Supplementary Material

Translational repression protects human keratinocytes from UVB-induced apoptosis through a discordant eIF2 kinase stress response

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Supplemental Figure S1: UVB-induced translational control occurs at early time points and following low doses of UVB in human keratinocytes.

Supplemental Figure S1: N-TERTs were irradiated with the indicated doses of UVB and subjected to polysome profile analysis at 1 (a) or 3 hours (b) of post-UVB irradiation. N-TERTs were irradiated with 600 J/m² UVB and harvested for immunoblot analysis following the indicated incubation times (c). Alternatively, cells were treated with 2 µM TM and harvested after 6 hours. N-TERTs were irradiated with the indicated dose of UVB or 2 µM TM, harvested after 6 hours, and total levels of _ATF4_ (d) and _CHOP_ (e) mRNA were measured by qRT-PCR. Values are presented as averages +/- standard deviation of three separate experiments (*, p<0.01).
Supplemental Figure S2. Sal-003 increases the onset of UVB-induced apoptosis in both N-TERT and primary human keratinocytes

Supplemental Figure S2: (a) Primary human keratinocytes were pretreated with sal-003 or vehicle for 6 hours and then irradiated with the indicated doses of UVB. Following 6 hours in the culture media, cells were assayed for caspase-3 activity. (b) N-TERTs were pretreated with sal-003 or vehicle for 6 hours prior to irradiation with 600 J/m² UVB and assayed for caspase-3 activity at the indicated times post-UVB. Values are presented as averages +/- standard deviation of three separate experiments (*, p<0.01 compared to UVB alone at the indicated dose, #, p<0.01 compared to no treatment group).

Materials and Methods

Cell culture
Normal human keratinocytes were isolated from neonatal foreskin tissue as described previously (Kuhn et al., 1999). N-TERT (Dickson et al., 2000) and normal human keratinocytes were grown in EpiLife media (Invitrogen, Carlsbad, CA) supplemented with human keratinocyte growth supplement (HKGS, Invitrogen) and 1000U Penicillin-Streptomycin (Roche, Indianapolis, IN). N-TERTs stably knocked down for ATF4 or CHOP were produced by transducing cells with lentivirus encoding shRNA against ATF4 or CHOP from validated mission shRNA TRC clones TRCN0000013575 and TRCN0000007264, respectively (Sigma-Aldrich, St. Louis, MO). The ATF4 knockdown cells and their wild-type counterparts were supplemented with extra amino acids and 55 μM β-mercaptoethanol due to an increased sensitivity of ATF4-depleted cells to oxidative stress (Harding et al., 2003).

GADD34 overexpressing cells were produced by transducing N-TERT keratinocytes with a lentivirus encoding a doxycycline-inducible full-length human GADD34 gene with a C-terminal HA tag. This lentivirus was constructed by first PCR-amplifying the GADD34 coding sequence (Thermo Fisher, prepared from plasmid MHS6278-202828370) using the following primers:

F: 5’-GGGGACAAGTTTGTACAAAAAAGCAGGCTGC-3’,
B: 5’-GGGGACCACTTTGTACAAGAAAGCTGGGTCCTA-3’

The resulting GADD34 PCR product was incubated with Donor Vector and BP Clonase enzyme mix (Invitrogen, #11789-020,) to create the GADD34 entry vector, following the vendor’s
instructions. The GADD34 entry vector was then incubated with the LR clonase mix (Invitrogen, #11791-020) and a p-LIX402 lenti vector (gift from David Root, Addgene plasmid # 41394, Cambridge, MA). Primers used to insert GADD34 into p-LIX402 were as follows:

pLIX R: 5’-phospho-CTTATCGTCATCCTTTGTAATC-3’,
pLIX F: 5’-phospho-GAAATGGAAGCCAAAGCTGAAGATTAA-3’,
GADD34 F: 5’-phospho-ATGGCCCCAGCAGCACCACCCAT-3’,
GADD34 R: 5’-phospho-TTATCAGCCACGCCTCCCACTGAG-3’.

GADD34 insertion into p-LIX402 was confirmed by sequencing. Transduced cells were selected for stable expression with 1 μg/mL puromycin. Expression of GADD34 was induced by 24 hours of treatment with 1μg/mL doxycycline hyclate (Sigma) that was confirmed by immunoblot analyses.

**UVB irradiation**

UVB irradiation of N-TERT and normal human keratinocytes was performed using two Philips FS20T12 UVB broadband light sources as described previously (Lewis et al., 2010). The UVB intensity was measured before each experiment using an IL1700 radiometer and a SED240 UVB detector (International Light, Newburyport, MA) at a distance of 8 cm from the UVB source to monolayer of cells. Cells were irradiated in EpiLife media, which filters out UVC wavelengths, and returned to standard incubation conditions (37°C and 5% CO₂).

**Polysome Profiling**

Polysome analysis used 10 to 50% sucrose gradients containing 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 5 mM MgCl₂, and 50 μg/ml cycloheximide as described previously (Palam et al., 2011; Teske et al., 2011). Gradients were made using a tilted tube rotation method on a gradient station equipped with a Piston Gradient Fractionator and a Gradient Master (BioComp, Fredericton, Canada). Irradiated and control cultured keratinocytes were incubated in EpiLife media containing 50 μg/ml cycloheximide for 10 minutes at 37°C prior to harvesting. Cells were rinsed twice with ice-cold PBS containing 50 μg/ml cycloheximide and then lysed with 500 μl of cold lysis buffer containing 20 mM Tris (pH 7.5), 100 mM NaCl, 10 mM MgCl₂, 0.4% Nonidet P-40, 50 μg/ml cycloheximide, and an EDTA-free protease inhibitor cocktail tablet (Roche, Indianapolis, IN). Lysates were sheared with a 1 mL syringe and 23-gauge needle, incubated on ice for 10 minutes, and clarified at 13,000 rpm for 10 minutes. Supernatants were added to the top of the gradients, which were then subjected to ultracentrifugation in a Beckman SW41Ti rotor at 40,000 rpm for 2 hours at 4°C. Polysome profiles and 14 sucrose fractions for each sample were collected with a Piston Gradient Fractionator and a 254-nm ultraviolet monitor with Data Quest software. To analyze specific mRNA transcript shifts following UVB irradiation, RNA was extracted from sucrose fractions via TRIzol Reagent LS (Invitrogen, Life Technologies). Firefly luciferase control RNA (Promega, Madison, WI) was added to each pooled sample before RNA isolation, so that the relative amounts of the transcript of interest to be standardized to an exogenous RNA control (Palam et al., 2011; Teske et al., 2011). To represent percentage total gene transcript for the seven fractions, the 2(−ΔΔCT) value for each fraction was divided by the 2(−ΔΔCT) value sum of all fractions.

**Immunoblot analysis**

For immunoblot analyses, cultured cells were washed with ice-cold PBS and lysed in a solution containing 50 mM Tris-HCl (pH 7.9), 150 mM NaCl, 0.1% SDS, 100 mM NaF, 17.5 mM glycerol phosphate, and 10% glycerol supplemented with protease inhibitors (100 μM phenylmethylsulfonyl fluoride, 0.15 μM aprotinin, 1 μM leupeptin and 1 μM pepstatin). Lysates
were sonicated for 30 seconds and precleared by centrifugation at 13,000 rpm in a microfuge for 10 minutes. Protein concentrations were measured using the Bio-Rad protein quantification kit for detergent lysis (Bio-Rad Laboratories, Inc.; Hercules, CA) and equivalent protein levels were subjected to electrophoresis by SDS/PAGE. Proteins were transferred to nitrocellulose membranes via wet transfer for 2 hours at 80 volts. Membranes were then incubated in PBS supplemented with 5% (w/v) nonfat dried milk for 30 minutes followed by overnight incubation with antibodies for phosphorylated eIF2α at serine 51, total p53, phosphorylated p53 at serine 15 (Cell Signaling Technologies; Danvers, MA; #9721, #9282 and #9284, respectively), monoclonal antibody for total eIF2α (Scott Kimball. Pennsylvania State University College of Medicine, Hershey, PA); CHOP and GADD34 (Santa Cruz Biotechnology; Dallas, TX; sc-575 and sc-8327, respectively), ATF4 prepared against recombinant protein (Zhou et al., 2008), and β-actin (Sigma-Aldrich A5441). Membranes were washed the next day in PBS-Tween for 1 hour followed by 1 hour in milk supplemented with a secondary antibody tagged with horseradish peroxidase, followed by 1 hour in PBS-Tween. Proteins bound to antibody on the membrane were visualized by incubation in a chemiluminescent solution followed by exposure to x-ray film.

**Measurement of mRNA levels by qPCR**

RNA was isolated from cultured keratinocytes using TRIzol reagent (Invitrogen, Life Technologies). Single-strand cDNA synthesis was conducted using the TaqMan reverse transcriptase kit (Applied Biosystems, Life Technologies). mRNA levels were measured by quantitative PCR using transcript-specific TaqMan probes or the SYBR Green (Applied Biosystems, Life Technologies) method for changes in polysome fraction distribution on a Realplex2 Master Cycler (Eppendorf, Hamburg, Germany). To measure the levels of target mRNAs, transcripts were normalized to either 18S rRNA or luciferase control RNA (Promega) for changes in polysome fraction distribution. Values represent three independent experiments, with standard deviations as indicated. Statistical significance was calculated using the two-tailed student’s t test. Taqman probe sets used were ATF4 and 18S rRNA (HS00909569_gl, HS99999901_sl). SYBR green primer sets are as follows:

- **ATF4**: F:5’-TCAAACCTCATGGGTCTCC-3’, R:5’-GTGTCACTCAACGTTGTCAG-3’,
- **CHOP**: F:5’-AGCCAAATCAGCTGGAA-3’, R:5’-ACAAGTTGGCAAGCTGGCTCT-3’,
- **luciferase**: F:5’-CCAGGGATTTCAGTCGATGT-3’, R:5’-AATCTCACGCAGGCAGTTCT-3’,
- **eIF4E**: F:5’-TGTGGCGCTGTTGTTAATGT-3’, R:5’-GCGTGGGACTGATAACCAAT-3’,
- **B2M**: F:5’-ATGAGTATGCGCTGGCTGA-3’, R:5’-GGCATCTCAGGTTTGGCGTACAG-3’,
- **ACTB**: F:5’-GGACTTCCAGCAAGAGATGG-3’, R:5’-AGCAGCTGTTTGGCGTACAG-3’.

**Caspase-3 Assay**

Caspase-3 activity was measured using a synthetic fluorogenic substrate (DEVD-AMC, Alexis Biochemicals, San Diego, CA). Cultured keratinocytes scraped from culture dishes were pelleted and suspended in caspase lysis buffer (50 mM PIPES pH 7.0, 50 mM KCl, 5 mM EGTA, 2 mM MgCl₂, 1 mM DTT) and subjected to three rounds of freeze-thawing (-80°C to 42°C). Cellular debris was removed by centrifugation at 13,000 rpm for 3 minutes. 50 µL of cell lysate was added to 55 µL of caspase-3 reaction buffer (100 mM HEPES, pH7.5, 10% sucrose, 0.1% CHAPS, 10 mM DTT, 0.1 mg/mL bovine albumin, and 50 µM DEVD-AMC substrate) and incubated at 37°C for 45 minutes. Release of the fluorescent AMC moiety was measured using
a Hitachi F2000 spectrophotofluorimeter (excitation, 380nm; emission, 460nm). The fluorescence intensity was converted to pmoles of AMC by comparison to the fluorescent intensity of standards of AMC (7-amino-4-methylcoumarin; Molecular Probes, Eugene, OR). The specific activity of caspase-3 was determined following measurement of the total protein concentration of the cell lysates (Bio-Rad Protein Assay Reagent). Values represent three independent experiments, with standard deviations as indicated. Statistical significance was calculated using the two-tailed student’s t-test.

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