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Supplemental Data

Inactivation of the SMN Complex

by Oxidative Stress

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Supplemental Experimental Procedures

In vitro transcription and labeling of RNAs

In vitro transcription and labeling of RNAs was carried out using MEGAshortscript T7 transcription kit (Ambion) with the modification that 0.65 mM biotin-16-UTP (Roche) and 2.6 mM UTP were used in the reactions to produce biotin-labeled RNAs (Wan et al., 2005).

Cell culture, drug treatment and preparation of cell extracts

HeLa S3 cell pellets were purchased from National Cell Culture Center. Cytoplasmic extracts competent for snRNP assembly were prepared as described (Pellizzoni et al., 2002). Total cell extracts were made by resuspending cell pellets in reconstitution buffer (20 mM HEPES/KOH pH7.9, 50 mM KCl, 5 mM $MgCl_2$, 0.2 mM EDTA) containing 50 μg/ml digitonin (Calbiochem) and 1x Complete EDTA-free Protease Inhibitor Cocktail (Roche), followed by sonication. Nonidet P-40 (NP-40) was added to a final concentration of 0.01%, followed by centrifugation at 10,000 rpm for 15 minutes at 4^oC. Glycerol was then added to the supernatant at a final concentration of 5%. For various drug treatments, two 10 cm plates of HeLa PV cells were treated with each drug at the indicated concentrations and times. Cells were harvested (~90% confluent) and total cell extracts were prepared using the aforementioned procedures. Protein concentrations of various extracts were determined using the Bradford protein assay (BioRad). All of the extracts for the same experiment were adjusted to the same final protein concentration.

Purification of native, RNA-free snRNP proteins

Native, RNA-free total snRNP proteins (TPs) were prepared from HeLa S3 cells (National Cell Culture Center) as described previously (Sumpter et al., 1992).

Gel mobility shift assay and magnetic beads assay for in vitro assembly of snRNPs

In vitro Sm core assembly on ^{32}P-labeled snRNAs by gel mobility shift assay or on biotin-labeled snRNAs by magnetic beads assay for snRNP assembly was performed as described previously (Wan et al., 2005).

Antibodies, quantitative Western blot analysis and indirect immunofluorescence microscopy

Cell extracts containing 20 μg of total proteins were mixed with NuPAGE LDS sample buffer (Invitrogen) with or without reducing agent (Invitrogen), and then separated on NuPAGE 4-12% Bis-Tris gels (Invitrogen) and transferred to nitrocellulose membranes. Quantitative Western blot analysis was performed as suggested by the manufacturer (Li-Cor) and described previously (Wan et al., 2005). Mouse monoclonal antibodies, anti-SMN (62E7), anti-Gemin3 (12H12), anti-Gemin4 (17D10), anti-Gemin5 (10G11), anti-JBP1 (6G8), anti-Magoh (18G12) and anti-Sm (Y12), were used as described previously (Battle et al., 2006; Lerner et al., 1981; Wan et al., 2005). The IRDye800 anti-mouse IgG secondary antibody (Rockland) was used at 1:5000. Indirect immunofluorescence microscopy using monoclonal antibodies 2B1 (anti-SMN) and Y12 was performed as described (Liu and Dreyfuss, 1996).

Fluorescence microscopic detection of ROS

HeLa cells were washed with buffer (modified Hank's balanced salt solution containing 10 mM HEPES, 1.0 mM $MgCl_2$, 2 mM $CaCl_2$, and 2.7 mM glucose adjusted to pH 7.3) and incubated with 10 μM ROS indicator fluorescein dye 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA, Molecular Probes) in buffer at 37°C for 30 minutes. The cells were then washed with buffer and treated with compounds for 30 minutes, followed by washing and immediate acquisition of the fluorescence images using a Zeiss microscope.

Plasmid construction and generation of mutations

Full-length wild-type (WT) human SMN sequence was cloned into pcDNA3 vector at EcoRI / XhoI sites. Deletion of sequences encoding exon 7 (ΔEx7) was made by inserting PCR fragments into UP234 vector at BamHI / XhoI sites. "no Cys" SMN mutant was generated by mutating all eight cysteines in human SMN to alanines by Quickchange site-directed mutagenesis kit (Stratagene). SMN mutants (C60, C98, C123, and C250), which contain single cysteine at amino acid positions 60, 98, 123, 250, respectively, were generated by mutating respective residue in SMN "no Cys" mutant back into cysteine. SMN mutants "Ex3-7" and "Ex1-4" were constructed by inserting SMN sequences corresponding to amino acid 92 to 294 and 1 to 198, respectively, to pcDNA3-myc-pyruvate kinase construct (Siomi and Dreyfuss, 1995) at EcoRI/XhoI site.

Supplemental References

Lerner, E. A., Lerner, M. R., Janeway, C. A., Jr., and Steitz, J. A. (1981). Monoclonal antibodies to nucleic acid-containing cellular constituents: probes for molecular biology and autoimmune disease. Proc Natl Acad Sci U S A *78*, 2737-2741.

Siomi, H., and Dreyfuss, G. (1995). A nuclear localization domain in the hnRNP A1 protein. J Cell Biol *129*, 551-560.