

**Translational Control of Nrf2 Protein in Activation of Antioxidant Response by  
Oxidants**

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**Running Title:** Oxidant Induced Nrf2 Protein Translation

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**Non-standard Abbreviations:**

ARE: Antioxidant Response Element

IRES: Internal Ribosomal Entry Site

NQO1: NAD(P)H : quinone oxidoreductase 1

Nrf2: Nf-E2 related factor-2

## **Abstract**

Nf-E2 related factor-2 (Nrf2) is a bZIP transcription factor that binds and activates the Antioxidant Response Element (ARE) in the promoters of many antioxidant and detoxification genes. We found that H<sub>2</sub>O<sub>2</sub> treatment caused a rapid increase in endogenous Nrf2 protein level in rat cardiomyocytes. Semiquantitative or real-time RT-PCR failed to show an increase of Nrf2 mRNA level by H<sub>2</sub>O<sub>2</sub> treatment. Measurements of Nrf2 protein stability excluded the possibility of Nrf2 protein stabilization. While inhibiting protein synthesis with cycloheximide prevented H<sub>2</sub>O<sub>2</sub> from elevating Nrf2 protein level, RNA synthesis inhibition with actinomycin D failed to do so. Measurements of new protein synthesis with <sup>35</sup>S-Methionine incorporation confirmed that H<sub>2</sub>O<sub>2</sub> increased the translation of Nrf2 protein. Inhibitors of phosphoinositide 3-kinase (PI3K) were able to abolish the induction of Nrf2 protein by H<sub>2</sub>O<sub>2</sub>. While H<sub>2</sub>O<sub>2</sub> increased phosphorylation of p70 S6 kinase, rapamycin failed to inhibit H<sub>2</sub>O<sub>2</sub> from elevating Nrf2 protein. H<sub>2</sub>O<sub>2</sub> also induced phosphorylation of eIF4E and eIF2 $\alpha$  within 30 and 10 mins respectively. Inhibiting eIF4E with siRNA or increasing eIF2 $\alpha$  phosphorylation with salubrinal did not affect Nrf2 elevation by H<sub>2</sub>O<sub>2</sub>. Our data present a novel phenomenon of quick onset of the antioxidant/detoxification response via increased translation of Nrf2 by oxidants. The mechanism underlying such stress induced de novo protein translation may involve multiple components of translational machinery.

## **Introduction:**

Heart failure is a leading cause of mortality worldwide. An initial event such as hypertension or cardiac ischemia can lead to an intermediate hypertrophic phenotype of the heart, which may eventually deteriorate into failure. An increased expression of several antioxidant genes has been detected in the early stage of heart failure. Hypertrophic hearts initially contain higher levels of superoxide dismutase (SOD), glutathione peroxidase and total glutathione (Dhalla et al., 1996; Gupta and Singal, 1989; Hill and Singal, 1996; Singh et al., 1995). Patients with dilated or hypertrophic cardiomyopathy show an increased expression of NAD(P)H : quinone oxidoreductase 1 (NQO1) (Hwang et al., 2000). Levels of SOD and the GSH to GSSG ratio both decrease when hearts transition from hypertrophy to failure (Dhalla et al., 1996; Gupta and Singal, 1989; Singh et al., 1995). The loss of antioxidant reservoir is observed in the late stage of heart failure. The molecules controlling the adaptive response associated with the early stage of cardiac hypertrophy remain unidentified.

NF-E2 related factor-2 (Nrf2) is a bZIP transcription factor that binds and activates the Antioxidant Response Element (ARE) after heterodimerizing with a binding partner. Many antioxidant and detoxification-related genes contain the ARE in the promoters, such as glutathione S-transferases, hemeoxygenase-1, SOD1, and thioredoxin (Lee et al., 2003). Activation of Nrf2 transcription factor has been linked to cytoprotection (Jaiswal, 2004; Lee et al., 2005; Nguyen et al., 2003). The inducers of Nrf2 activation come in a variety of forms, from natural product isothiocyanates and

coumarin to toxicants such as metals and quinones (Nguyen et al., 2003). Although Nrf2-mediated ARE activation has been studied in multiple tissues, this pathway has not been well characterized in the heart.

As a transcription factor, Nrf2 level and activity are regulated at several levels: transcription, degradation, translocation and post-translational modifications such as phosphorylation (Huang et al., 2000; Huang et al., 2002; Kong et al., 2001; Kwak et al., 2003; Nioi and Hayes, 2004; Zhang and Hannink, 2003). A positive feedback loop through an ARE-like element in the promoter has been shown to regulate Nrf2 gene transcription in the cellular response to the anti-cancer agent 3H-1,2-dithiole-3-thione (Kwak et al., 2002). Nrf2 can be phosphorylated at Ser40 *in vitro* by protein kinase C (Huang et al., 2002). There is evidence that PI3-kinase and ERKs regulate the phosphorylation and therefore the activity of Nrf2 (Kang et al., 2000; Zhang et al., 2006). An important mechanism controlling the increase of Nrf2 protein level is through a decreased rate of Nrf2 protein degradation. Keap1, an inhibitor of Nrf2, is known to bind Nrf2 and hold it in the cytoplasm, where Keap1 recruits an E3 ubiquitin ligase, resulting in Nrf2 ubiquitination and therefore degradation by the proteasome (Zhang and Hannink, 2003). Disrupting the interaction with Keap1 causes stabilization of Nrf2 (Jaiswal, 2004; Lee and Johnson, 2004; Motohashi and Yamamoto, 2004; Nguyen et al., 2003). However many of the studies examining the interaction between Nrf2 and Keap1 use gene overexpression approaches. How endogenous Nrf2 gene responds to chemical stress is less well understood.

MOL#35360

Recent evidence suggests that chemical stress can cause selective protein translation. We found that low to mild doses of oxidants activate the ARE in cardiomyocytes in an Nrf2-dependent manner (Purdom-Dickinson et al., 2006). A rapid increase of Nrf2 protein level has been observed with oxidant exposure in cardiomyocytes. We address here the critical regulatory mechanism controlling the rapid increase of Nrf2 protein.

### ***Materials and Methods:***

Cell Culture and H<sub>2</sub>O<sub>2</sub> or Pharmacological Inhibitor Treatment: Neonatal rat cardiomyocytes were prepared as described previously (Coronella-Wood et al., 2004; Purdom and Chen, 2005). Cells were seeded at 0.3x10<sup>6</sup> cells per well for 6-well plates or 2.5x10<sup>6</sup> per 100 mm dish. Freshly isolated cells were cultured in low glucose Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) for 3 days before 24 hr serum starvation (0.5%FBS/DMEM) and 10 mins treatment with 100 μM H<sub>2</sub>O<sub>2</sub>. Pharmacological inhibitors were dissolved in dimethyl sulfoxide in 1000x concentration. Vehicle or pharmacological inhibitors were added to cells one hour prior to H<sub>2</sub>O<sub>2</sub> treatment. After H<sub>2</sub>O<sub>2</sub>, the media were changed to fresh media (0.5%FBS/DMEM) with vehicle or inhibitors added back.

Transfection: ARE-luciferase plasmid (0.2 μg, kindly provided by Dr. Jeffery Johnson) was cotransfected with Renilla-luciferase plasmid (0.04 μg) using Fugene 6 transfection reagent (Roche) as described previously (Purdom-Dickinson et al., 2006). To test the effect of PI3-Kinase inhibition, dominant-negative p85 (0.2 μg) or corresponding empty vector (generous gifts from Dr. Wataru Ogawa) was included in the transfection. Firefly luciferase and Renilla luciferase were measured using a Dual Luciferase Assay System (Promega) and a Luminometer (Turner Designs).

Cardiomyocytes were transfected with two difference siRNA sequences against eIF4E (Ambion catalogue #16704, ID #56139 and #56229) using oligofectamine

(Invitrogen, Carlsbad, CA). siRNAs (100 nM each, total 200 nM) were mixed with oligofectamine in the Opti-MEM media and incubated with cells in DMEM for 6 hr before FBS was added to a final concentration of 10% for overnight recovery. At 48 hr after transfection, cells were treated 10 mins with 100  $\mu$ M H<sub>2</sub>O<sub>2</sub> before harvesting. The level of eIF4E was verified by Western blot and the approach results in about 50% knockdown of eIF4E expression.

Western Blot Protocol: Cytosolic lysates were obtained using extraction buffer (1% Triton X-100, 10 mM Tris, pH 7.4, 5 mM EDTA, 50 mM NaCl, 50 mM NaF, and freshly added 2 mM DTT, 1 mM Na<sub>2</sub>VO<sub>3</sub>, 1 mM phenylmethylsulfonyl fluoride, 100  $\mu$ g/mL leupeptin and 10  $\mu$ g/mL aprotinin) and retaining the supernatant after 10 min centrifugation at 13,000 g. Total cell lysates or nuclear enriched fractions obtained from pellets after centrifugation of the lysates were prepared in Laemmli Lysis Buffer [125 mM Tris, pH 6.8, 50% (v/v) glycerol, 2.4% (w/v) SDS, and freshly added 100  $\mu$ M phenylmethylsulfonyl fluoride and 10  $\mu$ g/mL aprotinin]. Samples were diluted by addition of ½ volume Laemmli Sample Buffer [65 mM Tris, pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS with 5% freshly added beta-mercaptoethanol] before 10 min boiling. After SDS-PAGE, Western Blot was performed using antibodies against Nrf2 (sc 722, Santa Cruz Biotechnology, CA), Thr389 phospho-p70S6 Kinase (#9205), total p70S6 Kinase (#9202), Ser209 phospho-eIF4E (#9741), total eIF4E (#9742), Ser51 phospho-eIF2 $\alpha$  (#9721), or total eIF2 $\alpha$  (#9722, Cell Signaling Technology, Beverly, MA). Secondary antibodies conjugated with the horseradish peroxidase (Invitrogen, Carlsbad, CA) were used for Enhanced Chemiluminescence Reaction.

Reverse-Transcription and PCR: Cells were harvested in TRIzol (Invitrogen, Carlsbad, CA) for extracting RNA. Total RNA (2  $\mu$ g) were used for reverse transcription (RT), and 1/10 of the resultant cDNAs were used for each PCR reaction using Nrf2 primer pair: forward 5'-GCCAGCTGAACTCCTTAGAC-3' and reverse 5'-GATTCGTGCACAGCAGCA-3'. For real-time RT-PCR, total RNA samples were used with probe and primer sets purchased from ABI (Nrf2: Rn00477784\_m1; beta-glucuronidase: Rn00566655\_m1).

<sup>35</sup>S-Methionine Labeling and Immunoprecipitation: Serum-starved cells in 100 mm dishes were incubated 20 mins in labeling medium (DMEM without methionine, cysteine and L-glutamine, catalog #21013-024, Invitrogen, CA) to reduce the intracellular pools of methionine. Inhibitors (LY294002 and cycloheximide) were added 5 mins prior to H<sub>2</sub>O<sub>2</sub> treatment (100  $\mu$ M, 10 min). After H<sub>2</sub>O<sub>2</sub>, the media was replaced with fresh labeling medium containing 200  $\mu$ Ci <sup>35</sup>S-methionine per dish and the corresponding inhibitors. Labeled cells were harvested 1 hr later in 200  $\mu$ L extraction buffer containing 50  $\mu$ M MG132. For immunoprecipitation, 500  $\mu$ g of cellular protein were incubated with Nrf2 antibody and Protein G beads in radioimmunoprecipitation buffer (50 mM Tris-HCl, pH7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, with freshly added 1 mM DTT, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 2  $\mu$ M leupeptin, 10 units/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride). The beads were then washed 8 times with the buffer before boiling in Laemmli Sample Buffer for SDS-PAGE. Proteins were stained using Silver Stain Plus reagents (#161-0449, Bio-Rad, Hercules, CA) and the gel was

dried for autoradiography to visualize the newly synthesized Nrf2 using a phospho-imager.

Statistics: Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Bonferroni analysis for cross comparisons with Stata 8.2 software. Each group of means that is not significantly different from each other is indicated by a common letter symbol. Therefore, means in the “a” group are significantly different than means in the “b” group, and so on.

## **Results:**

An increase in the level of Nrf2 protein has been observed within 10 min after a pulse treatment of H<sub>2</sub>O<sub>2</sub> (Fig. 1). The increase reached a peak of 2-fold at 1 to 2 hrs after H<sub>2</sub>O<sub>2</sub> treatment (Fig. 1A&B). Sample loading titration experiments confirmed about 2 fold induction of Nrf2 protein at 1 hr after H<sub>2</sub>O<sub>2</sub> treatment (Fig. 1C), consistent with band intensity quantifications (Fig. 1B). Within 10 mins time frame, H<sub>2</sub>O<sub>2</sub> activates PI3 Kinase pathway in cardiomyocytes (Tu et al., 2002). Pharmacological inhibition of PI3 Kinase using LY294002 [2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one] and Wortmannin blocked H<sub>2</sub>O<sub>2</sub> from inducing Nrf2 elevation (Fig. 2A). A luciferase reporter assay, which measures the activation of ARE, was used to further verify the effect of PI3 Kinase inhibition.(Purdom-Dickinson et al., 2006). Treatment with LY294002 or cotransfection with dominant negative p85, the regulatory subunit of PI3 Kinase, showed an inhibition of H<sub>2</sub>O<sub>2</sub>-induced ARE activation (Fig. 2B&C). These data indicate a role of PI3 Kinase in H<sub>2</sub>O<sub>2</sub>-induced rapid Nrf2 accumulation and activation.

An increase in the level of Nrf-2 protein suggests three possibilities: 1) increased transcription, 2) protein stabilization, or 3) an increase in Nrf-2 protein translation. To determine whether H<sub>2</sub>O<sub>2</sub> causes an increase in Nrf2 transcription, we performed semi-quantitative RT-PCR at various time points after H<sub>2</sub>O<sub>2</sub> treatment (Fig. 3A). The results failed to show elevated Nrf2 mRNA with H<sub>2</sub>O<sub>2</sub> treatment (Fig. 3A). As a comparison, the positive control, i.e. cardiomyocytes infected with replication-deficient adenoviral constructs encoding Nrf2, showed higher levels of Nrf2 mRNA (Fig. 3B). Real time RT-

PCR also confirmed no elevation of Nrf2 mRNA by H<sub>2</sub>O<sub>2</sub> treatment (Fig. 3C). To further indicate that Nrf2 was not regulated at the transcriptional level, we included RNA synthesis inhibitor actinomycin D. Actinomycin D at the dose used is capable of inhibiting transcription as shown by its effect in preventing H<sub>2</sub>O<sub>2</sub> from inducing cyclooxygenase-2 gene at mRNA and protein levels (Fig. 4A, bottom panel). However actinomycin D failed to block H<sub>2</sub>O<sub>2</sub> from inducing Nrf2 protein (Fig. 4A, upper panel). In contrast, when cells were treated with the protein synthesis inhibitor cycloheximide (CXM), H<sub>2</sub>O<sub>2</sub> could no longer increase the level of Nrf2 (Fig. 4A).

Nrf2 protein is known to be degraded by the proteasome. If H<sub>2</sub>O<sub>2</sub> inhibited Nrf2 degradation and LY294002 reversed the inhibition, a sustained elevation of Nrf2 protein should be observed in H<sub>2</sub>O<sub>2</sub> treated cells even when new protein synthesis is blocked and LY294002 would somehow prevent such an increase. We compared the stability of Nrf2 protein and the effect of LY294002 in a time course study. Cardiomyocytes were given time (1 hr) to increase the level of Nrf2 protein after H<sub>2</sub>O<sub>2</sub> treatment before addition of CXM to inhibit further protein synthesis (Lane 2 compared to Lane 1, Fig. 4B). Within 30 mins of CXM addition, H<sub>2</sub>O<sub>2</sub> treated cells showed a reduction of Nrf2 protein level compared to untreated cells (Lane 4 compared to Lane 3, Fig. 4B). The addition of LY294002 did not enhance the rate of Nrf2 degradation (Lane 6 compared to Lane 4, Fig. 4B). These data argue against the possibilities that H<sub>2</sub>O<sub>2</sub> increases Nrf2 level by preventing Nrf2 degradation and LY294002 accelerates Nrf2 protein degradation.

To demonstrate that H<sub>2</sub>O<sub>2</sub> causes an increase in Nrf2 translation, we used <sup>35</sup>S-methionine incorporation assay to measure newly synthesized Nrf2. Nrf2 antibody-immunoprecipitated samples contain a band corresponding to Nrf2 molecular weight (Fig. 5). An increase in the intensity of the band with H<sub>2</sub>O<sub>2</sub> treatment indicates an increase in newly-translated Nrf2 protein (Fig. 5). Quantification of band intensities indicates 2.0 ± 0.4 fold induction of newly synthesized Nrf2 protein by H<sub>2</sub>O<sub>2</sub> treatment from three independent experiments. As expected, CXM efficiently blocked Nrf2 as well as background protein synthesis (Fig. 5A). The increase in Nrf2 was weakened by the presence of LY294002 (Fig. 5A), showing an average 50% inhibition by LY294002 based on the intensities of the bands compared to that of H<sub>2</sub>O<sub>2</sub> treatment alone in three independent experiments (Fig. 5B). This data supports the hypothesis that PI3 Kinase is involved in regulating Nrf2 translation after oxidative stress. Measurements of the overall protein synthesis in 6 hrs after H<sub>2</sub>O<sub>2</sub> treatment by <sup>3</sup>H-leucine incorporation show that H<sub>2</sub>O<sub>2</sub> treatment did not cause significant increase or loss of overall protein synthesis (Table 1). LY294002 alone did not significantly inhibit the overall protein synthesis but decreased protein synthesis in H<sub>2</sub>O<sub>2</sub> treated cells (Table 1). The data suggest that H<sub>2</sub>O<sub>2</sub> treatment selectively enhances Nrf2 protein synthesis.

PI3 Kinase generally activates several intermediates that eventually funnel through p70S6-Kinase, which then phosphorylates the ribosomal protein S6 important for the assembly of 43S pre-initiation complex (Fingar and Blenis, 2004; Gingras et al., 2001). H<sub>2</sub>O<sub>2</sub> treatment caused p70S6 Kinase activation (Tu et al., 2002). Inhibiting PI3 Kinase with LY294002 or wortmannin blocked p70S6 Kinase phosphorylation (Tu et al.,

2002). Rapamycin, an inhibitor of the immediate upstream regulator of p70S6 Kinase mTOR, can block p70S6-Kinase phosphorylation effectively but failed to prevent H<sub>2</sub>O<sub>2</sub> from inducing Nrf2 elevation (data not shown).

Phosphorylation of Ekaryotic Initiation Factor 4E (eIF4E) or eIF2 $\alpha$  represents two mechanisms of translational initiation (Dever, 2002; Gebauer and Hentze, 2004; Holcik and Sonenberg, 2005; Preiss and M, 2003). We found that H<sub>2</sub>O<sub>2</sub> treatment induced phosphorylation of eIF4E at 20 mins (Fig. 6). This phosphorylation remained detectable 2 hr after H<sub>2</sub>O<sub>2</sub> treatment (Fig. 6). In comparison, phosphorylation of eIF2 $\alpha$  appeared within 10 mins of H<sub>2</sub>O<sub>2</sub> treatment (Fig. 6). The level of eIF2 $\alpha$  phosphorylation returned to baseline 2 hrs after H<sub>2</sub>O<sub>2</sub> treatment (Fig. 6). It appears that eIF2 $\alpha$  phosphorylation occurred earlier than eIF4E phosphorylation and the time course of eIF2 $\alpha$  phosphorylation is consistent with Nrf2 induction.

When inhibitors of PI3 Kinase were tested for phosphorylation of eIF4E or eIF2 $\alpha$ , LY294002 appeared to inhibit phosphorylation of eIF4E and eIF2 $\alpha$  (Fig. 7). For reasons unknown, wortmannin induced the level and phosphorylation of eIF4E and eIF2 $\alpha$  in the controls (Fig. 7). To test which translational pathway mediates Nrf2 protein synthesis, we used siRNA against eIF4E. Since siRNA against eIF2 $\alpha$  was not available, we used a specific inhibitor of eIF2 $\alpha$  dephosphorylation, salubrinal, which enhances eIF2 $\alpha$  activity (Boyce et al., 2005). Although levels of eIF4E protein were reduced about 50% using siRNA, Nrf2 accumulation was unaffected as shown by the intensities of the bands (Fig. 8A). Salubrinal alone induced eIF2 $\alpha$  phosphorylation similar to the level of H<sub>2</sub>O<sub>2</sub>

MOL#35360

treatment at 30 to 60 mins and appeared to enhance H<sub>2</sub>O<sub>2</sub> induced eIF2 $\alpha$  phosphorylation (Fig. 8B). However, Salubrinal failed to increase Nrf2 by itself or to enhance H<sub>2</sub>O<sub>2</sub>-induced Nrf2 (Fig. 8B). These data point to a complex mechanism of translational control of Nrf2 protein.

### ***Discussion:***

This study found that H<sub>2</sub>O<sub>2</sub> causes a rapid increase of Nrf2 protein in cardiomyocytes. Elimination of transcription and protein stabilization as contributors to oxidant-induced Nrf2 increase led us to explore protein translation as a primary means regulating this occurrence. We have provided evidence that H<sub>2</sub>O<sub>2</sub> treatment indeed results in an increase in newly-synthesized Nrf2 protein in cardiomyocytes. Our study focuses on the endogenous Nrf2 gene, unlike the experimental systems utilizing transfected Nrf2 gene. Although the literature argues for increased Nrf2 protein stability as a means of protein accumulation (Jaiswal, 2004; Kong et al., 2001; Motohashi and Yamamoto, 2004; Nguyen et al., 2003), a key piece of experimental evidence used overexpression approaches with a Nrf2 transgene that does not contain 5' untranslated region (UTR) (Zhang and Hannink, 2003). Endogenous Nrf2 gene may differ from Nrf2 transgenes in protein translation control.

The process of protein translation is divided into 3 stages: initiation, elongation and termination. The rate-limiting step of translation relies on the process of initiation predominantly. For 95-97% mRNA species in mammalian cells, translation initiation requires 5' m<sup>7</sup>GpppN cap structure in front of the start codon, 3' poly(A) RNA tail, at least 12 eIFs, and Poly (A) Tail Binding Proteins (PABPs) (Dever, 2002; Holcik and Sonenberg, 2005; Preiss and M, 2003). The process of initiation contains four physical steps: 1) formation of a 43 Svedberg (S) preinitiation complex from the small (40S) ribosomal subunit, eIFs, and Met-tRNA<sup>Met</sup>; 2) binding of the 43S complex to the vicinity of the 5' m<sup>7</sup>GpppN cap structure on mRNA; 3) scanning of the 5' untranslated region

(5'UTR) of the mRNA and start codon AUG. The consensus sequence around AUG, i.e. the Kozak sequence, is GCC(A/G)CCAUGG in most genes; and 4) joining of a large 60S subunit to assemble a complete (80S) ribosome. The eIF4E acts as a recruiter that first locates and sits on the 5' cap structure to attract the binding of the 43S pre-initiation complex (Gingras et al., 1999; Holcik and Sonenberg, 2005; Preiss and M, 2003).

An Internal Ribosomal Entry Site (IRES) promotes the binding of 40S ribosome to an internal portion of the mRNA to initiate translation in 3 - 5% mRNA species. Although IRES mediated protein translation was first discovered with viral proteins, about 50 cellular proteins have been found to exhibit IRES mediated translation (<http://www.iresite.org>; <http://ifr31w3.toulouse.inserm.fr/iresdatabase>). Examples of these proteins include c-myc, c-Jun, HIF-1 $\alpha$ , p27/Kip1, APC, Apaf1, XIAP and GRP78. The mRNA species of these cellular proteins contain 5'UTR varying in length from 83 (APC) or 152 (p27/Kip1) to 407 (c-myc) or 577 (Apaf1) nucleotides (<http://ifr31w3.toulouse.inserm.fr/iresdatabase>). The IRES sequences usually have high GC content, a feature essential for formation of secondary structures containing "stems and loops". Since the IRES sequences are heterogeneous and apparently each gene contains a distinct IRES sequence that forms a unique secondary structure (Merrick, 2004), it prohibits quick identification of an IRES in Nrf2 mRNA. According to GenBank sequence information, mouse, rat or human Nrf2 gene encodes mRNA containing 5' UTRs extending 82 (rat) to 114 (human) or 233 (mouse) nucleotides upstream of the start codon. Rat Nrf2 5'UTR is 94% homologous with the 3' portion of mouse Nrf2 5'UTR, while human Nrf2 5'UTR does not share significant sequence

homology with rat or mouse Nrf2 5'UTR. The common feature between these genes is the lack of well-defined Kozak sequence. Zuker's MFOLD software predicts stable secondary structure with "stems and loops" among these 5'UTR sequences. These features support the hypothesis of an IRES mediated protein translation in H<sub>2</sub>O<sub>2</sub> induced Nrf2 elevation.

Increasing evidence suggests that a rapid onset of selective protein translation serves as an important mechanism for cells to deal with stress (Holcik and Sonenberg, 2005; Sheikh and Fornace, 1999). IRES mediated protein translation enables cells to express a small number of proteins while the overall protein synthesis through 5' m<sup>7</sup>GpppN cap dependent mechanism has been shut down to conserve energy. There is evidence that in certain experimental systems eIF2 $\alpha$  phosphorylation is critical for IRES dependent translation. Amino acid depletion, UV irradiation, viral infection, heat shock, hypoxia and endoplasmic reticulum (ER) stress have been shown to induce eIF2 $\alpha$  phosphorylation and selective protein translation through IRES (Holcik and Sonenberg, 2005). The kinases that phosphorylate eIF2 $\alpha$  include the general control non-repressible-2 (GCN2), protein kinase RNA (PKR), heme-regulated inhibitor kinase (HRI), and PKR-like ER kinase (PERK) (Holcik and Sonenberg, 2005). There is evidence that PERK participates in Nrf2 expression under an ER stress condition (Cullinan and Diehl, 2004).

In our study, inhibitors of PI3 Kinase prevent H<sub>2</sub>O<sub>2</sub> from elevating the steady state Nrf2 protein levels and newly synthesized Nrf2 protein. Modulating individual

components of the usual pathways of translational initiation, i.e. p70S6 Kinase, eIF4E or eIF2 $\alpha$ , does not seem to affect H<sub>2</sub>O<sub>2</sub> from inducing Nrf2 protein. This suggests that either a combination of several pathways or a p70S6 kinase, eIF4E or eIF2a independent pathway downstream of PI3 Kinase regulates Nrf2 protein translation. Based on the data from <sup>35</sup>S-methionine labeling experiments (Fig. 5), LY294002 inhibited H<sub>2</sub>O<sub>2</sub> from inducing Nrf2 protein. The overall protein synthesis as measured by <sup>3</sup>H-leucine incorporation was reduced when LY294002 was added to H<sub>2</sub>O<sub>2</sub> treated cells (Table 1). This suggests that LY294002 may inhibit stress induced protein synthesis in general. Despite the negative data with rapamycin, eIF4E siRNA or an activator of eIF2 $\alpha$  in H<sub>2</sub>O<sub>2</sub> induced Nrf2 protein elevation, it is possible that multiple components of the translational machinery collaborate to turn on Nrf2 translation. Therefore inhibiting one component may not be sufficient to block Nrf2 induction. Along this line, although Salubrinal caused phosphorylation of eIF2 $\alpha$ , additional signals such as those induced by H<sub>2</sub>O<sub>2</sub> appear to be required to cooperate with eIF2 $\alpha$  for permitting efficient translation of Nrf2 protein. In other words, eIF2 $\alpha$  activity may be necessary but not sufficient in regulating stress induced protein translation. One caveat of eIF4E siRNA experiment is the lack of complete elimination of eIF4E protein. This is likely related to the fact that cardiomyocytes are difficult to transfect. Although eIF4E protein level is significantly reduced, the remaining eIF4E may be sufficient to work in concert with other eIFs in initiating protein translation. With 5' m<sup>7</sup>GpppN cap dependent protein translation, 12 eIFs carry out the process in multiple steps. Therefore although one or a few eIFs are important in initiating the coordination of the translational machinery, other eIFs may play a role in stress induced protein translation once the initiation complex is assembled.

MOL#35360

The fact that LY294002 inhibits Nrf2 induction and three key components of protein translation shown here, i.e. phosphorylation of p70S6K, eIF4E and eIF2 $\alpha$ , supports this multi-components argument of stress induced protein translation.

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**Footnotes:**

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### **Figure Legends:**

#### **Figure 1. H<sub>2</sub>O<sub>2</sub> treatment induces a rapid accumulation of Nrf2 protein.**

Cardiomyocytes were treated with 100  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 10 min, at which time the media was changed and cells were harvested for Western blot analysis (30  $\mu$ g protein/lane) at the indicated time points (A). A representative gel is shown (A). The intensity of bands was quantified using Image J software (NIH) and the data represents the averages and standard deviations from three independent experiments (B). H<sub>2</sub>O<sub>2</sub> sample harvested at 1 hr after treatment was diluted in series for Western blot to verify the fold of induction (C).

#### **Figure 2. Inhibition of PI3 Kinase prevents Nrf2 induction or ARE activation.**

ARE-luciferase reporter construct (0.2  $\mu$ g) was transfected along with TK-Renilla luciferase (0.04  $\mu$ g), which corrects for the transfection efficiency (B). Additional 0.2  $\mu$ g control or DNp85 plasmid was included for cotransfection (C). Cardiomyocytes without (A) or with plasmid transfection (B & C) were pretreated with LY294002 (LY, 20  $\mu$ M, A & B) or Wortmannin (WT, 2  $\mu$ M, A) for 1 hr before exposure to 100  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 10 min. The inhibitor was added back to the corresponding group for 1 hr incubation before harvesting for Western blot analyses (30  $\mu$ g protein per lane, A) or were harvested 4 hr later for Dual luciferase assay (B, C).

#### **Figure 3. H<sub>2</sub>O<sub>2</sub> treatment does not induce Nrf2 mRNA.**

Cardiomyocytes were treated with 100  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 10 mins and were harvested at indicated time points for RNA

preparation. Semi-quantitative RT-PCR (A, B) or real-time RT-PCR (C) was performed as described in the Methods. Cells were pretreated with 20  $\mu$ M LY294002 (LY) or 2  $\mu$ M wortmannin (WT) before H<sub>2</sub>O<sub>2</sub> treatment (A). Replication-deficient adenovirus-infected CMCs were used as a positive control (B, C).

**Figure 4. Cycloheximide but not actinomycin D blocks H<sub>2</sub>O<sub>2</sub> induced Nrf2 protein increase.** Cardiomyocytes were pretreated with cycloheximide (CXM, 0.5  $\mu$ g/mL) or actinomycin D (ActD, 0.5  $\mu$ M) for 1 hr before treatment with 100  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 10 min (A). CXM or ActD was added back when the media was changed and cells were harvested at 1 hr after H<sub>2</sub>O<sub>2</sub> treatment for Western blot to detect Nrf2 or cyclooxygenase-2 (COX-2) protein (30  $\mu$ g protein/lane) or RT-PCR to detect COX-2 mRNA (A). Cardiomyocytes were treated with 100  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 10 min and were allowed to recover for 1 hr, when CXM (0.5  $\mu$ g/mL) or CXM plus LY294002 (LY, 20  $\mu$ M) were added to the cells (B). The cells were then harvested at 30 min intervals for Western blot (30  $\mu$ g protein/lane) to measure Nrf2 protein (B).

**Figure 5. H<sub>2</sub>O<sub>2</sub> induces newly synthesized Nrf2 protein.** Cardiomyocytes were treated with 100  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 10 mins in the absence or presence of LY294002 (LY, 20  $\mu$ M) or CXM (0.5  $\mu$ g/mL). Cells were placed in <sup>35</sup>S methionine labeling medium immediately after H<sub>2</sub>O<sub>2</sub> treatment and were harvested 1 hr later for immunoprecipitation of Nrf2 protein followed by SDS-PAGE. The resultant bands were visualized using a phospho-imager (A). The intensity of bands was quantified using Image J software (NIH). The

band intensity is set to 1 for the control and all others are relative to the control. The data represents the averages and standard deviations from three independent experiments (B).

**Figure 6. H<sub>2</sub>O<sub>2</sub> induces time dependent phosphorylation of eIF4E and eIF2 $\alpha$ .**

Cardiomyocytes were treated with 100  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 10 mins and the cells were harvested at the indicated time points for measurement of phosphorylated or total eIF4E and eIF2 $\alpha$  by Western blot analyses (30  $\mu$ g protein/lane).

**Figure 7. Inhibition of PI3-Kinase blocks phosphorylation of both eIF4E and eIF2 $\alpha$ .**

Cardiomyocytes were pretreated with 20  $\mu$ M LY294002 (LY) or 2  $\mu$ M Wortmannin (WT) for 1 hr prior to treatment with 100  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 10 min. The inhibitors were added back and the cells were harvested 1 hr later for measurement of phosphorylated or total eIF4E or eIF2 $\alpha$  by Western blot (30  $\mu$ g protein/lane).

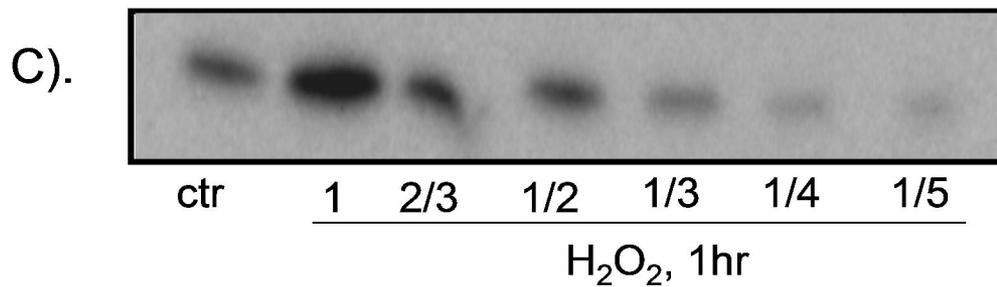
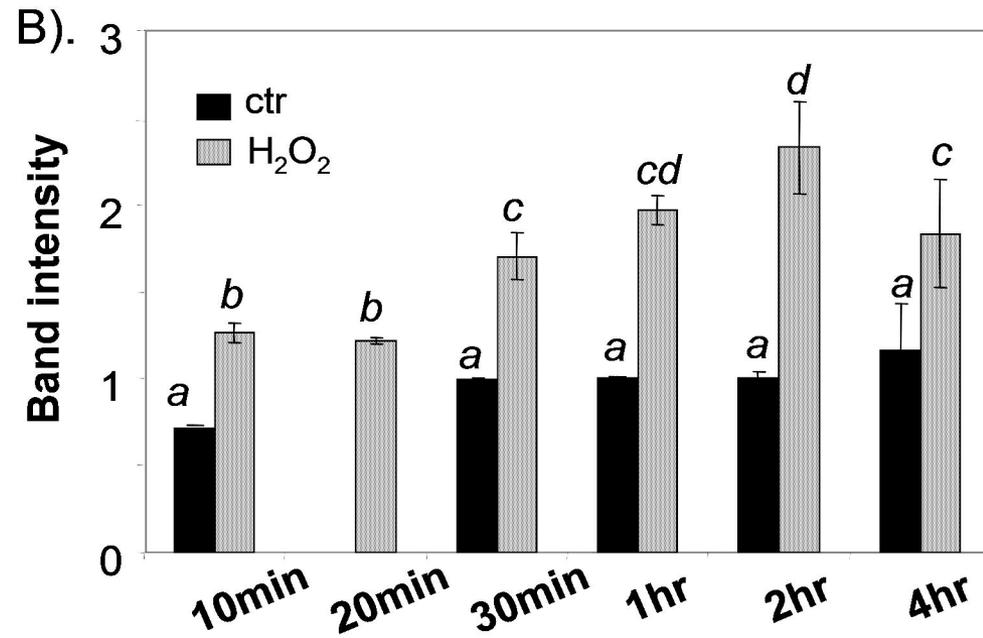
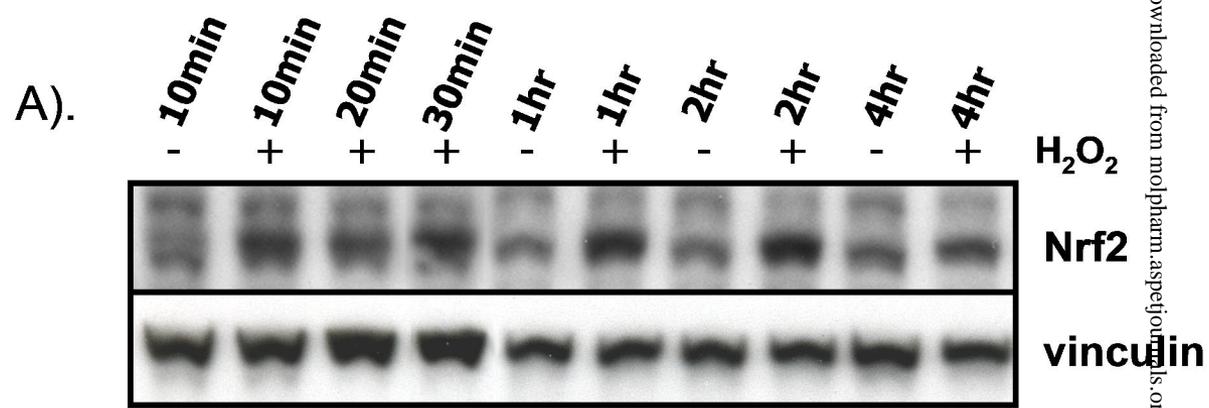
**Figure 8. Reducing eIF4E or increasing eIF2 $\alpha$  phosphorylation does not affect Nrf2**

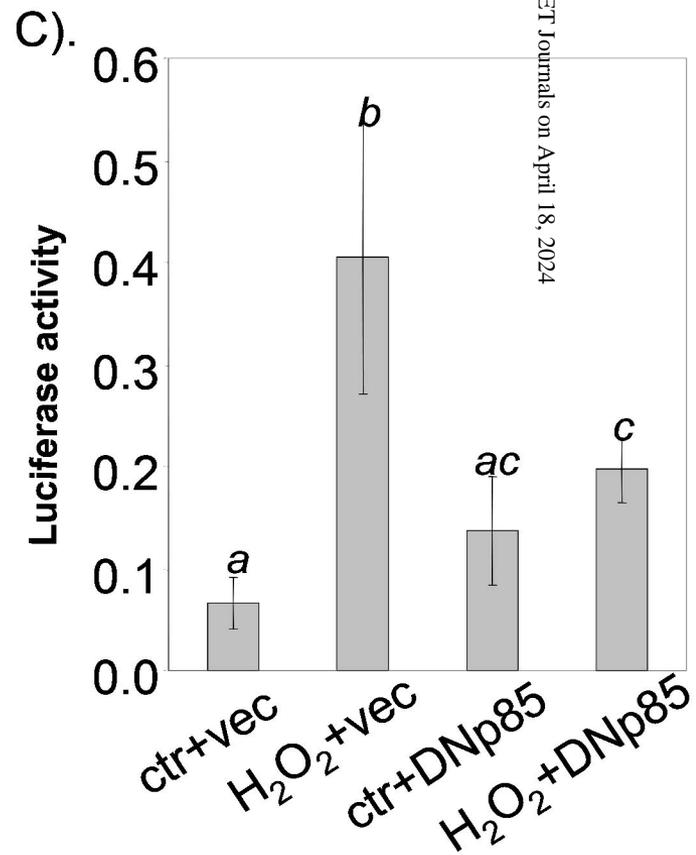
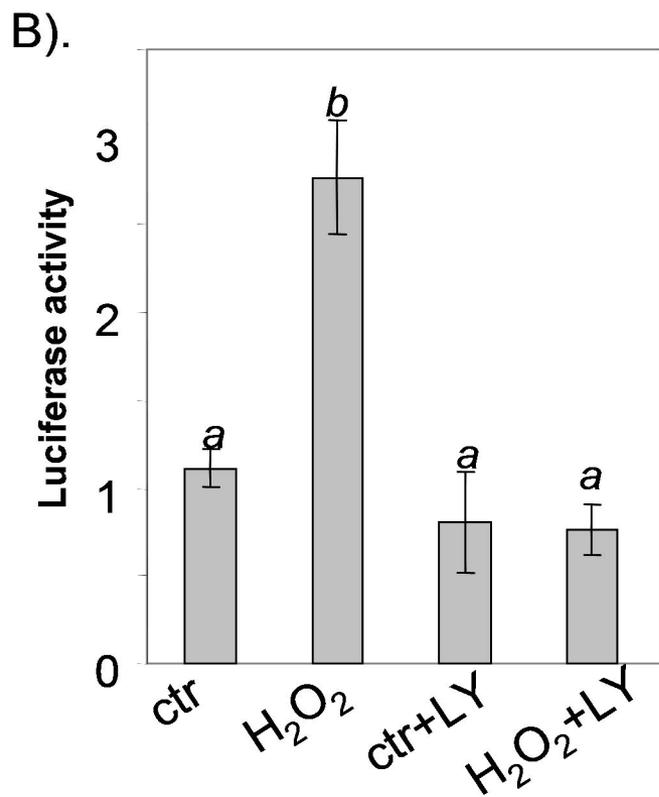
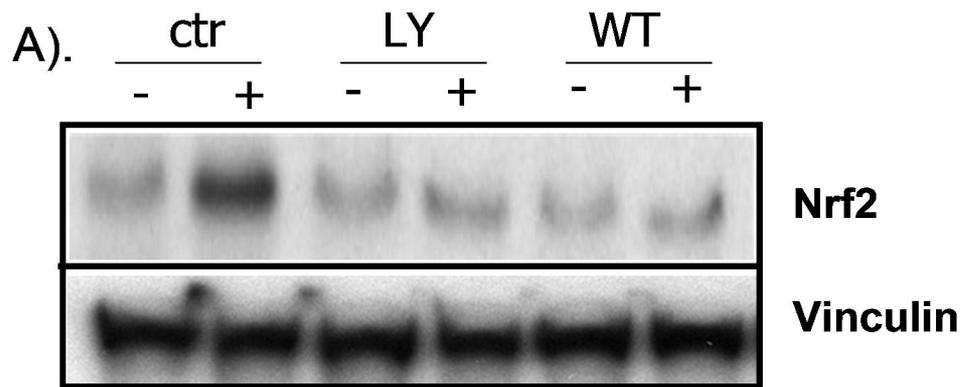
**induction.** Cardiomyocytes were transfected with siRNA against eIF4E as described in the Methods and were treated with 100  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 10 min at 48 hrs after siRNA transfection (A). Cardiomyocytes were pretreated with salubrinal (Sal, 50  $\mu$ M) for 30 min before 10 min treatment of 100  $\mu$ M H<sub>2</sub>O<sub>2</sub> (B). Cells were harvested at the indicated time points after H<sub>2</sub>O<sub>2</sub> treatment for measurements of Nrf2, phosphorylated or total eIF4E, and phosphorylated or total eIF2a using Western blot analyses (30  $\mu$ g protein/lane).

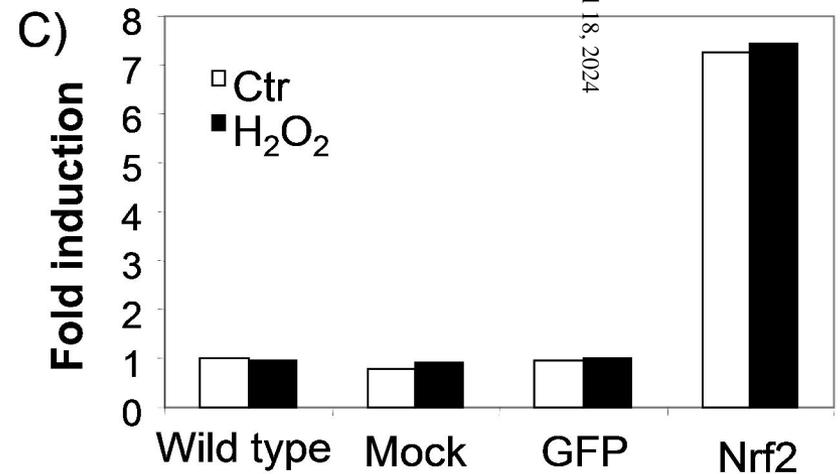
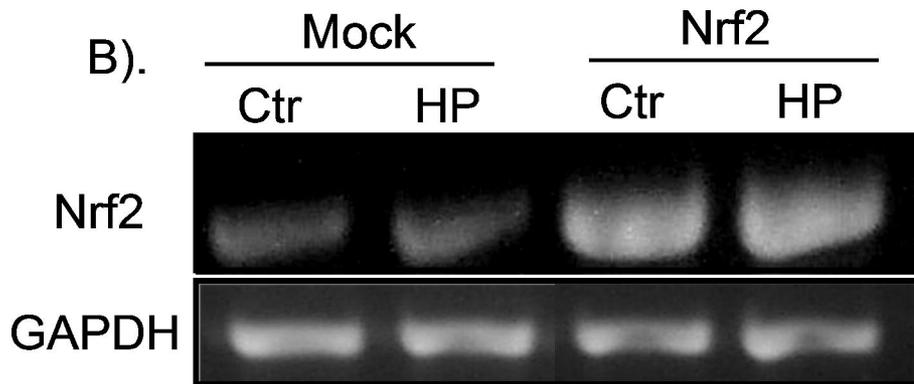
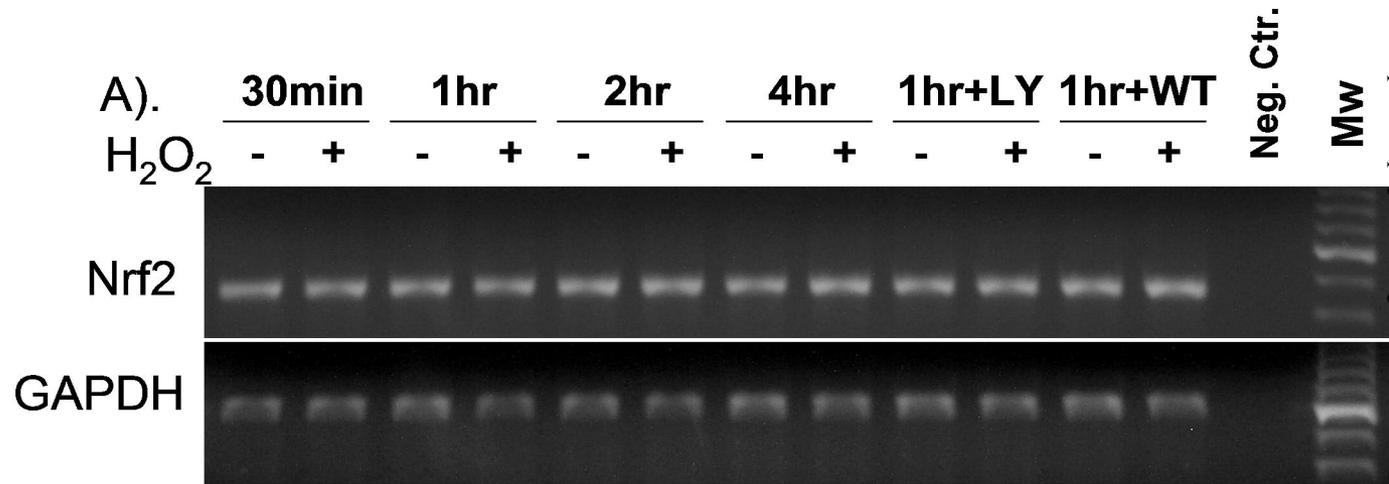
**Table 1. LY294002 Inhibits Overall Protein Synthesis in H<sub>2</sub>O<sub>2</sub> Treated Cells.**

	Relative cpm		
	<u>Ctrl</u>	<u>LY294002</u>	<u>CXM</u>
Control	1.00+0.08	1.07+0.22	0.36+0.05
H <sub>2</sub> O <sub>2</sub>	1.03+0.27	0.54+0.13	0.34+0.11

Cardiomyocytes in 24-well plates were treated with 100  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 10 mins in the absence or presence of LY294002 (20  $\mu$ M) or CXM (0.5  $\mu$ g/mL). Cells were placed in DMEM containing 0.2  $\mu$ Ci/well <sup>3</sup>H-leucine immediately after H<sub>2</sub>O<sub>2</sub> treatment and were harvested 6 hrs later for measuring incorporated <sup>3</sup>H-leucine by trichloroacetic acid precipitation.







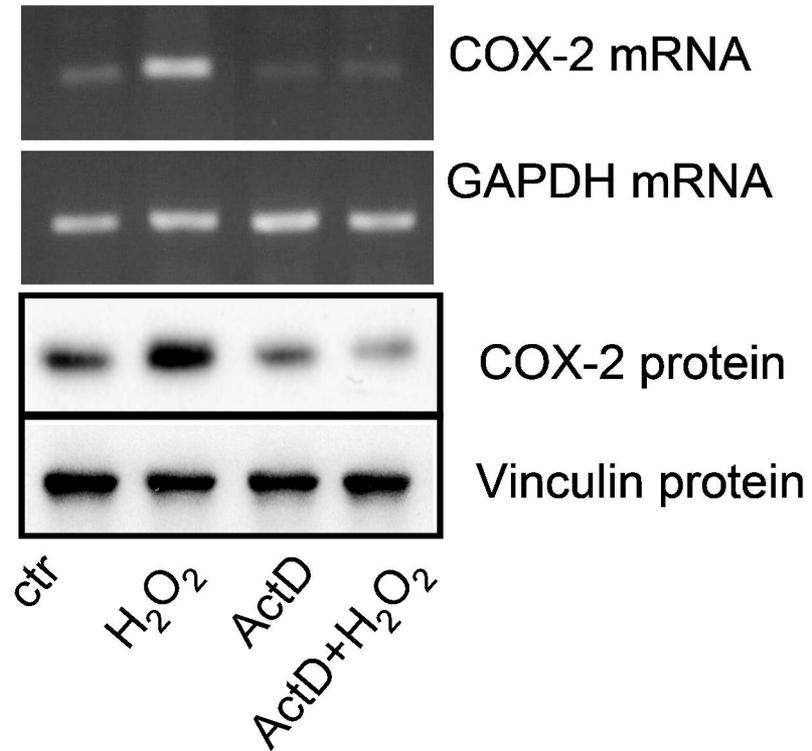
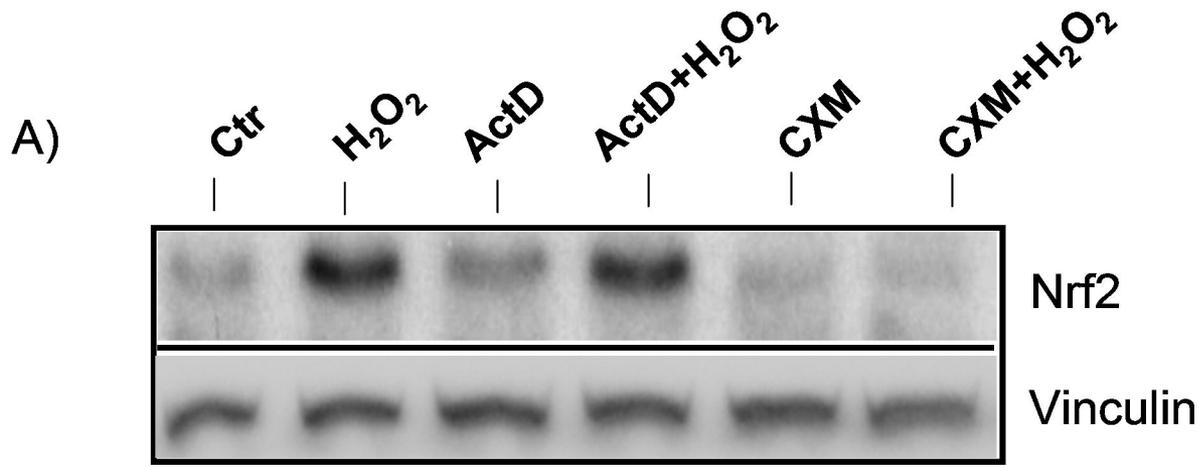
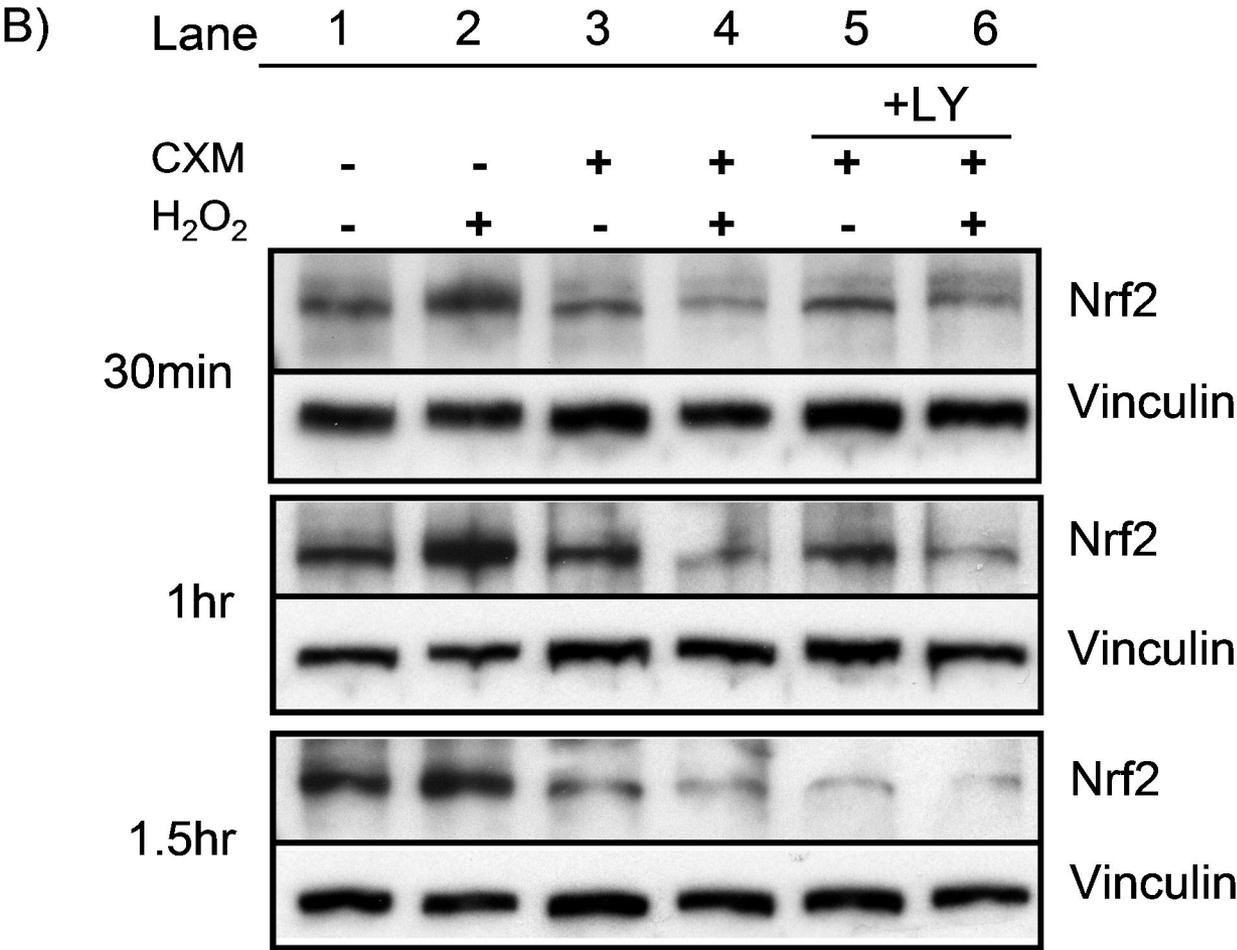


Figure 4A

Figure 4B



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Figure 5

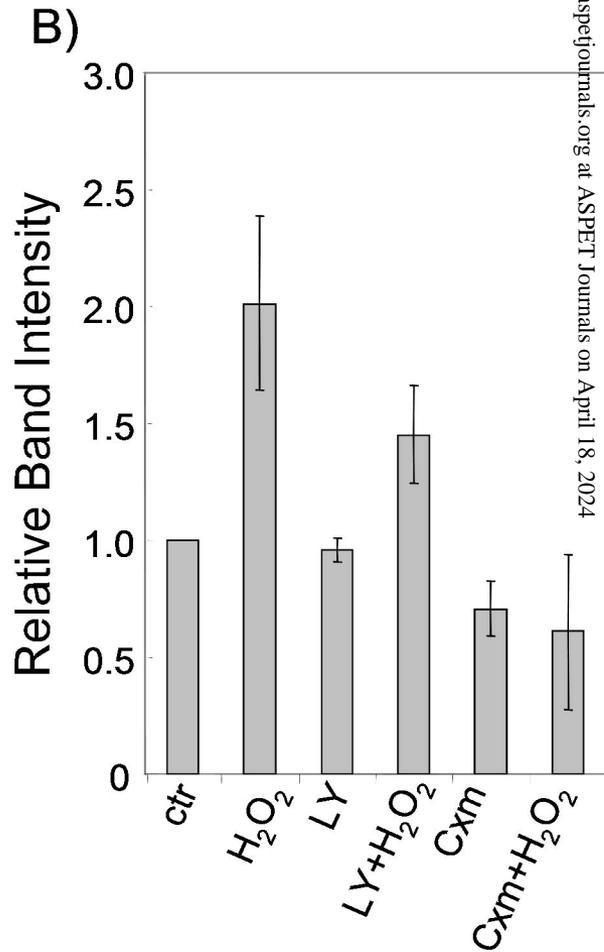
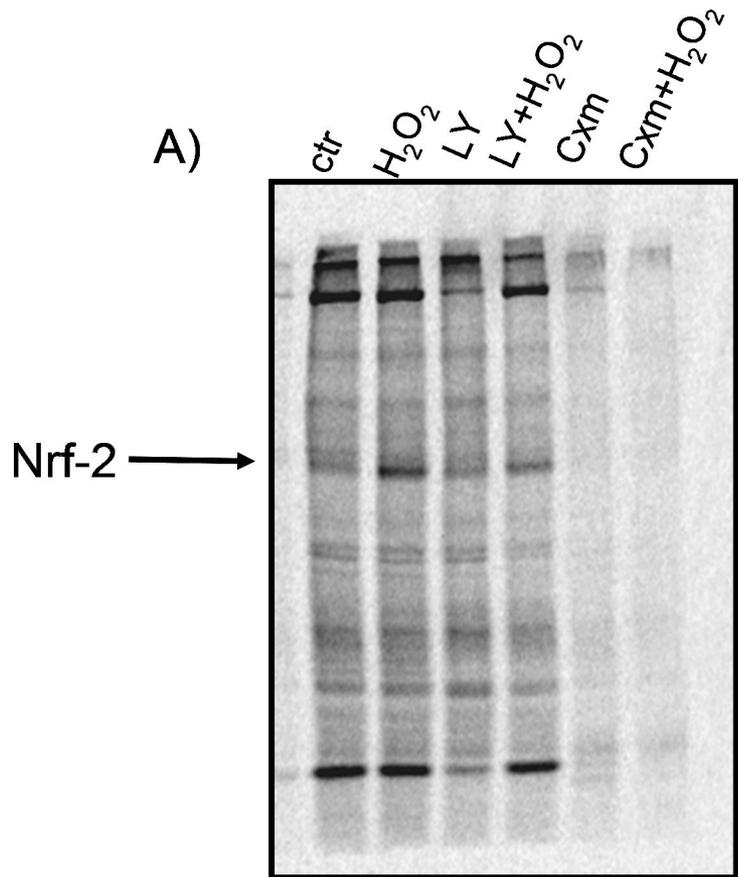
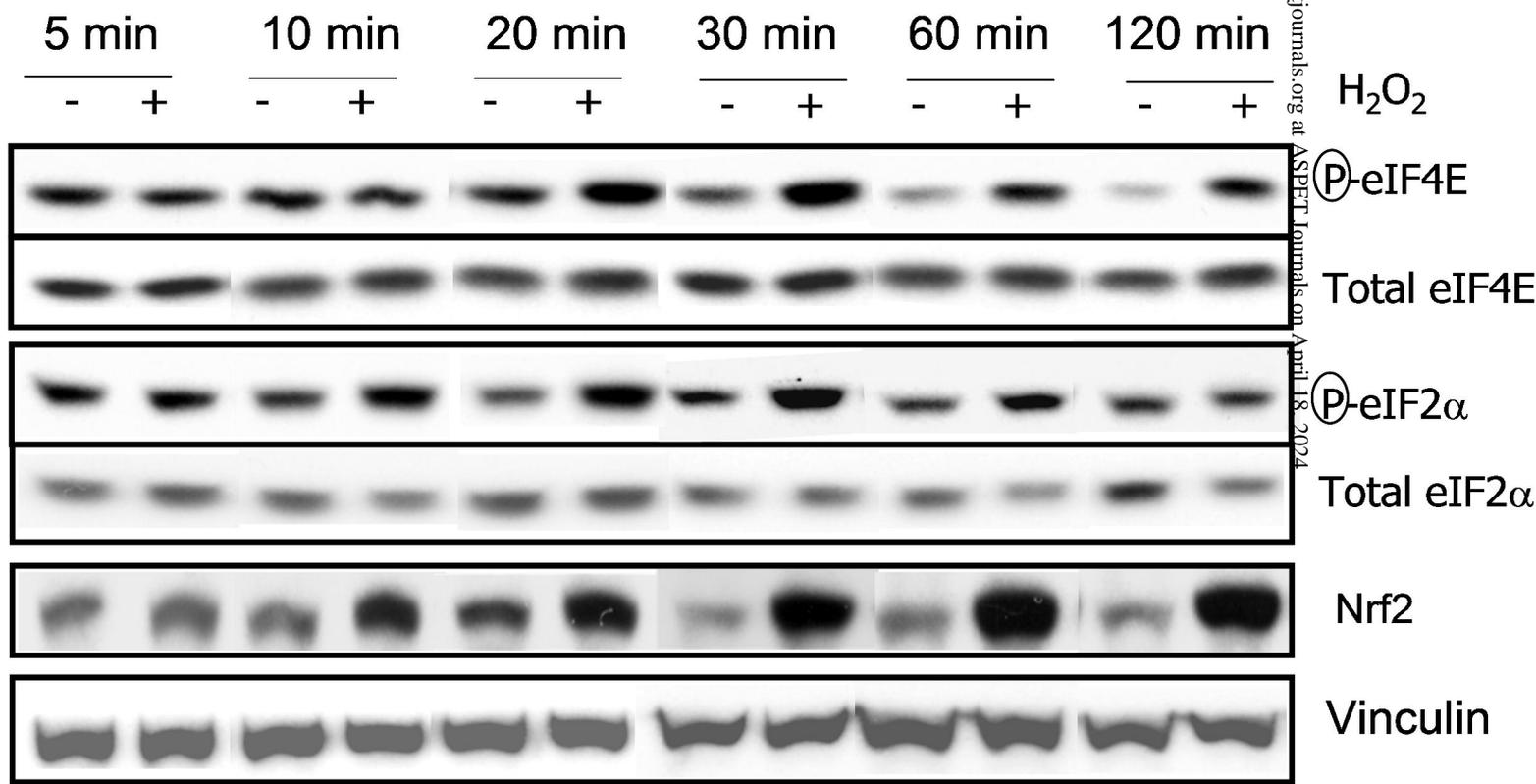
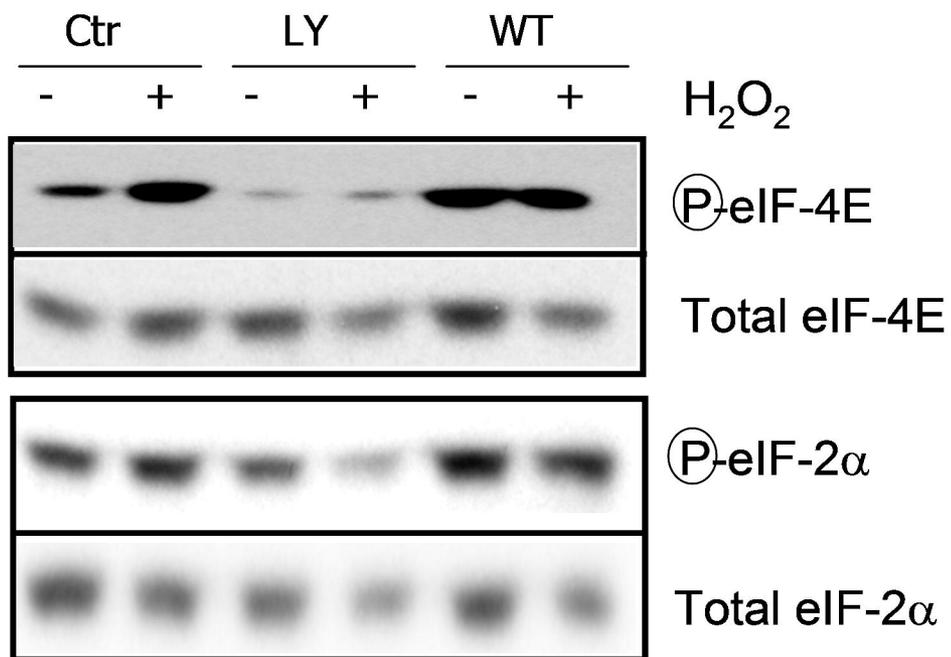
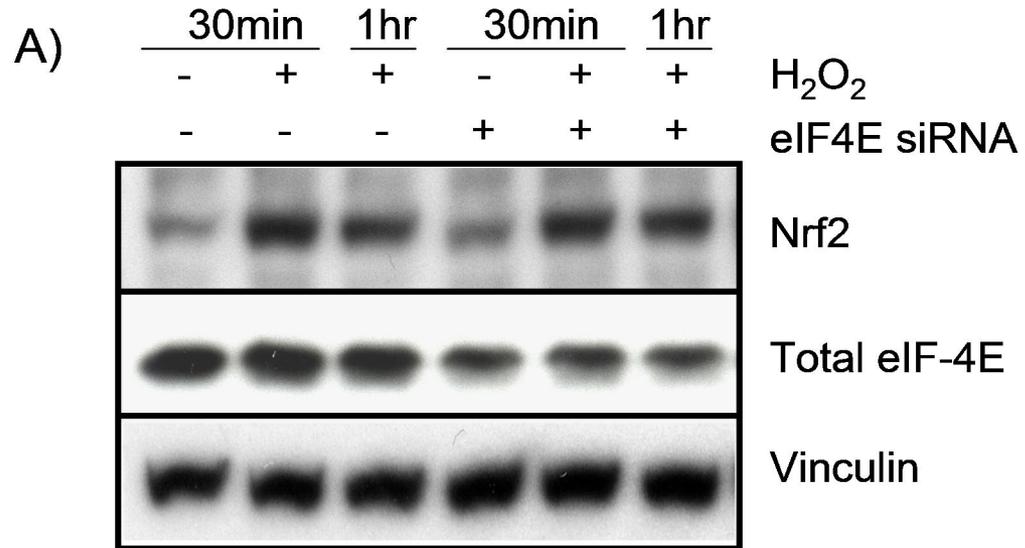


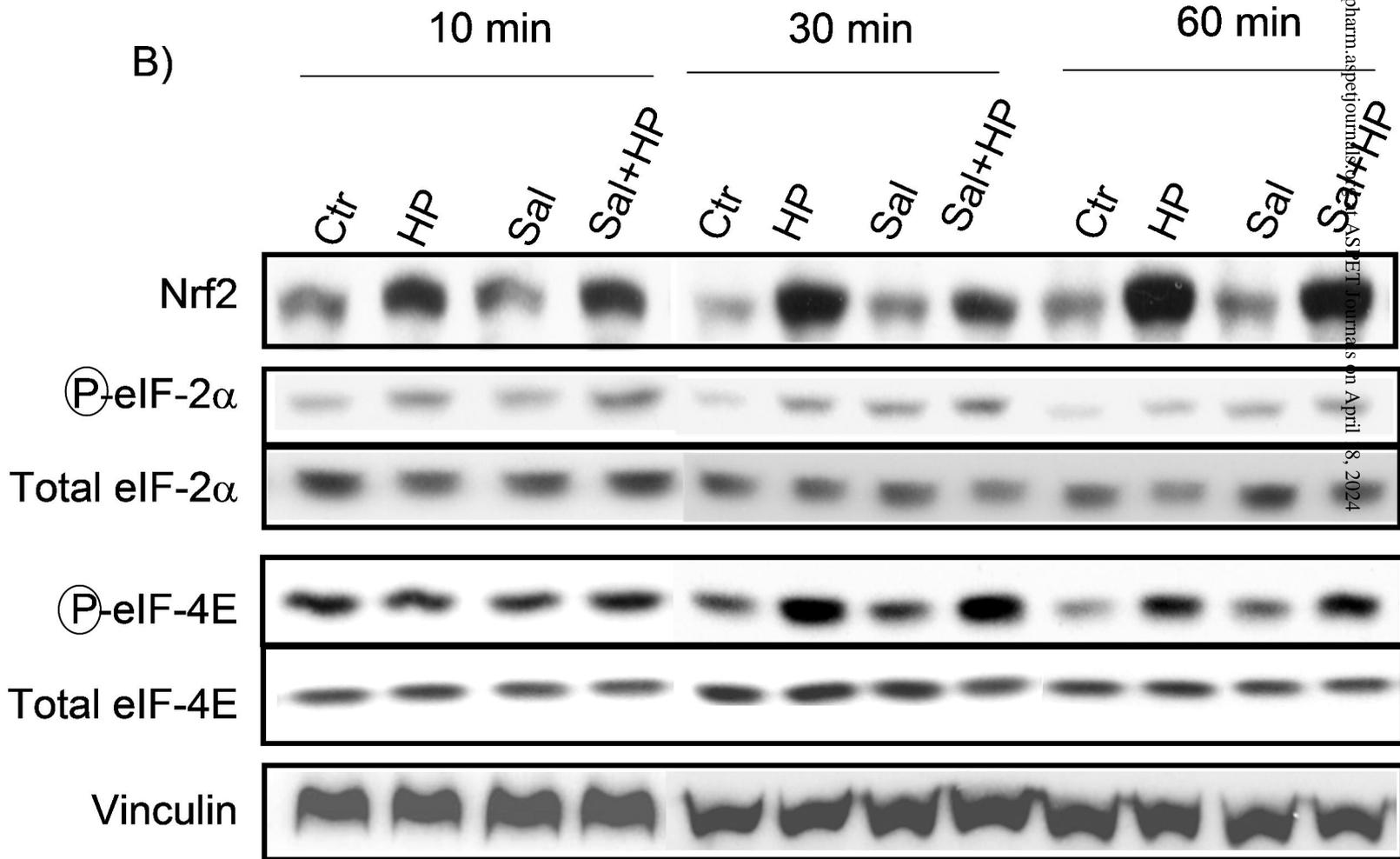
Figure 6



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