

## Supplementary materials

### **Lysosomal Chymotrypsin Induces Mitochondrial Fission in Apoptotic Cells by Proteolytic Activation of Calcineurin**

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## **Materials and Methods**

### ***Cell culture, DNA constructs, and transfection***

SH-SY5Y human neuroblastoma cells obtained from ATCC (American Type Culture Collection, Manassas, VA, USA) were cultured in DMEM/F12 (Life Technologies, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C with 5% CO<sub>2</sub>. To construct expression vectors containing full-length or truncated calcineurin, the cDNA for calcineurin was cloned by RT-PCR using human mRNA as the template. The cDNAs for full-length calcineurin and truncated calcineurin (1-410 lacking the C-terminal autoinhibitory domain) were subcloned into the mammalian expression vector pEGFP-C2 (Clontech, Mountain View, CA, USA). The shRNA targeting the calcineurin was provided by RiboBio (Guangzhou, China).

In some experiments SH-SY5Y cells were transfected using Fugene HD reagent (Promega, Madison, WI, USA) according to the manufacturer's instructions.

### ***Expression of recombinant proteins***

Recombinant human chymotrypsin was obtained as previously described (Zhao et al., 2010). Recombinant human calcineurin  $\alpha$  was expressed and purified as previously described (Mondragon et al., 1997).

### ***Intracellular delivery of recombinant chymotrypsin***

Recombinant chymotrypsin was delivered to SH-SY5Y cells using the BioPORTER protein transfection reagent (Gene Therapy Systems; San Diego, CA, USA) as described previously (Zhao et al., 2010).

### ***Immunofluorescence and confocal microscopy***

SH-SY5Y neuroblastoma cells on glass coverslips were fixed with 4% paraformaldehyde in PBS, permeabilized with 0.2% Triton X-100, and blocked for 1

h at room temperature with PBS containing 5% goat serum albumin. Cells were incubated with antibodies (anti-chymotrypsin, Santa Cruz Biotechnology, Santa Cruz, CA, USA; anti -Drp-1, Cell Signaling Technology, Danvers, MA, USA ) and then incubated with the appropriate secondary antibodies. To visualize mitochondria in living cells, cells were stained with MitoTracker Red CMXRos (Life Technologies) at 37°C for 30 min and washed twice with PBS. Nuclei were stained with DAPI (Sigma-Aldrich, St. Louis, MO, USA). Cells were visualized by Z-Stack imaging with a confocal microscope (Olympus FV1000; Tokyo, Japan) and processed using Fluoview software (Olympus). The length of mitochondria was measured using Image-Pro Plus software (Media Cybernetics, Rockville, MD, USA) as described previously (Zhao et al., 2013).

### ***Immunoblotting***

SH-SY5Y cells were lysed in ice-cold RIPA buffer (50 mM Tris-HCl, pH 7.4; 1% NP-40; 150 mM NaCl; 0.25% sodium deoxycholate; 1 mM EDTA; and a protease inhibitor mixture). After ultracentrifugation at 10,000×g for 30 min at 4°C, supernatants containing solubilized proteins were analyzed by immunoblotting. In some experiments, mitochondrial or cytosolic fractions were fractionized by protocols described previously (Zhao et al., 2010). Samples were resolved by SDS-PAGE, transferred to a polyvinylidene fluoride (PVDF) membrane, and blotted with proper primary antibodies (anti-β-actin, Santa Cruz; anti-PARP, anti-cytochrome *c* and anti-Drp1, Cell Signaling Technology; anti- N-terminus of calcineurin, Chemicon, Cambridge, UK; anti-C-terminus of calcineurin, anti-Tim23, BD Bioscience, Sparks, MD, USA. They were then incubated with appropriate peroxidase-conjugated secondary antibodies (Santa Cruz, Santa Cruz, CA, USA) and visualized using a chemiluminescent substrate (GE Amersham Pharmacia ECL; Beijing, China) with

Kodak X-OMAT film (Rochester, NY, USA).

### ***Apoptosis assays***

Cells were harvested by mild trypsinization, washed with cold PBS, and fixed with 70% ethanol. Cells were stained with propidium iodide (PI, Sigma-Aldrich, St. Louis, MO, USA), and the percentage of hypodiploid (apoptotic) cells was measured with a FACSCalibur flow cytometer.

### ***In vitro cleavage of endogenous calcineurin***

SH-SY5Y cells were lysed in ice-cold buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl) by sonication. After ultracentrifugation at 10,000×g for 10 min at 4°C, supernatants containing solubilized proteins were incubated with chymotrypsin at 37 °C for 1 h. Samples were resolved by SDS-PAGE and the specific cleavage of endogenous calcineurin was detected by immunoblotting with antibodies against N- or C-terminus of calcineurin, respectively.

### ***Phosphatase activity assay of calcineurin***

Calcineurin activity was assayed by measuring the hydrolysis of *p*-nitrophenyl phosphate (pNPP; Sigma-Aldrich) at 405 nm, as described below. Briefly, the assay buffer consisted of 50 mM Tris-HCl (pH 7.5), 2.5 mM DTT, 20 mM pNPP and one of the following: (i) 1 mM EDTA for measurement of background activity; (ii) 100 nM EGTA, 1 mM Ca<sup>2+</sup>, 1 mM Mn<sup>2+</sup> for basal activity; (iii) 100 nM EGTA, 1 mM Mn<sup>2+</sup>, 0.8 mM calmodulin for calmodulin-stimulated activity or (iv) 100 nM EGTA, 1 mM Ca<sup>2+</sup>, 1 mM Mn<sup>2+</sup>, 0.8 mM calmodulin for Ca<sup>2+</sup>/calmodulin-stimulated activity. Because calcineurin activity is enhanced in the presence of Mn<sup>2+</sup>, Mn<sup>2+</sup> was included in the assay buffer for conditions ii, iii and iv to aid detection.

## **References**

Mondragon, A., Griffith, E.C., Sun, L., Xiong, F., Armstrong, C., and Liu, J.O. (1997). Overexpression and purification of human calcineurin alpha from *Escherichia*

coli and assessment of catalytic functions of residues surrounding the binuclear metal center. *Biochemistry* 36, 4934-4942.

Zhao, J., Zhang, J., Yu, M., Xie, Y., Huang, Y., Wolff, D.W., Abel, P.W., and Tu, Y. (2013). Mitochondrial dynamics regulates migration and invasion of breast cancer cells. *Oncogene* 32, 4814-4824.

Zhao, K., Zhao, X., Tu, Y., Miao, Q., Cao, D., Duan, W., Sun, Y., Wang, J., Wei, T., and Yang, F. (2010). Lysosomal chymotrypsin B potentiates apoptosis via cleavage of Bid. *Cell Mol Life Sci* 67, 2665-2678.