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Translational control of Nrf2 within the open reading frame

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ABSTRACT

Nuclear Factor Erythroid 2-Related Factor 2 (Nrf2) is a transcription factor that is essential for the regulation of an effective antioxidant and detoxifying response. The regulation of its activity can occur at transcription, translation and post-translational levels. Evidence suggests that under environmental stress conditions, new synthesis of Nrf2 is required – a process that is regulated by translational control and is not fully understood. Here we described the identification of a novel molecular process that under basal conditions strongly represses the translation of Nrf2 within the open reading frame (ORF). This mechanism is dependent on the mRNA sequence within the 3′ portion of the ORF of Nrf2 but not in the encoded amino acid sequence. The Nrf2 translational repression can be reversed with the use of synonymous codon substitutions. This discovery suggests an additional layer of control to explain the reason for the low Nrf2 concentration under quiescent state.

1. Introduction

Nuclear Factor Erythroid 2-related factor 2 (Nrf2) is a basic leucine zipper transcription factor that is essential for the regulation of antioxidant enzymes and the phase II detoxifying response [1]. Nrf2 exerts its function in the nucleus by forming a heterodimer with members of the small maf protein family and promotes the transactivation of its target genes through a DNA enhancer motif known as the antioxidant response element (ARE) [2].

The regulation of the activity Nrf2 is a highly active process and involves tight control at different levels including transcriptional control, translational control, post-translational modifications and proteasomal degradation [3]. The most studied mechanism for Nrf2 regulation is based on fast proteasomal degradation and indicates that, under basal conditions, the detection of Nrf2 is difficult due to its low abundance. The half life for this transcription factor is <20 min [4], which is attributed to the interaction of the cytoplasmic Nrf2 with Kelch-like ECG-associated protein 1 (Keap1). This interaction facilitates ubiquitination by a Cul3-E3 ubiquitin ligase system and subsequent proteasomal degradation. When the cells are exposed to electrophilic or oxidative stressor molecules, the interaction between Keap1 and Nrf2 is disrupted through posttranslational modifications of reactive cysteines in Keap1 [5], thus preventing degradation and facilitating the nuclear translocation of Nrf2 and binding to ARE. ARE is a promoter element found in many antioxidant enzymes, including superoxide dismutase (SOD), peroxiredoxins, thioredoxins, catalase, glutathione peroxidase, and heme oxygenase-1 (HO-1). Nrf2 therefore plays a pivotal role in the ARE-driven cellular defense system against oxidative stress.

Translational control is one of the Keap1 independent mechanisms involved in the regulation of Nrf2 [6]. Rather than just the inhibition of protein degradation mediated by Keap1, evidence has shown that newly translated Nrf2 is also required to actively counteract the effect of electrophiles [7–9]. Mechanisms involving translational control allow the cells to quickly respond to noxious conditions by specifically regulating the translation of certain transcripts in space and time, which occurs by keeping the mRNA molecules in a repress state. This allows for their translation, when environmental signals indicate that it is appropriate, without requiring mRNA transcription, maturation and nuclear export. It has been shown that both the 5′ and 3′ untranslated regions (UTR) of Nrf2 mRNA contain regulatory elements that control Nrf2 translation. Specifically, the 5′ UTR of Nrf2 has an internal ribosome entry site (IRES) that is redox-sensitive [10] and the 3′ UTR is recognized by microRNAs that negatively regulate the expression of Nrf2 [11]. Translational control mechanisms acting on the coding region of various translationally repressed genes have been studied and described [12,13], however, translational control on the coding region of Nrf2 has not been explored.

In the present work, we describe the identification and characterization of a novel molecular process that regulates the translation of Nrf2 within the open reading frame (ORF). This regulatory process is dependent on the mRNA sequence within the 3′ portion of the Nrf2 ORF, and imposes a strong translational repression on the whole transcript. The regulatory element is able to control

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the expression of the reporter gene eGFP and its effect can be reversed if the 3′ sequence is altered with synonymous codon substitutions.

2. Materials and methods

2.1. Recombinant constructs

A plasmid containing the cDNA of Nrf2 was obtained from Thermo fisher (accession no. BC011558 clone ID: 4548874) and was used as a template for PCR reactions. Also the plasmid pLVTHM (addgene.org clone 12247) was used as a template for eGFP PCR reactions.

All the recombinant constructs described in this work were cloned in the plasmid PLEX-MCS (Thermo fisher) that was modified to include in the C-term of the recombinant proteins, a strep tag II and a His 6X tag [13]. The recombinant constructs were created with the following primer sets, and contained, in the forward primer, a restriction site for BamHI (Underlined) plus a kozak sequence (lower case), and in the reverse primer a restriction site for AgeI (Underlined); the integrity of all the construct described was confirmed by sequencing. Nrf2 F: 5′ CGG GAT CCg ccg cca ccA TGA TGG ACT TGG AGC TGC C 3′ R: 5′ TCC CAC CGG TGT TTT TCT TAA CAT CTG GCT TCT T 3′; Δ17–32 Nrf2 F: 5′ CGG GAT CCg ccg cca ccA TGA TGG ACT TGG AGC TGC CGC CGC CGG GAC TCC CGT CCC AGC AGG ACA GTC GAG AAG TAT TTG ACT TCA GTC A 3′; Segment 1 F: 5′ CGG GAT CCg ccg cca ccA TGA TGG ACT TGG AGC TGC C 3′ R: 5′ TCC CAC CGG TCT CAA CCA GCT TGT CAT TTT CA 3′; Segment 2 F: 5′ CGG GAT CCg ccg cca ccAT GAC TAC CAT GGT TCC AAG TCC AG 3′ R: 5′ TCC CAC CGG TTC CAG GGG CAC TAT CTA GCT CTT 3′; Segment 3 F: 5′ CGG GAT CCg ccg cca ccA TGA GTG TCA AAC AGA ATG GTC CTA AA 3′ R: 5′ TCC CAC CGG TGT TTT TCT TAA CAT CTG GCT TCT T 3′; Segment1–2 F: 5′ CGG GAT CCg ccg cca ccA TGA TGG ACT TGG AGC TGC C 3′ R: 5′ TCC CAC CGG TTC CAG GGG CAC TAT CTA GCT CTT 3′; Segment 2–3 F: 5′ CGG GAT CCg ccg cca ccAT GAC TAC CAT GGT TCC AAG TCC AG 3′ R: 5′ TCC CAC CGG TGT TTT TCT TAA CAT CTG GCT TCT T 3′. All these PCR products were gel-purified (Promega), digested with BamHI and AgeI (Fermentas) and ligated into PLEX-MCS previously digested with the same enzymes.

The creation of the constructs containing eGFP fused to Segment 2 and Segment 3 was performed in three steps: First, a PCR product for eGFP containing a C-term His 6X followed by two stops codons and a KpnI recognition site was created with the primer set F: 5′ CGG GAT CCg ccg cca ccA TGG TGA GCA AGG GCG AG 3′ R: 5′ TCC CAC CGG TGG TAC CTT ACT AAT GAT GGT GAT GGT GGT GTC GAG ATC TGA GTC CGG ACT T 3′. This PCR product contained the recognition sites for BamHI and AgeI and was cloned into PLEX-MCS as described above to over express eGFP with C-term His tag. The same PCR product was used to create the fusion constructs eGFP-Segment 2 and eGFP-Segment 3 by using the KpnI recognition site. Second, a PCR product for Segment 2 and Segment 3 containing a KpnI recognition site in the 5′ was obtained with the following set of primers: KpnI-Segment 2 F: 5′ GGG GTA CCAC TAC CAT GGT TCC AAG TCC AG 3′ R: the primer described above for Segment 2; KpnI-Segment 3 F: 5′GGG GTA CCA GTG TCA AAC AGA ATG GTC CTA AA 3′ R: the primer described above for Segment 3. Third, the PCR products for eGFP, KpnI-Segment 2 and KpnI-Segment 3 were digested with KpnI and a ligation was performed between eGFP and Segment 2 and Segment 3. These ligations were used as templates to obtain the fusion clones eGFP-Segment 2 and eGFP-Segment 3 by using the Forward primer to amplify eGFP and the Reverse primers for Segment 2 and Segment 3 described above. These PCR products were digested with BamHI and AgeI and were cloned into PLEX-MCS.

The generation of the modified Segment 3 with all codons substituted with synonym codons was performed manually using a standard codon table as a reference. The construct containing the modified sequence was synthesized by IDT DNA technologies using gblock technology. This synthetic construct was fused with an eGFP PCR product obtained from the eGFP His construct described above using the primers F: 5′ ATA GAA GAC ACC GAC TCT ACT AGA GGA TCC GCC GCC ACC ATG GTG AG 3′ and R: 5′ GGG CGT CTT TGG GCC GTT TTG CTT AAC CGA TTA CTA ATG ATG GTG ATG GTG GT 3′. The gibson assembly method [14] with the gibson enzyme mix from New England Biolabs was used, and was then cloned into PLEX-MCS previously digested with BamHI-AgeI. The sequence of the modified Nrf2 segment 3 is as follows:

TCGGTTAAGCAAAACGGCCCAAAGACGCCCGTCCACTCGTCAGG TGACATGGTCCAGCCACTGTCCCCCTCGCAAGGACAAAGTACGCATGT ACACGACGCTCAGTGCGAAAATACCCCCGAAAAGGAGCTACCCGTGTC CCCCGGGCACAGAAAGACGCCCTTTACGAAGGATAAGCACTCCTCCAG GTTAGæTCGGTTAAGCAAAACGGCCCAAAGACGCCCG TCCACTCGTC AGGTGACATGGTCCAGCCACTG TCCCCCTCGCAAGGACAAAGTACG CATGTA TCGGTTAAGCAAAACGGCCCAAAGACGCCCGTCCACTCGT CAGGTGACATGGTCCAGCCACTGTCCCCCTCGCAAGGACAAAGTACGC ATGTACACGACGCTCAGTGCGAAAATACCCCCGAAAAGGAGCTACCCG TGTCCCCCGGGCACAGAAAGACGCCCTTTACGAAGGATAAGCACTCCT CCAGGTTAGæTCGGTTAAGCAAAACGGCCCAAAGACGCCCGTCCACTC GTCAGGTGACATGGTCCAGCCACTGTCCCCCTCGCAAGGACAAAGTAC GCATGTA TCGGTTAAGCAAAACGGCCCAAAGACGCCCGTCCACTCGT-CAGGTGACATGGTCCAGCCACTGTCCCCCTCGCAAGG. To create a full length Nrf2 with the mutated segment 3 described above, two PCR fragments corresponding to a product containing the wild type sequence for segment 1 and 2 and the other containing the sequence of the mutated segment 3 were fused together using the gibson assembly mix (New England biolabs). The fragment containing segments 1–2 was obtained with the primer set: F: 5′ ATA GAA GAC ACC GAC TCT ACT AGA GGA TCC GCC GCC ACC ATG ATG GAC T 3′ R: 5′ GGG CGT CTT TGG GCC GTT TTG CTT AAC CGA TCC AGG GGC ACT ATC TAG CTC TT 3′ and the mutant segment 3 with the set: F 5′ TCG GTT AAG CAA AAC GGC 3′ R: 5′ GTT GGC GCA GCA GCC GGG GCA GCA ACC GGT ATT CTT TTT CAC GTC GGG TTT 3′. The fusion of these PCR fragments was performed in the same vector as described above.

2.2. Cell culture and transfections

HEK-293T cells were obtained from ATCC and were grown in Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum. The recombinant plasmids reported in this work were extracted using the Pureyield Maxiprep system from Promega and were transfected using Jetprime (Polyplus) following the manufacturer's recommendations without modifications. The protein expression levels were evaluated 48 h after transfection with Western blotting or fluorescence laser scanning.

2.3. Western blotting

Transfected HEK-293T cells were lysed by using M-PER mammalian protein extraction reagent (Thermo scientific) containing Halt protease inhibitor cocktail (Thermo scientific). Lysates were centrifuged at 16,000*g* at 4 °C for 15 min. The supernatants (40 micrograms) from each sample were separated by SDS–PAGE and transferred into nitrocellulose membranes. The following antibodies were used: mouse monoclonal Anti strep-tagII labeled with HRP (Genescript Cat. A01742-100), mouse monoclonal anti His C-term labeled with HRP (Life technologies cat. R931-25) and mouse monoclonal anti beta actin labeled with HRP (Santa Cruz Biotechnologies Cat. sc-47778 HRP). All antibodies were diluted 1:5000 in 1× PBS with 0.1% tween 20 and 5% nonfat dry milk.

2.4. Fluorescence laser scanning

For fluorescence imaging, HEK-293T cells were grown and transfected in 48 well tissue culture plates. NucRed Live 647 (Life technologies) was added to label the cell nuclei following the manufacturer's recommendations. Cells fluorescence was determined using a Fujifilm FLA-5000 Laser scanner. The 473 nm laser and the LPB filter was used for eGFP detection and the 635 nm laser in combination with the LPR filter was used to detect nuclei fluorescence. Densitometry measurements were obtained with the Fujifilm image analysis software Multi Gauge.

2.5. Software analysis

The codon adaptation Index (CAI) was calculated using the application developed by Puigbo et al. [15] available at http://genomes.urv.es/CAIcal/.

3. Results

3.1. The translation of the open reading frame of Nrf2 is low despite having a good codon usage frequency

The codon adaptation index (CAI) [16] is a measurement of codon bias that allows the comparison of the codons present in a specific gene versus a reference codon usage set from the organism in which the protein is expressed. This index ranges from 0 to 1 and correlates with protein translation efficiency. An index of 1 indicates that a gene uses the most common codons for a particular amino acid in the set. We found a CAI of 0.73 for Nrf2, suggesting a codon composition that is expected to be highly expressed.

In agreement with previous reports [9], we also found that while Nrf2 can be detected by western blot (Fig 1A), the expression is low, and is only slightly elevated if a degradation-resistant Nrf2 mutant previously described (Δ17–32aa) [17] is used for overexpression (Fig 1A). This low Nrf2 expression is more evident when compared to the recombinant expression with the same vector and transfection conditions of Grp78 (HSPA5), a protein that has a similar size and a similar CAI (0.77) (Fig 1B). These results suggest that the low expression is due the presence of an unidentified Keap-1 independent mechanism regulating the expression of Nrf2 within the ORF.

3.2. Nrf2 expression is regulated by a translational control mechanism within the open reading frame

Because there was no previous information suggesting the location of potential regulatory elements for protein translation within the ORF of Nrf2, we decided to explore the translation potential by dividing the whole transcript into three segments in order identify a segment with repressed translation. The Nrf2 ORF is 1815 bp excluding the stop codon and therefore the three segments were composed of the following base pairs: Segment 1 = 1–627 bp, Segment 2 = 628–1158 bp and Segment 3 = 1159–1815 bp (Fig. 2A). Their length was selected according to the possibility of designing good primers pairs for PCR amplification. We also verified that the 3 segments have similar CAI (Segment1 = 0.71, Segment 2 = 0.75 and Segment 3 = 0.73), which indicated that their ability to be efficiently translated was similar.

To exclude the possibility of poor protein detection by fast proteosomal degradation, the constructs were overexpressed with and without the proteasome inhibitor MG132. We first verified that the three constructs were efficiently transcribed (Fig. 2B bottom panel). Next, we determined the expression levels of the 3 segments of Nrf2 by western blot with anti strep tag II antibody. We found that the expression of segment 1 was low (Fig. 2B lane 1), but was rescued with the use of the proteasomal inhibitor. This result is as expected because segment 1 contains the amino acids sequence that interacts with Keap1 to promote proteasomal degradation [9,17]. In contrast, the expression of segment 2 was elevated and was independent of the proteasomal degradation (Fig. 2B lane 2). Surprisingly, the expression of segment 3 could not be detected (Fig. 2B lane 3), even after the use of proteasomal inhibitor, suggesting the presence of an unknown mechanism preventing the expression of this segment. To corroborate this finding, we decided to create other constructs to evaluate the effect on protein expression by fusing segment 2, which we found to be highly over expressed, with segment 3. As a control, we also evaluated the translation of segment 1 fused together with segment 2. The expression of all the constructs was evaluated with and without the use of a proteasomal inhibitor. We found that while segment 1 dramatically reduced the expression of the fused segment 2 (Fig 2C lane 1), the expression can be rescued with the use of the proteasomal inhibitor. On the other hand we confirmed that segment 3 prevented the expression of segment 2 even with the inhibition of the proteasomal degradation (Fig 2C lane 3). Collectively, these results suggest that segment 3 contains a novel translational repressor mechanism that regulates the expression of Nrf2.

3.3. The regulation of the expression of Segment 3 is dependent on the mRNA sequence and not by the amino acids encoded by the sequence

To confirm that the mRNA sequence of segment 3 contains regulatory elements for protein translation, and to exclude the possibility that an unknown mechanism was promoting protein degradation by targeting amino acids present in the segment 3,

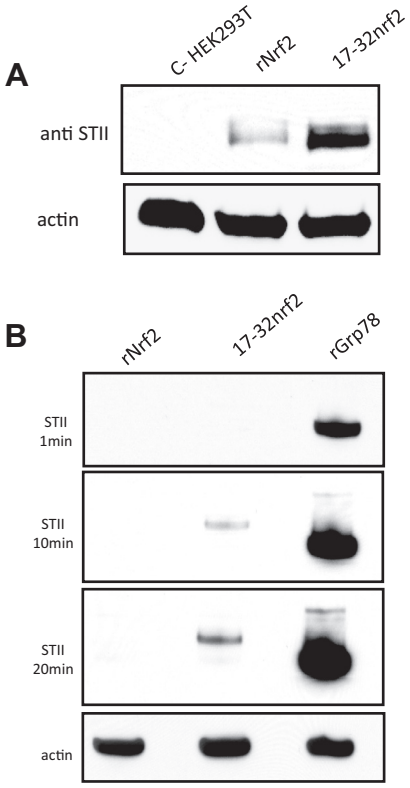

Fig. 1. The translation of the Nrf2 open reading frame is low. (A) Detection of recombinant overexpression of Nrf2 and a mutant (Δ17–32aa) resistant to degradation after 48 h in HEK293T cells. (B) A comparison of recombinant expression levels of Nrf2 and Δ17–32-nrf2 vs Grp78. The three panels indicate different exposition times of the X-ray films used to detect the antibody signal.

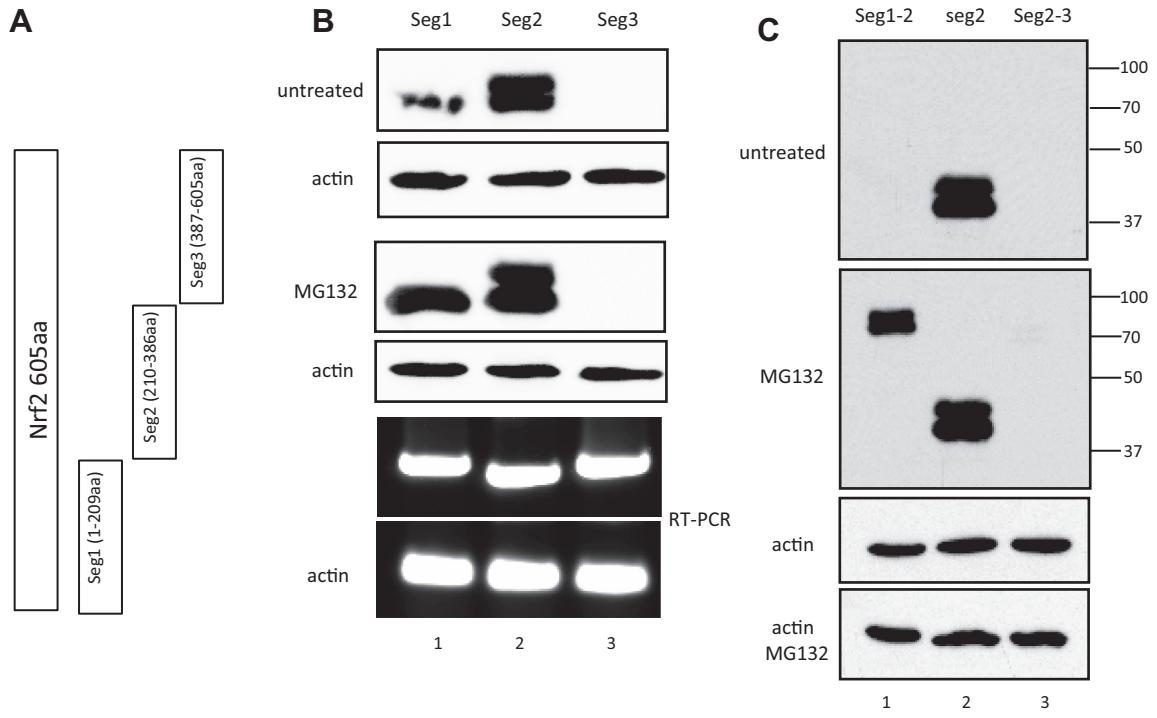

Fig. 2. The expression of Nrf2 is regulated within the coding region. (A) Diagram showing the three segments of the Nrf2 protein with similar CAI. (B) Recombinant overexpression of Nrf2 segments 1, 2 and 3 with and without the proteasome inhibitor MG132 after 48 h of transient transfection of HEK293T cells as detected with anti Strep tag II antibody. The lower panel confirmed the transcription of the three recombinant constructs by semi quantitative PCR. (C) Evaluation of recombinant overexpression of Nrf2 segment 2 fused with segment 1 or Segment 3 with and without a proteasome inhibitor.

we evaluated the effect of fusing eGFP with the mRNA sequences of segment 3. The experimental design included two stop codons in between the sequences of eGFP and segment 3 to prevent the translation of the amino acids encoded by segment 3 (Fig 3A). As a control, we generated a similar construct by fusing eGFP with segment 2 (Fig. 3A). The constructs were transfected into HEK-293T cells and eGFP was detected by western blot using an anti 6X-His tag included in the C-term of eGFP.

We found that the mRNA sequence of segment 2 did not alter the expression of eGFP (Fig. 3B lane 2). On the other hand, we verified that the segment 3 mRNA sequence dramatically reduced the translation of eGFP (Fig. 3B lane 3), even if the translation of the amino acids of segment 3 did not occur. Our results suggest that the mechanism inhibiting the translation of segment 3, alone or fused to other sequences is not by an unidentified protein degradation process.

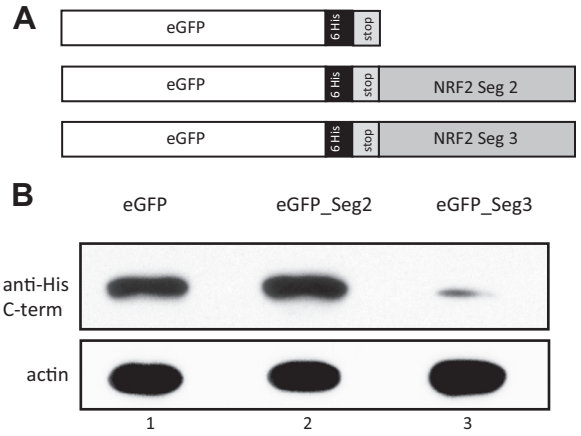

Fig. 3. The regulation of the expression of Segment 3 is dependent on the mRNA sequence and not by the amino acids encoded by the sequence. (A) A diagram of the constructs used to evaluate the effect of the mRNA sequence of Nrf2 Segment 2 and 3 fused with eGFP. (B) Evaluation of overexpression of eGFP fused to Nrf2 segment 2 and 3 by western blot using an anti-His C-term antibody.

3.4. Synonym mutations of Segment 3 reverse the translational repression

Next, we asked whether the translational repression of Segment 3 could be reversed by a mutant with synonymous substitutions of all the codons present in Segment 3. The experimental design was to fuse this mutant in a similar way to the eGFP study described above (Fig. 3A). We were able to create a Segment 3 sequence with synonym mutations that was only 61% similar to the wild type sequence. After transfection of this construct into HEK-293T cells, it was confirmed that the translational repression regulating the expression of Segment 3 could be completely reversed by changing the sequence of the mRNA without requiring alteration in the amino acids encoded by the sequence (Fig. 4A well in lane 3 vs well in lane 2). These mutations were also able to revert the translational repression when they were included into the full length Nrf2 open reading frame (Fig. 4B) and promoted a 6-fold increase in translation when compared to the wild type ORF sequence.

4. Discussion

The detection of cellular Nrf2 under basal conditions is difficult, due to its low abundance. The large and rapid stress-induced increases in nuclear Nrf2 originating only from an existing pool of Keap1-bound Nrf2 suggests an alternate mechanism involving translational control regulating the expression of Nrf2 [6,7]. The translational control process can occur either within the UTR and/or within the ORF of the regulated genes [18]. While UTR associated Nrf2 translational control has been described [10,11], there

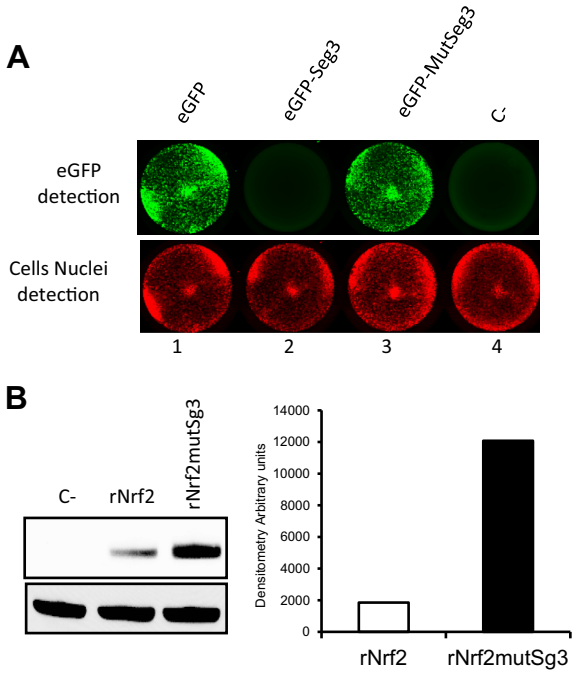

Fig. 4. Synonym mutations of Segment 3 reverse translational repression. (A) eGFP fluorescence detection by laser scanning to evaluate the expression eGFP derived from recombinant constructs that contained the sequence of wild type Nrf2 segment 3 or the mutant with synonym codon substitutions. (C) Evaluation of recombinant expression of wild type Nrf2 vs a full length Nrf2 where the sequence corresponding to segment 3 was mutated using the synonym codons described in (A). The bar graph to compare the signal intensity of the expression of this two constructs after laser scanning densitometry.

was no information about translational control within the ORF. Our data, for the first time, shows that Nrf2 translational regulation occurs within the ORF and results in the repression of the translation.

Gene-specific translational control is a highly active process that can involve the participation of multiple cis-acting and trans-acting factors [18]. The cis-acting factors are located within the mRNA sequence itself and include upstream open reading frames, RNA secondary structures such as hairpin loops, or IRES [18]. The trans-acting factors are external components that impose regulation on a transcript and can be proteins or RNA molecules such as microRNAs. It is common to find that the regulation of a gene at the translational level involves a close interaction between cis-acting and trans-acting factors. These regulatory elements for translation are generally found in the UTRs [19]. In the particular case of Nrf2, these regions have been studied for their role in translational control, and have resulted in the identification of an IRES at the 5′ UTR and multiple microRNA binding sites at the 3′ UTR [10,11].

Translational control elements regulating the expression of specific genes within their coding region have also been reported for other proteins but not in Nrf2 [12,13]. Our rationale for exploring this possibility of the presence of translational control elements within the ORF was based on the fact that the mRNA sequence of Nrf2 lacks codon bias that potentially could reduce the expected translation efficiency of this transcript. Our results indicate that the translation of Nrf2 was low even in a mutant lacking amino acids essential for its rapid proteasomal degradation (Fig 1A and B). We used an innovative approach by dividing the ORF into 3 segments that had similar CAI in order to independently determine the translational efficiency of these segments. This unconventional approach allowed us to identify a Nrf2 translational control dependent mechanism within the open reading frame. Our data convincingly show that the repressor mechanism requires the mRNA nucleotide sequences or tertiary structure of the 3′ ORF, but not the encoded amino acids. We believe that the identification of this novel regulatory element within the ORF adds to the knowledge of the previously described Nrf2 translation control mechanisms. More importantly, it points out to the sophistication of the translational control of Nrf2 and suggests the importance of a tight regulation of Nrf2 levels.

The molecular mechanism regulating the translation of Nrf2 imposed by the sequence contained in its 3′ ORF is poorly understood. Based on the available literature for other genes regulated in a similar way, we expect other trans-acting factors such as RNA-binding proteins or other RNA molecules to play a role in regulating Nrf2 expression at the 3′ ORF. Although our results show a novel repressor mechanism under quiescent state, the environmental conditions that activate Nrf2 translation via this mechanism acting on the 3′ ORF are yet to be determined. Future work using both established and modern techniques in the field of RNA-interactions will be needed to characterize this novel translational control mechanism. This could potentially lead to the identification of new drugs to increase Nrf2 translation, which could be used to treat or prevent human diseases where oxidative stress plays a central role.

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References

[1] T.W. Kensler, N. Wakabayash, S. Biswal, Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway, Annual Review of Pharmacology and Toxicology 47 (2007) 89–116.
[2] K. Itoh, T. Chiba, S. Takahashi, T. Ishii, K. Igarashi, Y. Katoh, T. Oyake, N. Hayashi, K. Satoh, I. Hatayama, M. Yamamoto, Y. Nabeshima, An Nrf2 small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements, Biochemical and Biophysical Research Communications 236 (1997) 313–322.
[3] H.K. Bryan, A. Olayanju, C.E. Goldring, B.K. Park, The Nrf2 cell defence pathway: Keap1-dependent and -independent mechanisms of regulation, Biochemical Pharmacology 85 (2013) 705–717.
[4] A. Kobayashi, M.I. Kang, H. Okawa, M. Ohtsuji, Y. Zenke, T. Chiba, K. Igarashi, M. Yamamoto, Oxidative stress sensor Keap1 functions as an adaptor for Cul3-based E3 ligase to regulate for proteasomal degradation of Nrf2, Molecular and Cellular Biology 24 (2004) 7130–7139.
[5] S. Fourquet, R. Guerois, D. Biard, M.B. Toledano, Activation of NRF2 by nitrosative agents and H2O2 involves KEAP1 disulfide formation, Journal of Biological Chemistry 285 (2010) 8463–8471.
[6] S.E. Purdom-Dickinson, E.V. Sheveleva, H. Sun, Q.M. Chen, Translational control of Nrf2 protein in activation of antioxidant response by oxidants, Molecular Pharmacology 72 (2007) 1074–1081.
[7] K.P. Shay, A.J. Michels, W. Li, A.-N.T. Kong, T.M. Hagen, Cap-independent Nrf2 translation is part of a lipoic acid-stimulated detoxification stress response, Biochimica Et Biophysica Acta-Molecular Cell Research 2012 (1823) 1102–1109.
[8] K. Itoh, K.I. Tong, M. Yamamoto, Molecular mechanism activating Nrf2-Keap1 pathway in regulation of adaptive response to electrophiles, Free Radical Biology and Medicine 36 (2004) 1208–1213.
[9] K. Itoh, N. Wakabayashi, Y. Katoh, T. Ishii, T. O'Connor, M. Yamamoto, Keap1 regulates both cytoplasmic-nuclear shuttling and degradation of Nrf2 in response to electrophiles, Genes to Cells 8 (2003) 379–391.
[10] W.G. Li, N. Thakor, E.Y. Xu, Y. Huang, C. Chen, R. Yu, M. Holcik, A.N. Kong, An internal ribosomal entry site mediates redox-sensitive translation of Nrf2, Nucleic Acids Research 38 (2010) 778–788.
[11] M. Narasimhan, D. Patel, D. Vedpathak, M. Rathinam, G. Henderson, L. Mahimainathan, Identification of novel microRNAs in post-transcriptional control of Nrf2 expression and redox homeostasis in neuronal, SH-SY5Y cells, PLoS One 7 (2012).
[12] E. Chu, D. Voeller, D.M. Koeller, J.C. Drake, C.H. Takimoto, G.F. Maley, F. Maley, C.J. Allegra, Identification of an rna-binding site for human thymidylate synthase, Proceedings of the National Academy of Sciences of the United States of America 90 (1993) 517–521.
[13] O. Perez-Leal, C.A. Barrero, A.B. Clarkson, R.A. Casero, S. Merali, Polyamine-regulated translation of spermidine/spermine-N-1-acetyltransferase, Molecular and Cellular Biology 32 (2012) 1453–1467.

[14] D.G. Gibson, L. Young, R.Y. Chuang, J.C. Venter, C.A. Hutchison, H.O. Smith, Enzymatic assembly of DNA molecules up to several hundred kilobases, Nature Methods 6 (2009) 341–343.

[15] P. Puigbo, I.G. Bravo, S. Garcia-Vallve, CAIcal: a combined set of tools to assess codon usage adaptation, Biology Direct 3 (2008).

[16] P.M. Sharp, W.H. Li, The codon adaptation index – a measure of directional synonymous codon usage bias, and its potential applications, Nucleic Acids Research 15 (1987) 1281–1295.

[17] M. McMahon, N. Thomas, K. Itoh, M. Yamamoto, J.D. Hayes, Redox-regulated turnover of Nrf2 is determined by at least two separate protein domains, the redox-sensitive Neh2 degron and the redox-insensitive Neh6 degron, Journal of Biological Chemistry 279 (2004) 31556–31567.

[18] N. Sonenberg, J.W.B. Hershey, M. Mathews, Translational Control of Gene Expression, second ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 2000.

[19] F. Gebauer, M.W. Hentze, Molecular mechanisms of translational control, Nature Reviews Molecular Cell Biology 5 (2004) 827–835.