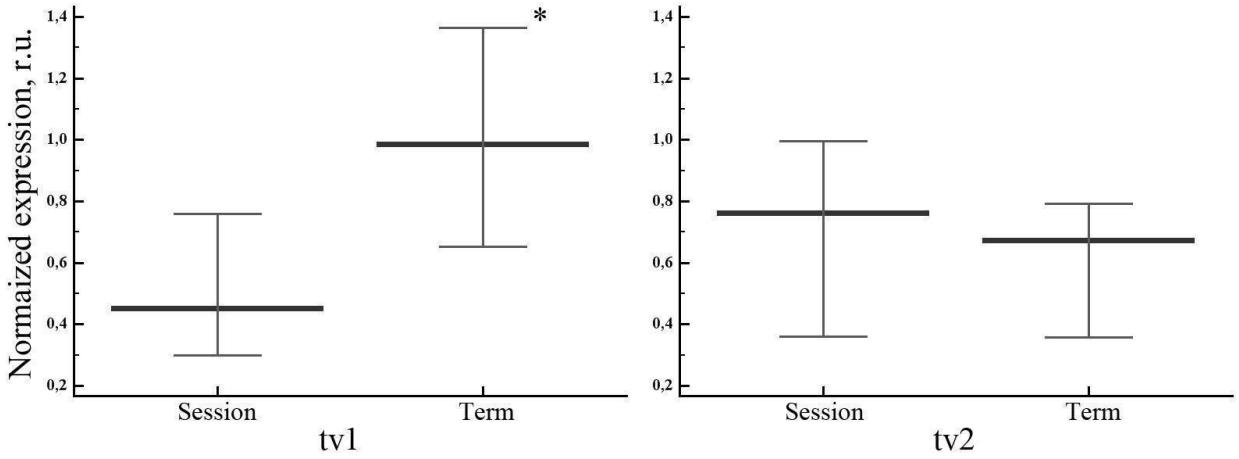

*Figure 2 — RNA expression of TXN transcript variants 1 and 2 between the two stress conditions. Intra-conditional data were combined; analysis involved independent group statistics. * — p=0.0093 differences are significant between the examination session and the academic term for the TXN tv1 expression.*

We found no significant differences between the two independent mild stress time points (the two term stages, month apart) in the expression of TXN tv1 nor tv2. Expanding the previous discussion of the set theory application in the gene expression regulation studies, whenever the weight of the common variable decreases, the interconnection between factors should drop unless other common variables are present. As, for today's knowledge, NFE2L2 significantly contributes to the constitutive expression of only NQO1 (up to 90%) and HMOX1 (up to 70%, cell-type specific) among selected genes, it is anticipated that most of the inducedstate similarities (correlations) will become insignificant under the mild stress conditions. As it was expected, non of the classical inducible NFE2L2dependent genes are correlated under the term stress conditions (data not shown).

This is also true for TXN tv1, which further supports the similarity in the qualitative inducible RNA expression control between TXN tv1 and proven NFE2L2-dependent genes.

Taking together all the data and the conclusions of the present study, TXN appears to have the differentially regulated transcript variants. Natural limitation of the used here cost-effective approach is in obvious inability to prove directly the dependence of thioredoxin transcript variant 1 on NFE2L2-pathway, though reliable indirect data are collected allowing to suggest this and that TXN tv2 is NFE2L2-independent. In the outlook, elucidation of the true NFE2L2-dependent transcript of thioredoxin 1 gene would led to completion of the build-up of the practically valuable analytical TXN feed-forward circuit map.

Thioredoxin 1 feed-forward circuit may have important adaptive role in the cell functioning by facilitating: 1) rapid antioxidant induction; 2) direct blockade of AP1/NF-kappaB by preventing their cytoplasmic prooxidative activation, and 3) blockade of NF-kappaB prooxidative feed-forward circuits [7] in case of antioxidant system's failure and nuclear TXN pool exhaustion. In the present study, the dynamic-under-induction expression profiling (24-hours between two blood collection stages during academic session) of the TXN tv1 alongside classic NFE2L2 targets suggests the locking-up of the NFE2L2 positive feed-forward circuits, and TXN tv1 may represent a part of feed-forward systems of the central fast reacting antioxidant system of the human cell.

Conclusion

As thioredoxin 1 is rather the important nuclear regulator of the gene expression than immediate antioxidant, complex regulation of the TXN expression may be required for the cell in order to isolate very distinct protective and regulatory functions of TXN. We herein demonstrate the experimental evidences of the differential regulation of both constitutive and inducible expression of the TXN gene transcript variants. Correlation analysis reveals the similarities in the inducible expression regulation of TXN tv1 with the classic NFE2L2 targets, while TXN tv2 expression character is not related to TXN tv1 nor any of the studied NFE2L2 targets.

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