EBP1 regulates Suv39H1 stability via the ubiquitin-proteasome system in neural development

Running title: EBP1 controls protein stability of Suv39H1

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SUPPLEMENTAL MATERIALS

MATERIALS AND METHODS

MEF, HEK293T, and PC12 cell culture

E13.5 mouse embryos were isolated and placed in ice-cold phosphate-buffered saline (PBS). For tissue digestion, 500 μL of trypsin-EDTA (25200-072, Gibco) was added to each embryo for 1 min and then minced, followed by the addition of 3 mL complete medium containing 10% fetal bovine serum (FBS, 16000-044, Gibco). The supernatant was transferred to a new tube after 3 min and centrifuged. The pellet was retained and cells were resuspended in 6 mL complete medium. HEK293T cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS (35-015-CV, Corning) and 100 units of penicillin/streptomycin solution. PC12 cells were cultured in DMEM containing 10% FBS (Gibco), 5% horse serum (16050-122, Gibco), and 100 units of penicillin/streptomycin. Cells were incubated at 37 ℃ with 5% CO_2.

Plasmid constructs, si-*Ebp1*, and antibodies

Human *EBP1* was cloned in a pCDNA3 vector with Flag and Myc tags and in a pEGFP-c2 vector. Mouse Suv39H1 WT, domain constructs, and deletion forms were generated in a pCDNA3 vector with GST and in a pEGFP-c2 vector. The △chromo construct was missing amino acids 43 - 88 and the △SET mutant was missing amino acids 243 - 370 of the full-length Suv39H1. *Ebp1* siRNAs (si-*Ebp1*) were provided by IDT MBiotech. Duplex sequences were 5'-GCAGGACAGAGAACCACUAUUUACA-3'. Anti-EBP1 (ab180602) and anti-H3K27ac (ab4729) antibodies were acquired form Abcam (Cambridge, MA, USA). Anti-Suv39H1 (D11B6) antibody was purchased from Cell Signaling Technology (Danvers, MA, USA), whereas anti-β-actin (sc-47778), anti-GST (sc-138), anti-GFP (sc-9996), anti-HA (sc-7392), and anti-c-Myc (sc-40) antibodies were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). The anti-Flag antibody (M185-3L) was purchased from MBL International (Woburn, MA 01801), and the

anti-H3K9 me3 antibody (07-442) was obtained from Millipore (Burlington, MA, USA).

GST pull-down assay

Cells were rinsed with PBS and lysed in protein-lysis buffer containing 50 mM Tris-Cl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, 10 mM β-glycerol phosphate, and a cocktail of protease inhibitors (1.5 mM Na_3VO_4 and 1 mM PMSF). Cell extracts were centrifuged at 25,000 rpm for 10 min at 4 ℃. Proteins were quantified using Bradford solution (#5000006, Bio-Rad, Hercules, CA, United States) and mixed with glutathione agarose resin (#G-250-10, GoldBio U.S Registration No 3,257,926) with 0.5 - 1 mg of lysate. After 3 h incubation at 4 ℃ with gentle agitation, the resin was washed in lysis buffer, mixed with 2× SDS sample buffer, and boiled. An anti-GST antibody was used for immunoblotting.

Co-immunoprecipitation assay

Cells were rinsed and lysed as described in the previous section. Following quantification, 0.5 - 1 mg protein was mixed with A/G beads (1104-3, Incospharm) and primary antibody, and incubated at 4℃ for 3 h with gentle agitation. The beads were washed in protein lysis buffer and mixed with 2× SDS sample buffer.

Subcellular fractionation

The subcellular fractionation protocol was modified from (1). Transfected cells were rinsed in PBS and mixed in 500 μL cytosolic buffer containing 250 mM sucrose, 20 mM HEPES (pH 7.4), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, and proteinase cocktail. A 1 mL syringe (25 gauge) was used to resuspend the lysates and the mixture was agitated during 30 min at 4 ℃, followed by centrifugation at 720 × *g*. The supernatant was transferred to a new tube. The process was repeated using 500 μL of cytosolic buffer with the remaining pellet and

the second supernatant was added to the first one. The cell pellet was then resuspended in nuclear buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 10% glycerol, and proteinase cocktail, and agitated at 4 ℃ for 15 min. The mixture was centrifuged at 10,000 × *g* for 10 min at 4 ℃. Nuclear and cytosolic extracts were quantified, and samples were prepared for western blotting.

Immunocytochemistry

MEF cells for immunocytochemistry were raised on coverslips in 24-well plates and fixed in 4% paraformaldehyde for 5 min. Cells were permeabilized in PBS containing 0.25% Triton X-100 for 1 h at room temperature, followed by blocking with 1% BSA for 1 h at room temperature. Next, cells were immunostained with primary antibodies (H3k9 me3 and H3k27ac), followed by secondary antibodies conjugated with Alexa-Fluor 594. The nuclei of cells were counterstained with DAPI. A confocal microscope (LSM710, Carl Zeiss) was used to acquire immunostained images.

Statistical analysis

ImageJ(https://imagej.nih.gov/ij/) was used to measure the density of figures. The generation of associated graphs and statistical analysis were performed using GraphPad Prism ver 9.0.1 (La Jolla, CA, USA). Experiments were conduced at least three times for statistical validity, and comparisons between two experimental groups were analyzed using two-tailed Student's t-tests. P-values < 0.05 were considered statistically significant.

REFERENCES

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