Prothymosin-α mediates nuclear import of INrf2/Cul3-Rbx1 complex to degrade nuclear Nrf2
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Running Title: Prothymosin-α mediated nuclear import of INrf2
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Abstract
Nrf2-mediated coordinated induction of a battery of defensive genes is a critical mechanism in cellular protection and survival. INrf2 (Keap1), an inhibitor of Nrf2, functions as an adaptor for Cul3-Rbx1 mediated degradation of Nrf2. A majority of the INrf2/Cul3-Rbx1 complex is localized in the cytosol that degrades cytosolic Nrf2. However, 10-15% of INrf2 is also localized inside the nucleus. INrf2 does not contain a defined nuclear import signal and the mechanism of nuclear import and its function inside the nucleus remain obscure. Present studies demonstrate that the DGR region of INrf2 is required for nuclear import of INrf2. Studies also demonstrate that Cul3 and Rbx1 are also imported inside the nucleus in complex with INrf2. Interestingly, Nrf2 and prothymosin-α both bind to the DGR region of INrf2. However, it is prothymosin-α and not Nrf2 that mediates nuclear import of INrf2/Cul3-Rbx1 complex. Antioxidant treatment increases nuclear import of INrf2/Cul3-Rbx1 complex. The INrf2/Cul3-Rbx1 complex inside the nucleus exchanges prothymosin-α with Nrf2 resulting in degradation of Nrf2. These results led to the conclusion that prothymosin-α mediated nuclear import of INrf2/Cul3-Rbx1 complex leads to ubiquitination and degradation of Nrf2 inside the nucleus presumably to regulate nuclear level of Nrf2 and rapidly switch off the activation of Nrf2 downstream gene expression.

Introduction
Nrf2:INrf2 serve as sensor of chemical and radiation induced oxidative and electrophilic stress (1-3). Nrf2 is a nuclear transcription factor that regulates expression of several defensive genes including detoxifying enzymes and antioxidant genes (1-3). The induction of these enzymes is important for neutralizing the cellular stresses, providing cellular protection and survival (1-3). Nrf2 resides predominantly in the cytoplasm where it interacts with actin-associated cytosolic protein, INrf2 (inhibitor of Nrf2) or Keap1 (Kelch-like ECH-associated protein 1) (4-6). INrf2 functions as a substrate adaptor protein for a Cul3-Rbx1-dependent E3 ubiquitin ligase complex to ubiquitinate and degrade Nrf2 thus maintaining a steady-state levels of Nrf2 (7). The exposure to oxidative/electrophilic stress leads to dissociation of Nrf2 from INrf2. Nrf2 is stabilized, translocates into the nucleus, and activates the transcription of several defensive genes. Recently, the mechanisms by which Nrf2 is released from INrf2 under stress have been actively investigated. One mechanism is that cysteine thiol groups of INrf2 were shown to function as sensors for oxidative stress which are modified by the chemical inducers, causing formation of disulfide bonds between cysteines of two INrf2 peptides. This results in conformational change that renders INrf2 unable to bind to Nrf2 (8-10). On the other hand, antioxidant-induced protein kinase C mediated phosphorylation of serine40 in Nrf2 leads to
dissociation of Nrf2 from INrf2 (11-12). It is possible that two mechanisms work in concert or independently of each other. However, the evidence to support either is lacking. Interestingly we have recently demonstrated that an autoregulatory loop between Nrf2 and INrf2 controls their abundance inside the cells (13). In other words, Nrf2 induces INrf2 gene expression for its own degradation.

Several reports suggest that persistent accumulation of Nrf2 in the nucleus is harmful. INrf2-null mice demonstrated persistent accumulation of Nrf2 in the nucleus that led to postnatal death from malnutrition resulting from hyperkeratosis in the esophagus and forestomach (14). Reversed phenotype of INrf2 deficiency by breeding to Nrf2-null mice suggested tightly-regulated negative feedback might be essential for cell survival (15). The recent systemic analysis of INrf2 genomic locus in human lung cancer patients and cell lines showed that deletion, insertion, and missense mutations in functionally important domains of INrf2 results in reduction of INrf2 affinity for Nrf2 and elevated expression of cytoprotective genes (16-17). Taken together, unrestrained activation of Nrf2 in cells increases a risk of adverse effects including tumorigenesis. On the other hand, stress-induced activation of the Nrf2 pathway in normal cells is tightly regulated and confers cytoprotection against oxidative and electrophilic stress and carcinogens. Therefore, it appears that cells contain mechanisms that auto-regulate cellular abundance of Nrf2.

A majority of the INrf2 is present in the cytosol (7). However, 10-15% of INrf2 is also localized inside the nucleus (7). Procite analysis revealed that INrf2 does not contain a defined nuclear import signal and the mechanism of nuclear import of INrf2 and its function inside the nucleus remain unknown. In this report we investigated the mechanism of nuclear import of INrf2 and its role in Nrf2 degradation inside the nucleus. The studies demonstrated that the DGR region of INrf2 and prothymosin-α (PTMα) are required for nuclear import of INrf2. Studies also demonstrated that INrf2 is not transported alone but in complex with Cul3 and Rbx1. Interestingly, PTMα containing a nuclear localization signal binds to the DGR region of INrf2 and transports whole complex of INrf2/Cul3-Rbx1 inside the nucleus. The complex inside the nucleus releases PTMα, binds to Nrf2, and triggers Nrf2 degradation. This is presumably to rapidly switch off the activation of Nrf2 downstream gene expression.

Materials and Methods

Cell Cultures— Mouse hepatoma (Hepa-1) cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, penicillin (40 units/ml), and streptomycin (40 µg/ml). The cells were grown in monolayer in an incubator at 37 °C in 95% air and 5% CO2.

Plasmid Constructs— Mammalian expression vector pEGFP-N1 was purchased from Clontech (CA USA). This vector upon transfection in cells expresses green fluorescence protein (GFP). The vector was designated as 1X-GFP. We modified this vector by cloning another coding sequence of GFP. The modified vector was designated as 2XGFP. A Full-length mouse INrf2 coding sequence (1872 bp) was cloned into 1X GFP as well as 2X GFP vector. The primer sequences used for amplification of full-length INrf2, different deletion domains and mutations of INrf2 are shown in Supplement Table 1. Two leucine residues (L308 and L310) present between functional nuclear export signals 2 (NES2) of INrf2 were mutated to alanine by using specific mutant primers and site directed mutagenesis kit (Invitrogen). cDNA of prothymosin-α (PTMα) was purchased from OriGene (Rockville, MD) and a full-length coding sequence was amplified by PCR and subcloned into pcDNA3.1/V5-His/Topo vector by TA cloning. The construction of pGL2B-NQO1-ARE, pcDNA-Flag-INrf2, pcDNA-Flag-Nrf2, pcDNA-INrf2-V5 and HA-UB has been described previously (15).
site directed mutations and deletions mutations were confirmed by DNA sequencing.

In vitro transcription/translation- In vitro transcription/translation of the plasmids encoding domains/deletions of INrf2-GFP and PTMα–V5 constructs were performed using the TNT-coupled rabbit reticulocyte lysate system (Promega). 0.2 µg of plasmid DNA was incubated with 25 µl of TNT-coupled rabbit reticulocyte lysate supplied with 40 µM of L-methionine at 30°C for 90 min. The plasmid encoding luciferase provided in the kit was used as a control for the transcription/translation reaction. After the coupled transcription/translation, the proteins were checked for their correct size by SDS-PAGE followed by immunoblotting. All of the in vitro transcribed/translated proteins gave the expected size bands.

Generation of stable Flp-In T-REx HEK293 cells expressing tetracycline-inducible Flag INrf2, Flag-ΔDGR-INrf2 and Flag-DGR-INrf2— Full-length INrf2, ΔDGR-INrf2 and DGR-INrf2 regions were amplified by PCR using specific sets of primers (Supplement Table 1) and cloned into modified pcDNA5/FRT/TO (Flag) X2-(His) 8 vector. Flp-In T-REx HEK-293 cells were purchased from Invitrogen and co-transfected separately with 0.1 µg of Flag-INrf2-pcDNA5/FRT/TO, Flag-ΔDGR-INrf2-pcDNA5/FRT/TO and Flag-DGR-INrf2-pcDNA5/FRT/TO along with 0.9 µg pOG44 plasmids (Invitrogen) by Effectene (Qiagen, Valencia, CA) method, as described by the manufacturer’s instructions. Forty eight hours after transfection, the cells were grown in medium containing 100 µg/ml hygromycin B and 30 µg/ml blasticidin (Invitrogen). The 293/FRT/TO cells stably expressing tetracycline-inducible Flag-INrf2 proteins. Flag-ΔDGR-INrf2 and Flag-tagged DGR-INrf2 proteins.

Subcellular fractionation and Western blotting— Hepa-1 cells, seeded in 100-mm plates and treated or transfected as displayed in the figures, were washed twice with ice-cold phosphate-buffered saline, trypsinized, and centrifuged at 1500 rpm for 5 min. For making whole cell lysates, the cells were lysed in RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.2 mM EDTA, 1% Nonidet P-40, 0.5% deoxycholic acid, 1 mM phenylmethylsulfonyl fluoride, and 1 mM Sodium orthovanadate supplemented with protease inhibitor mixture (Roche Applied Science). Cytoplasmic and nuclear cell lysates were separated by using the Active Motif nuclear extract kit (Active Motif, Carlsbad, CA) by following the manufacturer’s protocol. The protein concentration was determined using the protein assay reagent (Bio-Rad). 60 to 80 micrograms of proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 3% non fat dry milk and incubated with anti-INrf2 (E-20) (1:1000), anti-Nrf2 (H-300) (1:500), anti Prothymosin α (H-50) (1:1000) and anti fetal alz-50 clone 1 (FAC1) (1:1000) antibodies, all purchased from Santa Cruz Biotechnology (CA). Anti Cul3 and anti Rbx1 antibodies were purchased from Cell Signaling (Boston, MA). Anti-Flag-HRP (1:10000), anti-HA-HRP (1:10000) and anti-actin antibodies were obtained from Sigma. Anti-GFP and anti-V5 HRP antibodies obtained from Invitrogen. The membranes were washed three times with TBST and immunoreactive bands were visualized using a chemiluminescence’s system ECL (Amersham). The intensity of protein bands after Western blotting were quantitated by using QuantityOne 4.6.3 Image software (ChemiDoc XRS, Bio-Rad) and normalized against proper loading controls. To confirm the purity of nuclear-cytoplasmic fractionation, the membranes were reprobed with cytoplasm-specific, anti-lactate dehydrogenase (Chemicon) and nuclear specific, anti-lamin B antibodies (Santa Cruz Biotechnology). In related experiments, the cells were treated with 50 to 100 µM t-BHQ, 10 ng/ml LMB and 20 µM...
genestein or DMSO as a vehicle for different time intervals.

**Immunoprecipitation**—1 mg of whole cell lysate or nuclear/cytoplasmic extract were equilibrated in RIPA buffer, pre-cleaned with protein-AGplus-agarose (Santacruz Biotechnology) and incubated with respective antibodies (1 µg) at 4°C for overnight. Immune-complexes were collected by addition of protein AG-agarose again after centrifugation. The immune-complexes were washed three times with RIPA buffer containing 0.25% NP-40, and proteins were resolved by 10% reducing SDS-PAGE and transferred to nitrocellulose membrane. The membranes were blocked with 3% non fat dry milk and incubated with their respective primary and secondary antibodies. Immunoreactive bands were visualized using a chemiluminescence's system ECL (Amersham).

**Transient transfection and luciferase assay**— Hepa-1 cells were plated in 100 mm plates at a density of 1x10^5 cells/plate 24h prior to transfection. In the related experiments, the cells were transfected with 1 µg of the indicated plasmids using Effectene transfection reagent (Qiagen) according to the manufacturer’s instructions. After 36h of transfection, cells were harvested and cellular specific protein regulation was examined by Western blotting. For luciferase reporter assay, Hepa-1 cells were grown in monolayer cultures in 12-well plates in DMEM medium supplemented with 10% fetal bovine serum. Then cells were co-transfected with 0.1 µg of reporter construct human NQO1-ARE-Luc and 10 times less quantities of firefly Renilla luciferase encoded by plasmid pRL-TK. Renilla luciferase was used as the internal control in each transfections. After 24h of transfection, the cells were washed with 1X phosphate-buffered saline and lysed in 1X Passive lysis buffer from the Dual-Luciferase® reporter assay system kit (Promega, Madison, WI). The luciferase activity was measured using the procedures described previously (15) and plotted.

**siRNA interference assay**—Control, Nrf2 and INrf2 siRNA purchased from Dharmacon were used to inhibit Nrf2 and INrf2 proteins respectively by a procedure described previously (10). Hepa-1 cells were transfected with 5, 25 and 50 nM of control or Nrf2 or INrf2 siRNA using Lipofectamine RNAiMAX reagent (Invitrogen) according to the manufacturer’s instructions.Thirty two hours after transfection, cells were harvested and localization of INrf2, Cull3, RBX1 was analyzed by Western blotting by probing the membranes with INrf2, Nrf2 and Cull3 or RBX1 antibodies. Fac1 and PTMα siRNA was also purchased from Ambion (CA) and effect of these siRNA on localization of INrf2 was analyzed. Effect of PTMα siRNA on localization of INrf2 and NQO1 ARE luciferase activity was carried out after control siRNA or PTMα siRNA transfections.

**Immunofluorescence**—Hepa-1 cells were grown in Lab-Tek II chamber slides in DMEM supplemented with 10% fetal bovine serum. In related experiments, cells were transfected with INrf2-GFP, INrf2-ΔDGR-GFP or INrf2 DGR 2X GFP for 32h. After fixing with 2% formaldehyde, cells were stained with DAPI and directly observed under fluorescence microscope. Effect of PTMα siRNA on localization of INrf2 was performed by transient transfection of PTMα siRNA using the procedures described above. Twenty-four hours later, cells were fixed with 2% formaldehyde at 4°C for 10 min and permeabilized by 0.25% Triton X-100. Cells were washed twice with PBS and incubated with 1:1000 dilution of goat INrf2 primary antibodies in 2% BSA at 4°C for 12h. Then cells were washed twice with PBS and incubated with FITC conjugated second antibody for 2 h at room temperature. Cells were washed and stained with DAPI and mounted. Effect of PTMα overexpression on localization of INrf2-V5 was also visualized by immunofluorescence. Hepa-1 cells were transfected with INrf2-V5 alone or in combination with pcDNA-PTMα. Twenty-four hours later, cells were treated with 50 µM t-BHQ for 2h, and cells were fixed and
permeabilized by 0.25% TritonX-100. After washing, cells were incubated with 1:1000 dilution of FITC conjugated anti-V5 tag antibodies for 12 h. Cells were washed twice with PBS, stained with DAPI and mounted. After immunostaining, cells were observed under Nikon fluorescence microscope and photographs were captured. The cytoplasmic and nuclear FITC fluorescence intensities were quantified from 10 different cells (N=10) by using NIS-Elements BR2.30 SP4 software (Nikon). The green fluorescence intensity of the images were quantified by using Nikon Elements Advanced Research Software (Melville, NY). The entire green region of nuclear and cytosolic compartment was first marked and delineated, and the average fluorescence intensity of the green channel was measured. The experiments were repeated three times.

Isolation and purification of INrf2/Cul3-Rbx1/PTMα/Nrf2 complex— Human embryonic kidney FRT-HEK293 cells stably expressing Flag-INrf2 were treated with 0.5 μg/ml of tetracycline for 24 hour followed by treatment with DMSO or tBHQ (50 µM) for 1 and 2h. Cells were harvested and cytoplasmic and nuclear extracts prepared by using Active Motif Kit. 5 mg of cytoplasmic and nuclear extracts were mixed with anti-Flag agarose beads (200 μl) at 4°C for 8 h, beads washed three times with RIPA buffer and bound protein were eluted with 100 μl of 1X Flag peptide (Sigma). The native protein complex was electrophoresed by 6% Native gel and stained with Coomassie Brilliant Blue. The same native complex was immunoblotted with anti-Flag, Nrf2, PTMα, Cul3 and Rbx1 antibodies.

Ubiquitination assay—Hepa-1 cells were transfected with control or PTMα siRNA (50 nM) for 10h followed by co-transfection with Flag-INrf2 (0.5 μg), Nrf2-V5 (1.0 μg) and HA-Ub (0.2 μg). Nuclear and cytoplasmic extracts were prepared using active motif kit. To check the effect of PTMα siRNA on localization of INrf2 and PTMα protein, 60 μg of the cytoplasmic and nuclear extracts were immunoblotted with anti-Flag, anti-V5 and anti-PTMα antibodies. To check Nrf2 ubiquitination in cytoplasmic and nuclear compartments, one mg of cytoplasmic and nuclear lysates were immunoprecipitated with anti-V5 antibody (Invitrogen). Immune-complexes were resolved by 10% SDS-PAGE followed by immunoblotting with anti-HA-HRP antibody.

Statistical Analyses— Statistical analysis of the data from luciferase assays and immunoblotting band intensities were performed by using the SPSS-16 software. Error bars indicate standard error of the means (s.e.m) of triplicate samples and presented in figures. Statistical analysis was performed by one way ANOVA, followed by the Tukey-Kramer’s post hoc test for multiple comparisons. Significance P values were also calculated and presented.

Results

DGR is required for nuclear localization of INrf2- INrf2 and INrf2 domain deletion mutants were separately cloned into GFP vector (Fig 1A). In vitro transcription/translation reactions confirmed, these plasmids produced the expected size of GFP tagged INrf2 protein (Fig. 1B). The various plasmids were transfected in Hepa-1 cells. The transfected cells were lysed to generate whole cell lysate (W) or fractionated to cytosolic (C) and nuclear (N) fractions. The purity of cytosolic and nuclear fractions were assessed by Western blotting and probing for cytosolic marker lactate dehydrogenase (LDH) and nuclear marker Lamin B (Fig. 1C, panels 13 and 14). Western analysis of transfected cells revealed that deletion of the DGR region led to abrogation of nuclear localization of INrf2 (Fig. 1C, compare top 10 panels). All other deletions showed mutant INrf2 protein in the nucleus. The mutation of NES2 region had no effect on localization of DGR deficient mutant INrf2 (Fig. 1C, compare panels 7
and 8). In the same experiment, DGR region attached to 2XGFP localized in the nucleus (Fig. 1C, panel 12). These results demonstrated that DGR region is required for nuclear localization of INrf2. This interpretation was also supported by immunocytochemical analysis (Fig. 1D). INrf2-GFP and DGR-2XGFP and not INrf2ΔDGR-GFP localized in the nucleus.

Further support to the role of DGR region in nuclear localization of INrf2 was obtained by analysis of human kidney carcinoma FRT 293 cells permanently expressing tetracycline inducible Flag-tagged wild type INrf2, DGR and INrf2ΔDGR in separate experiments. Western analysis of tetracycline treated FRT 293 cells showed expression of expected sizes of tetracycline-induced INrf2, DGR and INrf2ΔDGR protein (Fig. 2A). Western analysis of cytosolic and nuclear fractions revealed presence of INrf2 and DGR but not INrf2ΔDGR in nuclear fraction of FRT 293 cells (Fig. 2B). Treatment with t-BHQ enhanced nuclear import of wild type INrf2 and DGR domain of INrf2 but not INrf2ΔDGR. Nuclear export inhibitor leptomycin B (LMB) treatment led to nuclear accumulation of INrf2. This was similar to LMB mediated nuclear accumulation of endogenous Nrf2, a positive control used in the experiment. However, LMB had no effect on the nuclear localization of DGR and INrf2ΔDGR (Fig. 2C). Similar results were also obtained in Hepa-1 cells transfected with GFP-tagged constructs (Fig 2D). FRT293 cells expressing Flag-tagged INrf2 were treated with t-BHQ or LMB, and nuclear and cytoplasmic INrf2 levels were quantified from three independent experiments. As shown in Figure 2E, 15 to 20% more INrf2 accumulates in the nucleus upon treatment with either t-BHQ or LMB compared to DMSO treated cells (Fig. 2B, 2E).

INrf2 is known to contain a functional nuclear export signal (NES2) (19). The absence of an effect on deletion of NES2 (Fig. 1C and 1D) and LMB on nuclear localization of INrf2ΔDGR and DGR-2XGFP and the loss of nuclear localization of INrf2ΔDGR suggested that nuclear localization of INrf2 is independent of nuclear export of INrf2.

**Cul3 and Rbx1 translocation to nucleus in complex with INrf2**

Immunoprecipitation and Western analysis of Hepa-1 cells treated with either DMSO or t-BHQ revealed that INrf2 antibody immunoprecipitated INrf2, Cul3 and Rbx1 (Fig. 3A, upper panel DMSO lane). In same experiment, the Cul3 antibody immunoprecipitated Cul3, INrf2 and Rbx1 (Fig. 3A, middle panel, DMSO lane), and Rbx1 antibody immunoprecipitated Rbx1, Cul3 and INrf2 (Fig. 3A, lower panel, DMSO lane). These results suggested that INrf2, Cul3 and Rbx1 existed in a complex. This finding is similar as reported earlier that Cul3 in complex with Rbx1 binds to INrf2 to ubiquitinate and degrade Nrf2 (7). It might be noteworthy that Rbx1 binds to Cul3 and not INrf2 (7). Rbx1 is immunoprecipitated with INrf2 because of its binding with Cul3 that binds to INrf2. Interestingly, treatment with t-BHQ led to significant loss in immunoprecipitation of Cul3 and Rbx1 with INrf2 antibody (Fig. 3A, upper panel, compare t-BHQ lane with DMSO lane). Similar results were also observed in immunoprecipitation with Cul3 and Rbx1 antibodies (Fig. 3A, middle and lower panels). These results indicated that t-BHQ destabilized INrf2/Cul3-Rbx1 interaction to stabilize Nrf2. The replacement of whole cell lysate with cytosolic and nuclear fractions showed similar results (data not shown).

We used INrf2 siRNA to demonstrate that inhibition of INrf2 led to nuclear translocation of not only INrf2 but also Cul3 and Rbx1 (Fig. 3B and C). Hepa-1 cells were transfected with increasing concentrations of either control siRNA or INrf2 siRNA. INrf2 siRNA and not control siRNA showed concentration dependent decrease in INrf2 protein level (Fig. 3B). In related experiment, cytoplasmic and nuclear proteins were isolated from mock, control or
INrf2 siRNA transfected Hepa-1 cells and immunoblotted with INrf2, Cul3 and Rbx1 antibodies. Immunoblots were also probed for cytosolic marker LDH and nuclear marker Lamin-B. The results revealed that INrf2 siRNA significantly reduced whole cell lysate (W), cytosol (C) and nuclear (N) INrf2, as compared to mock and control siRNA transfected Hepa-1 cells (Fig. 3C, top panel). Interestingly, decrease in siRNA-mediated INrf2 in the nucleus also decreased nuclear Cul3 and Rbx1 and corresponding increase in cytosolic Cul3 and Rbx1 (Fig. 3C, compare INrf2 siRNA lane with mock and control siRNA lanes in Cul3 and Rbx1 panels). These results suggested that loss of INrf2 reduced nuclear import of Cul3-Rbx1 or also suggested Cul3-Rbx1 translocate to nucleus in complex with INrf2. The effect of INrf2 siRNA on the overall localization of Cul3 and Rbx1 in cytoplasmic and nuclear compartments was quantified from three different experiments and presented (Fig. 3D). Further immunoprecipitation experiments confirmed that knocking down INrf2 by siRNA significantly decreased INrf2/Cul3-Rbx1 or also suggested Cul3-Rbx1 translocate to nucleus in complex with INrf2. The results are shown in Fig. 4B. Anti-V5 immunoprecipitated both INrf2-V5 and INrf2ΔDGR-V5. Interestingly, Flag-Nrf2 was pulled down along with INrf2-V5 but not with INrf2ΔDGR (Fig. 4B, Upper two left panels). Reverse IP with anti-Flag immunoprecipitated Flag-Nrf2 (Fig. 4B, Lowest left panel). In reverse IP, INrf2-V5 but not INrf2ΔDGR-V5 was pulled down (Fig. 4B, third left panel from the top). Interestingly, only DGR domain of INrf2 interacts with Flag Nrf2 (Fig. 4B, right upper and lower panels). The interaction of endogenous Nrf2 with INrf2GFP, and INrf2ΔDGRGFP was examined by GFP immunoprecipitation followed by Nrf2 immunoblotting. Endogenous Nrf2 interacts with INrf2-GFP but not with INrf2ΔDGR-GFP or GFP vector (Fig. 4C). These results combined demonstrated that Nrf2 interacts with the DGR domain of INrf2.

**Nrf2 is not required for nuclear translocation of INrf2.** Hepa-1 cells were transfected with INrf2-GFP, DGR-GFP or INrf2ΔDGR with or without Flag-Nrf2 in the ratio of 1:4 to determine the effect of the overexpression of INrf2 and INrf2ΔDGR on degradation of Nrf2. As shown in Figure. 4A, overexpression of INrf2 led to degradation of greater than 90% of Nrf2. However, in the same experiment, INrf2ΔDGR deficient in DGR domain failed to degrade Nrf2. Overexpression of the DGR by itself had little effect on degradation of Nrf2. Further, we performed reverse immunoprecipitation from Hepa1 cell extracts which were co-transfected with INrf2-V5 or INrf2ΔDGR-V5 or INrf2DGR-V5 and Flag-Nrf2 in 1:4 ratio. The results are shown in Fig. 4B. Anti-V5 immunoprecipitated both INrf2-V5 and INrf2ΔDGR-V5. Interestingly, Flag-Nrf2 was pulled down along with INrf2-V5 but not with INrf2ΔDGR (Fig. 4B, Upper two left panels). Reverse IP with anti-Flag immunoprecipitated Flag-Nrf2 (Fig. 4B, Lowest left panel). In reverse IP, INrf2-V5 but not INrf2ΔDGR-V5 was pulled down (Fig. 4B, third left panel from the top). Interestingly, only DGR domain of INrf2 interacts with Flag Nrf2 (Fig. 4B, right upper and lower panels). The interaction of endogenous Nrf2 with INrf2GFP, and INrf2ΔDGRGFP was examined by GFP immunoprecipitation followed by Nrf2 immunoblotting. Endogenous Nrf2 interacts with INrf2-GFP but not with INrf2ΔDGR-GFP or GFP vector (Fig. 4C). These results combined demonstrated that Nrf2 interacts with the DGR domain of INrf2.

Next, we determined if Nrf2 interaction with the DGR domain of INrf2 is required for nuclear localization of INrf2. Hepa-1 cells were transfected with different concentrations of control or Nrf2 siRNA and analyzed by immunoblotting with Nrf2. The results showed Nrf2 siRNA concentration dependent decrease in Nrf2 (Fig. 4D). Control siRNA in the same experiment had no effect on expression of Nrf2 protein. siRNA inhibition of Nrf2 was used to determine if the decrease in Nrf2 had an effect on nuclear localization of INrf2. The results demonstrated that siRNA-mediated decrease in Nrf2 did not alter nuclear localization of INrf2 (Fig. 4E). This indicated that Nrf2 is not essential for nuclear import of INrf2.

**PTMα is required for nuclear import of INrf2.** Human 293 cells permanently expressing tetracycline inducible Flag-INrf2Flag, Flag-DGR and Flag-INrf2ΔDGR-Flag were grown in culture, treated with tetracycline to induce respective proteins, lysed, immunoprecipitated with anti-Flag antibody and immunoblotted with
prothymosinα (PTMα) and fetal alz-50 clone 1 (Fac1) antibodies to determine the interaction of INrf2 with PTMα and Fac1. The results showed an interaction of both PTMα and Fac1 with INrf2 and DGR domain since anti-Flag immunoprecipitated INrf2 and DGR. Both PTMα and Fac1 immunoprecipitated along with INrf2 and DGR (Fig. 5A, left panel). Interestingly, immunoprecipitation of INrf2ΔDGR-Flag with anti-Flag antibody failed to immunoprecipitate either PTMα or Fac1 (Fig. 5A, left panel). Reverse immunoprecipitation with anti-PTMα and anti-Fac1 immunoprecipitated INrf2-Flag and DGR-Flag but failed to immunoprecipitate INrf2ΔDGR-Flag (Fig. 5A, right panels). These results showed that PTMα and Fac1 both interact with the DGR domain of INrf2. Similar experiment was performed in cytosolic and nuclear fractions of Hepa-1 cells to determine interaction of endogenous PTMα and INrf2 and associated Cul3-Rbx1 proteins (Fig. 5B). INrf2 antibody immunoprecipitated INrf2 from both cytosolic and nucleus fractions (Fig. 5B, second blot from top). PTMα was pulled down along with INrf2 from both the fractions (Fig. 5B, top blot). In reverse IP, PTMα antibody immunoprecipitated PTMα (Fig. 5B, second blot from bottom). INrf2, Cul3, and Rbx1 but not Nrf2 were pulled down along with PTMα (Fig. 5B, blot 3-6 from the top). These results clearly suggested that PTMα and INrf2/Cul3-Rbx1 exist in a complex in cytosolic and nuclear fractions.

Procite search revealed that both PTMα and Fac1 contain strong nuclear localization signals. We used PTMα and Fac1 specific siRNA to decrease respective proteins in separate experiments with the aim to determine if PTMα or Fac1 interaction with DGR region leads to nuclear localization of INrf2. PTMα and Fac1 siRNA showed concentration dependent inhibition of PTMα and Fac1 in Hepa-1 cells (Fig. 5C). In a related experiment, Hepa-1 cells were transfected with either PTMα siRNA or Fac1 siRNA and cytosolic and nuclear extracts were immunoblotted for endogenous INrf2. As shown in Figure 5D, siRNA-mediated inhibition of PTMα and not Fac1 led to abrogation of nuclear localization of INrf2. Further, we used three different concentrations (25, 50 and 75 nM) of PTMα siRNA that led to siRNA concentration dependent decrease in PTMα (Fig. 5C, top panel) and significant loss of nuclear localization of INrf2 was also observed (Fig. 5E). These results clearly indicate that PTMα interaction with DGR domain of INrf2 leads to nuclear localization of INrf2 in the nucleus. We reasoned that since Cul3-Rbx1 move to the nucleus through Cul3 interaction with INrf2, then inhibition of PTMα-mediated nuclear localization should also lead to inhibition of nuclear localization of Cul3 and Rbx1.

Immunocytochemical analysis of INrf2 in PTMα siRNA transfected Hepa-1 cells supported the role of PTMα in nuclear translocation of INrf2 (Fig. 6A). Seventy-five nM PTMα siRNA caused almost complete loss of PTMα (Fig. 5C, top panel) and abrogation of nuclear import of INrf2 (Fig. 6A, Immunocytochemistry left panels and intensity measurement right panels). Moreover, siRNA-mediated inhibition of PTMα led to siRNA concentration dependent stabilization of Nrf2 and increase in endogenous Nrf2 downstream NQO1 gene expression (Fig. 6B). This was presumably due to a decrease in import of INrf2 in the nucleus and stabilization of Nrf2. Additionally we measured NQO1 ARE-luciferase activity from Hepa-1 cells which were co-transfected with either control siRNA or PTMα siRNA. The results demonstrated that PTMα siRNA dependent increase in ARE-luciferase gene expression was significant compared with control siRNA (Fig. 6C, P<0.004). This was due to stabilization of Nrf2 in PTMα siRNA transfected Hepa-1 cells.

Overexpression of PTMα-V5 increases nuclear localization of INrf2- Hepa-1 cells were either transfected with vector control
or PTM-α-V5, lysed and immunoblotted with V5 antibody. Significant overexpression of PTMα-V5 was observed in cells transfected with PTMα-V5 (Fig. 7A). To examine the effect of overexpression of PTMα on nuclear localization INrf2, we overexpressed PTMα-V5 in Hepa-1 cells and cytosolic and nuclear extracts were immunoblotted with anti-V5 antibody for PTMα-V5, anti-PTMα for endogenous PTMα and anti-V5 for INrf2-V5 transfected protein. As shown in Figure 7B, overexpression of PTMα-V5 led to increased nuclear localization of INrf2-V5 in presence and absence of t-BHQ (Fig. 7B, Western analysis upper panels and relative band intensity lower panels, P>0.005). Immunocytochemical analysis (Fig 7C upper panels) and intensity measurement (Fig 7C lower panels) showed significantly increased nuclear localization of INrf2 in cells overexpressing PTMα-V5 (P>0.0001). This again supported a role of PTMα in nuclear import of INrf2.

**Antioxidant treatment induces exchange of PTMα with Nrf2 in the nucleus.** Hepa-1 cells were treated with either DMSO or t-BHQ for indicated periods as shown in Figure 8A. Endogenous INrf2 protein was immunoprecipitated from equal amounts (1 mg) of cytosolic and nuclear extracts and immunoblotted for endogenous INrf2, PTMα and Nrf2 proteins for analyzing their interactions. INrf2 antibody immunoprecipitated INrf2 from nuclear and cytosolic fractions (Fig. 8A, Western analysis left panels and relative binding as determined by band intensity measurements right panels). It showed an increase in immunoprecipitated INrf2 in cytosol but not in nucleus in response to t-BHQ treatment. This was expected as t-BHQ is known to increase INrf2 gene expression (13). Nrf2 and PTMα were pulled down along with INrf2 because of their interaction with INrf2 (Fig. 8A). Interestingly, Immunoprecipitation with INrf2 antibody demonstrated that in the nucleus Nrf2 interaction with INrf2 was more or less unchanged while PTMα interaction with INrf2 was increased at 1 hour of t-BHQ treatment as compared to DMSO treated control. The magnitude of Nrf2 and PTMα interaction with INrf2 reversed in the nucleus at two hours of t-BHQ treatment. The Nrf2 interaction increased and PTMα interaction decreased with INrf2. These results suggested that t-BHQ treatment in the first hour enhanced PTMα:INrf2 interaction and nuclear import of INrf2. The results also suggest that in the nucleus Nrf2 replaces PTMα to bind to INrf2 presumably for degradation in the second hour of t-BHQ treatment of Hepa-1 cells. The Nrf2 interaction with INrf2 in the cytosol during first hour of t-BHQ treatment decreased due to t-BHQ-mediated release of Nrf2 from INrf2 and nuclear translocation of Nrf2 to activate ARE-mediated defensive gene expression. The Nrf2 interaction with INrf2 showed an increase during the second hour of t-BHQ treatment to start degrading Nrf2. The PTMα interaction with INrf2 in the cytosol during first and second hour of t-BHQ treatment showed only marginal increase presumably to enhance nuclear translocation of INrf2 to capture and degrade nuclear Nrf2.

The PTMα exchange with Nrf2 in the nucleus was further investigated by analyzing the immunoprecipitated INrf2/Cul3-Rbx1/PTMα/Nrf2 complex from the cytosol and nucleus on native blue gel (Fig. 8B) and immunoblotting (Fig. 8C). The INrf2/Cul3-Rbx1/PTMα/Nrf2 complex increased in nucleus at 1hr of t-BHQ treatment and then decreased at 2hr of t-BHQ treatment (Fig. 8B). Western analysis of the cytosolic and nuclear complex revealed interaction of both PTMα and Nrf2 with INrf2 in varying amounts (Fig. 8C). As expected, the cytosol from cells treated with t-BHQ for 1hr showed increased binding of PTMα with Nrf2 and almost complete loss of interaction of Nrf2 with INrf2. This presumably is due to t-BHQ mediated release of Nrf2 to activate gene expression and to increase in PTMα-mediated import of INrf2 in the nucleus. The cytosol from cells treated with t-BHQ for 2hr demonstrated interaction of both PTMα and Nrf2
presumably to keep sending INrf2 to the nucleus and degradation of Nrf2 in the cytosol. Interestingly, the nuclear fractions from cells treated with DMSO and 1hr t-BHQ showed INrf2 interaction with both PTMα and Nrf2. However, at 2hr of t-BHQ treatment, nuclear fraction demonstrated almost complete loss of PTMα interaction with INrf2 and significant increase in interaction of Nrf2 with INrf2 indicating that INrf2 exchanged PTMα with Nrf2. These results provide further support to the theory of PTMα exchange with Nrf2 in complex with INrf2 inside the nucleus. The PTMα exchange with Nrf2 in complex with INrf2 was observed only in the nucleus and not in the cytosol in three independent experiments.

The pretreatment of Hepa-1 cells with tyrosine kinase inhibitor genestein inhibited Nrf2 exchange with PTMα for binding to INrf2 in the nucleus (Fig. 8D). Immunoprecipitation with INrf2 antibody pull down PTMα but not Nrf2 at both 1 and 2 hr of t-BHQ treatment in the nucleus. (Fig. 8D, Western analysis upper panel and band intensities in lower panel).

PTMα-mediated nuclear import of INrf2 leads to degradation of nuclear Nrf2. Hepa-1 cells co-transfected with HA-UB and Nrf2-V5 demonstrated very low level of ubiquitination of Nrf2 (Fig. 9A). The inclusion of Flag-INrf2 increased ubiquitination of Nrf2 (Fig.9A) and slight degradation of Nrf2 (Fig. 9B). Prior transfection of cells with control siRNA had no effect on PTMα and ubiquitination/degradation of Nrf2 (Fig. 9A and B). Prior transfection with PTMα siRNA inhibited PTMα in the cytosol as well as nucleus (Fig. 9B). The inhibition of PTMα in nucleus but not in the cytosol led to decreased ubiquitination and degradation of Nrf2 (Fig. 9A and B).

**Discussion**

INrf2 is a negative regulator of Nrf2 (2). INrf2 functions as an adapter for bringing Nrf2 closer to Cul3-Rbx1 complex for ubiquitination and degradation of Nrf2 (7). This mechanism controls Nrf2 level inside the cells. INrf2/Cul3-Rbx1 complex is predominately localized in the cytosol. Therefore, cytosol is the prime site for ubiquitination and degradation of Nrf2. However, 10-15% of INrf2 is reported in the nucleus. The nucleus also contains Cul3-Rbx1 complex and our data confirmed that INrf2-Cul3-Rbx1 complex are also present in nucleus. Antioxidant t-BHQ is known to induce INrf2 through a proximal promoter ARE on the reverse strand (13). Interestingly, t-BHQ induces INrf2 in the cytosol as well as in the nucleus. In the current investigation we demonstrated the unique mechanism of nuclear import of INrf2 and Cul3-Rbx1 complex and its function in the nucleus. An analysis of INrf2 primary sequence did not reveal the presence of a putative nuclear import signal or nuclear localization signal (NLS). Therefore, we reasoned that the nuclear localization of INrf2 has to be mediated through an associate protein with a defined nuclear import signal or NLS.

Domain deletion analysis clearly demonstrated that DGR region of INrf2 is required for nuclear translocation of INrf2. Deletion of DGR region from INrf2 abrogated nuclear localization of INrf2. In addition, attachment of DGR region with GFP led to nuclear translocation of GFP protein. The studies also revealed that PTMα bound to DGR region and mediated nuclear translocation of INrf2. INrf2ΔDGR mutant deficient in DGR failed to bind to PTMα and also failed to localize in the nucleus. In addition, siRNA inhibition of PTMα resulted in significant loss of nuclear localization of INrf2. Further, the overexpression of PTMα enhanced nuclear localization of INrf2. These results concluded that PTMα interaction with DGR region led to nuclear localization of INrf2. Procite search revealed that PTMα contains a strong nuclear localization signal at the C-
terminal of the protein that presumably helps PTMα to mediate nuclear localization of INrf2. The role of PTMα in nuclear translocation of INrf2 is also supported by an earlier observation that PTMα interaction with INrf2 increases nuclear INrf2 (20). Our data suggested that Nrf2 and Fac1 also bound to the DGR domain of INrf2 but were not required for nuclear localization of INrf2.

Cul3 is known to bind to the BTB domain of INrf2 (7). Rbx1 is known to bind to Cul3 but not INrf2 (7). The present studies demonstrated that the Cul3-Rbx1 complex is present in the nucleus. INrf2 knock down studies and immunoprecipitation assays revealed that Cul3-Rbx1 is imported in the nucleus along with INrf2 presumably due to Cul3 interaction with INrf2. Therefore, it is reasonable to conclude that PTMα through its binding with DGR region of INrf2 mediates the nuclear import of INrf2/Cul3-Rbx1 complex.

The results also showed that the treatment of cells with antioxidant t-BHQ resulted in the release of Nrf2 from INrf2 and Nrf2 translocates to the nucleus. This coincided with an increase in INrf2 in the cytosol and nucleus. The PTMα interaction with INrf2 led to an increase in nuclear translocation of INrf2, followed by exchange of PTMα with Nrf2 on INrf2. Interestingly, tyrosine kinase inhibitor genestine inhibited the exchange of PTMα with Nrf2 on INrf2. This was expected since it was reported earlier that Nrf2 is phosphorylated by tyrosine kinase Fyn at Y568. The Nrf2Y568 phosphorylation is required for binding with INrf2 (21). Genestine inhibited tyrosine kinase-mediated phosphorylation of Nrf2 and exchange of PTMα with Nrf2. Therefore, it is possible that Nrf2Y568 has to be phosphorylated to bind to INrf2 in exchange for PTMα. What happens to PTMα after exchange with Nrf2 inside nucleus remains unknown.

The exchange of PTMα with Nrf2 inside the nucleus on INrf2 leads to ubiquitination and degradation of Nrf2. The Cul3-Rbx1 complex are present in the nucleus that ubiquitinate Nrf2 leading to degradation of Nrf2. Therefore, PTMα-mediated nuclear import of INrf2/Cul3-Rbx1 complex is required for rapid degradation of used Nrf2 inside the nucleus. The nuclear degradation of Nrf2 along with cytosolic degradation of Nrf2 provides a complex but fine mechanism to regulate Nrf2 in the nucleus and cytosol. Nrf2 is known to promote cell survival by reducing apoptosis (22). This means that the damaged cells could escape apoptosis and survive if Nrf2 in the nucleus is not checked. Indeed, mutations in INrf2 result in loss of INrf2 and accumulation of nuclear Nrf2 in lung cancer (16-17). Nrf2 has to be rapidly degraded after its function is over to give opportunity to other cellular pathways of surveillance and the decision to survive, die, or undergo senescence.

In conclusion, we demonstrated that PTMα interaction with INrf2 leads to nuclear translocation of INrf2/Cul3-Rbx1 complex that exchanges PTMα with Nrf2 leading to ubiquitination and degradation of Nrf2. This process is late response to oxidative/electrophilic stress following the release and nuclear translocation of Nrf2. The function of nuclear import of INrf2/Cul3-Rbx1 complex is to rapidly degrade Nrf2 inside nucleus and to switch off the activation of Nrf2 downstream genes to promote normal growth and survival of cells.
ACKNOWLEDGMENTS
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KEY WORDS
INrf2, Nrf2, antioxidant response element, phosphorylation and dephosphorylation, transcriptional regulation, ubiquitination and degradation.

Abbreviations: Nrf2, NF-E2 related factor, INrf2; Cytosolic inhibitor of Nrf2 also known as Keap1; ARE, Antioxidant response element; NQO1, NAD(P)H:quinine oxidoreductase 1; PTMα, Prothymosin α; t-BHQ, tert-butyl hydroquinone; IP, immunoprecipitation.

REFERENCES
FIGURE LEGENDS

FIGURE 1. Schematic presentation and localization of INrf2 deletion domains. A. Schematic presentation of INrf2 domains and deletion mutants. B. The INrf2 and deletion mutants were in vitro transcribed, translated and analyzed by immunoblotting with anti-GFP antibodies. C. Hepa-1 cells were transfected with 0.5 μg empty vector (1XGFP) or INrf2 or deletion mutants of INrf2. Whole cell lysate (W), cytoplasmic (C) and nuclear (N) protein fractions were prepared and analyzed by immunoblotting and probing with anti-GFP, anti-LDH and anti-lamin-B antibodies. D. Hepa-1 cells transfected with INrf2-1XGFP, INrf2ΔDGR-1XGFP and DGR-2X GFP on slides were fixed in 2% formaldehyde, washed with PBS, stained with Vectashield containing nuclear DAPI stain and mounted. Cells were observed under Nikon fluorescence microscope and photographs were captured. The cytoplasmic and nuclear GFP fluorescence were quantified from 10 different transfected cells (N=10) by using NIS-Elements BR2.30 SP4 software. All the experiments were repeated three times. Error bars indicates standard error of means (s.e.m).of fluorescence intensities.

FIGURE 2. Subcellular localization of INrf2, INrf2ΔDGR and INrf2DGR. A. HEK293 cells stably expressing tetracycline-inducible Flag-INrf2, Flag-INrf2ΔDGR and Flag-INrf2DGR were treated with 0.5 μg/ml of tetracycline for 24 h and immunoblotted with anti-Flag and β-actin antibodies. B and C. HEK293 cells as in A were first treated with tetracycline followed by treatment with DMSO or t-BHQ (50 μM) for 2h (B), or LMB (10 ng/ml) for 12 h (C). Whole cell lysate (W), cytoplasmic (C) and nuclear (N) lysates were prepared and immunoblotted with anti-Flag-HRP, anti-LDH and anti-Lamin-B antibodies. D. Hepa-1 cells were transfected with plasmids as shown, untreated or treated with 10 ng/ml LMB for 12 hrs. Whole cell lysate (W), cytosolic (C) and nuclear (N) extracts were immunoblotted with anti-GFP, anti-LDH and anti-lamin-B antibodies. E. Densitometry of INrf2 bands from three independent experiments in B and C. Error bars indicate s.e.m. of triplicate samples. Statistical analysis was performed by one way ANOVA, followed by the Tukey-Kramer’s post hoc test for multiple comparisons * P<0.004; ** P<0.005 (compared with DMSO treated samples).

FIGURE 3. INrf2/Cul3-Rbx1 translocates from cytosolic to nuclear compartment in a complex under physiological conditions. A. Hepa-1 cells were treated with DMSO or 100 μM of t-BHQ for 2h and cellular interaction of INrf2, Cul3 and Rbx1 were analyzed by immunoprecipitation and immunoblotting. B. Hepa-1 cells were transfected with control or INrf2 siRNA and immunoblotted with anti-INrf2 anti-β-actin antibodies. C. Hepa-1 cells were transfected with control or INrf2 siRNA (50 nM) and 50 μg Whole (W), cytosolic (C) and nuclear (N) extracts were immunoblotted with anti-INrf2, anti-Cul3, anti-Rbx1, anti-LDH and anti-Lamin-B antibodies. D. Densitometric analysis of Cul3 and Rbx1 bands from three different experiments in C. Error bars indicates s.e.m .of triplicate samples. * P<0.004; (compared with control siRNA transfected nuclear Cul3 samples);** P<0.003 (compared with control siRNA transfected nuclear Rbx1 samples). E, Hepa-1 cells were transfected with control and INrf2 siRNA (50 nM) for 24 h, harvested and cytosolic and nuclear extracts prepared. One mg of extracts was immunoprecipitated with INrf2, Cul3 and Rbx1 antibodies and immunoblotted with indicated antibodies. All experiments were repeated three times.

FIGURE 4. Nrf2 is not required for nuclear import of INrf2. A. Hepa-1 cells were co-transfected with 0.5 μg 1XGFP vector, INrf2-GFP, INrf2ΔDGR-GFP and DGR-2X GFP along with Flag-Nrf2 in ratio 1:4 and immunoblotted with anti-Flag-HRP, anti-GFP and anti-β-actin antibodies. B. Hepa-1 cells co-transfected with 0.5 μg INrf2-V5, INrf2ΔDGR-V5 and
INrf2DGR-V5 along with 2 μg Flag-Nrf2 plasmids. 1 mg cell lysates form transfected cells were immunoprecipitated with anti-V5 or anti-Flag antibody and immunoblotted with antibodies as shown. C. Hepa-1 cells were transfected with 0.5 μg of 1XGFP vector, INrf2-GFP and INrf2ΔDGR-GFP plasmid DNA. One mg cell lysates were immunoprecipitated using anti-GFP antibodies and immunoblotted with anti-Nrf2 and anti-β-actin antibodies. D, Hepa-1 cells were transfected with 5, 25 and 50 nM control and Nrf2 siRNA and analyzed by immunoblotting with anti Nrf2 and anti-β-actin antibodies. E, Effect of Nrf2 siRNA on subcellular localization of INrf2. Hepa-1 cells were transfected with 50 nM control or Nrf2 siRNA. Whole, cytosolic and nuclear extracts were prepared and immunoblotted with anti-Nrf2, anti-INrf2, anti-LDH and anti-Lamin B antibodies. All experiments repeated three times.

FIGURE 5. Interaction of PTMα with INrf2 is required for nuclear import of INrf2. A, HEK293 cells stably expressing tetracycline-inducible Flag-INrf2, Flag-INrf2ΔDGR and Flag-INrf2DGR were treated with 0.5 μg/ml of tetracycline for 24 hour. 1 mg whole cell lysates were immunoprecipitated with anti-Flag (left panel) or anti-PTMα and anti-Fac1 (right panel) and immunoblotted with indicated antibodies as shown. B. One mg of cytosolic and nuclear extracts of Hepa-1 cells were immunoprecipitated with PTMα or INrf2 antibodies, immune complexes were immunoblotted with anti-INrf2, anti-Nrf2, anti-Cul3, anti-Rbx1 and anti-PTMα antibodies C. Hepa-1 cells were transfected with 75 nM control siRNA and different concentrations of PTMα (upper panel) or Fac1 (lower panel) siRNA. Twenty-four hours later, cells were harvested and endogenous protein levels were visualized by immunoblotting with anti-PTMα, anti-Fac1, INrf2 and anti-β-actin antibodies. D, Effect of PTMα and Fac1 siRNA on endogenous localization INrf2. Hepa-1 cells were transfected with 50 nM control or PTMα or Fac1 siRNA. Cytosolic and nuclear extract prepared from transfected cells and 50 μg of whole, cytosolic and nuclear extracts were immunoblotted with anti-INrf2, anti-LDH and anti-Lamin B antibodies. E, Hepa-1 cells were transfected with 75 nM control siRNA or 25, 50 and 75 nM PTMα siRNA. Whole, cytosolic and nuclear extracts were analyzed by immunoblotted with INrf2, LDH and Lamin-B antibodies.

FIGURE 6. PTMα siRNA-mediated inhibition of endogenous PTMα reduced nuclear import of INrf2 and activation of endogenous and transfected NQO1 gene expression A. Hepa-1 cells were grown on coverslip and transfected with control or PTMα siRNA. Twenty-four hours later, cells were fixed, permeabilized and incubated with 1:1000 dilution of goat INrf2 primary antibodies for 12 h followed by FITC conjugated second antibody for 2h. Cells were washed with PBS, stained with DAPI and observed under Nikon fluorescence microscope and photographs were captured. The cytoplasmic and nuclear FITC fluorescence intensities were quantified from 10 different cells (N=10) by NIS-Elements BR2.30 SP4 software (Nikon) and plotted (right panel). Error bars indicate s.e.m of samples. Statistical analysis was performed as described in methods section. * P<0.0005; (compared with mock or control siRNA transfected nuclear INrf2 samples). B. Hepa-1 cells were transfected with control or PTMα siRNA and immunoblotted antibodies as shown. C. Hepa-1 cells transfected with control or PTMα siRNA. Ten hours after, cells were transfected with 0.1 μg pGL2B-hARE-NQO1 plasmid DNA and 10 ng of pRL-TK plasmid encoding Renilla luciferase plasmid DNA (Promega). Twenty-four hours later of second transfection, the cells were lysed in 1X passive lysis buffer (Promega) and relative luciferase activity was measured and plotted. Error bars indicate s.e.m of triplicate samples. Statistical analysis was performed as described in methods section. * P<0.0004; ** P<0.004 (compared with control siRNA or mock transfected samples).
FIGURE 7. Effect of overexpression of PTMα and tBHQ treatment on PTMα mediated nuclear import of INrf2. A. Hepa-1 were transfected with empty vector pcDNA or PTMα–V5 plasmid DNA and immunoblotted with anti-V5-HRP and anti β-actin antibodies. B. Effect of PTMα overexpression and tBHQ treatment on localization of INrf2. Hepa-1 cells were transfected with INrf2-V5 or PTMα–V5 plasmid DNA as shown. After for 36 h, cells were treated with t-BHQ (50 µM) for 3h. Cytosolic and nuclear extracts were prepared, 60 µg of extracts were immunoblotted with anti-lINrf2-V5, PTMα-V5 or anti-PTMα (endogenous), anti-LDH and anti –Lamin-B antibody. The relative INrf2 band intensities were plotted in lower panel. Error bars indicate s.e.m .of triplicate samples. Statistical analysis was performed by one way ANOVA, followed by the Tukey-Kramer’s post hoc test for multiple comparisons * P<0.005 (compared with nuclear INrf2 of cells transfected with pcDNA control vector with tBHQ); ** P<0.005 (compared with nuclear INrf2 of cells transfected with pcDNA control vector without tBHQ).

C. Hepa-1 cells grown on coverslip were transfected with INrf2-V5 alone or in combination with pcDNA-PTMα. Twenty-four hours later cells were treated with 50 µM t-BHQ. Cells were fixed, permeabilized and incubated with 1:1000 dilution of anti-V5-FITC conjugated antibody at 4°C for 12h. Cells were washed and stained with DAPI and mounted. Cells were observed under Nikon fluorescence microscope and photographs were captured. The cytoplasmic and nuclear FITC fluorescence were quantified from 10 different cells (N=10) by using NIS-Elements BR2.30 SP4 software (Nikon) as described in method section and plotted. Error bars indicate s.e.m .of different samples. * P<0.0001 (compared with nuclear INrf2 of cells transfected with INrf2-V5 only).

FIGURE 8. t-BHQ-mediated exchange of PTMα with Nrf2 in the nucleus. A. Hepa-1 cells were treated with DMSO or tBHQ for 1 and 2h. Cytosolic and nuclear extracts were prepared. One mg of extracts was immunoprecipitated with anti-INrf2 antibodies and Western blotted with Nrf2, INrf2 and PTMα antibodies (left panel). The relative binding/mg of protein profile of INrf2-Nrf2 and INrf2-PTMα in cytosolic and nuclear compartments were plotted after measuring band densities (right panel). Error bars indicate s.e.m .of triplicate samples. B. HEK293 cells stably expressing Flag-INrf2, were treated with 0.5 µg/ml of tetracycline for 24 hour followed by exposure to DMSO or tBHQ (50 µM) for 1 and 2h and harvested. INrf2/Cul3-Rbx1/PTMα/Nrf2 native complex purified, electrophoresed by 6% Native gel and stained with Coomassie Brilliant Blue. C. The native purified complex immunoblotted with anti INrf2, Nrf2, PTMα, Cul3 and Rbx1 antibodies. D. Hepa-1 cells were exposed with genestine 20 µM for 2 hrs before treatment with DMSO or t-BHQ. One mg of cytosolic or nuclear extracts were immunoprecipitated with anti-INrf2 antibodies and Western blotted with anti-Nrf2, anti-INrf2 and anti-PTMα antibodies (upper panel). The effect of genestine on binding profile of INrf2-Nrf2 and INrf2-PTMα in cytosolic or nuclear compartments were plotted after quantification of band intensities. All experiments were repeated three times. Error bars indicate s.e.m .of triplicate samples. Statistical analysis was performed as described in methods section, and P values are presented inside in the graph.

FIGURE 9. Effect of PTMα siRNA on ubiquitination and degradation of cytosolic and nuclear Nrf2. A, Hepa-1 cells were transfected with 75 nM control or PTMα siRNA for 10 h and then co-transfected with HA-ubiquitin, Flag-INrf2 and Nrf2-V5 plasmid DNA for 36 hrs. The cells were harvested and cytosolic and nuclear extracts prepared. 1mg of extracts was immunoprecipitated with anti-V5 antibodies and Western blotted with HA-HRP tag antibody (Upper panel). Densitometry of the Nrf2 ubiquitinated bands were plotted (lower panel). Error bars indicate s.e.m .of triplicate samples. Statistical analysis was performed by one way ANOVA, followed by the Tukey-Kramer's post hoc test for multiple comparisons. * P<0.003 (compared with ubiquitinated nuclear Nrf2 of cells transfected with mock or control siRNA). B. Sixty micrograms of cytosolic and nuclear extracts in the above experiment were
immunoblotted with anti-Flag-HRP and, anti-V5-HRP antibodies. The same extracts also probed with, anti-PTMα, anti LDH and anti Lamin-B antibodies for detection of endogenous proteins.
**Fig. 1**

A) Schematic representation of INrf2 and its mutants. INrf2-GFP, INrf2-ΔNTR-GFP, INrf2-ΔBTB-GFP, INrf2-ΔDGR-GFP, INrf2-ΔIVR-GFP, INrf2-ΔCTR-GFP, INrf2-ΔBTB-NES2-GFP, INrf2-ΔDGR-NES2-GFP, INrf2-ΔCTR-NES2-GFP, and INrf2-2XGFP.

B) Western blot analysis of INrf2 and its mutants with 1XGFP Vector, INrf2-GFP, INrf2-ΔNTR-GFP, INrf2-ΔBTB-GFP, INrf2-ΔDGR-GFP, INrf2-ΔIVR-GFP, INrf2-ΔCTR-GFP, INrf2-ΔBTB-NES2-GFP, INrf2-ΔDGR-NES2-GFP, INrf2-ΔCTR-NES2-GFP, INrf2-2XGFP. kDa: 150, 100, 75, 50.

C) WB: GFP bands for INrf2-GFP, INrf2-ΔNTR-GFP, INrf2-ΔBTB-GFP, INrf2-ΔDGR-GFP, INrf2-ΔIVR-GFP, INrf2-ΔCTR-GFP, INrf2-ΔBTB-NES2-GFP, INrf2-ΔDGR-NES2-GFP, INrf2-ΔCTR-NES2-GFP, INrf2-2XGFP. Bands for W, C, N.

D) Fluorescence images of INrf2-GFP, INrf2-ΔDGR-GFP, INrf2-DGR-2XGFP. Staining for Lamin B, LDH. % GFP Fluorescence intensity graph.
Fig. 2

A

FRT 293 cells + TET

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Flag-HRP
β-actin

B

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1XGFP Vector
INrf2-GFP
INrf2-DGR-GFP
INrf2ΔDGR-GFP
INrf2ΔDGR-NES2-GFP
Lamin B
LDH

E

INrf2 (% Distribution)

Cytosol
Nucleus
**Fig. 5**

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**B**

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<th>PTMα siRNA</th>
<th>25 nM</th>
<th>50 nM</th>
<th>75 nM</th>
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<tbody>
<tr>
<td>PTMα</td>
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<td></td>
<td></td>
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<tr>
<td>INrf2</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>β actin</td>
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**D**

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<tr>
<th>Mock</th>
<th>Con siRNA 50 nM</th>
<th>PTMα siRNA 50 nM</th>
<th>Fac1 siRNA 50 nM</th>
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<tbody>
<tr>
<td>LDH</td>
<td>Lamin B</td>
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**E**

<table>
<thead>
<tr>
<th>Mock</th>
<th>Con siRNA 75 nM</th>
<th>PTMα siRNA 75 nM</th>
<th>Fac1 siRNA 75 nM</th>
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<tbody>
<tr>
<td>LDH</td>
<td>Lamin B</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Fig. 6**

**(A)** Images of FITC, DAPI, and Merge under different conditions:
- **Mock**
- **Control siRNA**
- **PTMα siRNA 50 nM**
- **PTMα siRNA 75 nM**

**(B)** Graph showing % FITC Fluorescence intensity:
- **Mock**
- **Con siRNA**
- **50 nM**
- **75 nM**

**(C)** Graph showing Relative Fold Luciferase Activity:
- **Mock**
- **Con siRNA**
- **50 nM**
- **75 nM**

**Nucleus** vs **Cytosol**

**FITC**

**DAPI**

**Merge**

**PTMα**

**Nrf2 H-300**

**NQO1**

**INrf2**

**β-actin**

**Relative Fold Luciferase Activity**
INrf2-V5

PTMα-V5

C N C N C N C N

INrf2-V5

pcDNA

PTMα-V5

t-BHQ (50 μM)

C N C N C N C N

INrf2-V5

PTMα-V5

PTMα endo.

LDH

Lamin B

Vector

PTMα-V5

PTMα + INrf2-V5

+ + + + + + + + INrf2-V5

+ + - - + + + + pcDNA

- - + + - - + + PTMα-V5

+ + + + - - - + t-BHQ (50 μM)

Fig. 7

A B C

FITC

DAPI

Merge

% FITC Fluorescence intensity

INrf2-V5

PTMα + INrf2-V5

PTMα + INrf2-V5 + t-BHQ

INrf2 Band Intensity

Relative INrf2 Band Intensity

0 10 20 30 40 50 60 70 80 90 100

Nucleus Cytosol
**Fig. 9**

**A**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>WB: HA HRP</th>
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<tbody>
<tr>
<td>HA-UB</td>
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</tr>
<tr>
<td>Nrf2-V5</td>
<td>+ + + + + + + + +</td>
</tr>
<tr>
<td>Flag-INrf2</td>
<td>- - + + + + + + +</td>
</tr>
<tr>
<td>Cont. siRNA</td>
<td>- - - + - - - + -</td>
</tr>
<tr>
<td>PTMα siRNA</td>
<td>- - - + - - - + -</td>
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**IP:**

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<th>Condition</th>
<th>Anti-V5 ab</th>
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<tbody>
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<td>Con IgG</td>
<td>+</td>
</tr>
<tr>
<td>Anti-V5 ab</td>
<td>+</td>
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</table>

**WB: HA HRP**

- Cytoplasm
- Nucleus

**UB-Nrf2**

**B**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>WB: HA HRP</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA-UB</td>
<td>+ + + + + + + + +</td>
</tr>
<tr>
<td>Nrf2-V5</td>
<td>+ + + + + + + + +</td>
</tr>
<tr>
<td>Flag-INrf2</td>
<td>- + + + - + + + +</td>
</tr>
<tr>
<td>Cont. siRNA</td>
<td>- - + - - - + -</td>
</tr>
<tr>
<td>PTMα siRNA</td>
<td>- - - - - - - +</td>
</tr>
</tbody>
</table>

**IP:**

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<tr>
<th>Condition</th>
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<tr>
<td>Anti-V5 ab</td>
<td>+</td>
</tr>
</tbody>
</table>

**WB: HA HRP**

- Cytoplasm
- Nucleus

**Cytoplasm**

**Nucleus**
EXPRESSSION OF CONCERN

SRC subfamily kinases regulate nuclear export and degradation of transcription factor Nrf2 to switched off Nrf2-mediated antioxidant activation of cytoprotective gene expression.
Suryakant K. Niture, Abhinav K. Jain, Phillip M. Shelton, and Anil K. Jaiswal

PROTHYMOSIN-α mediates nuclear import of the INrf2/Cul3-Rbx1 complex to degrade nuclear Nrf2.
Suryakant K. Niture and Anil K. Jaiswal

Phosphorylation and dephosphorylation of tyrosine 141 regulate stability and degradation of INrf2. A novel mechanism in Nrf2 activation.
Abhinav K. Jain, Shilpi Mahajan, and Anil K. Jaiswal

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Suryakant K. Niture and Anil K. Jaiswal

J. Biol. Chem. published online March 11, 2009

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