Supplemental Data

c-Jun Downregulation by HDAC3-Dependent Transcriptional Repression Promotes Osmotic Stress-Induced Cell Apoptosis

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Supplemental Experimental Procedures

Cells and Cell Culture Conditions

NIH3T3 mouse fibroblasts; 3Y1 rat fibroblasts; MEK1$^{+/+}$ and MEK1$^{-/-}$ (Giroux et al., 1999), caspase-8$^{+/+}$ and caspase-8$^{-/-}$ (Sakamaki et al., 2002), caspase-9$^{+/+}$ and caspase-9$^{-/-}$ (Kuida et al., 1998), caspase-12$^{+/+}$ and caspase-12$^{-/-}$, and c-Jun$^{+/+}$ and c-Jun$^{-/-}$ mouse 3T3 fibroblasts; MEK2$^{+/+}$ and MEK2$^{-/-}$ (Belanger et al., 2003) and JNK1/2$^{+/+}$ and JNK1/2$^{-/-}$ mouse fibroblasts immortalized with human papilloma virus 16 gene products E6 and E7; and 293T cells were all maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (HyClone, Logan, UT). Chicken B lymphoma cell line DT40 cells and DT40 caspase-7$^{-/-}$ cells were cultured as described previously (Korfali et al., 2004).

Materials

U0126, SP600125, caspase inhibitor Z-VAD-FMK, caspase-3 inhibitor V (Z-DQMD-FMK), and caspase-3/7 inhibitor I were from Calbiochem (San Diego, CA). Active recombinant caspases-1, -2, -3, -4, and -7 were from BioVision (Mountain View, CA). Polyclonal antibodies against HDAC1, HDAC2, HDAC3 (H-99) recognizing aa 330–428, HDAC3 (N-19) recognizing the N terminus, actin, ERK1 (K-23), phospho-MEK1/2, MEK2, HDAC8, and PARP and monoclonal antibodies against phospho-ERK1/2, phospho-c-Jun (KM-1), and FAS-ligand (Kay-10) were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody recognizing HDAC3 aa 411–428 and acetyl-histone H3 were from Upstate Biotechnology (Lake Placid, NY). CHX, sorbitol, mannitol, TSA, NaB, and monoclonal antibodies against FLAG and tubulin were from Sigma (St. Louis, MO). Monoclonal antibodies against c-Jun, c-Fos, and caspase-7, polyclonal antibody against

cytochrome c, and caspase-8 inhibitor Ac-IETD-CHO were from BD Biosciences Pharmingen (San Diego, CA). Polyclonal antibody against caspase-8 was from R&D systems (Minneapolis, MN). Hoechst 33342, Alexa Fluor 488 goat anti-mouse antibody, and Alexa Fluor 594 goat anti-rabbit antibody were from Molecular Probes (Eugene, OR). Polyclonal antibody against V5 was from Abcam (Cambridge, MA).

Immunoprecipitation and Immunoblotting Analysis

Extraction of proteins with a modified buffer from cultured cells was followed by immunoprecipitation and immunoblotting with corresponding antibodies, as described previously (Lu et al., 1998).

Luciferase Reporter Gene Assay

pc-Jun-Luc containing the full-length c-*jun* promoter (Wei et al., 1998) was used for measuring the transcriptional level of c-*jun* using the Dual-Luciferase Reporter Assay kit from Promega (Madison, WI), as described previously (Lu et al., 2003). A Renilla luciferase-expressing plasmid (pRL-TK) was used as an internal control of transfection efficiency. Cells were harvested at the end of sorbitol treatment. The relative levels of luciferase activity were normalized to the levels of untreated cells and to the levels of luciferase activity of the Renilla control plasmid.

DNA Constructs and Mutagenesis

A PCR-amplified human c-*jun* cDNA was cloned into pFLAG vector between *Bam*HI and *Not*I. A PCR-amplified full-length human HDAC3 cDNA and a truncated mutant (aa 1–391) were cloned into pHis8, pCDNA6/V5-HisB, and pFLAG vectors between *Bam*HI and *Not*I. His-HDAC3 D391A, FLAG-HDAC3 D391A, FLAG-c-Jun Δ256-258, and FLAG-c-Jun Δ270-272 were made using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA).

pRETRO-SUPER caspase-7 (pRS caspase-7) was generated with oligonucleotides 5′-GATCCCCATGCCTCTATGTGCCCCGTTTCAAGAGAACGGGGCACATAGAGGCATTTTTTGGAAA-3′ (forward) and 5′-

AGCTTTTCCAAAAAATGCCTCTATGTGCCCCGTTCTCTTGAAACGGGGCACAT AGAGGCATGGG-3′ (reverse). The pRS control was generated with oligonucleotides 5′-GATCCCCAGATGGTGTCACACCAATATTCAAGAGATATTGGTGTGACACCAT CTTTTTTGGAAA-3′ (forward) and 5′-AGCTTTTCCAAAAAAGATGGTGTCACACCAATATCTC TTGAATATTGGTGTGACACCATCTGGG-3′ (reverse).

Purification of Recombinant Proteins

WT His-HDAC3 and His-HDAC3 D391A were expressed in bacteria and purified, as described previously (Lu et al., 2002).

Preparation of Proteins and trichloroacetic acid (TCA) Precipitation

Non-serum medium (3 ml/60 cm dish) was collected and centrifuged at 1200 x g to pellet cell debris. Total protein in the culture medium was precipitated with TCA (final concentration, 15%). The samples were centrifuged at 12,000 x g, and the pellets were washed three times with ice-cold 80% acetone. The precipitated proteins were dried and resuspended in sample loading buffer for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

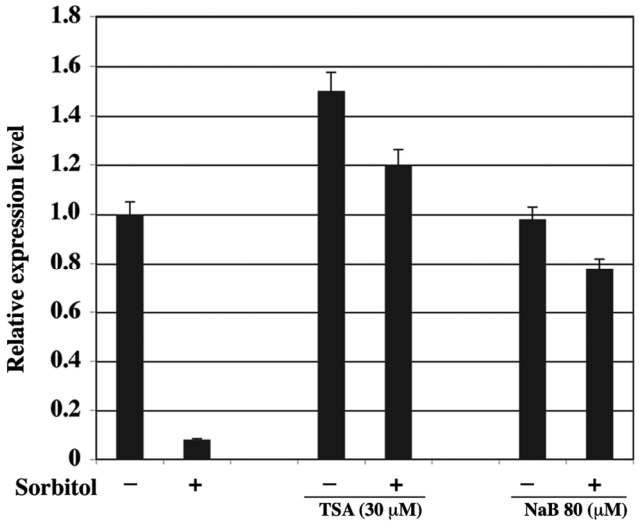

Figure S1. TSA and NaB Blocked Sorbitil-Induced c-Jun Dowrnregulation

Figure 3A was quantified by scanning densitometry. The relative expression levels of c-Jun with or without TSA (30 μM) or NaB (80 μM) were normalized to the levels of c-Jun and actin of untreated cells.

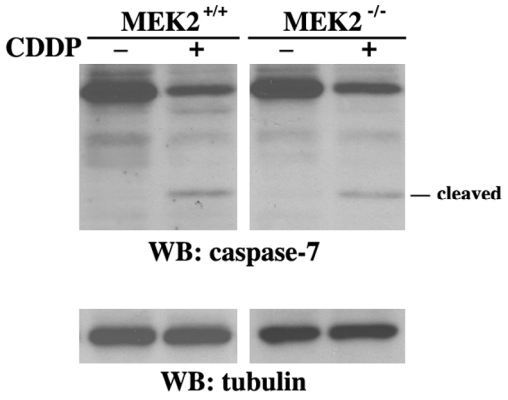

Figure S2. CDDP Treatment Induces MEK2-Independent Caspase-7 Activation

MEK2$^{+/+}$ and MEK2$^{-/-}$ cells were treated with or without CDDP (50 μg/ml) for 12 h followed by immunoblotting analyses with the indicated antibodies.

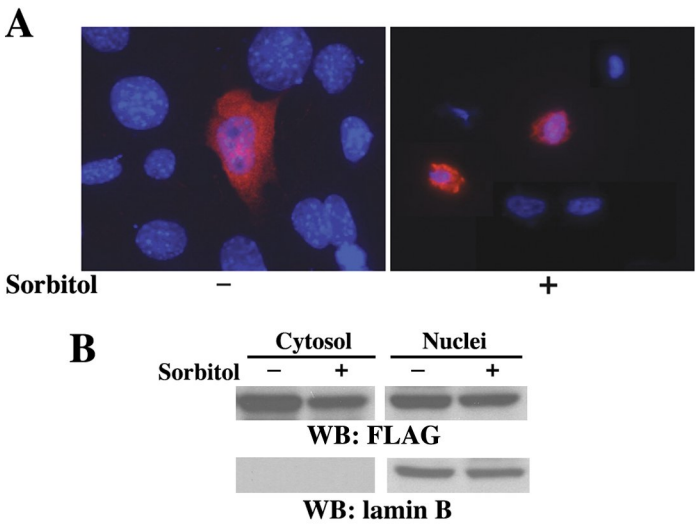

Figure S3. The Cellular Distribution of HDAC3 Is Not Significantly Changed in Response to Osmotic Stress

(A) NIH3T3 cells were transfected with pCDNA6/V5-His-HDAC3, in which V5 is tagged at the C-terminus of HDAC3, for 48 h and then treated with sorbitol for 6 h. Cells were stained with an anti-V5 antibody (red) and Hoechst 33342 (blue).

(B) NIH3T3 cells transiently expressing FLAG-HDAC3 (1-391) were treated with sorbitol for 4 h and cytosol and nuclei fractions were prepared and subjected to immunoblotting analyses with the indicated antibodies. Lamin B is a control nuclear protein.

References:

Belanger, L. F., Roy, S., Tremblay, M., Brott, B., Steff, A. M., Mourad, W., Hugo, P., Erikson, R., and Charron, J. (2003). Mek2 is dispensable for mouse growth and development. Mol Cell Biol *23*, 4778-4787.

Giroux, S., Tremblay, M., Bernard, D., Cardin-Girard, J. F., Aubry, S., Larouche, L., Rousseau, S., Huot, J., Landry, J., Jeannotte, L., and Charron, J. (1999). Embryonic death of Mek1-deficient mice reveals a role for this kinase in angiogenesis in the labyrinthine region of the placenta. Curr Biol *9*, 369-372.

Korfali, N., Ruchaud, S., Loegering, D., Bernard, D., Dingwall, C., Kaufmann, S. H., and Earnshaw, W. C. (2004). Caspase-7 gene disruption reveals an involvement of the enzyme during the early stages of apoptosis. J Biol Chem *279*, 1030-1039.

Kuida, K., Haydar, T. F., Kuan, C. Y., Gu, Y., Taya, C., Karasuyama, H., Su, M. S., Rakic, P., and Flavell, R. A. (1998). Reduced apoptosis and cytochrome c-mediated caspase activation in mice lacking caspase 9. Cell *94*, 325-337.
Lu, Z., Ghosh, S., Wang, Z., and Hunter, T. (2003). Downregulation of caveolin-1 function by EGF leads to the loss of E-cadherin, increased transcriptional activity of beta-catenin, and enhanced tumor cell invasion. Cancer Cell *4*, 499-515.
Lu, Z., Liu, D., Hornia, A., Devonish, W., Pagano, M., and Foster, D. A. (1998). Activation of protein kinase C triggers its ubiquitination and degradation. Mol Cell Biol *18*, 839-845.
Lu, Z., Xu, S., Joazeiro, C., Cobb, M. H., and Hunter, T. (2002). The PHD domain of MEKK1 acts as an E3 ubiquitin ligase and mediates ubiquitination and degradation of ERK1/2. Mol Cell *9*, 945-956.
Sakamaki, K., Inoue, T., Asano, M., Sudo, K., Kazama, H., Sakagami, J., Sakata, S., Ozaki, M., Nakamura, S., Toyokuni, S.*, et al.* (2002). Ex vivo whole-embryo culture of caspase-8-deficient embryos normalize their aberrant phenotypes in the developing neural tube and heart. Cell Death Differ *9*, 1196-1206.
Wei, P., Inamdar, N., and Vedeckis, W. V. (1998). Transrepression of c-jun gene expression by the glucocorticoid receptor requires both AP-1 sites in the c-jun promoter. Mol Endocrinol *12*, 1322-1333.