Inhibitor of Nrf2 (INrf2 or Keap1) degrades Bcl-xL via Phosphoglycerate mutase 5 and controls cellular apoptosis

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Running title: INrf2 degradation of PGMA5-Bcl-xL

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SUMMARY

INrf2 (Keap1) is an adaptor protein that facilitates INrf2/Cul3-Rbx1-mediated ubiquitination/degradation of Nrf2, a master regulator of cytoprotective gene expression. Here, we present evidence that members of the phosphoglycerate mutase family 5 (PGAM5) proteins are involved in the INrf2-mediated ubiquitination/degradation of anti-apoptotic factor Bcl-xL. Mass spectrometry and co-immunoprecipitation assays revealed that INrf2, through its DGR domain interacts with PGAM5 which in turn interacts with anti-apoptotic Bcl-xL protein. INrf2/Cul3-Rbx1 complex facilitates ubiquitination and degradation of both PGAM5 and Bcl-xL. Overexpression of PGAM5 protein increased INrf2-mediated degradation of Bcl-xL, whereas knocking down PGAM5 by siRNA decreased INrf2 degradation of Bcl-xL resulting in increased stability of Bcl-xL. Mutation of PGMA5E79AS80A abolished INrf2/Pgam5-Bcl-xL interaction. Therefore, PGAM5 protein acts as a bridge between INrf2 and Bcl-xL interaction. Further studies showed that overexpression of INrf2 enhanced degradation of PGAM5-Bcl-xL complex, led to etoposide mediated accumulation of Bax, increased release of cytochrome C from mitochondria, activated caspase-3/7, and enhanced DNA fragmentation and apoptosis. In addition, antioxidant (tBHQ) treatment destabilized the Nrf2-INrf2/Pgam5-Bcl-xL complex, which resulted in release of Nrf2 in cytosol and mitochondria, release of Bcl-xL in mitochondria, increase in Bcl-xL heterodimerization with Bax in mitochondria and reduced cellular apoptosis. These data provide the first evidence that INrf2 controls Bcl-xL via PGAM5 and controls cellular apoptosis.

INTRODUCTION

INrf2/Keap1:Nrf2 complex serves as sensor of chemical and radiation induced oxidative and electrophilic stress (reviewed in 1, 2). INrf2 (inhibitor of Nrf2) or Keap1 (Kelch-like ECH-associated protein 1) functions as a substrate adaptor protein for a Cul3-Rbx1-dependent E3 ubiquitin ligase complex to ubiquitinate and degrade Nrf2, thus maintaining steady-state levels of Nrf2 (1, 2). Nrf2 is a nuclear transcription factor that in response to stress is released from INrf2 (1, 2). Chemical modification of INrf2/C151 or/and protein kinase C delta phosphorylation of Nrf2S40 leads to the release of Nrf2 from INrf2 (1, 2). Nrf2 is stabilized, translocates into the nucleus, binds with antioxidant response element (ARE) and activate a battery of cytoprotective gene expression. This provides protection against oxidative and electrophilic stress and promotes cell survival (2). Nrf2-null mice are prone to acute damages induced by acetaminophen, ovalbumin, pentachlorophenol and 4-vinylcyclohexene diepoxide (3-6). Nrf2-null mice showed increased pulmonary DNA adducts and bladder tumors when exposed to diesel exhaust and N-nitrosobutyl (4-hydroxybutyl) amine, respectively (7-9).

INrf2-null mice demonstrated persistent accumulation of Nrf2 in the nucleus, and led to postnatal death from malnutrition resulting from hyperkeratosis in the esophagus and fore stomach (10). Reversed phenotype of INrf2 deficiency by breeding to Nrf2-null mice suggested that a tightly-regulated negative feedback might be essential for cell survival (11). The 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 which resulted in drug resistance and cell survival in lung cancer cells (12,13). Unrestrained activation of Nrf2 in cells increases a risk of adverse effects including survival of damaged cells, tumorigenesis and drug resistance (2). Therefore, it appears that cells contain mechanisms that auto-regulate cellular abundance of Nrf2 (14,15). Structural and functional analyses of INrf2 identified an evolutionarily conserved Kelch (DGR) domain which interacts with several proteins. Although Nrf2 is a well known substrate for INrf2, the DGR domain of INrf2 has been reported also to bind other proteins including Nrf1, PGAM5, prothymosin-α, fetal Alz clone 1 and IKKβ (16-20). It is noteworthy that binding of a protein with INrf2 DGR region does not always lead to degradation of the protein. Recently, we have shown that prothymosin-α interacts with the DGR domain of INrf2 and this interaction is required for nuclear localization of INrf2 (21). Therefore, INrf2 and its interacting partners play several different roles in cell signaling and survival.

Cellular apoptosis is a critical process that is dysregulated in tumourigenesis (22). Bcl-2 family proteins regulate cell death and survival (23,24). Bcl-2 family includes more than 6 anti-apoptotic including Bcl-2 and Bcl-xL proteins and many pro-apoptotic members (25-26). Bcl-2 and Bcl-xL share regions of sequence similarity, as well as a C-terminal hydrophobic region required for membrane localization (27). Bcl-2 and Bcl-xL appear to function in the same apoptotic pathway and both confer resistance to multiple chemotherapy agents. Over-expression of either protein is usually associated with poor prognosis in many human cancers. However, in some cancer types multiple anti-apoptotic proteins are expressed (28, 23), and have opposite effects on prognosis indicating that there may be subtle, but clinically and biologically relevant functional differences between family members. Experiments in mice with deletion of individual anti-apoptotic genes indicate that the phenotypes are not identical presumably because of differential tissue expression of the various members (29). The mechanisms of action of Bcl-2 and Bcl-xL are complex, with many postulated interactions with other proteins, and the role of any single interaction in the final phenotype at the cellular level remains unknown.

Recently, INrf2 is shown to target anti-apoptotic Bcl-2 protein for degradation and control cellular apoptosis (30). In the present report, we investigated the novel role of INrf2 in the regulation of anti-apoptotic factor Bcl-xL. INrf2 through its DGR domain interacts with PGAM5 proteins, which interact with Bcl-xL. Interestingly, we show that INrf2/Cul3-Rbx1 complex facilitates both PGAM5 and Bcl-xL ubiquitination and degradation. The data also revealed that PGAM5 proteins control INrf2 mediated degradation of Bcl-xL. Therefore, PGAM5 protein acts as a bridge between INrf2 and Bcl-xL interaction. Further, studies showed that overexpression of INrf2 degrade both PGAM5 and Bcl-xL proteins which increases/activates cellular pro-apoptotic factors and apoptosis. However, antioxidant (tBHQ) treatment destabilized Nrf2-INrf2-PGAM5-Bcl-xL complex in mitochondria leading to the release of Nrf2 and increased Bcl-xL heterodimerization with Bax and reduced cellular apoptosis.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructs**—PGAM5 exists in two forms (31). Long form (PGAM5L) is 34 amino acids longer at C-terminus than short form (PGAM5S). PGAM5L cDNA was obtained from Origene and coding sequence was amplified by using forward 5’-AACCCCATGGCGTTCCGGCAGGCCTG-3’ and reverse 5’-GGATCGAGTGTCTCTGGAGGAGCAT-3’ primers and PCR product was cloned in pcDNA3.1-V5 tagged vector by TA-cloning and the construct was designated as PGAM5L-V5. We generated PGAM5LE79A-V5 double mutant plasmid by site directed mutagenesis GeneTailor kit (Invitrogen) using forward...
primer 5’-CGGAAGAGGAACGTGGCTGCTGGGGAA GAAGAGCTG-3’ and reverse prime 5’-CAGTTTCTTCTCCGACACCTTGATCAGAC ACGTGG-3’ to determine the role of mutated residues in PGAM5 interaction with INrf2. Mouse Bcl-xL plasmid was obtained from addgene (ID # 8772) and coding sequence was amplified by using forward primer 5’-AATGGCTCTAGAATGTCTCAGAGCAACC GGGAGCTGGTG-3’ and reverse primer 5’-TTCAGGGCTCAGCTTCGACTGAAGAGT GAGCCCGAGCAAGACCA-3’ and cloned into pcmxFlag-2X vector using Xba I and Xho I restriction sites. The resultant plasmid was designated as Flag-Bcl-xL. The construction of Flag-INrf2, INrf2-V5, Flag-INrf2, and HA-Cul3, Myc-Rbx1 and HA-Ub were described previously (14, 15, 30). All plasmids were confirmed by DNA sequencing.

Cell Culture and Generation of stable Flp-In T- REx HEK293 cells expressing tetracycline-inducible Flag-INrf2 Flag-INrf2DGR Flag-INrf2ΔDGR—Mouse Hepatoma (Hepa-1) 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). Jurkat cells kindly provided by Dr. Ronald Gartenhaus (Greenebaum Cancer Center, University of Maryland, Baltimore) were grown in RPMI medium containing 10% fetal bovine serum. The cells were grown in the cell culture incubator at 37°C in 95% air and 5% CO₂. Generation of stable Flag-INrf2, Flag-INrf2DGR and Flag-INrf2ΔDGR cells were described previously (30).

Subcellular fractionation and Western blotting—Hepa-1 cells were seeded in 100-mm plates and transfected/treated as displayed in the figures. 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% Sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, and 1 mM Sodium orthovanadate supplemented with 1X protease inhibitor mixture (Roche Applied Science). Mitochondrial and cytosolic lysates were prepared by standard procedures. The isolated mitochondria were washed with HEPES buffer and lysed in RIPA buffer. 60 to 80 micrograms of proteins were separated on 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-INrf2 (H-300) (1:1000), anti-Bcl-xL (H5) (1:1000) anti-Bax (N20) (1:1000), anti-Bcl-2(N-19) (1:1000), anti-Mcl-1(S-19) (1:1000) and anti-ubiquitin (P4D1) 1:1000) antibodies, all purchased from Santa Cruz Biotechnology (CA). Anti-Flag-HRP, anti-HA-HRP and anti-β-actin antibodies were obtained from Sigma and used in 1:10000 dilutions to probe the western blots. Anti-V5 antibody and anti-V5-HRP antibody were obtained from Invitrogen and anti-Caspase-3 antibody was purchased from cell signaling. To confirm the purity of cytoplasmic and mitochondrial protein fractionation, the membranes were re-probed with cytoplasm-specific, anti-lactate dehydrogenase (LDH) (Chemicon) and mitochondrial specific, anti-cytochrome C or Cox IV antibodies (Cell signaling). Jurkat cells were treated with 100 ng/ml Killer TRAIL soluble human recombinant protein (Enzo life sciences, Cat. #ALX-201073). We generated and purified bacterial PGAM5L-His tagged protein. The polyclonal antibodies against PGAM5L form were generated in rabbits (Pacific Immunology; CA, USA) and purified. The membranes were washed three times with TBST and immunoreactive bands were visualized using a chemiluminescence ECL system (Amersham). The intensity of protein bands after Western blotting were quantified by using QuantityOne 4.6.3 Image software (ChemiDoc XRS, Bio-Rad) and normalized against proper loading controls. In related experiments, the cells were treated with 50 µM of t-BHQ or DMSO as a vehicle for different time intervals.

Immunoprecipitation—For immunoprecipitation, one mg of whole cell extracts or 300 µg mitochondrial lysates were equilibrated in RIPA buffer, pre-cleaned by protein-AG plus-agarose (Santacruz Biotechnology) and then extracts were incubated with respective antibodies (1 µg) at 4°C for
overnight. Immune-complexes were collected by addition of protein AG-agarose and centrifugation. The immune-complexes were washed three times with RIPA buffer containing 0.25% NP-40, and proteins were resolved by 10 to 12% 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 ECL system (Amersham).

Ubiquitination assay— INrf2-FRT293, INrf2DGR-FRT293 and INrf2ΔDGR-FRT293 cells were co-transfected with PGAM5-V5 and HA-Ub, treated with tetracycline and analyzed for PGAM5 ubiquitination. Effect of overexpression of INrf2 in INrf2-293 and Hepa-1 cells or effect of INrf2 siRNA on endogenous ubiquitination of Bcl-xL was also analyzed.

Cell pellets were lysed in RIPA buffer containing 1% SDS. One mg of the lysate (~100 µl) was diluted to 10 fold with RIPA buffer. After pre-clearing, samples were immunoprecipitated with 2µg of antibody or anti-Flag beads (15µl) as indicated in figures. Immune-complexes were collected by addition of protein AG-agarose. Immune-complexes were boiled with SDS sample buffer and denatured samples were resolved by SDS-PAGE followed by immunoblotting with anti-HA and anti-ubiquitin antibodies.

Transient transfection/siRNA interference 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 immunoblotted. INrf2 siRNA, PGAM5L siRNA and Bcl-xL siRNA were used to inhibit INrf2, PGAM5L and Bcl-xL proteins respectively. Control GAPDH siRNA and INrf2 siRNA were purchased from Dharmacon. PGAM5L siRNA and BclxL siRNA were obtained from Ambion. In most cases, Hepa-1 cells were transfected with 25, 50 and 75 nM of INrf2, PGAM5L and Bcl-xL siRNA separately using Lipofectamine RNAiMAX reagent (Invitrogen) according to the manufacturer’s instructions. Thirty-two hours after transfection, cells were harvested and INrf2, PGAM5L, Bcl-xL and actin proteins were analyzed by Western blotting. In related experiments, Jurkat cells were transfected with 25 and 50 nM INrf2 siRNA. siRNA was mixed with Lipofectamine RNAiMAX reagent in Opti-MEM medium (1ml) and mixture was incubated at room temperature for 15 min. Then the reaction mixture was coated to 100 mm tissue culture plates for 10 min. Exponentially grown Jurkat cell suspensions in RPMI medium without antibiotics (4 ml; 10^6 cells) were added into the plates and cells were incubated at 37°C for 24h followed by treatment with TRAIL protein (100 ng/ml) for 30h. Cells were harvested, lysed and immunoblotted.

Immunofluorescence— Hepa-1 cells were grown in Lab-Tek II chamber slides. Cells were fixed in 2% formaldehyde and permeabilized by the treatment of 0.25% Triton X-100. Cells were washed twice with PBS and incubated with 1:1000 dilution of sheep cytochrome C antibody along with goat-INrf2, rabbit-PGAM5 and mouse-Bcl-xL antibody separately at 4°C for 12 hrs. Then cells were washed twice with PBS and incubated with anti-sheep FITC-conjugated second antibody or Alexa Fluor-594 conjugated anti-goat, anti-rabbit and anti-mouse second antibodies (Invitrogen). After immunostaining, cells were washed twice with PBS, stained with Vectashield containing nuclear DAPI stain, and mounted. Cells were observed under Nikon fluorescence microscope and photographed.

Real Time PCR— Hepa-1 cells were transfected with increasing concentration of Flag-INrf2 or endogenous INrf2 expression was knocked down by siRNA (25 to 75 nM) for 30h and cells were harvested. The total RNA was isolated using the RNeasy mini kit (Qiagen). 250 ng of total RNA was subjected to reverse transcription using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystem). After synthesis of cDNA at 37°C for 60 min, the PCR was performed using 7500 Real Time PCR system as per manufacturer’s instructions. Bcl-xL (ID: Mm00437783_m1) and control Gusb (ID:Mm...
DNA fragmentation assay— Hepa-1 cells or control 293 cells or INrf2-293 cells were plated at a density of 2000 cells per well in 96 well plates. After 20 hours, Hepa-1 cells were transfected with pcDNA or INrf2-V5 construct for 12h. Similarly, control 293 cells and INrf2-293 cells were treated with 0.5μg/ml of tetracycline for 12h and all cells were exposed to varying concentrations of etoposide for 72h. A photometric enzyme immunoassay was performed for the quantitative in vitro determination of cytoplasmic histone-associated DNA fragments (mono and oligonucleosomes) after etoposide exposure to cells using Cell Death Detection ELISA kit (Roche) and as per manufacturer’s instructions. Each combination of cell line and drug concentration was set up in eight replicate wells, and the experiment was repeated thrice. Each data point represents a mean ± SD and normalized to the value of the corresponding control cells.

TUNNEL assay— For TUNNEL assay, Hepa-1 cells were transfected with indicated plasmids treated with etoposide or etoposide+tBHQ (50 μM) for 48h. A Dead End Fluorometric TUNNEL assay kit (Promega) was used as per manufacturer’s protocol. Tunnel positive cells were counted from three independent experiments and plotted.

MTT cell survival assay— Hepa-1, Hek293, HepG2 and Jurkat cells were plated at a density of 5000 cells per well in 24 well plates and allowed to recover for 12h. Then cells were transfected with indicated constructs or control siNRA or INrf2 siRNA for 20h and cells were treated with etoposide or etoposide+tBHQ for 48h as indicated in figures. Jurkat cells were also transfected with control siRNA or INrf2 siRNA and treated with TRAIL (100 ng/ml) for 30h. Cells were incubated with fresh MTT solution (200 μl/well; stock 5 mg/ml in PBS) at 37°C for 2h and absorbance at 570 nm was measured. Each combination of cell line and drug concentration was set up in eight replicate wells, and the experiment was repeated thrice. Each data point represents a mean±SD and normalized to the value of the corresponding control cells.

Statistical Analyses— The data real time PCR, and immunoblotting band intensities were analyzed using a two-tailed Student’s t test. Data are expressed as mean ± S.D. of three independent experiments. Error bars indicate standard error of the means (s.e.m) of triplicate samples and comparisons were made using the two-tailed Student’s test for repeated measures. Differences between means were accepted as statistically significant at the 95% level (p<0.04).

RESULTS

INrf2-mediated degradation of PGAM5 and anti-apoptotic Bcl-xL protein— Control 293 and INrf2-293 cells were treated with tetracycline for indicated time and analyzed for Flag-INrf2, endogenous PGAM5 and Bcl-xL (Fig. 1A, top left panels). The results showed time dependent increase in Flag-INrf2 in INrf2-293 but not in control 293 cells. Interestingly, the increase in INrf2 led to concentration dependent decrease in PGAM5 and Bcl-xL. In similar experiments, control 293 and INrf2-293 cells were transfected with PGAM5L-V5 and repeated the experiments (Fig. 1A, top right panels). Similar results as described above were also observed for transfected PGAM5L-V5. The increase in INrf2 showed concentration dependent decrease in transfected PGAM5L-V5 and Bcl-xL proteins in transfected INrf2-293 cells. In another experiment, Hepa-1 cells showed similar results as INrf2-293 cells. The increase in INrf2 led to INrf2 concentration dependent decrease in endogenous PGAM5 and Bcl-xL (Fig. 1A, middle left panels) and transfected PGAM5L-V5 and Bcl-xL (Fig. 1A, middle right panels). Fig. 1A, lower panels demonstrate densitometry analysis of INrf2-mediated decrease in Bcl-xL in top and middle panels. The data clearly demonstrate INrf2 dependent decrease in Bcl-xL. In related experiments, siRNA dependent decrease in INrf2 resulted in increased PGAM5L-V5 and Bcl-xL (Fig. 1B). Further
experiments demonstrated marginal decreases in Bcl-xL mRNA with increasing INrf2 (Fig. 1C, upper panel). Similar experiments showed siRNA inhibition of INrf2 and marginal increases in Bcl-xL mRNA (Fig. 1C, lower panel). The results together suggested that INrf2 mediated degradation of PGAM5 and Bcl-xL.

**INrf2 interacts with PGAM5-BclxL complex**—The cell lysates from tetracycline treated control 293 and Flag-INrf2-293 cells were immunoprecipitated with anti-Flag antibody to identify INrf2 interacting proteins. The immunecomplexes were separated on SDS-PAGE and stained with CBB (Fig. 2A). Several protein bands that specifically interacted with INrf2 were identified by LC-MS/MS (Fig. 2A, lane 7 and Supplement Table 1). Band A (69 kDa) was identified as INrf2 (Keap1) (NCBI accession number NM_001110306) with ProteinProphet probability score 1 (34 unique peptides, 37% sequence coverage) in two independent experiments. The Bands B and C (32 and 30 kDa) were identified as PGAM5L and PGAM5S, respectively (NCBI accession number NP_612642) with ProteinProphet probability score 1 (25 unique peptides, 62% sequence coverage) in two independent experiments. The positions, sequences and Xcorr of the INrf2 and PGAM5 peptides identified by LC-MS/MS mass spectrometry are shown in Supplement Table 1. The LC-MS/MS data clearly suggested that INrf2 interacted with PGAM5. It is noteworthy that mass spectrometry analysis did not reveal Bcl-xL peptide that is known to interact with PGAM5 (20). We believe this is due to over expression of Flag-INrf2 in INrf2-293 cells that might have degraded Bcl-xL protein thus escaping its detection. Furthermore, in order to analyse INrf2 and PGAM5-Bcl-xL interaction, control 293 and INrf2-293 cells were transfected with PGAM5-V5 and treated with tetracycline for 12h. Cell lysates (1 mg) were immunoprecipitated with anti-Flag or anti V5 antibody and immunoblotted. The data demonstrate that, Flag-INrf2 pulled down PGAM5-V5 and PGAM5-V5 immunoprecipitated with Flag-INrf2 in INrf2-293 cells, but not in control 293 cells (Fig. 2B). Since PGAM5 proteins possessed highly conserved Bcl-xL binding PGAM domain, we further examined INrf2-PGAM5 complex interaction with endogenous Bcl-xL protein by probing the blots with Bcl-xL antibody. The results clearly showed that Flag-INrf2 and PGAM5-V5 pulled down Bcl-xL protein. Furthermore, immunoprecipitation of endogenous INrf2, PGAM5L, or Bcl-xL also pulled down endogenous PGAM5, Bcl-xL, and INrf2 in Hepa-1 cells (Fig. 2C). Anti-INrf2 antibodies immunoprecipitated INrf2, and PGAM5 and Bcl-xL were pulled down with it (Fig. 2C, upper three panels). Similarly, in reverse IP anti-PGAM5 antibodies immunoprecipitated INrf2 and BclxL proteins (Fig. 2C, middle three panels), and anti-Bcl-xL antibodies immunoprecipitated Bcl-xL, INrf2 and PGAM5 proteins (Fig. 2C, lower three panels). Collectively these results demonstrated that INrf2 interacts with PGAM5-Bcl-xL complex.

**INrf2/Cul3-Rbx1 complex ubiquitinate and degrade both PGAM5 and Bcl-xL**—INrf2 is known to degrade several proteins including Nrf2, PGAM5 and IKK (2, 17, 20). Therefore, we investigated INrf2-mediated ubiquitination and degradation of PGAM5-Bcl-xL. Flag-INrf2-293 cells were transfected with PGAM5-V5 followed by treatment with tetracycline for indicated time points to examine whether INrf2 ubiquitinates and degrades PGAM5-Bcl-xL (Fig. 3A). The cells were analyzed for Flag-INrf2, PGAM5L-V5 and Bcl-xL and ubiquitination of PGAM5L-V5 and Bcl-xL. The treatment of Flag-INrf2-293 with tetracycline showed time dependent increase in INrf2 and decrease in PGAM5 and Bcl-xL (Fig. 3A, left panel). Dose dependent overexpression of Flag-INrf2 also increased PGAM5L-V5 and Bcl-xL ubiquitination (Fig. 3A, right four panels), suggesting that INrf2 degrades both PGAM5 and Bcl-xL by ubiquitination. Furthermore, to examine whether the INrf2/Cul3-Rbx1 complex is involved in the ubiquitination and degradation of PGAM5-V5 and Flag-Bcl-xL, we overexpressed INrf2, Cul3 and Rbx1 proteins in Hepa-1 cells by transient transfection and ubiquitination and degradation of PGAM5-V5 and Flag-Bcl-xL was examined (Fig. 3B). Hepa-1 cells transfected with INrf2/Cul3-Rbx1 plasmids showed higher magnitude of
degradation of PGAM5-V5 and Flag-Bcl-xL as compared with INrf2 alone or pcDNA transfected cells (Fig. 3B, left panel). The results also demonstrated that, overexpression of INrf2/Cul3-Rbx1 increased PGAM5-V5 and Flag-Bcl-xL ubiquitination (Fig. 3B, right panels). Furthermore, siRNA-mediated dose dependent knock down of INrf2 significantly reduced ubiquitination of endogenous PGAM5 and Bcl-xL leading to stabilization of both PGAM5 and Bcl-xL proteins (Fig. 3C). This suggested that the INrf2-Cul3-Rbx1 complex is involved in the ubiquitination and degradation of PGAM5 and Bcl-xL proteins.

**DGR domain of INrf2 is required for interaction and ubiquitination/degradation of PGAM5-Bcl-xL.** INrf2 DGR domain is known to interact with Nrf2 leading to ubiquitination and degradation of Nrf2 (21). We generated two additional stable FRT-Heck-293 cells that upon exposure to tetracycline express Flag-INrf2DGR and Flag-INrf2ΔDGR (Fig. 4A) for use to examine the requirement of DGR domain for interaction with PGAM5-Bcl-xL. Control 293, INrf2-293, INrf2DGR-293 and INrf2ΔDGR-293 cells were treated with tetracycline for 24h. Ten mg cell lysates were immunoprecipitated using anti-Flag antibodies, the immune-complexes were separated by SDS-PAGE, and gels were stained with Coomassie Brilliant blue (Fig. 4A). Flag-INrf2, Flag-INrf2DGR and Flag-INrf2ΔDGR proteins strongly expressed in these cells as denoted by * over the bands (Fig. 4A). Interestingly, PGAM5 bands were pulled down along with Flag-INrf2 and Flag-INrf2DGR but not with Flag-INrf2ΔDGR as determined by mass spectrometry analysis (Fig. 4A). This suggested that DGR domain is required for interaction with PGAM5-Bcl-xL. This was also supported by immunoprecipitation and immunoblotting experiments (Fig. 4B-C). INrf2-293, INrf2DGR-293 and INrf2ΔDGR-293 cells were transfected with PGAM5-V5 constructs, treated with tetracycline for 12h and one mg cell lysates were immunoprecipitated with anti-Flag or anti-V5 antibodies, and immunoblotted with same antibodies (Fig. 4B). Immunoprecipitation of INrf2 and INrf2DGR pull down both PGAM5L-V5 and Bcl-xL proteins (Fig. 4B, left panel). In addition, immunoprecipitation of PGAM5-V5 pull down Flag-INrf2 and Flag-INrf2DGR (Fig. 4B, right panel). However, in same experiments, INrf2ΔDGR failed to pull down either PGAM5L-V5 or Bcl-xL protein and PGAM5-V5 failed to pull down INrf2ΔDGR (Fig. 4B, left and right panels).

Next, we analyzed the ubiquitination and degradation of PGAM5L-V5 and Bcl-xL proteins in INrf2, INrf2DGR, and INrf2ΔDGR expressing cells after transfection with PGAM5-V5 and HA-ubiquitin constructs (Fig. 4C). Overexpression of INrf2 in INrf2-293 cells by tetracycline degraded both PGAM5-L-V5 and Bcl-xL protein, and significantly increased PGAM5 and Bcl-xL ubiquitination (Fig. 4C). Interestingly, overexpression of INrf2ΔDGR domain or INrf2ΔDGR-protein both failed to degrade PGAM5L-V5 or Bcl-xL protein (Fig. 4C, left panel), as well as also failed to ubiquitinate PGAM5L and Bcl-xL proteins (Fig. 4C, right panels). These data clearly demonstrated that the DGR domain of INrf2 was required for binding with PGAM5L-Bcl-xL complex. However, the DGR domain of INrf2 was not sufficient for ubiquitination and degradation of PGAM5L and Bcl-xL protein since Cul3-Rbx1 ubiquitin E3 ligases complex binds with the BTB domain at N-terminus of INrf2 and mediate ubiquitination and degradation. Therefore the BTB and DGR both domains of INrf2 are required for ubiquitination and degradation of PGAM5-Bcl-xL complex.

**INrf2 physically interacts with PGAM5 but not Bcl-xL.** Several experiments were performed to examine if INrf2 directly interacts only with PGAM5 or with both PGAM5 and Bcl-xL. To investigate, we knocked down PGAM5L protein by siRNA in Hepa-1 cells, and the levels of PGAM5, Bcl-xL, INrf2 and actin were analyzed by western blotting (Fig. 5A). Transient transfection of PGAM5 siRNA (50 to 75 nM) decreased PGAM5 protein by 60 to 80%. However Bcl-xL protein levels significantly increased (2 to 2.5 fold) upon PGAM5 knockdown (Fig. 5A, left panel). Using the same cell lysates we analysed INrf2-PGAM5 and INrf2-Bcl-xL interaction by immunoprecipitation and immunoblotting (Fig. 5A, right panel). Transfection of PGAM5
siRNA decreased the interaction between INrf2 and PGAM5, as expected, whereas it also decreased INrf2-Bcl-xL interaction to the same magnitude (Fig. 5A, right panel), suggesting that PGAM5L is required for INrf2-Bcl-xL interaction. PGAM5 at N-terminus is known to contain a motif NXESGE that is similar to Nrf2 motif DEETGE (17). Both these motifs are binding sites for other proteins. To test whether the 77NXESGE82 motif in PGAM5-L is involved in the binding to INrf2, a mutant PGAM5-L protein was generated in which two alanine substitutions were introduced in place of Glu-79 and Ser-80. V5-tagged plasmids of the wild-type PGAM5-L and PGAM5-L-E79A/S80A mutant proteins were transfected in Hepa-1 cells along with HA-INrf2 and Flag-Bcl-xL constructs for 30h and cell lysates were immunoblotted (Fig. 5B, upper panel). Immunoblotting data itself indicate that in mutant PGAM5-L transfected cells the levels of mutant PGAM5 and Bcl-xL protein were ~1.5 fold more than wild type PGAM5-L transfected cells, suggesting that NXESGE motif of PGAM5-L is required for the binding to INrf2. To support this observation, we performed the forward and reverse immunoprecipitation and immunoblotting experiments. The mutant PGAM5-L-E79A/S80A protein did not bind with INrf2 (Fig. 5B, lower left panel), whereas, Bcl-xL interaction was same with wild type and mutant PGAM5. Flag-Bcl-xL interaction with INrf2 was observed in wild type PGAM5-V5 transfected cells. However, Flag-Bcl-xL and HA-INrf2 interaction was abolished in mutant PGAM5L-E79A/S80A-V5 transfected cells (Fig. 5B, right lower panel). In addition, we further investigated whether Bcl-xL has any role in INrf2-PGAM5L interaction. For this, we knocked down Bcl-xL protein by siRNA, and interactions between INrf2-PGAM5L and PGAM5L-Bcl-xL were analysed (Fig. 5C, left and right panels). Silencing of Bcl-xL protein by siRNA decreased Bcl-xL protein, whereas PGAM5L and INrf2 levels remained the same (Fig. 5C, left panel). Importantly, immunoprecipitation/immunoblotting data clearly indicate that knockdown of Bcl-xL protein has no effect on INrf2 and PGAM5L interactions (Fig. 5C, right panels). These results together suggested that INrf2 physically interacts with PGAM5 but not Bcl-xL. In addition, INrf2 interaction with Bcl-xL required PGAM5.

**PGAM5 is required for mitochondrial localization of Nrf2:INrf2:PGAM5:L complex.** PGAM5L contains mitochondrial localization signal between amino acids 9-29 and shown to localize in the mitochondria (31). Therefore, we performed immunohistochemistry analysis to investigate the co-localization of Nrf2:INrf2:PGAM5:Bcl-xL complex to the mitochondria. Immunocytchemistry analysis clearly showed the co-localization of INrf2, PGAM5 and Bcl-xL proteins with mitochondrial cytochrome C protein (Fig. 6A). Interestingly, immunohistochemistry analysis of Nrf2 in the same experiment demonstrated that Nrf2 also co-localized with INrf2:PGAM5:Bcl-xL complex in the mitochondria (Fig. 6A). These results suggested that Nrf2:INrf2:PGAM5:Bcl-xL complex localizes to the mitochondria. This is also supported by a single report earlier (31). Next we examined the role of PGAM5 on the localization of Nrf2:INrf2:PGAM5:Bcl-xL complex in the mitochondria (Fig. 6B). Hepa-1 cells were transfected with control and PGAM5 siRNA, cytoplasmic and mitochondrial fractions were isolated and immunoblotted for PGAM5, Bcl-xL, INrf2 and Nrf2. The immunoblot was also probed with anti-LDH (cytosolic marker) and anti-CoxIV (mitochondrial marker). Results revealed that PGAM5 knock down by siRNA significantly reduced the mitochondrial localization of Nrf2:INrf2:PGAM5:Bcl-xL (Fig 6B). This suggested that PGAM5 proteins are involved in the trafficking of Nrf2:INrf2:PGAM5:Bcl-xL complex to the mitochondria.

**Antioxidant treatment led to the release of Bcl-xL**—INrf2 contains reactive cysteines (C151, C272 and C282) that in response to chemicals/radiation are oxidized leading to destabilization of dimeric structure of INrf2, degradation of INrf2 and release of Nrf2 (1, 2). In the present report, we studied the effect of antioxidant tBHQ on the INrf2:PGAM5L:Bcl-xL interaction. Hepa-1 cells transfected with PGAM5-V5 were exposed to DMSO (vehicle control) or tBHQ (50 μM) for different time
periods (2 to 8h), and cell lysates were immunoblotted for INrf2, PGAM5L-V5 and Bcl-xL (Fig. 7A). DMSO showed more or less no effect on endogenous levels of INrf2, PGAM5L-V5, and Bcl-xL. However, cells upon treatment with t-BHQ (between 2 to 4h) showed decreased levels of INrf2 and PGAM5-V5 and increased levels of Bcl-xL (Fig. 7A). Forward and reverse immunoprecipitation followed by immunoblotting in similar experiments also analyzed INrf2:PGAM5:Bcl-xL interactions (Fig. 7B). Forward IP results revealed t-BHQ exposure time dependent loss in interaction of INrf2 with PGAM5 and Bcl-xL (Fig. 7B, right upper three panels). Reverse IP in similar experiments also showed loss of interaction of PGAM5 with INrf2 and Bcl-xL (Fig. 7B, right lower three panels). The various results indicated that t-BHQ destabilized INrf2:PGAM5:Bcl-xL complex leading to degradation of PGAM5 and stabilization of Bcl-xL.

Next we investigated the effect of t-BHQ on Nrf2, INrf2, PGAM5, Bcl-xL and Bax interactions in cytosol and mitochondria (Fig. 7C). Hepa-1 cells were treated with DMSO or tBHQ for 2h and cytoplasmic and mitochondrial fractions isolated. Immunoprecipitation followed by immunoblotting analyzed the various interactions. Immunoprecipitation of PGAM5 pull down Bcl-xL, INrf2 and Nrf2 in both DMSO treated cytosol and mitochondria (Fig. 7C, left panels). The treatment with t-BHQ led to reduced interaction of PGAM5 with INrf2 and release of Nrf2 in cytosol as well as in mitochondria. t-BHQ treatment also led to release of Bcl-xL in mitochondria but not in cytosol (Fig. 7C, left panels). Similar results were obtained in case of INrf2 immunoprecipitation (Fig 7C, second left panels). Bcl-xL immunoprecipitation demonstrated that Bcl-xL-PGAM5 and Bcl-xL-INrf2 interaction was decreased significantly in mitochondrial but not in cytosolic compartment upon treatment with tBHQ (Fig 7C, left third panels). Bcl-xL protein did not show a strong interaction with Nrf2 in both compartments of the cells. In addition, Nrf2 immunoprecipitation data clearly showed that Nrf2 interacts with INrf2 and PGAM5 in DMSO treated cells in cytoplasm and mitochondria, however, the interaction was decreased more than 90% when cells were treated with tBHQ and again no interaction with Bcl-xL (Fig 7C, right panels).

Interestingly, immunoprecipitation of Bcl-xL and Bax data clearly showed that the mitochondrial interaction of Bcl-xL and Bax was increased after tBHQ treatment as compared with DMSO treatment (Fig. 7D) and no change in cytoplasmic Bcl-xL and Bax interaction. Collectively, the results demonstrated that antioxidant t-BHQ led to release of Nrf2 in the cytosol and Nrf2 and Bcl-xL in the mitochondria. The release of Bcl-xL in mitochondria led to increased interaction with Bax that is expected to contribute to altered apoptosis.

Overexpression of INrf2 led to degradation of PGAM5:Bcl-xL and enhancement of etoposide-induced cytochrome C release, up-regulation of pro-apoptotic Bax and increase in activated Caspases -3/7— Our data suggested that INrf2 mediated degradation of PGAM5:Bcl-xL protein (Fig. 1). This indicated that INrf2 through regulation of anti-apoptotic Bcl-xL protein might influence apoptotic cell death/survival. Therefore, we examined the role of INrf2 mediated degradation of PGAM5 and Bcl-xL in cellular apoptosis. INrf2-293 cells were transfected with PGAM5-V5 and treated with tetracycline and Hepa-1 cells transfected with INrf2 were treated with two different concentrations of etoposide and analyzed for PGAM5:Bcl-xL degradation, cytochrome C release, caspase-3/7 activity and cleaved caspase-3 (Fig. 8). INrf2-293 and Hepa-1 cells overexpressing INrf2 showed degradation of PGAM5:Bcl-xL and increase in Bax (Fig. 8A). Etoposide treatment marginally increased alterations in the various molecules. INrf2 overexpression followed by etoposide treatment in Hepa-1 cells also induced cytochrome C release from mitochondria to cytosol by 1.8 fold (Fig. 8B, compare lanes 2/3/4, data shown only for Hepa-1), increased 1.5 to 2 fold caspases -3/7 activity (Fig. 8C) and cleaved caspase 3 (Fig. 8D), as compared with etoposide treated cells expressing endogenous levels of INrf2. These data suggested that INrf2 overexpression degrades PGAM5 and Bcl-xL proteins, promotes etoposide mediated increases in
cellular Bax level, release cytochrome from mitochondria and activates caspases -3/7. In similar experiments, we also examined the role of INrf2 in the regulation of anti-apoptotic factors Bcl-2 and Mcl-1. Increasing INrf2-V5 levels in Hepa-1 cells by transient tranfection degraded not only Bcl-xL as described above but also Bcl-2 and Mcl-1 (Supplement Figure S1A). Moreover, a dose dependent siRNA-mediated INrf2 knock down stabilized anti-apoptotic proteins Bcl-xL, Bcl-2 and Mcl-1 (Supplement Figure S1B). These results together indicate that INrf2 down-regulates these anti-apoptotic factors in addition to Bcl-xL to control apoptosis. It is noteworthy that we have recently reported INrf2 degradation of anti-apoptotic factor Bcl-2 (30).

**Overexpression of INrf2 increased and treatment with antioxidant t-BHQ decreased etoposide-mediated DNA fragmentation and cellular apoptosis—** The biochemical significance of INrf2 mediated degradation of PGAM5-Bcl-xL complex and up-regulation of pro-apoptotic marker proteins by INrf2 raised an interesting question, whether INrf2 regulated cellular apoptosis. Hepa-1 cells transfected with pcDNA (vector control) or INrf2-V5 were treated with the various concentrations of etoposide. Similarly, control 293 and INrf2-293 cells were treated with tetracycline first for 12h and then treated with various concentrations of etoposide. The histone associated DNA fragmentations were analysed (Fig. 9A). The results demonstrated that overexpression of INrf2 followed by etoposide treatments enhanced DNA fragmentation at least 1.4 to 2.1 fold in both cell lines compared with control cells (Fig. 9A, Upper and lower panels).

We also examined the role of PGAM5, a bridge protein between INrf2 and Bcl-xL, in Hepa-1 cell survival. Overexpression of PGAM5-V5 protein and etoposide treatments decreased cell survival by 20% compared with pcDNA transfected cells (Fig. 9B, left panel). In contrast, PGAM5 knock down by siRNA followed by etoposide treatments increased cell survival by 15 to 20% as compared with control siRNA transfected cells (Fig. 9B, right panel) again suggesting that PGAM5 proteins are involved in Bcl-xL regulation and cell survival.

Next we determined the effect of antioxidant t-BHQ on etoposide-mediated apoptosis. This was analyzed by TUNNEL assay (Fig. 9C). Hepa-1 cells overexpressing INrf2 or Nrf2 were treated with etoposide and t-BHQ as shown in Fig. 9C and the TUNNEL positive cells were observed under a microscope that were counted and plotted (Fig. 9C). Hepa-1 cells overexpressing INrf2 upon treatment with etoposide showed 12% more TUNNEL positive cells, as compared with etoposide treated control cells. Interestingly, Nrf2 overexpression and BHQ treatment both protected the cells from etoposide mediated DNA damage since the TUNNEL positive cells declined (Fig. 9C). DNA fragmentation/TUNNEL data were further supported by cell survival assays (Fig. 9D). Overexpression of Flag-INrf2 in Hepa-1 cells significantly reduced (~18%) cell survival upon treatment with etoposide, as compared to etoposide treated control cells expressing endogenous levels of INrf2. Nrf2 overexpression or t-BHQ treatment showed increased cell survival (20 to 30%) compared with etoposide alone treated cells (Fig. 9D).

**siRNA-mediated knock down of INrf2 increased etoposide and TRAIL mediated cell survival.** We investigated the effect of siRNA-mediated inhibition of INrf2 on Bcl-xL stabilization and etoposide mediated cell survival in three different cancer cell lines. Hepa-1, Hek-293 and HepG2 cells were transfected with different concentrations of either control siRNA or INrf2 siRNA for 24h, treated with etoposide for 36h, and cell survival analysis was performed by MTT assay (Fig. 10A). A dose dependent knock down of INrf2 by siRNA in all there cell lines followed by etoposide treatment showed 20 to 40% increased cell survival compared with control siRNA transfected cells (Fig 10A, upper, middle and lower panels). The reasons for increasing cell survival after knock down of INrf2 was further examined in Hepa-1 cells (Fig. 10B). INrf2 knock down and etoposide treatment stabilized cellular Bcl-xL and also decreased INrf2 mediated Bcl-xL ubiquitination compared with control siRNA transfected cells (Fig. 10B, left and right panels). These results together suggested that knock down of INrf2 stabilized Bcl-xL and increased cell survival.
Interestingly, we also used cell killer TRAIL protein, which is known to activate extrinsic apoptotic pathway in cells by the activation of caspase-8. For this we used Jurkat (T cell Lymphoma) cells. Cells were transfected with control siRNA or INrf2 siRNA and treated with TRAIL protein as indicated (Fig. 10C, lower panel) and cell survival was measured. Treatment of cells with TRAIL decreased cell survival by 40% compared with untreated cells. However, knockdown of INrf2 followed by TRAIL treatment increased cell survival by 20 to 25% compared with control siRNA transfected and TRAIL treated cells (P < 0.01) (Fig.10C, lower panel). In addition, we also confirmed INrf2 knockdown and Bcl-xL stabilization in Jurkat cells by immunoblotting of the same cell lysates with INrf2, Bcl-xL and actin antibodies (Figure 10C upper panels). These data indicate that increased levels of Bcl-xL by knocking down of INrf2 contribute to some resistance against extrinsic apoptotic pathway in Jurkat cells.

The above results together suggested that overexpression of INrf2 degraded PGAM5:Bcl-xL complex, activated pro-apoptotic factors, and promoted etoposide mediated cellular apoptosis. In contrast, the knockdown of INrf2 resulting in increased Nrf2 or overexpression of Nrf2 or tBHQ treatment leading to stabilization of Nrf2 all promoted cell survival. Therefore, INrf2 and Nrf2 both play opposite roles in the regulation of cellular apoptosis.

**DISCUSSION**

Recently, we have shown that INrf2 interacts and degrades anti-apoptotic protein Bcl-2 and controls cellular apoptosis (30). In this report, we demonstrate INrf2 regulation of another anti-apoptotic protein Bcl-xL and its role in cellular apoptosis. INrf2 is known to directly interact with Bcl-2 to control apoptosis (30). However, unlike Bcl-2, INrf2 does not directly interact with Bcl-xL. It interact Bcl-xL through PGAM5 protein. Results also revealed that DGR region of INrf2 is required for interaction with PGAM5. PGAM5 protein not only mediated INrf2 interaction with Bcl-xL but also directed localization of Nrf2:INrf2:PGAM5:Bcl-xL complex to the mitochondria. These observations are also supported by previous studies (17, 31). Our data also agree with previous report that INrf2 dimer binds to Nrf2 on one strand and PGAM5 on the other strand. However, this clearly requires further investigation. Further studies revealed that INrf2 along with E3 ubiquitin ligase complex Cul3-Rbx1 ubiquitinate and degrade PGAM5 and Bcl-xL. Antioxidant t-BHQ destabilizes Nrf2:INrf2:PGAM5:Bcl-xL complex especially in mitochondria leading to the release of Bcl-xL and increase in Bcl-xL:Bax complex. The release of Bcl-xL and increase in Bcl-xL:Bax complex in cytosol if any is limited for unknown reasons.

Results also demonstrate that INrf2 by regulating PGAM5:Bcl-xL control cellular apoptosis. Overexpression of INrf2 upon exposure to etoposide led to increased degradation of PGAM5-Bcl-xL, increased cytochrome C release, increased cleaved caspase-3 and caspase-3/7 activity. These events led to increased apoptotic cell death and decreased cell survival. In similar experiments, siRNA inhibition of INrf2 led to stabilization of Bcl-xL and increased cell survival. Therefore, up and down regulation of INrf2 is directly related to apoptosis and inversely related to cell survival. Interestingly, our results also demonstrated that INrf2 through PGAM5-Bcl-xL also controls TRAIL-mediated extrinsic apoptotic pathways.

INrf2 mediated degradation of anti-apoptotic proteins Bcl-xL (current report) and Bcl-2 (30). Therefore, under normal conditions, INrf2 control of Bcl-xL and Bcl-2 keeps a homeostatic balance between cell death and cell survival. Exposure to antioxidant or other chemical stress leads to release of Nrf2 in the cytosol that translocates to the nucleus and release of Nrf2 and Bcl-xL in mitochondria. This leads to Nrf2-mediated coordinated activation of cytoprotective genes and increase in Bcl-xL: Bax complex leading to reduced apoptotic cell death and increased cell survival. Antioxidant is also known to stabilize Bcl-2 that forms complex with Bax and contributes to decreased apoptosis and increased cell survival (30). Therefore, it is expected that antioxidant induced stabilization of anti-apoptotic proteins Bcl-xL
and Bcl-2 leads to reduced apoptotic cell death and increased cell survival.

PGAM5 exists in two isoforms that are identical in N-terminus 239 amino acids (32). The longer form of (PGAM5L) contains 289 amino acids and the shorter form (PGAM5-S) contains 255 amino acids. The 16 C-terminal amino acids in PGAM5S are not similar with the PGAM5L isoform. The N-terminus of the PGAM5 proteins contains a conserved NXESGE motif (amino acid 77-82), similar to Nrf2, that binds to the DGR region of INrf2, whereas, the C-terminal PGAM domain (amino acid 125-156) binds anti apoptotic factor Bcl-xL. Interestingly, both isoforms of human PGAM5 contain a large PGAM domain which begins at amino acid 98 and extends to the C-terminal end (32). In addition to this, PGAM5 proteins also possess N-terminal mitochondrial localization signal (amino acid 9-29), which is involved in the mitochondrial localization of PGAM5 and its binding partners to the mitochondria. The present studies used PGAM5L form for the studies. PGAML5E79AS80A failed to bind with INrf2 and ubiquitinate/degrade Bcl-xL. This indicated that PGAM5 through 77NXESGE82 domain binds to INrf2. Since this domain is present in both isoforms, we believe PGAM5L and PGAM5S both function as bridge between INrf2 and Bcl-xL.

A hypothetical model demonstrating the role of INrf2 control of Bcl-xL and apoptosis is depicted in Figure 11. INrf2 homodimer bound to Nrf2 on one monomer and PGAM5:BclxL on other monomer exists in the cytosol and mitochondria. Under physiological conditions INrf2 homodimers promote a Cul3-Rbx1-mediated degradation of Nrf2 and PGAM5:BclxL, thereby contributing to the maintenance of normal level of Bcl-xL and apoptosis. Oxidative/electrophilic stress leads to the release of Nrf2 and PGAM5-Bcl-xL complex from INrf2 dimers in the cytosol. Nrf2 translocates to nucleus leading to activation of cytoprotective gene expression (2). PGAM5-Bcl-xL is directed to mitochondria where PGAM5 is degraded to release Bcl-xL. In addition, oxidative/electrophilic stress also leads to release of Nrf2 and Bcl-xL in the mitochondria resulting in activation of unknown mitochondrial gene expression and increase in Bcl-xL:Bax dimers. The Nrf2 mediated increased expression of nuclear and mitochondrial cytoprotective gene expression and induction of Bcl-xL:Bax dimers leads to reduced apoptosis and increased cell survival. Further studies are required to investigate Nrf2 regulated genes in mitochondria. Oxidative/electrophilic stress is also known to stabilize Bcl-2 in the cytosol that forms dimers with Bax and contributes to reduced apoptosis and increased cell survival (not shown in Fig. 10, ref. 30). It is noteworthy that Nrf2:INrf2:PGAM5:Bcl-xL complex in the cytosol might be in addition to previously characterized Nrf2:INrf2 complex (2) and remains to be further studied.

The stabilization of Bcl-xL (current study) and Bcl-2 (ref. 30) from INrf2 and prevention of apoptosis is presumably an important mechanism to save cells from dying in acute stress due to exposure to antioxidants, xenobiotics, drugs and radiation. Once the exposure effect subsides, the level of Bcl-xL, Bcl-2 and cytoprotective proteins are brought back to normal and normal level of apoptotic cell death is restored. Recent studies have reported increased stabilization/accumulation of Nrf2 due to mutations in INrf2 resulting in loss of function in lung and breast tumors (12, 13, 33, 20). Lung cancer cell line A549 contains INrf2G333C mutant protein that has lost its capacity to bind/degrade Nrf2 leading to accumulation of Nrf2 in nucleus (13). It has been suggested that higher levels of Nrf2 in A549 cells might have contributed to the survival of these cells in lung cancer. Similarly, our studies demonstrate that during stress destabilization of INrf2-PGAM5 interaction also stabilized cellular Bcl-xL proteins resulted in down regulation of cellular apoptosis. In conclusion we demonstrate that both INrf2 and PGAM5 protein contribute to the regulation of Bcl-xL protein and apoptosis.
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KEY WORDS: Nrf2, INrf2 (Keap1), Bcl-xL, PGAM5, Anti-apoptotic, Apoptosis

ABBREVIATIONS: Nrf2, NF-E2 related factor; INrf2. Cytoplasmic inhibitor of Nrf2 also known as Keap1; PGAM5, phosphoglycerate mutase family 5; ARE, Antioxidant response element; NQO1, NAD(P)H:quinone oxidoreductase; t-BHQ, tert-butyl hydroquinone; IP, immunoprecipitation; WB, Western blotting.

REFERENCE


FIGURE LEGENDS

FIGURE 1. Overexpression of INrf2 leads to PGAM5 and Bcl-xL degradation. A, Overexpression of Flag-INrf2 degrades endogenous/transfected PGAM5 and endogenous Bcl-xL. Control 293 or INrf2-293 cells were either untransfected (upper left panel) or transfected with PGAM5L-V5 plasmid (upper right panel). The cells were treated with tetracycline (0.5µg/ml) for different time periods and immunoblotted (upper panels). In similar experiments, Hepa-1 cells were transfected with Flag-INrf2 or Flag-INrf2 with PGAM5L-V5 plasmids and immunoblotted (middle panels). The bands were quantified from three independent experiments and plotted (lower panels). B, siRNA inhibition of INrf2 stabilized PGAM5L and Bcl-xL protein. Hepa-1 cells were transfected with control (75 nM) or INrf2 siRNA (25-75 nM) for 36h and immunoblotted. C, RT-PCR analysis. Hepa-1 cells were transfected with increasing amount of Flag-INrf2 plasmid (upper panel) or INrf2 siRNA (lower panel) for 30h. Cells were harvested and Bcl-xL mRNA quantified by real time PCR. All experiments were repeated three times.

FIGURE 2. INrf2 interacts with PGAM5-Bcl-xL complex. A, Mass spectroscopic identification of PGAM5 proteins as interacting partners of INrf2. Control 293 cells and INrf2-293 cells were treated with tetracycline and cell lysates were immunoprecipitated with anti-Flag antibodies, immune complexes separated by SDS-PAGE and gels stained with CBB. Gel slices containing bands indicated by arrows were reduced, alkylated, and digested with trypsin. Tryptic peptides were desalted and subjected to LC-MS/MS analysis. The Mascot software package was used to match the mass of the peptides with predicted tryptic peptides generated from the translated human genome. B, INrf2 interacts with PGAM5-Bcl-xL complex. Control 293 cells and INrf2-293 cells expressing tetracycline inducible Flag-INrf2 were transfected with PGAM5-V5 plasmid and treated with tetracycline. One mg cell lysates were immunoprecipitated with anti-Flag, or anti-V5 antibodies and immunoblotted. C, Endogenous INrf2 interacts with endogenous PGAM5L-Bcl-xL proteins. One mg Hepa-1 cell lysates were immunoprecipitated with anti-INrf2 antibodies (upper panel) or anti-PGAM5 antibodies (middle panel) or anti-Bcl-xL antibodies (lower panel) and immunoblotted. All experiments were repeated three times.
FIGURE 3. INrf2/Cul3-Rbx1 complex ubiquitinate and degrade both PGAM5 and Bcl-xL proteins. A, INrf2-293 cells were co-transfected with PGAM5-V5 along with HA-Ub plasmid and treated with tetracycline for different time periods. Sixty μg proteins were immunoblotted with anti-Flag, anti-V5 and anti-Bcl-xL antibodies (left panel). One mg of same cell lysates were immunoprecipitated with control IgG or anti-V5 or anti-Bcl-xL antibodies and immune-complexes were immunoblotted with anti-HA-HRP and anti-ubiquitin antibodies (right four panels). B, Hepa-1 cells were co-transfected with pcDNA-INrf2, PGAM5-V5, Flag-Bcl-xL, HA-Cul3, Myc-Rbx1 and HA-Ub in combinations as shown and immunoblotted (left panel). For visualization of ubiquitination of PGAM5 and Bcl-xL, same one mg lysates were immunoprecipitated with anti-V5 antibody and anti-Flag antibody respectively and immunoblotted with anti-HA-HRP antibodies (right panels). C, Effect of INrf2 siRNA on endogenous ubiquitination of PGAM5 and Bcl-xL. Hepa-1 cells were transfected with control (75 nM) or INrf2 siRNA and immunoblotted with indicated antibodies (left panel). Same one mg lysates were immunoprecipitated with PGAM5 and Bcl-xL antibodies in separate experiments and immunoblotted with anti-ubiquitin (UB) antibodies (middle and right panels). All experiments were repeated three times.

FIGURE 4. INrf2-DGR domain is required for interaction and ubiquitination/degradation of PGAM5-Bcl-xL. A, Tetracycline induced expression of Flag-INrf2, Flag-INrf2DGR and Flag-INrf2ΔDGR in 293 cells. The cells were treated with tetracycline and ten mg cell lysates were immunoprecipitated with anti-Flag antibodies and the immune complexes were separated by SDS-PAGE and stained with CBB. INrf2, INrf2DGR and INrf2ΔDGR protein bands are labeled with * and interacting PGAM5 proteins are shown by arrows. B, INrf2 DGR domain is required for interaction with PGAM5L-Bcl-xL. Flag-INrf2-293, Flag-INrf2DGR and Flag-INrf2ΔDGR cells were transfected with PGAM5-V5 plasmid and treated with tetracycline. One mg of lysates was immunoprecipitated with anti-V5 antibody (left panel) or anti-Flag antibodies (right panel) and immunoblotted with anti-Flag or anti-V5 or anti-Bcl-xL antibodies. C, INrf2 DGR domain is essential for ubiquitination and degradation of PGAM5 and Bcl-xL protein. Flag-INrf2, Flag-INrf2DGR and Flag-INrf2ΔDGR-293 cells were co-transfected with PGAM5-V5 and HA-UB plasmids, treated with tetracycline and immunoblotted with anti-Flag, anti-V5 and anti-Bcl-xL antibodies (left panel). One mg of lysates was immunoprecipitated with anti-V5 or anti Bcl-xL antibodies and immunoblotted with HA-HRP antibodies (right panels).

FIGURE 5. PGAM5L is required for INrf2 and Bcl-xL interaction. A, Western analysis/Immunoprecipitation. Hepa-1 cells were transfected with control or PGAM5 siRNA for 36h, lysed and immunoblotted (left panel). One mg of same cell lysates from siRNA transfected cells were immunoprecipitated with anti-INrf2 antibody and immunoblotted with antibodies as shown. B, Western analysis/Immunoprecipitation. Mutation of PGAM5L/E79S80 abolished INrf2-Bcl-xL interaction. Hepa-1 cells were transfected with PGAM5L-V5 or mutant PGAM5L-E79A/S80A-V5 along with HA-INrf2 and Flag-Bcl-xL plasmids and immunoblotted (upper panel). Interactions of PGAM5L-V5 and mutant PGAM5L-LE79AS80A-V5 with HA-INrf2 and Flag-Bcl-xL was analysed by immunoprecipitation/immunoblotting (lower panels). C, Western analysis/Immunoprecipitation. Hepa-1 cells were transfected with control or Bcl-xL siRNA and immunoblotted with anti-Bcl-xL, anti-PGAM5L and anti-INrf2 antibodies (left panel). One mg lysates from transfected cells were immunoprecipitated with anti-INrf2 or anti-PGAM5L antibody and immunoblotted for Bcl-xL, PGAM5L and INrf2.

FIGURE 6. PGAM5 is required for localization of Nrf2-INrf2-PGAM5-Bcl-xL complex to the mitochondria. A, Immunocytochemistry for localization of Nrf2-INrf2-PGAM5-Bcl-xL complex to the mitochondria. Hepa-1 cells were grown on cover-slips, fixed, permeabilized, washed and first incubated with a 1:500 dilution of anti-cytochrome C sheep antibody for 12h. After washing, cells were incubated with 1:1000 dilution of anti-Nrf2 rabbit, anti-INrf2 goat, anti-Bcl-xL mouse and anti-PGAM5 rabbit antibody for 12h separately. After washing with PBS, cells were incubated with anti-sheep FITC conjugated second antibody or Alexa Fluor-594 conjugated anti-goat, anti-rabbit and anti-mouse second
antibody for 1h. After immunostaining, cells were observed under Nikon fluorescence microscope and photographs were captured. B, Hepa-1 cells were transfected with 100 nM control or PGAM5 siRNA and cytosolic and mitochondrial fractions prepared. Sixty microgram extracts were immunoblotted with anti-PGAM5L, anti-Bcl-xL, anti-INrf2, anti-Nrf2, anti-LDH and anti-Cox IV antibodies.

FIGURE 7. Antioxidant tBHQ destabilized Nrf2-INrf2-PGAM5-BclxL complex in the cytosol and on the mitochondria leading to the release of Bcl-xL. A, tBHQ treatment leads to degradation of PGAM5 and stabilization of anti-apoptotic factor Bcl-xL. Hepa-1 cells transfected with PGAM5-V5 were treated with DMSO or tBHQ (50 μM) for 2 to 8h, lysed and immunoblotted. Band intensities are shown below the immunoblots. B, t-BHQ treatment causes destabilization of Nrf2-INrf2-PGAM5-Bcl-xL complex and release of Bcl-xL. Hepa-1 cells were co-transfected with pcDNA or Flag-INrf2 and PGAM5-V5 and treated with DMSO or tBHQ (50 μM) for 2h and 4h, lysed and immunoblotted (left panel). One mg of same cell lysates from transfected cells were immunoprecipitated with anti-Flag or anti-V5 antibody and the immune complexes were immunoblotted with anti-Bcl-xL, anti-Flag or anti-V5 antibodies (right panels). C, t-BHQ causes Nrf2 release in the cytosol and Nrf2 and Bcl-xL lysate in the mitochondria. Hepa-1 cells were treated with DMSO or tBHQ for 2h, and 1 mg cytosolic and 300 μg of mitochondrial lysates were immunoprecipitated with anti-PGAM5L, anti-INrf2, anti-Bcl-xL anti-Nrf2 antibodies and immunoblotted with indicted antibodies (upper panels). The band intensities of the respective panels are shown (lower panels). C- cytosolic; M- mitochondrial. D, tBHQ treatment increased heterodimerization of Bcl-xL and Bax protein on mitochondria. Hepa-1 cells were treated with DMSO or tBHQ for 2h, and one mg cytosolic and 300 μg mitochondrial lysates were immunoprecipitated with anti-Bcl-xL or anti-Bax antibodies and immunoblotted with indicted antibodies. C- cytosolic; M- mitochondrial. All experiments were repeated two times and one representative set of data is presented.

FIGURE 8. Overexpression of INrf2 leads to degradation of PGAM5-Bcl-xL and activation of pro-apoptotic proteins. A, INrf2-293 cells transfected with PGAM5-V5 plasmid and treated with tetracycline for 24h and Hepa-1 cells co-transfected with Flag-INrf2 and PGAM5L-V5 plasmids for 24h were treated with indicated concentrations of etoposide for additional 24h. Cells were lysed and immunoblotted (left and right panels). B, Hepa-1 cells were transfected with pcDNA vector or Flag-INrf2 and treated with etoposide (20 μM) for 36h. Cells were harvested and mitochondria isolated and mitochondrial and cytosolic lysates were immunoblotted with anti-cytochrome C, Cox IV and actin antibodies. C, Hepa-1 cells were transfected with pcDNA vector or Flag-INrf2 and treated with etoposide (20 μM) as indicated for 36h. Similarly, INrf2-293-cells were treated with tetracycline followed by treatment with 1 μM etoposide for additional 36h. Cells were lysed and 20 μg cell lysates were mixed with Caspase Glo 3/7 substrate (Promega) and Caspase 3/7 activity was measured and plotted (upper and lower panels). Data are mean±SD from three independent experiments. D, Sixty micrograms of INrf2 overexpressed or etoposide treated cell lysates of Hepa-1 cells and INrf2-293 cells form figure “C” were immunoblotted with anti-caspase 3, anti-Flag and anti-actin antibodies (left and right panels).

FIGURE 9. Nrf2-INrf2-PGAM5-Bcl-xL and cellular apoptosis. A, Overexpression of INrf2 leads to increase in etoposide-mediated apoptosis. Hepa-1 cells were transfected with pcDNA or INrf2-V5 plasmids and treated with etoposide for 48h (Upper panel). Control 293 and INrf2-293 cells were treated with tetracycline and then treated with indicated concentration of etoposide for 48h (Lower panel). Transfected/treated Hepa-1 and treated 293/INrf2-293 cells were analyzed for apoptotic cell death by DNA fragmentation assay. The cytoplasmic histone-associated DNA fragments (mono and oligonucleosomes) were quantified using Cell Death Detection ELISA kit (Roche) and plotted (upper & lower panels). B, Alterations in PGAM5 levels leads to inverse relationship with cell survival. Hepa-1 cells were plated at a density of 5000 cells per well in 24 well plates, and transfected with different concentration of PGAM5L-V5 constructs (0.5, 1 and 2 μg) (left panel) or transfectioned with different concentration of siRNA against PGAM5 (25, 50, 100 nM) for 24h (right panel). The cells were then treated with DMSO or etoposide (20 μM) for 36h. Cells were incubated with fresh MTT solution for 2h

16
at 37°C and absorbance at 570 nm was measured. The experiment was repeated thrice. Each data point represents a mean ± SD and normalized to the value of the corresponding control cells. 

C, TUNEL assay. 
t-BHQ treatment reduces etoposide-mediated apoptosis. Hepa-1 cells were transfected with Flag-INrf2 or Flag-Nrf2 and treated with etoposide for 48h. One set of cells were further treated with tBHQ for additional 24h, cells were fixed, permeabilized and TUNEL assay was performed. TUNEL positive cell were observed under fluorescence microscope, quantified and plotted. The data represented as the mean ±SD from two experiments. 

D, Cell survival assay. Hepa-1 cells were plated at a density of 5000 cells per well in 24 well plates, and transfected with Flag-INrf2 or Flag-Nrf2 and treated with DMSO or etoposide for 48h and tBHQ for 24h. Cells were incubated with fresh MTT solution for 2h at 37°C and absorbance at 570 nm was measured.

FIGURE 10: siRNA-mediated knockdown of INrf2 stabilized Bcl-xL and promoted etoposide or TRAIL mediated cell survival. 

A, Cell survival assay. Hepa-1, Hek-293 and HepG2 cells were plated at a density of 5000 cells per well in 24 well plates, and transfected with control or INrf2 siRNA. The cells were then treated with etoposide (Hepa-1 cells 20 μM, Hek-293 cells 1μM and HepG2 cells, 30 μM) for 36h. Cells were incubated with fresh MTT solution for 2h at 37°C and absorbance at 570 nm was measured. 

B, Hepa-1 cells transfected with control or INrf2 siRNA (50μM) for 24 h and then treated with DMSO or etoposide (20 μM) for 36h and harvested. Sixty micrograms of the lysates were immunoblottedted (left panel). One mg of same cell lysates were immunoprecipitated with anti-Bcl-xL antibody and immune complexes were western blotted for anti-ubiquitin (UB-ubiquitin) antibodies (right panel). 

C, Cell survival assay. Jurkat cells were plated at a density of 5000 cells per well in 24 well plates, and transfected with control siRNA or INrf2 siRNA as indicated for 24h. The cells were then treated with human recombinant TRAIL protein (100ng/ml) for 30h. Cells were incubated with fresh MTT solution for 2h at 37°C and absorbance at 570 nm was measured (Lower panel). From the same experiments eight micrograms of Jurkat cell lysates were immunoblotted with anti-INrf2, anti-Bcl-xL and anti-β-actin antibodies (upper panel). Each data point represents a mean ± SD and normalized to the value of the corresponding control cells.

Figure 11. A hypothetical model showing the role INrf2 in control of cellular apoptosis through PGAM5 and Bxl-xL degradation.
### Figure 1

#### A

<table>
<thead>
<tr>
<th>TET Time (h)</th>
<th>pcDNA (1μg)</th>
<th>PGAM5L-V5 (1μg)</th>
<th>Flag-INrf2</th>
<th>PGAM5L</th>
<th>Bcl-xL</th>
<th>β-actin</th>
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<td>+</td>
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<td>+</td>
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#### B

- Con. siRNA 75 nM
- INrf2 siRNA

#### C

- Relative Quantitation of Bcl-xL mRNA
- pcDNA 0.2 0.4 0.6 0.8
- Flag-INrf2 (μg)

- Relative Quantitation of Bcl-xL mRNA
- Mock Con. 25 50 75
- INrf2 siRNA (nM)
Figure 2

A: INrf2
B: PGAM5L
C: PGAM5S

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</tr>
<tr>
<td>Flag-beads</td>
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<td>+</td>
</tr>
<tr>
<td>Input</td>
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<td>+</td>
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<tr>
<td>Con. IgG</td>
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<td>+</td>
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<tr>
<td>Flag-Beads</td>
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</tbody>
</table>

Figure:

- **A**: CBB staining
- **B**: Western Blot (WB) of INrf2 and PGAM5L
- **C**: Immunoprecipitation (IP) and Western Blot (WB) of INrf2 and Bcl-xL
Figure 3

A

**Input**

<table>
<thead>
<tr>
<th>INrf2-293 cells</th>
<th>TET (h)</th>
<th>PGAM5L-V5</th>
<th>HA-Ub</th>
<th>Flag-INrf2</th>
<th>PGAM5L-V5</th>
<th>Bcl-xL</th>
<th>β-actin</th>
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**WB:** HA-Ub

**IP:** IgG
**IP:** PGAM5L-V5

**WB:** HA-HRP

**IP:** IgG
**IP:** Bcl-xL

**WB:** Ub

**IP:** IgG
**IP:** PGAM5L-V5

**WB:** HA-HRP

**IP:** IgG
**IP:** Flag-Bcl-xL

**WB:** HA-HRP

B

**Input**

<table>
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<th>HA-Cul3</th>
<th>Myc-Rbx1</th>
<th>PGAM5L-V5</th>
<th>Flag-Bcl-xL</th>
<th>HA-UB</th>
<th>INrf2</th>
<th>PGAM5L-V5</th>
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<td>+ + +</td>
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</table>

**WB:** HA-Ub

**IP:** IgG
**IP:** PGAM5L-V5

**WB:** HA-HRP

**IP:** IgG
**IP:** Flag-Bcl-xL

**WB:** HA-HRP
Figure 3 Cont.
Figure 4

A

Input

WB: Flag

WB: PGAM5L-V5

WB: Bcl-xL

PGAM5L-V5

Flag-INrf2

Flag-INrf2DGR

Flag-INrf2ΔDGR

IP: anti-Flag ab

IP: IgG

B

Input

WB: Flag

WB: PGAM5L-V5

WB: Bcl-xL

PGAM5L-V5

Flag-INrf2

Flag-INrf2DGR

Flag-INrf2ΔDGR

IP: IgG

IP: PGAM5L-V5

C

Input

WB: Flag

WB: PGAM5L-V5

WB: Bcl-xL

PGAM5L-V5

Flag-INrf2

Flag-INrf2DGR

Flag-INrf2ΔDGR

IP: anti-Flag ab

IP: IgG

IP: Bcl-xL

IP: HA-Ub

IP: PGAM5L-V5

IP: Bcl-xL

IP: HA-Ub
**Figure 5**

**A**

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- PGAM5L
- Bcl-xL
- INrf2

**B**

<table>
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<th>Input</th>
<th>HA-INrf2</th>
<th>PGAM5-V5</th>
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- INrf2-HA
- PGAM5L-V5
- Flag-Bcl-xL
- β-actin

**C**

<table>
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</table>

- Bcl-xL
- PGAM5L
- INrf2
- β-actin
Figure 6

A

INrf2

Bcl-xL

PGAM5L

Nrf2

Cyto. C  DAPI  Merge

B

Con. siRNA  +  -  +  -
PGAM5 siRNA  -  +  -  +

Cyto.  Mito.

PGAM5L

Bcl-xL

INrf2

Nrf2

LDH

Cox IV
Figure 7 Cont.

- **DMSO 2h**
- **tBHQ 2h**

**IP: Bcl-xL**
- **Bax**
- **Bcl-xL**

**Input**
- **C. IgG**
- **C**
- **M**

**Bax**
- **Bcl-xL**

**Bcl-xL:Bax**
- **Bax:Bcl-xL**

**Relative Interaction**
- **Graphs showing relative interaction**

- **Line graph**
  - **DMSO 2h**
  - **tBHQ 2h**
  - **Input**
  - **C. IgG**
  - **C**
  - **M**
Figure 8

A

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Flag-INrf2
PGAM5L-V5
Bcl-xL
Bax
β-actin

B

<table>
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<tr>
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Pc-DNA
Flag INrf2
Etoposide
Flag-INrf2
Flag-INrf2

Cytosolic Cytochrome C
Mitochondrial Cytochrome C
Cox IV

β-actin

C

Hepa-1

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INrf2-293 cells

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pcDNA Flag INrf2 Etoposide
Flag-INrf2 Etoposide
Flag-INrf2+ Etoposide

Flag-INrf2
Caspase 3
Cleaved Caspase 3
β-actin

D

Hepa-1

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INrf2-293 cells

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pcDNA Flag INrf2 Etoposide
Flag-INrf2 Etoposide
Flag-INrf2+ Etoposide

Flag-INrf2
Caspase 3
Cleaved Caspase 3
β-actin
**Figure 10**

**A**

**Hepa-1 cells**

- INrf2 siRNA
- Con. siRNA

% Cell Survival

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**Hek293 cells**

- INrf2 siRNA
- Con. siRNA

% Cell Survival

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**HepG2 cells**

- INrf2 siRNA
- Con. siRNA

% Cell Survival

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**B**

**Con. siRNA (nM)**

- 50 50 -

**INrf2 siRNA (nM)**

- - 50 50

**Etoposide**

- + - +

**IP: IgG**

**IP: Bcl-xL**

**Con. siRNA (nM)**

- 50 50 -

**INrf2 siRNA (nM)**

- - 50 50

**Etoposide**

- + - +

**WB: UB**

**C**

**Jurrat cells**

- INrf2
- Bcl-xL
- β-actin

% Cell Survival

<table>
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**P<0.01**