Dual Specificity Phosphotase 18, Interacting with SAPK, Dephosphorylates SAPK and Inhibits SAPK/JNK Signal Pathway *in vivo*

Qihan Wu [1], Shengdong Huang [2], Yaqiong Sun [1], Shaohua Gu [1], Fanglin Lu [2], Jianfeng Dai [1], Gang Yin [1], Liyun Sun [1], Dan Zheng [1], Chao Dou [1], Congjing Feng[1], Chaoneng Ji [1], Yi Xie [1] and Yumin Mao [1]

[1] State Key Laboratory of Genetic Engineering, Institute of Genetics, School of Life Science, Fudan University, Shanghai 200433, People's Republic of China, [2] Department of Cardiothoracic Surgery, Changhai Hospital, Second Military Medical University, Shanghai, People's Republic of China

1. ABSTRACT

The SAPK/JNKs play important roles in numerous cellular processes, and for this reason they have become putative drug targets. Most dual-specificity protein phosphatases (DSPs) play important roles in the regulation of mitogenic signal transduction and cell cycle control in response to extracellular stimuli. Dual-specificity phosphatase 18 (DUSP18), a newly recognized SAPK/JNK phosphatase, is widely expressed. This expression is modulated in response to extracelluar stimuli. By phosphorylation assay, pull down and coimmunoprecipetation experiments, it is shown here that DUSP18 interacts with SAPK/JNK and dephosphorylates it both *in vitro* and *in vivo*. DUSP18 does not dephosphorylate p38 or p44ERK1. Furthermore, DUSP18 inhibits SAPK/JNK pathway *in vivo*. Based on these findings, DUSP18 appears to serve an important role by regulation of SAPK/JNK pathway.

2. INTRODUCTION

Reversible protein phosphorylation is a ubiquitous mechanism for the control of signal transduction networks that regulate diverse biological processes of almost all aspects of cell life in eukaryotes including response to extracellular stimuli, DNA damage and cell growth and division. In particular, protein phosphorylation is a major mode of respone in signal transduction pathways. Processes that are reversibly controlled by protein phosphorylation require not only a protein kinase but also a protein phosphatase (1).

Dual-specificity protein phosphatases (DSPs) constitute a new family of protein tyrosine phosphatases (PTPs) characterized by their ability to dephosphorylate both phospho-tyrosyl and phospho-seryl/threonyl residues (2). They may also play an important role in mitogenic signal transduction and cell cycle control (3).

More than 20 mammalian DSPs have now been identified, most of which are reported to be able to dephosphorylate the phospho-threonyl and –tyrosyl residues in the T-X-Y motif within kinase domain VIII of the mitogen-activated protein kinase (MAPK) family and as a result cause inactivation of their kinase activity (2). These enzymes display differences in relative specificity for MAPK family members, tissue distribution and subcellullar localization (3). Moreover, some DSPs are expressed constitutively, whereas others are induced only by some types of cell stimulation (3).

The Mitogen-Activated Protein Kinase is a low abundance serine/threonine protein kinase, which can be transiently activated in many cell types by a variety of "mitogens", including insulin, epidermal growth factor, phorbol esters, antigens, fluoride, etc (4). It is an important signal transducing enzyme that is involved in many facets of cellular regulation (5, 6). The MAPK pathways transduce a large variety of external signals, leading to a wide range of cellular responses, including growth, differentiation, inflammation and apoptosis (7).

We cloned DUSP18 and initially characterized it in 2003 (8). And we took further study to understand how DUSP18 functions *in vivo*. Here we report that DUSP18 could interact with SAPK/JNK and dephosphorylate it both *in vitro* and *in vivo*. Further more, DUSP18 could inhibit SAPK/JNK pathway *in vivo*.

3. MATERIALS AND METHODS

3.1. Cell cultures, transfection, cell lysis and western blotting

HEK-293, NIH3T3,COS-7, Hela, L-02, QGY-7703, BEL-7402 and HepG2 cells obtained from Shanghai Institute of Cell Biology and Biochemistry (Shanghai, China), were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. The cells were split on 35-mm dishes at 1×10^6/dish. After 24 h, the cells were transfected using lipofectAMINE 2000 (Invitrogen) according to the manufacturer's protocol. Prior to stimulation, cells were serum starved for at least 18 h. The transfected cells were lysed as described (9) and subjected to SDS-page followed by blotting onto PVDF membranes (Schleicher & Schuell). The membranes were probed with the indicated antibodies and visualized with an enhanced chemiluminescence (ECL) detection kit (Amersham Pharmacia Biotech).

3.2. RNA isolation and reverse transcriptase PCR amplification

Total cellular RNA was isolated using Trizol (Biodev) according to the manufacturer's protocol. One μg of total RNA from cells was used to generate first strand cDNA after an initial annealing reaction to 0.1 μg of random hexamers at 70 °C for 10 min. Following equilibration to ambient temperatures, a buffer containing 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl2, 10 mM dithiothreitol, 500 μM of each of four dNTPs, and 200 units/μg of Superscript reverse transcriptase (Gibco BRL) was added, and the mix was incubated at 37 °C for 1 h. The reaction was terminated by placing the tubes on ice, and the cDNA was recovered by ethanol precipitation. The pellet was washed with 70% ethanol and resuspended in 100 μl of 5 mM Tris, 0.5 mM EDTA mix.

3.3. Expression pattern analysis of DUSP18

The first strand cDNAs generated from HEK-293, NIH3T3, COS-7, Hela, L-02, QGY-7703, BEL-7402 and HepG2 cells and two human Multiple Tissue cDNA panels (Clontech) were used as PCR templates. The DUSP18 and G3PDH primers are as listed in Table 1. Twenty-four cycles (for G3PDH) or thirty-two cycles (for DUSP18) of amplification (30 s at 94°C, 30 s at 65°C and 1 min at 72°C) were performed using Taqplus DNA polymerase (Sangon). The PCR products of DUSP18 and G3PDH were then electrophoresed on a 2% agarose gel.

3.4. Analysis of expression change of DUSP18 in response to serum stumili

Real-Time PCR was performed to investigate the expression level of DUSP18 and MAPKs after the stumili of serum. HEK293 cells were treated with 10% serum after 18 hour starvation and collected in different time after stumili. Total RNA samples were then extracted and reverse-transcribed according to the protocol described above. The resulting cDNA was used in subsequent real-time PCR reaction after ten times dilution. Real-time PCR was carried out using an ABI 7900HT sequence Detection System in 384-well reaction plates using ABSOLUTE™ QPCR SYBR GREEN LOW ROX MIXES (ABgene UK). All primers were designed using Prime Primer 5 software (PREMIER Biosoft International) (Table 1). Samples were analyzed in triplicate; the threshold cycle (Ct) method, as described in the manufacturer's protocol, was used to generate relative expression values. Results were expressed relative to G3PDH control. All the experiments repeated 3 times independently.

3.5. Subcellular localization of DUSP18

HEK-293 cells were transiently transfected with pCDNA4/His/Myc-DUSP18. After 48h, transfected cells were fixed with 3.7% formaldehyde in PBS for 10min at room temperature. After PBS washing, the cells were permeabilized in 0.5% Triton X-100 in PBS and washed with PBS three times. The cells were in sequence incubated with an anti-Myc antibody (CLONTECH) and an anti-rabbit IgG-FITC secondary antibody (Santa Cruz Biotechnology) in 5% bovine serum albumin in PBS. After three washes with PBS and two washes with Milli-Q water, coverslips were mounted. Fluorescence was viewed with a Leica fluorescence microscope.

3.6. Bacterial expression and purification of recombinant DUSP18

For bacterial expression, the open reading frame of DUSP18 was amplified by PCR and subcloned into pGEX4T1 (Amersham Pharmacia Biotech). E.coli cells transformed with pGEX-DUSP18 were grown overnight to saturation in 10ml of LB medium containing 50μg/ml ampicillin. The cells were grown in 3 liters of LB medium containing 50μg/ml ampicillin at 37°C to reach A600 nm=0.6. One hour after the temperature shift to

Table 1. Sequences of primers

Primers	Sequence
G3PDH Forward	5'-TGAAGGTCGGAGTCAACGGATTTGGT-3'
G3PDH Reverse	5'-CATGTGGGCCATGAGGTCCACCAC-3'
DUSP18 Forward	5'-TGCTATGGAAACCAAAGCGTTA-3'
DUSP18 Reverse	5'-GTCAGCAGTCAGCGAAGCAC-3'
p54SAPKbeta Forward	5'-AGCGGACTCCGAGCACAATAA-3'
p54SAPKbeta Reverse	5'-TCCACTTCGGCTGGGTCATAC-3'
p44ERK1 Forward	5'-CGCTACACGCAGTTGCAGTACA-3'
p44ERK1 Reverse	5'-AAGCGCAGCAGGATCTGGA-3'
p38 Forward	5'-TGTTCCCAAATGCTGACTCCAA-3'
p38 Reverse	5'-TCGGGTCGTAATACTGCTCCAGATA-3'

25°C, isopropyl-beta-D-thiogalactopyranoside was added to a final concentration of 400μM, and cells were cultured for 9h. Purification of GST-DUSP18 was performed by the method described previously (8). The yield of GST-DUSP18 was about 18 mg from 3 liters culture.

3.7. Phosphorylation assay *in vitro*

pMT3-HA-p38, pCDNA1-HA-p44ERK1, and pMT2-HA-p54SAPKbeta were kindly provided by Drs. D. Chadee (Harvard University, Boston, USA), J. Pouyssegur (University of Nice, Nice, France), and J. Woodgett (Ontario Cancer Institute, Ontario, Canada), respectively. HEK-293 cells were transfected with HA-p44ERK1, HA-p38, or HA-p54SAPKbeta. After stimulation, the cells were lysed in 500μl lysis buffer for immunoprecipitation (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 40 mM bglycerophosphate, 10 mM NaF, 1% [vol/vol] IGEPAL, 0.1% deoxycholate, 100μM ATP, 100 μMMgCl2, 2mM pefabloc, and complete protease inhibitor cocktail; Roche Diagnostics). HA tagged MAPKs were immumoprecipitated and then subjected to an *in vitro* dephosphorylation assay. HA-tagged proteins were probed by anti-HA antibody (Santa Cruz Biotechnology). Phosphorylated p44-ERK1, p38 and p54SAPKbeta were detected with specific antibodies (Cell Signaling Technology and Santa Cruz Biotechnology).

3.8. Phosphorylation assay *in vivo*

HA-tagged p44ERK1, HA-tagged SAPKbeta and HA-tagged p38 expression plasmids were co-transfected with Myc-DUSP18 expression plasmid or pCDNA4/His/Myc control plasmid respectively. 48 hours after transfection, cells were stimulated and lysed in lysis buffer. Extracts were prepared by centrifugation at 20,000xg for 15min. Each sample (50ug of protein) was separated by SDS-polyacrylamide gel and transferred to PVDF membranes. The phosphorylation status of activated MAPKs was monitored by anti-phospho-p44/42ERK, anti-phospho-JNK and anti-phospho-p38 antibody (Cell Signaling Technology and Santa Cruz Biotechnology), followed by a horse radish peroxidase conjugated donkey anti-rabbit IgG secondary antibody.

3.9. Pull Down

HEK293 cells were transfected with HA-p54SAPKbeta expression plasmid (2μg/5 x 106 cells) by lipofectamine2000TM (Invitrogene) and cultured in complete medium for 48 h. Cells were lysed in lysis buffer, and 1 ml of lysate containing 0.5 mg of protein was incubated with 10 μl of packed glutathione-agarose beads coated with 4 μg of GST-DUSP18 fusion or GST proteins for 30 min. Beads were washed three times in lysis buffer, and GST fusion proteins were eluted in 10 mM glutathione. Coeluted HA-tagged SAPKbeta were detected by western blotting as above using anti-HA mAb (Santa Cruz Biotechnology).

3.10. Co-immunoprecipitation

HEK293 cells grown on 60mm dishes transfected with Myc-DUSP18 and HA-p54SAPKbeta expression plasmids were collected and the cells were lysed in 500μl lysis buffer for immunoprecipitation. For immune coprecipitations, 2μg of rabbit polyclonal anti-Myc antibody (Clontech) and 20 μl protein G/A Sepharose (Santa Cruz) were incubated with 500 μl lysis buffer for 2 h at 4°C. Beads were washed twice with 1 ml lysis buffer and then twice with 1 ml wash buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 40 mM bglycerophosphate, 10 mM NaF, 0.1% [vol/vol] IGEPAL, and 1 mM Pefabloc). Bound proteins were eluted in 50 μl of sample buffer and then analyzed by Western blotting.

3.11. Pathway profiling assay

A Mercury pathway profiling system (Clontech) and a Dual-Luciferase reporter assay system (Promega) was used to assess signal transduction pathways that could be influenced by DUSP18. HEK293 cells were split on 24-well plate at 4X104/well. After 24 h, pCDNA4-DUSP18 or pMT2-HA- p54SAPKbeta and each cis-acting luciferase reporter vector of the Mercury Pathway Profiling System (Clontech) were co-transfected into cells by the lipofectAMINE 2000 (Invitrogen) according to the manufacturer's protocol. Relative transfection efficiency was determined by cotransfection with plasmid Renilla luciferase reporter vector (Promega). After 48 h, the transfected cells were lysed and the Luciferase activity was measured using the Dual-Luciferase reporter assay system (Promega), and separate luciferase readings were taken for both the reporter plasmid and the Renilla luciferase reporter plasmid. All the experiments repeated 3 times independently.

4. RESULTS

4.1. Expression pattern of DUSP18

After the cloning of *DUSP18* in 2002, we have focused on the functional research of this gene *in vivo*. As most DSPs act on MAPKs, the relation between MAPKs and DUSP18 was primarily studied.

First the expression pattern of *DUSP18* was investigated. RT-PCR revealed that the *DUSP18* was widely expressed in 16 normal adult tissues tested (which was shown in our paper published in 2003) (8). And further study also showed that *DUSP18* was expressed in 8 normal fetal tissues (Heart, Spleen, Kidney, Liver, Lung, Brain, Thymas and Skeletal Muscle), 8 tumor tissues (Breast Carcinoma GI-101, Lung Carcinoma LX-1, Colon
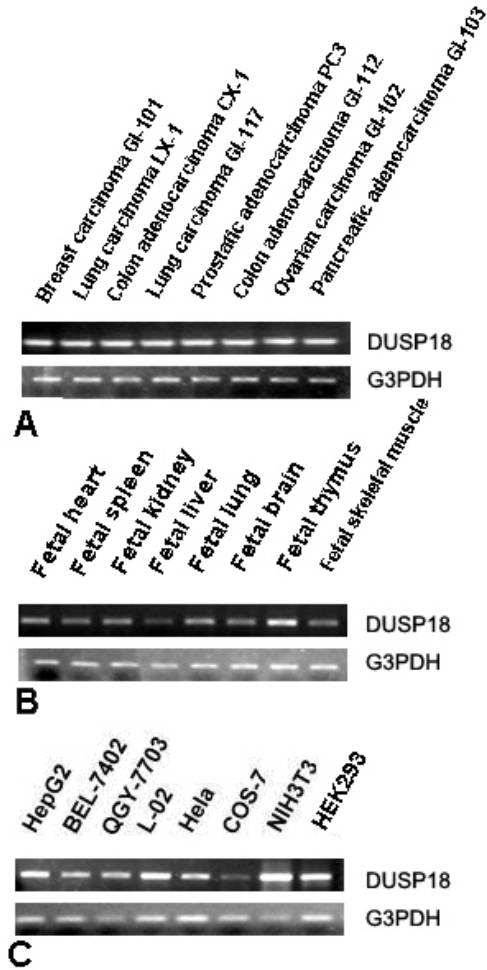
Figure 1. Expression pattern of *DUSP18* investigated by RT-PCR. Reverse transcription-PCR analysis of human cDNA for *DUSP18* and *G3PDH* (as a control). (A) Prenormalized cDNAs from eight human tumor tissues was purchased from Clontech and employed as a template in PCR reactions.(B) Prenormalized cDNAs from eight human fetal tissues was purchased from Clontech and employed as a template in PCR reactions. (C) First strands of cDNA from eight cell lines were employed as a template in PCR reactions.

Adenocarcinoma CX-1, Lung Carcinoma GI-117, Prostatic Adenocarcinoma PC3, Colon Adenocarcinoma GI-112, Ovarian Carcinoma GI-102 and Pancreatic Adenocarcinoma GI-103) and 8 cell lines (HEK-293, NIH3T3, COS-7, Hela, L-02, QGY-7703, BEL-7402 and HepG2)(Figure 1).

By searching public genechip database GeneAtlas (http://symatlas.gnf.org/SymAtlas/), *DUSP18* proved to be expressed in every tissue and cell line at similar expression levels (Figure 2.A). The database was constructed as described in the article published in *PNAS* in 2002 (10). The expression levels of *p54SAPKbeta*, *p44ERK1* and *p38* were also searched in that database. They also expressed in all the tissues and cell lines examined. The mRNA amount of *p38* and *p44ERK1* differentiated in different cell lines and tissues. However,

p54SAPKbeta was expressed similarly in all cell lines and tissues, the same as DUSP18 (Figure 2.B; Figure 2.C; Figure 2.D).

Because that the MAPK pathway responds to stimuli and the expression level of MAPKs would change during the stimuli, we also investigated the expression pattern of *DUSP18* during stimuli with serum in HEK293 cells by real time PCR. The result revealed that *DUSP18* had a 4-fold increase in expression 15 min after stimulation and a great decrease 30 min later. After that, it showed a steady expression with slight decline (Figure 3.A). *p54SAPKbeta* had the similar expression pattern (Figure 3.B). *p44ERK1* and *p38* were different, for they had relatively high expression from 15 min to 90 min Figure 3.C; Figure 3.D

4.2. Subcellular localization of DUSP18

Subcellular localization was also performed to determine where DUSP18 functioned. Subcellular localization of DUSP18 was done in HEK293 cells by transient transfection of Myc-tagged DUSP18. An anti-Myc antibody revealed the expression of DUSP18 in both cytoplasm and nucleus (Figure 4). Nucleus was stained with 4', 6'-diamidino-2-phenylindole dihydrochloride (DAPI) (Kirkegaard & Perry Laboratories) as a control. The subcellular localization of DUSP18 didn't change after stimulation by serum (figures not shown).

4.3. DUSP18 dephosphorylates p54SAPKbeta but not p38 or p44ERK1 *in vitro* and *in vivo*

The activity of DUSP18 against MAPKs was tested *in vitro* to examine if DUSP18 could dephosphorylate MAPKs. HEK-293 cells, which had been transfected with HA-p38, HA-p44ERK1, and HA-p54SAPKbeta, were stimulated with anisomysin and EGF (10μg/ml anisomycin for 30 min for p38 and p54SAPKbeta and 50nM epidermal growth factor for 20 min for p44ERK1) and then phosphorylated HA-p38, HA-p44ERK1 and HA-p54SAPKbeta were immunoprecipitated with anti-HA antibody. Each immune complex was incubated with GST-DUSP18 and the phosphorylation levels of p38, p44ERK1 and p54SAPKbeta were analyzed by Western blotting with anti-phospho-p38, anti-phospho-p44/42ERK and anti-phospho-SAPK/JNK antibodies, respectively. The result showed that p54SAPKbeta was dephosphorylated by DUSP18 while p38 and p44ERK1 were not (Figure 5).

The effect of DUSP18 on activation of HA-p38, HA-p44ERK1, and HA-p54SAPKbeta *in vivo* was analyzed to confirm the substrate specificity of DUSP18 as a MAPK phosphatase. In fact, DUSP18 showed an inhibitory effect on phosphorylation of HA-p54SAPKbeta. Co-transfection of 2 μg of DUSP18 expression plasmids reduced the phosphorylation level of HA-p54SAPKbeta (Figure 6). In contrast, HA-p38 and HA-p44ERK1 was not dephosphorylated by DUSP18.

**4.4. DUSP18 interacts with p54SAPKbeta *in vitro* and *in vivo***

Since DUSP18 dephosphorylated p54SAPKbeta *in vivo*, we asked whether DUSP18 could bind p54SAPKbeta.
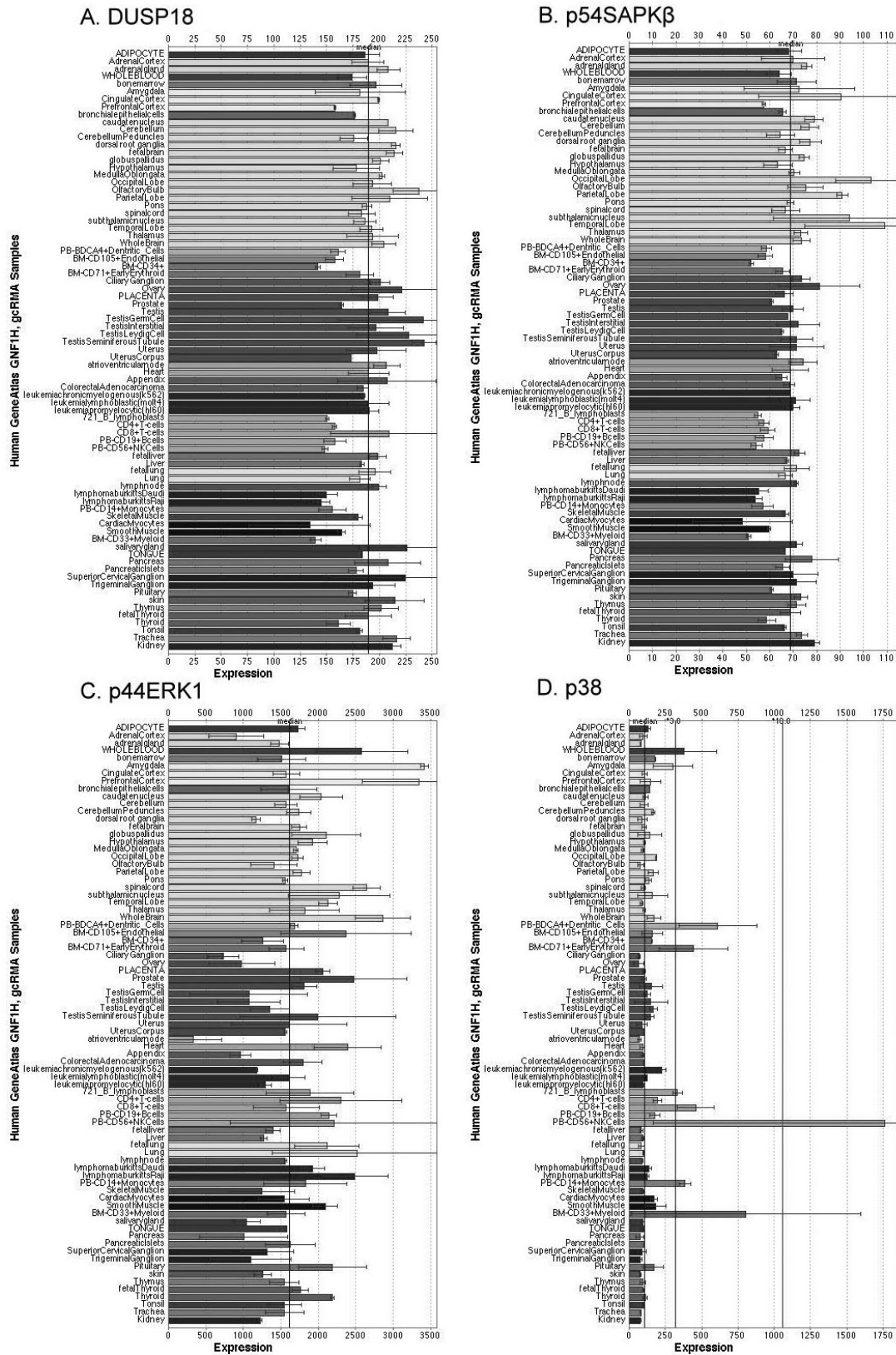
Figure 2. Expression pattern of *DUSP18* and *MAPKs* obtained from GeneAtlas database. The database was constructed as described in the article published in *PNAS* in 2002 (10). The scales in X-bar presented the relative expression level and a value for 200 has been estimated to represent 3-5 copies per cell. A, *DUSP18*; B, *p54SAPKbeta*; C, *p44ERK1*; D, *p38*.
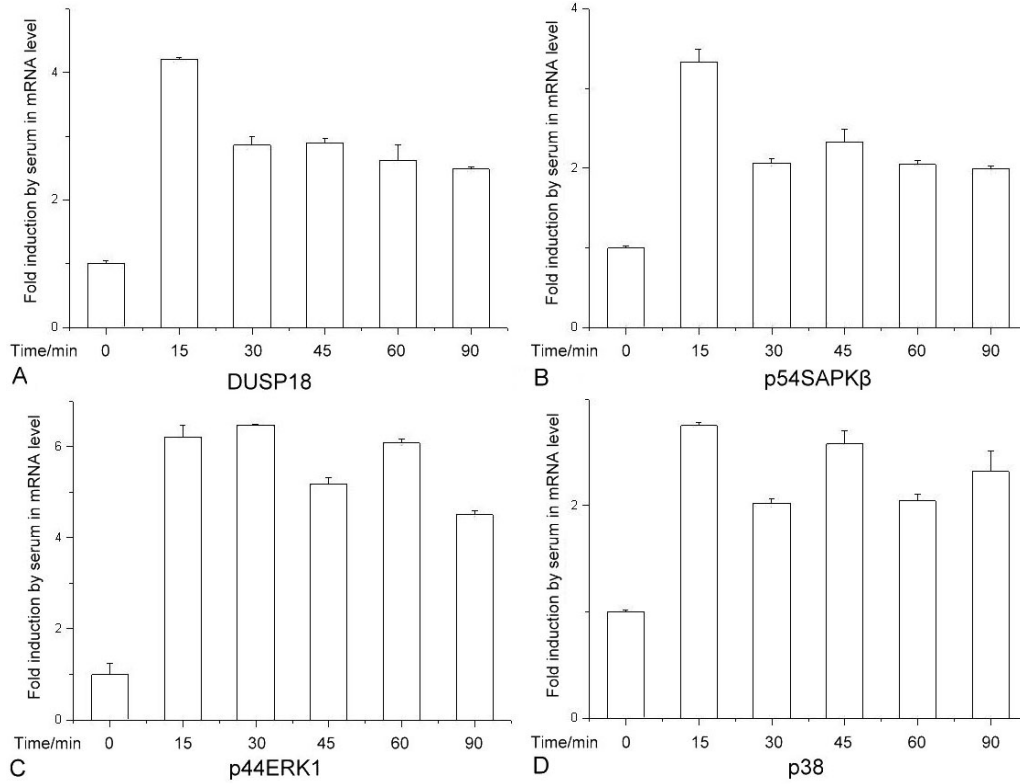
Figure 3. Expression pattern of *DUSP18* and *MAPKs* during the stimuli with serum in HEK293 cells. Real-Time PCR was performed to investigate the expression levels of *DUSP18* and *MAPKs* after the stumili of serum. HEK293 cells were treated with serum after 18 hour starvation and collected in different time after stumili. A, *DUSP18*; B, *p54SAPKbeta*; C, *p44ERK1*; D, *p38*.
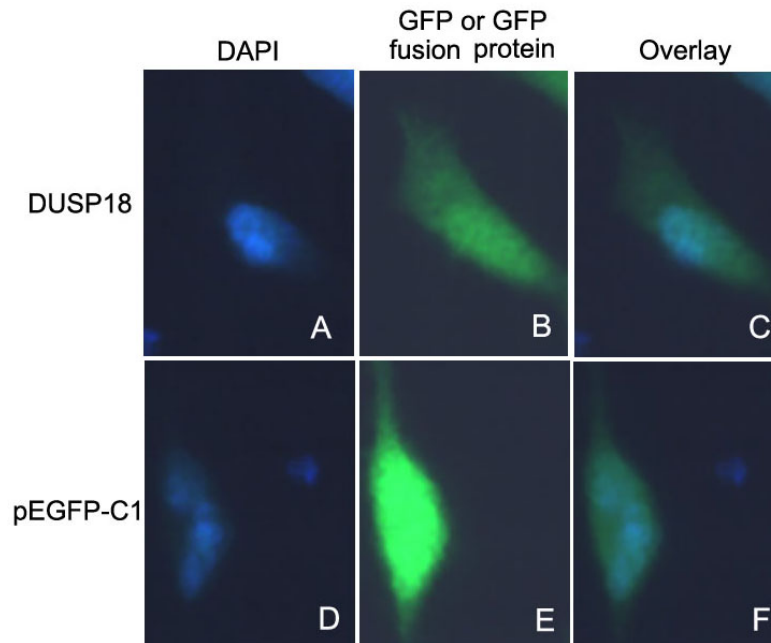

Figure 4. Subcellular localization of DUSP18. HEK-293 cells that had been transfected with Myc-DUSP18 were fixed and immunoprobed with anti-Myc antibody followed by incubation with FITC-labeled anti-rabbit IgG antibody. At the same time, DAPI staining was performed. The pEGFP-C1 vector was used as control.
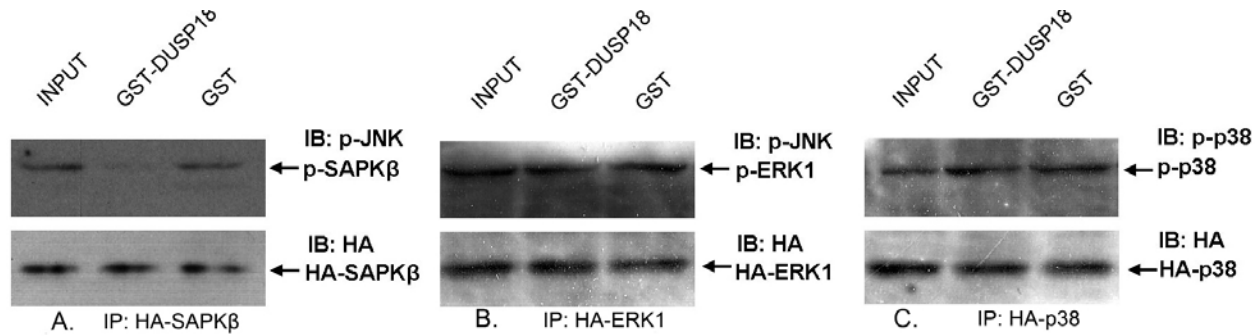
Figure 5. *In vitro* dephosphorylation of MAPKs by recombinant GST-DUSP18. HEK-293 cells were transfected with expression plasmids for HA-p38, HA-p44ERK1 or HA-p54SAPKbeta and stimulated with EGF (50nM for p44ERK1 for 20 min) and anisomycin (10 _g/ml for p38 and p54SAPKbeta for 30 min) after serum starvation. HA tagged MAPKs were immunoprecipitated, washed with lysis buffer, and then subjected to an *in vitro* dephosphorylation assay. After termination of the incubation, proteins were separated by SDS-PAGE and analyzed with anti-phospho-MAPKs antibody. Another blot was performed by using the HA probe to evaluate the amount of the HA tagged MAPKs in the assay.
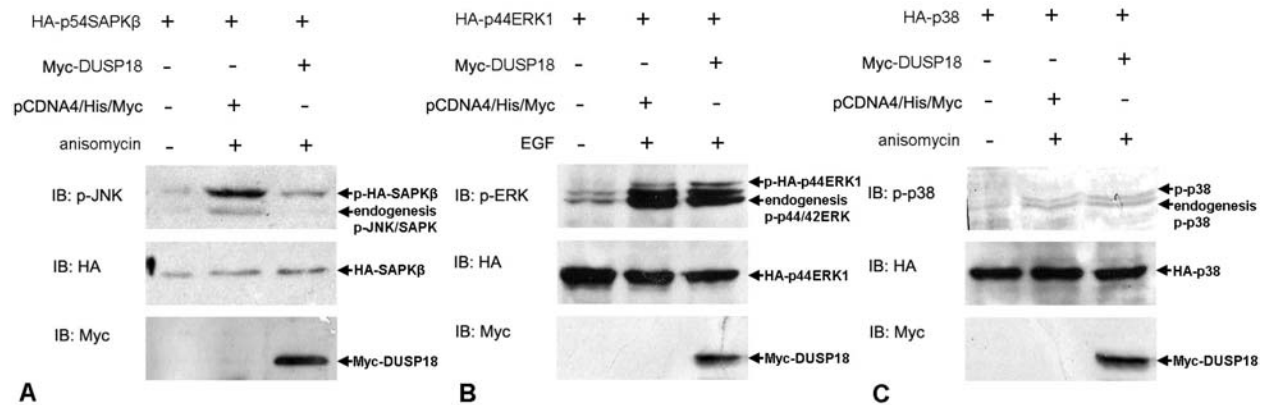
Figure 6. *In vivo* dephosphorylation of MAPKs by DUSP18. HEK-293 cells were transfected with expression plasmids for HA-p38, HA-p44ERK1 or HA-p54SAPKbeta and Myc-DUSP18 respectively, then stimulated with EGF (50nM for p44ERK1 for 20 min) and anisomycin (10 μg/ml for p38 and p54SAPKbeta for 30 min) after serum starvation. The cells were lysed and analyzed with western blot using anti-phospho-MAPKs antibody. Two other blots were performed by using the HA and Myc probe to evaluate the expression amount of the HA tagged MAPKs and the Myc tagged DUSP18.

In vitro and *in vivo* interaction between DUSP18 and p54SAPKbeta was tested by pull down and co-immunoprecipitation experiments to determine if a direct interaction between DUSP18 and p54SAPKbeta occurs. Pull down experiment revealed that bacteria expressed GST-DUSP18 could interact with HA-p54SAPKbeta *in vitro* (Figure 7). Furthermore, when myc-DUSP18 and HA-p54SAPKbeta were co-expressed in HEK293 cells, HA-p54SAPKbeta was co-immunoprecipitated with Myc-DUSP18 as expected (Figure 8).

4.5. DUSP18 inhibits SAPK/JNK related pathway *in vivo*

Many transcription factors are substrates of SAPK/JNK and are regulated by SAPK/JNK, thus they control a complicated signal net involving cell proliferation and apoptosis (11). Therefore, to examine the influence of DUSP18 on the SAPK/JNK related pathway in HEK293 cells, a Mercury pathway profiling system and a Dual-Luciferase reporter assay system were used. First, 9 cis-acting luciferase reporter vectors

(cAMP response element (CRE), Serum response element (SRE), Nuclear factor of κB cells(NFκB), Heat shock response element(HSE), Activator protein 1(AP1), Glucocorticoid response element(GRE), E-box DNA binding element(Myc), p53 response element(p53)) and a control vectors (TAL) were used to cotransfect into HEK293 cells with DUSP18. Relative transfection efficiency was determined by cotransfection with plasmid Renilla luciferase reporter vector. As expected, AP1 pathway, which was known to be regulated mainly by SAPK/JNK (12), was inhibited by DUSP18 in about 6.5 folds. Other 7 pathways were slightly inhibited by DUSP18 except that HSE pathway was slightly activated (Figure 9.A). The dose-dependent assays were then performed to verify the activation of DUSP18 to AP1 pathway (Figure 9.B). Six pathways which were known to involve SAPK/JNK pathway including AP1, SRE (13), CRE (14), p53 (15), Myc (16) and HSE (17) were then tested to verify if they would be regulated by SAPK/JNK and to investigate if
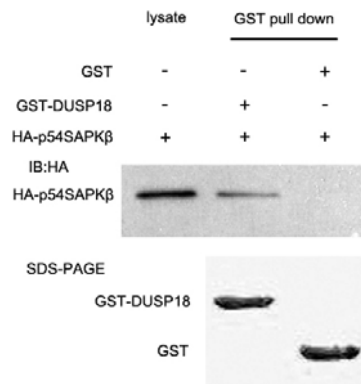
Figure 7. DUSP18 and p54SAPKbeta interacting *in vitro*. GST-DUSP18 or GST alone was expressed in E. coli. HEK293 cells were transfected with HA- p54SAPKbeta and the lysates were immunoprecipitated with anti-HA antibody. Each immune complex was incubated with GST-DUSP18 fusion protein or GST and analyzed by Western blotting with anti-HA antibodis. The complexes were also subjected to an SDS-page.
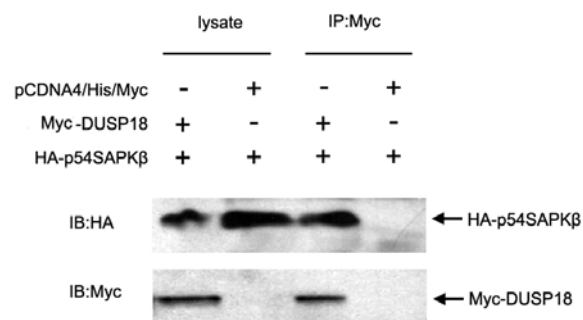
Figure 8. DUSP18 and p54SAPKbeta interacting *in vivo*. Co-immunoprecipitation was performed to investigate if HA-p54SAPKbeta and Myc-DUSP18 interacted *in vivo*. HA- p54SAPKbeta and Myc-DUSP18 were transiently expressed in HEK293 cells. Total cellular lysate and HA-p54SAPKbeta immunoprecipitates (IP: HA) were probed with anti-Myc and anti-HA antibodies.

DUSP18 could inhibit this regulation by cotransfection with p54SAPKbeta and DUSP18. As expected, 5 pathways were activated by SAPKbeta and HSE was slightly inhibited. And the regulation of these pathways by SAPK/JNK could be inhibited by DUSP18 (Figure 9.C).

5. DISCUSSION

DSPs display differences in tissue distribution and subcellular localization, suggesting that they may have different biological functions. hVH5 was expressed predominantly in brain, heart and skeletal muscle (18) while MKP-4 was only detected in placenta kidney and embryonic liver (19). Some DSPs were expressed constitutively and others were induced by some types of cell stimulation (3). Our results revealed that DUSP18 was expressed in all the tissues and cells examined including infant and tumor tissues and cell lines in which the expression levels were similar. The data from public

genechip database (http://symatlas.gnf.org/SymAtlas/) confirmed our results. We also found MAPKs were widely expressed and SAPKbeta had similar expression level in most tissues and cells. DUSP18 displays both nuclear and cytoplasmic localization in HEK293 cells. This finding was consistent with other members of the DSP family including VHR and MKP-5 (20, 21). The MAPKs occupy both the cytoplasmic and nuclear compartments and have the ability to translocate between compartments in response to stress or stimulation. Therefore, the activity of DUSP18 is not restricted to one subcellular compartment and has the potential to affect both cytoplasmic and nuclear targets. When HEK293 cells stimulated by serum, the amount of the expression of DUSP18 dramatically increased in about 4 folds in 15min and decreased about a half in the next 75min. The similar result did SAPKbeta have. Whereas, p38 and ERK1 also increased sharply in response to stimuli but decrease more slowly, which suggested that DUSP18 and SAPKbeta had some similarity in the mechanism for expression regulation.

In vivo, DSPs may play an important role in mitogenic signal transduction and cell cycle control since many of them could dephosphorylate MAPKs and as a result cause inactivation of their kinase activity (3). Further more, each DSP has its own substrate specificity for MAPKs. Through this series of experiments we have identified DUSP18 as a MAPKs phosphatase. SAPKbeta but not p38 or p44ERK1 was the direct substrate of DUSP18 and interacted with DUSP18 both *in vitro* and *in vivo*. MKP-7 is another SAPK/JNK specific phosphatase (22). It is more effective toward phosphorylated and activated SAPK/JNK than ERK and p38. hVH-5/M3/6 (23), MKP-2 (24) and MKP-5 (21) can also dephosphorylate SAPK/JNK but not specifically, whereas MKP3 (25), MKP4 (19), and PAC1 (24) appear more specific for ERK. And MKP1 appears to have equal activity against JNK, p38, and ERK (26).

The SAPK/JNK group of MAPKs is important for many cellular responses including apoptosis, growth, differentiation, embryonic development, and immune response (11). The phosphorylation level of SAPK/JNK is very important for its kinase activity and we supposed that DUSP18 could regulate the SAPK/JNK activity by dephosphorylation of SAPK/JNK and further take part in the control of SAPK/JNK pathway. Once activated, SAPK/JNK phosphorylates and regulates the activity of a number of transcription factors including the activator protein 1 family member c-Jun (12) and several other proteins. In our experiments, AP1, SRE, CRE, p53, Myc pathway was activated and HSE pathway was inhibited when co-transfected with SAPKbeta plasmid as expected. And when these 6 pathway-profiling plasmids were co-transfected with both SAPKbeta and DUSP18 plasmids, the influence of SAPKbeta were almost counteracted. These results confirmed that DUSP18 could inactivate SAPK/JNK and as a result influence the down stream pathway of SAPK/JNK.

The SAPK/JNKs, which are encoded by three different Sapk/Jnk loci, play important roles in numerous
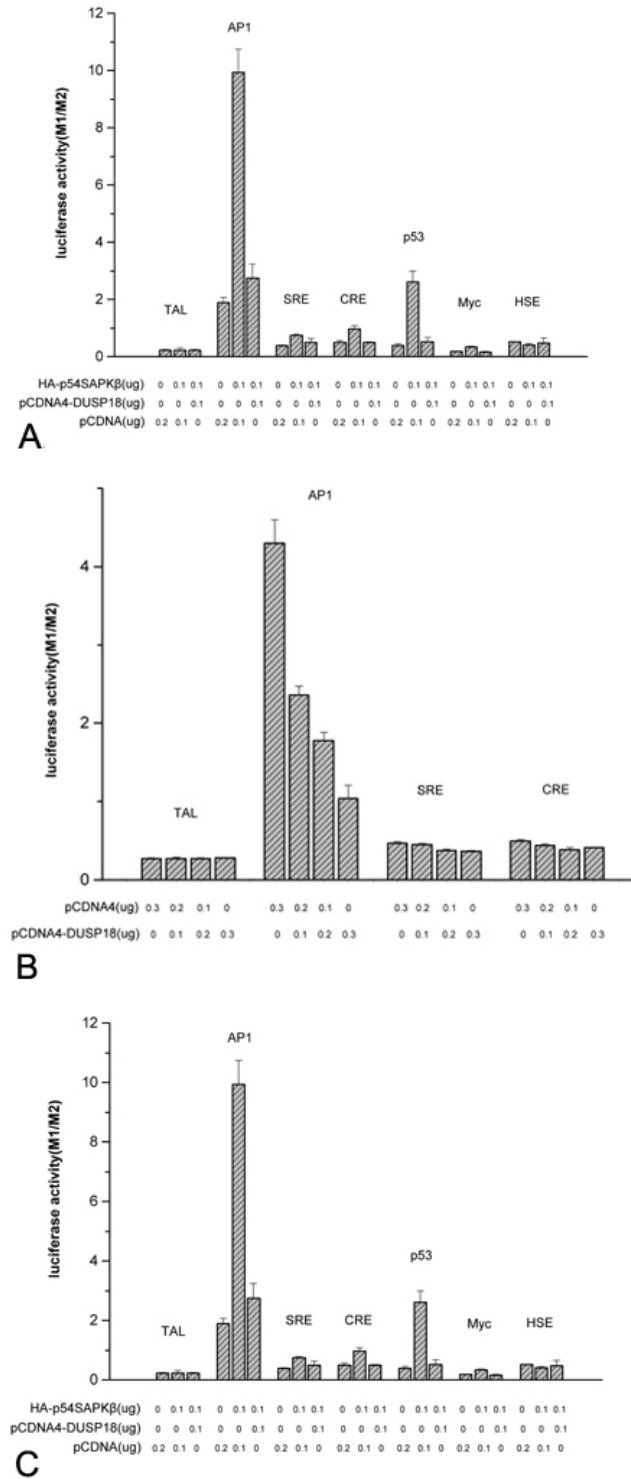
Figure 9. DUSP18 inhibits SAPK/JNK related pathway *in vivo*. (A)Empty vector pCDNA4 or pCDNA4-DUSP18 was cotransfected into HEK293 cells together with 8 cis-acting luciferase reporter vectors or a TAL control luciferase report vector vector and pRL-SV40 vector (a transfection efficiency control reporter). Cells were harvest 48 h later after transfection and luciferase activity was measured. Each bar represents the mean + SE of at three independent treatments. (B) Activation of AP1 pathway by DUSP18 in dose-dependent manner in HEK293 cells. Four luciferase reporter vectors were cotransfected with different amount of pCDNA4-DUSP18. (C) Six pathways which were known to involve SAPK/JNK pathway including AP1, SRE, CRE, p53, Myc and HSE were then tested to verify if they would be regulated by SAPK/JNK and if DUSP18 could inhibit this regulation by cotransfection with p54SAPKbeta and DUSP18.

cellular processes. Recently, the JNKs have become putative targets for drug development in several important clinical areas, including inflammation, diabetes, and cancer (27). Considering the similar expression pattern of DUSP18 and SAPKbeta in response to serum stimuli, we hypothesize that DUSP18 might function as an general inhibitor of SAPK/JNK pathway through direct binding and dephosphorylation of SAPK/JNK *in vivo*. When expression level of SAPK/JNK alters in response to stimulation, the expression of DUSP18 changes along with SAPK/JNK to slow the dramatically increase or decrease of SAPK/JNK activity. However, more experiments are required to confirm the hypothesis.

In conclusion, DUSP18 was testified as a widely expressed SAPK/JNK phosphatase by directly interacting with SAPK/JNK. Further more, DUSP18 inhibited the regulation of SAPK/JNK in SAPK/JNK pathway *in vivo*.

6. ACKNOWLEDGEMENTS

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Abbreviation: DUSP18: Dual-specificity protein phosphotase 18; SAPK/JNK: stress-activated protein kinase/c-Jun-NH2 kinase; DSP: dual-specificity protein phosphatases; PTP: protein tyrosine phosphatase; MAPK: mitogen-activated protein kinase; ECL: enhanced chemiluminescence; G3PDH: glyceraldehyde-3-phosphate dehydrogenase; ERK: extracellular signal-regulated kinase; EGF: endothelial growth factor; CRE: cAMP response element; SRE: Serum response element; NFκB: Nuclear factor of κB cells; HSE: Heat shock response element; AP1: Activator protein 1; GRE: Glucocorticoid response element; MKP: MAPK phosphatase

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Send correspondence to: Dr. Yumin Mao, Room 606, Science Building, Institute of Genetics, Fudan University, Shanghai 200433, P. R. China, Tel.: 86-21-6564-3573, Fax: 86-21-6564-2502, E-mail: ymmao@fudan.edu.cn