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Regulation of signal transducer and activator of transcription 3 activation by dual-specificity phosphatase 3

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Running Title: DUSP3 Regulates STAT3 by dephosphorylation at Y705

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SUPPLEMENTARY MATERIALS AND METHODS

Reagents and antibodies

Monoclonal anti-FLAG antibody, anti-FLAG conjugated M2 beads, sodium orthovanadate (Na_3VO_4), sodium fluoride (NaF), phenylmethane sulfonyl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA), Triton X-100, IGEPAL, and glycerol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Calf intestinal alkaline phosphatase (CIAP) was from Takara Bio Inc. (Takara, Shiga, Japan). Anti-phospho (p)-STAT3 (Tyr705) antibodies were from Cell Signaling Technology (Danvers, MA). Protein A/G PLUS-agarose immunoprecipitation reagent, anti-STAT3, anti-tubulin, anti-DUSP3 (VHR) antibodies, and anti-glutathione S transferase (GST) antibodies were from Santa-Cruz Biotechnology (Santa Cruz, CA). Goat anti-mouse and goat anti-rabbit secondary antibodies were from AbFrontier (Seoul, Korea). Recombinant human IL-6 protein was from R&D Systems (Minneapolis, MN, USA).

Plasmid constructs and transfection

For the overexpression of each gene in mammalian cells, FLAG-DUSP3 wild type (WT), HA-STAT3, and FLAG-STAT3 were constructed in pcDNA3.1/Zeo plasmid (Invitrogen, Carlsbad, CA). FLAG-DUSP3 C124S (CS), a catalytically inactive mutant, was generated from FLAG-DUSP3 WT using a QuikChange II Site-Directed Mutagenesis Kit (Agilent Technology, Santa Clara, CA, USA). For the construction of

GST-tagged STAT3 domains, the domains of STAT3 were amplified by PCR and constructed in pEBG plasmid (Addgene #22227). For the purification of recombinant His-tagged DUSP3 proteins, DUSP3 WT or CS was constructed in pET28a plasmid (Novagen, Madison, WI).

For the transient transfection, the cells were seeded in 60 mm cell culture dishes at a density of 70% and transfected with linear polyethylenimine (PEI; Polysciences, Inc., Warrington, PA) or Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA) following the manufacturer's instructions.

Immunoprecipitation and immunoblot analysis

HEK 293 cells were seeded in 60 mm dishes and co-transfected with 2 μg of FLAG-DUSP22 or FLAG-DUSP3 and 2 μg of HA-STAT3. After 48 h, transfected cells were lysed in PTP lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 0.5% IGEPAL, 10% glycerol, 1 mM PMSF). Cell lysates were incubated with anti-FLAG conjugated M2 beads for 4 h at 4℃ on a rotator. After washing the beads six times with fresh PTP lysis buffer, the proteins were eluted by boiling with 1 X SDS-PAGE sample buffer (62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 1% SDS, 1% β-mercaptoethanol, 0.01% bromophenol blue).

For the immunoprecipitation of endogenous DUSP3 and STAT3, Hep3B cells were seeded in 150 mm dishes. The cells were harvested, and then the collected cells were lysed by PTP lysis buffer. Cell lysates were incubated with normal mouse

IgG and protein A/G PLUS-Agarose beads for 30 min at 4 ℃ on a rotator. After centrifugation, the transferred supernatants were incubated with anti-DUSP3 antibody or normal mouse IgG (for a negative control) for 1 h at 4 ℃ by rotation. The samples were incubated for an additional 4 h at 4 ℃ with the addition of protein A/G PLUS-Agarose beads. The elution of samples was performed as described above.

Boiled samples were separated in 12% SDS-PAGE gel and transferred onto a nitrocellulose membrane. The membrane was blocked with 5% skim milk and incubated with appropriate primary antibodies (1:1,000-2,500) overnight at 4 ℃. Then, the membrane was washed twice with 1 X tris-buffered saline containing 0.5% Tween20 (TBST) and incubated for 1 h with secondary antibodies (1:5,000) at room temperature. The membranes were developed by an enhanced chemiluminescence reagent (Pierce, Rockford, IL).

Purification of recombinant proteins and *in vitro* phosphatase assay

For the purification of recombinant proteins, pET28a His-tagged DUSP3 WT or CS was transformed into *E.coli* (BL21 strain) and the transformed bacteria were grown overnight with a kanamycin selection marker (50 μg/mL). When the bacterial concentration was between 0.6-0.8 at OD_{600}, 1 mM of isopropyl-β-D-1 thiogalactopyranoside (IPTG) was treated to the cells to induce the expression of the recombinant proteins for 3 h at 37 ℃. After the IPTG induction, the cells were

harvested and lysed with *E.coli* lysis buffer [50 mM Tris-HCl (pH 6.8), 0.3 M NaCl, 1% IGEPAL, 1 mM PMSF] by sonication. Soluble cell lysates were collected by centrifugation and incubated with Ni-NTA beads for 1 h at 4 ℃ on a rotator. After incubation, beads were washed three times with wash buffer [20 mM Tris-HCl (pH 6.8), 0.5 M NaCl, 50 mM imidazole] and the recombinant proteins were eluted with elution buffer [20 mM Tris-HCl (pH 6.8), 0.5 M NaCl, 250 mM imidazole]. The phosphatase activity was evaluated by fluorescence value according to the phosphatase-dependent hydrolysis of 3-*O*-methylfluorescein phosphate (OMSF).

For the *in vitro* phosphatase assay, HEK 293 cells were transfected with FLAG-STAT3 for 48 h and stimulated with IL-6 (20 ng/mL) for 1 h. After stimulation, cells were harvested and lysed by PTP lysis buffer. Soluble cell lysates were incubated with anti-FLAG conjugated M2 beads for 4 h, and then FLAG-STAT3 bound beads were aliquoted to each sample tube. Each sample was mixed with His-DUSP3 WT or CS (1 μg) in 100 μl PTP reaction buffer [100 mM Tris-HCl (pH 7.5), 40 mM NaCl, 1 mM DTT] and incubated at 37 ℃ for 1 h. The reaction was stopped by boiling with 5 X sample buffer. The eluted samples were then separated on 12% SDS-PAGE gel and analyzed by immunoblot analysis with specific antibodies.

Luciferase assay system

HEK 293 cells were seeded in 100 mm cell culture dishes and incubated overnight. Cells were co-transfected with STAT-luc (300 ng/well) and gWIZ-GFP (100 ng/well).

After 6 h transfection, cells were split to 12 well plates and grown overnight. Then, cells were transfected with FLAG-DUSP3 WT, FLAG-DUSP3 CS, or FLAG-DUSP22 for 24 h. The culture medium was changed to a fresh medium and the transfected cells were incubated in the absence or presence of IL-6 (20 ng/mL) for 24 h. Luciferase assay system (Promega, Madison, WI, USA) was used according to the manufacturer's instructions. Luminescence was measured using a Synergy H1 Microplate Reader (BioTek Instruments).

Reverse transcription PCR (RT-PCR)

HEK293 cells were seeded in 12-well plates and transfected with the indicated plasmid. Cells were treated with IL-6 (20 ng/mL) for 48 h. After the isolation of RNA and the synthesis of cDNA, PCR was run for 18-30 cycles at 94 ℃ for 30 s, 60 ℃ for 30 s, and 72 ℃ for 1 min using a thermal cycler (Bioer Technology Co., Hangzhou, China). Following amplification, 10 μl of the amplified PCR products were separated in 2% (w/v) agarose gels and visualized by EtBr staining. The PCR primers used in these experiments were as follows: *HK2* sense primer, 5'-GGCTCTGATCCGGAAGGCCA-3' and antisense primer, 5'-CGGAGGAAGCGGACATCGCA-3'; *HIF1A* sense primer, 5'-GCGGCGCGAACGACAAGAAA-3' and antisense primer, 5'-TCCAGGCTGTGTCGACTGAGGA-3'; *MYC* sense primer, 5'-GAGTTTCATCTGCGACCCGGAC-3' and antisense primer, 5'-CCTTTTGCCAGGAGCCTGCC-3'; *MMP2* sense primer, 5'-

CCTGAACACCTTCTATGGCTGC-3' and antisense primer, 5'-CCCATACTTCACACGGACCAC-3'; *GAPDH* sense primer, 5'-GCTCTCTGCTCCTCCTGTTC-3' and antisense primer, 5'-ACGACCAAATCCGTTGACTC-3'.

Construction of DUSP3 WT or CS stable SK-Hep1 cell line

For making FLAG-DUSP3 WT or CS-stably expressing SK-Hep1 cells, HEK 293T cells were transiently transfected with FLAG-DUSP WT or CS-containing lentiviral transduction plasmid (pLVX-EF1-IRES-Puro; Clonetech, Japan) with packaging vectors (pMD2.G and psPAX2; Clonetech). After 24 h of transfection, the cells were washed twice with PBS and fed with fresh cell culture medium. After incubation for 2 days, the culture medium was collected and filtered with a 0.2 μm syringe filter (Sartorius, USA). SK-Hep1 cells were incubated with the filtered medium containing lentivirus for 24 h and washed three times with PBS. After incubation with fresh culture medium for 3 days, the infected SK-Hep1 cells were selected by puromycin (2 μg/mL) over 2 weeks. The evaluation of DUSP expression in selected SK-Hep1 cells was performed using immunoblot analysis with FLAG specific antibody.

Wound healing assay

SK-Hep1 cells that stably express FLAG-DUSP3 WT or CS were seeded in 6-well plates (1.2×10^6 cells/well). The wound was created with a scratcher tip (0.5 mm;

SPL Life Sciences, Gyeonggi-do, Korea). Cells were incubated in 1% FBS media for 36 h with 20 ng/mL of IL-6. The cell migration was observed with a JuLI Stage Real-Time Cell History Recorder (NanoEnTek Inc., Seoul, Korea). The wound closure represents the percent of wound recovery. All experiments were performed in triplicate.