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Feedback regulation of *DUSP6* transcription responding to MAPK1 via ETS2 in human cells

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ABSTRACT

DUSP6/MKP-3 is a dual specificity phosphatase exclusively specific to MAPK1/ERK2 for its substrate recognition and dephosphorylating activity. DUSP6 is demonstrated to play a negative regulatory role in MAPK1 in a feedback loop manner; however, the regulation mechanisms of its expression in human cells have been largely unknown. We previously found that human pancreatic cancer cells frequently lost DUSP6 expression, which could induce constitutively active MAPK1, and the loss was associated with hypermethylation of the CpG cluster region of intron 1 of *DUSP6*. In this study, we investigated the promoter activity of intron 1 of *DUSP6* in human cells. We demonstrated that the intron indeed had promoter activity and this activity was associated with MAPK1 activity. Moreover, promoter activity depended on a consensus binding sequence of ETS transcription factors and ETS2 was specifically associated with the intron. Because ETS2 is a direct target of MAPK, these results indicate that intron 1 of *DUSP6* plays a crucial role in transcriptional regulation of *DUSP6* in a feedback loop manner responding to MAPK1 via ETS2 in human cells.

DUSP6/MKP-3/PYST1 is a dual specificity phosphatase exclusively specific to MAPK1/ERK2/ERK for its substrate recognition and dephosphorylating activity [1,2]. DUSP6 has been demonstrated to be a negative feedback regulator of MAPK1 [3–5]; however, the regulation mechanisms of its expression in human cells have been largely unknown. The encoding gene, *DUSP6*, is located on 12q21-q22, the region commonly deleted hemizygously in pancreatic cancer [6,7]. DUSP6 is frequently underexpressed in pancreatic ductal adenocarcinoma although it is overexpressed in pancreatic intraepithelial neoplasia, one of precursor lesions of ductal adenocarcinoma [8]. Its underexpression is associated with constitutive activation of MAPK1 [9]. Exogenous overexpression of DUSP6 in DUSP6-abrogated pancreatic cancer cells results in inactivation of MAPK1 and eventual apoptosis [9]. This evidence indicates that pancreatic cancer cells losing DUSP6 expression are addicted to active MAPK1 for their survival and proliferation, and that DUSP6 plays an antagonistic role in this addiction; hence, a tumor suppressive role. The underexpression of DUSP6 in pancreatic ductal adenocarcinoma is associated with the hypermethylation of CpG cluster region of intron 1 of *DUSP6* [10], which suggest that the intron may be a key control region for DUSP6 expression. In this study, we investigated the expression mechanism of DUSP6 by examining the promoter activity of intron 1 of *DUSP6* in human cells.

Materials and methods

Molecular cloning of intron 1 of DUSP6. A portion of human genome consisting of 614-bp region of intron 1 of *DUSP6* was amplified by PCR employing a KOD-plus DNA polymerase kit (TOYOBO Co. Ltd., Osaka, Japan) with paired primers of 5′-TTTACGCGTGTACGCGCCCAGGGAACTC-3′ and 5′-TTTCTCGAGCTGCCAGAACGAGAAAAGCAA-3′, respectively harboring *Mlu*I and *Xho*I sites at 5′ ends to facilitate cloning, and an aliquot of 100 ng total human genomic DNA as a template. The PCR condition was initial denaturation for 2 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 60 °C and 30 s at 68 °C, and final extension for 5 min at 68 °C. The amplified product was purified with a High Pure DNA purification kit (Roche Diagnostics, Basel, Switzerland) and digested with *Mlu*I and *Xho*I (Roche Diagnostics). Then the digested product was purified with a High Pure DNA purification kit and cloned into the reporter vector, pGL3 (Promega Corporation, Madison, WI, USA), at *Mlu*I and *Xho*I sites. Four vectors harboring truncated portions of intron 1 of *DUSP6*, termed M-K, P-X, M-P, and P-K as illustrated in Fig. 1B, were generated by digesting the full-length vector with a combination of *Mlu*I, *Pvu*II, *Kpn*I, or *Xho*I, blunting digested ends and self-ligation. A reporter vector harboring a mutated sequence of the consensus binding site of ETS transcription factors was generated by site-directed mutagenesis performed with a Site-directed Mutagenesis kit (Stratagene, La Jolla, CA, USA) and the mutagenic primer 5′-ATCCACTCGCAAAAGGCTGCGGGTACCGGCGG-3′ corresponding

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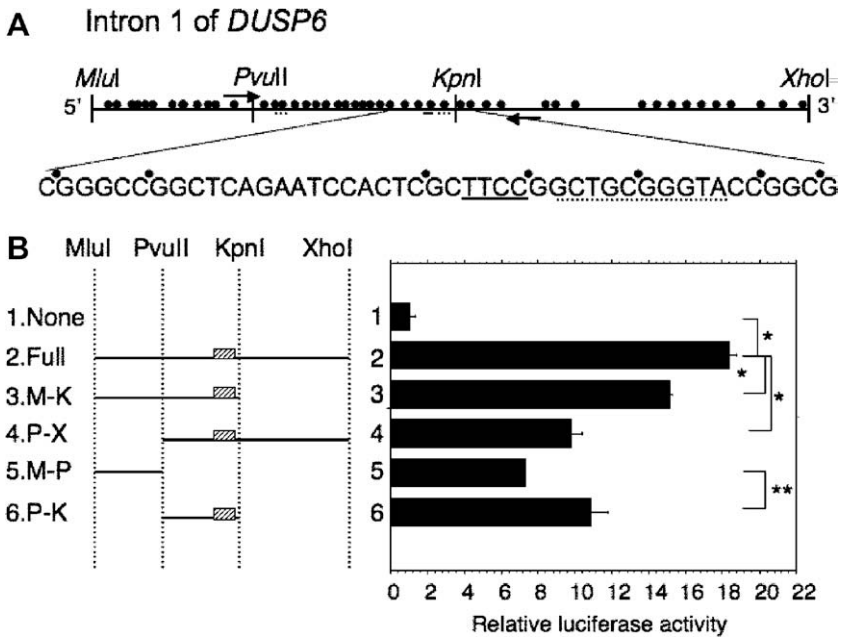

Fig. 1. (A) Intron 1 of *DUSP6* with recognition sites of restriction enzymes and the consensus binding sequences for ETS (solid underbar) and AP-2 (dotted underbars) transcription factors. Recognition sequences of *Mlu*I and *Xho*I were inserted at 5′ and 3′ ends of the intron, respectively, for cloning into pGL3 reporter vector. Arrows indicate the locations of primers used in the chromatin immunoprecipitation assay. Closed circles indicate CpG sites. (B) Reporter vectors harboring (1) none, (2) full-length, or (3–6) various truncations of intron 1 of *DUSP6* indicated in the left panel were transfected into cells of PK-8, a human pancreatic cancer cell line, and assayed for reporter activities. Hatched boxes in the left panel indicate the consensus binding site for ETS transcription factors. An asterisk indicates $p < 0.01$ and two asterisks indicate $p < 0.05$ by unpaired *t*-test. $n = 3$ for each assay. All data are means and standard errors.

to IVS+195 and +226 with mutation at the underlined four bases, according to the manufacturer's instructions. The cloned vectors were verified by sequencing using a Bigdye terminator and ABI Genetic Analyzer (Applied Biosystems Inc., Foster City, CA, USA).

Reporter assay. Cells of PK-8, a human pancreatic cancer cell line, were seeded in a 6-well plate at 5×10^3/well and cultured as described previously [7]. Twenty-four hours after seeding, 0.5 μg of either of the cloned reporter vectors and 0.05 μg of phRL-TK vector (Promega) were co-transfected mediated by Lipofectamine Plus reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Cells of 293, a human embryonic kidney epithelial cell line transformed with adenovirus 5 DNA, obtained as described previously [11], were seeded at 5×10^5/well and transfected with a combination of expression vectors of pcDNA3.1-V5/His, pcDNA3.1-active MAP2K1 (MAP2K1Δ44-51/S218E/S222E)-V5/His, or pcDNA3.1-DUSP6-V5/His, obtained as described previously [11], and reporter vectors. The transfected cells were maintained in appropriate culture medium for 48 h. The cells were then washed with PBS and lysed in Lysis buffer (Promega). A dual luciferase assay using a Dual Luciferase Assay Kit (Promega) and Luminoskan Ascent (Thermo Fisher Scientific Inc., Waltham, MA, USA) was performed according to the manufacturers' instructions.

Immunoblotting. Immunoblotting was performed as described previously [12]. The antibodies were a polyclonal anti-phospho-p44/42 MAP kinase (Cell Signaling Technology Inc., Danvers, MA, USA), a monoclonal anti-ERK2 (clone G263-7, BD Biosciences, San Jose, CA, USA), a polyclonal anti-MKP-3 (C-20, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), a monoclonal anti-V5 (Invitrogen), a monoclonal anti-beta actin (clone AC-15, Sigma, St. Louis, MO, USA), a horseradish peroxidase (HRP)-conjugated anti-mouse immunoglobulin (GE Healthcare UK Ltd., Buckinghamshire, England), and a HRP-conjugated anti-gout immunoglobulin (Santa Cruz Biotechnology Inc.). Blocking conditions and concentrations of antibodies followed the manufacturers' instructions. Signals were visualized by the reaction with ECL Detection Reagent (GE Healthcare UK Ltd.) and digitally processed using LAS 4000 mini (Fuji Photo Film Co. Ltd., Minamiashigara, Japan).

Chromatin immunoprecipitation assay. Cells of PK-8 were seeded in a 10-cm culture dish at 4×10^6 and maintained in RPMI 1640 supplemented with 10% fetal bovine serum. Twenty-four hours after seeding, cells were fixed with 1% formaldehyde solution and collected. Cells were sonicated in a Biorupter (Cosmobio, Tokyo, Japan) and used for immunoprecipitation reaction with a ChIP-IT-Express kit (Active Motif, Carlsbad, CA, USA) and a polyclonal anti-ETS2 antibody (Santa Cruz Biotechnology Inc.), a polyclonal anti-AP2 antibody (Active Motif), or nonspecific immunoglobulin (Santa Cruz Biotechnology Inc.). Paired primers of 5′-ACC TCTGCTCCGCTCAGCTG-3′ and 5′-AAAACAGGGTGATGGTGGAG-3′ for amplification of IVS1+78 and IVS1+287 of *DUSP6* and Accuprime PCR system (Invitrogen) were used for the PCR reaction.

Statistical analysis. Statistical analysis was performed using Statview 5.0 software (SAS Institute Inc., Cary, NC, USA).

Results

Promoter activity of intron 1 of DUSP6

Our previous study indicated that underexpression of DUSP6 in pancreatic cancer cells and tissues was associated with hypermethylation of CpG cluster region in intron 1 of *DUSP6* [10]. From this information, we analyzed the promoter activity of the intron. We constructed reporter vectors containing full-length and various truncated sequences of the intron (Fig. 1B) and transfected them into cultured human pancreatic cancer cells of PK-8, where an endogenous transcriptional mechanism of *DUSP6* functioned [10]. Transfection of the full-length construct revealed strong promoter activity while that of truncated constructs revealed reduced activity. This series of experiments elucidated that an area between the *Pvu*II and *Kpn*I sites of the intron had relatively strong promoter activity (Fig. 1B).

Association between promoter activity of DUSP6 and MAPK1 activity

DUSP6 is supposed to form a functional negative feedback loop with MAPK1; therefore, it is interesting to know whether the

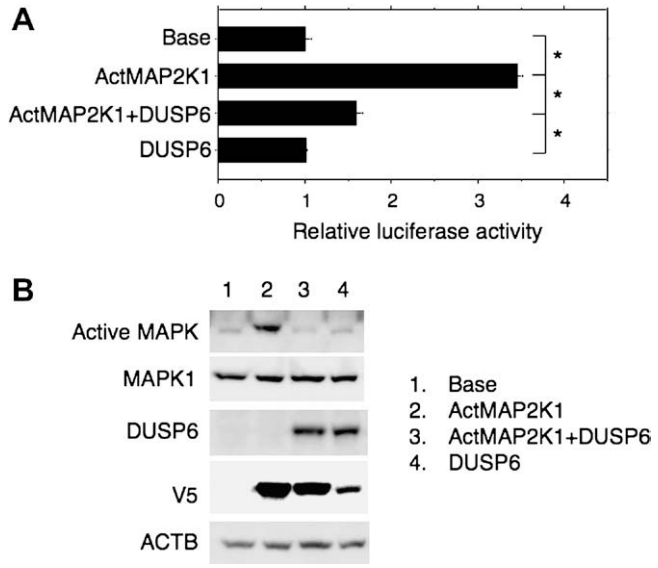

Fig. 2. (A) The reporter vectors harboring intron 1 of *DUSP6* and phRL-TK were co-transfected into cells of 293 with pcDNA3.1-V5/His (Base), pcDNA3.1-active MAP2K1-V5/His (ActMAP2K1), pcDNA3.1-DUSP6-V5/His (DUSP6), or pcDNA3.1-active MAP2K1-V5/His and pcDNA3.1-DUSP6-V5/His (ActMAP2K1+DUSP6), and then reporter activities were measured by the dual luciferase assay. Asterisks indicate $p < 0.01$ by unpaired t-test. $n = 3$ for each assay. All data are means and standard errors. (B) Immunoblotting of lysates of transfected cells with (1) Base, (2) ActMAP2K1, (3) ActMAP2K1+DUSP6, or (4) DUSP6.

promoter activity of intron 1 of *DUSP6* is associated with MAPK1 activity. We employed 293 cells transfected with reporter vectors and a plasmid expressing active MAP2K1/MEK (MAP2K1Δ44-51/S218E/S222E) [13] with or without a plasmid expressing DUSP6. By transfection of the active MAP2K1, activated (phosphorylated) MAPK was induced and promoter activity was increased (Fig. 2). On the other hand, when active MAPK1 was dephosphorylated by the exogenous expression of DUSP6, promoter activity was reduced (Fig. 2). These data indicated that the promoter activity of intron 1 of *DUSP6* was positively regulated by active MAPK1.

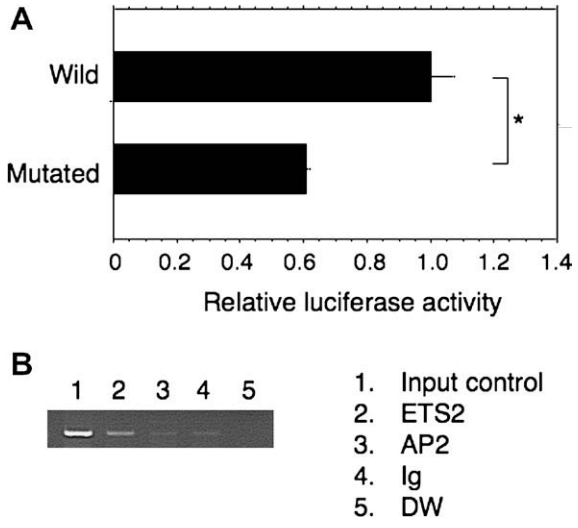

Fig. 3. (A) Reporter vectors harboring intron 1 of wild or mutated sequence of the consensus binding site for ETS transcription factors were transfected into cells of PK-8 and assayed for luciferase activities. An asterisk indicates $p < 0.01$ by unpaired t-test. $n = 3$ for each assay. All data are means and standard errors. (B) Chromatin immunoprecipitation assay. PCR products of formalin-fixed and sonicated DNA (lane 1), that of immunoprecipitated with anti-ETS2 antibody (lane2), anti-AP2 antibody (lane 3), or nonspecific immunoglobulin (lane4) were electrophoresed in 3% agarose gel containing ethidium bromide. Lane 5 was a PCR product with distilled water instead of DNA as a template.

ETS binding site associated with promoter activity

Next we searched for possible binding sites of transcription factors in the active promoter region. With help from the web-based searching program for transcription factor-binding sites, Match (http://www.gene-regulation.com/cgi-bin/pub/programs/match/bin/match.cgi), we found the consensus binding sequence of ETS transcription factors, GGAA, in the active region (see Fig. 1A). We then constructed a reporter vector containing a mutated sequence of the consensus binding site, i.e., from GGAA to TTTT, and analyzed its activity. The mutant vector showed less activity than the vector of the wild sequence, which indicated that ETSs were candidate transcription factors associated with intron 1 of *DUSP6* (Fig. 3A).

Association of ETS2 with intron 1 of DUSP6

To identify the association of ETS transcription factors with intron 1 of *DUSP6* in human cells, the chromatin immunoprecipitation assay was performed. We tested ETS2, which is known to be a target of MAPK [14]. We also tested AP-2, whose possible binding sequences existed in the active portion of the intron (see Fig. 1A). The result indicated that ETS2, but not AP-2, was specifically associated with the active region of intron 1 of *DUSP6* (Fig. 3B).

Discussion

In this study, we found that intron 1 of *DUSP6* had promoter activity and that this activity was associated with MAPK1 activity. Promoter activity depended on the consensus binding site of ETS transcription factors and ETS2 bound the intron in cultured human cells. These results indicate that intron 1 of *DUSP6* plays a promoter role mediated by ETS2 in response to MAPK1 activity in DUSP6 expression.

DUSP6 plays important roles in the phenotypes of various cancers including pancreatic cancer, one of the most devastating human malignancies [15–20]. Human pancreatic cancer cells frequently lose DUSP6 expression, and this loss is associated with hypermethylation of the CpG cluster region of intron 1 of *DUSP6*, which suggests that the intron would play a regulatory role in DUSP6 expression [10]. Consistent with this observation, the current study elucidated the role of the intron as an active promoter in transcriptional regulation of DUSP6. This activity could respond to MAPK activity. The intron harbored the consensus binding sequence of ETS transcription factors and ETS2 was associated with the intron. Because ETS2 is known to be a transcriptional target of MAPK1 [14], our results indicate that transcription of *DUSP6* is controlled in a feedback loop manner responding to MAPK1 activity via ETS2. The hypermethylation of intron 1 of *DUSP6* may disrupt the feedback loop regulation between DUSP6 and MAPK1, which can induce constitutive activation of MAPK1 in pancreatic cancer cells. Active MAPK1 actually induces the expressions of a number of downstream genes that can play crucial roles in the growth, invasion and metastasis of cancer cells [11], therefore, control of MAPK1 activity may be a key measure for cancer treatment. Exogenous overexpression of DUSP6 in pancreatic cancer cells lacking endogenous expression of DUSP6 induces the inactivation of MAPK1, growth suppression, and apoptotic death of the cells [9]. Methylase inhibitor 5-aza-2′-deoxycytidine induces endogenous re-expression of DUSP6 and growth delay in pancreatic cancer cells [21]. Thus, resurgence of DUSP6 expression can suppress malignant phenotypes of cancer cells. The current study will contribute to the design of a novel strategy against the resurgence of

endogenous expression of DUSP6 and the control of MAPK1 activity for effective therapy of human cancer.

Ekerot et al. [22] demonstrated that a murine genomic region upstream of *Dusp6* harboring the consensus binding site of Ets had promoter activity responding to the activity of Erk2 in murine cells [22]. Ets2 was also demonstrated to be associated with a region of murine genomic DNA. This murine study and our current human study both indicated that ETS2 (Ets2) is a key regulatory factor in the transcriptional control of *DUSP6* (*Dusp6*) in responding to activity of MAPK1 (Erk2).

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