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DUSP6: Potential interactions with FXR1P in the nervous system

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Fragile X syndrome (FXS) is a leading genetic cause of autism intellectual disorder and autism spectrum disorder (ASD), with either limited treatment options or incurable. Fragile X-related gene 1 (*FXR1*) is a homolog of the Fragile X mental retardation gene 1 (*FMR1*), the causative gene of FXS, and both are highly homologous and functionally identical. In FXS, both PI3K (AKT/mTOR signaling pathway) and ERK1/2 (MAPK signaling pathway) expression levels were abnormal. Dual specificity phosphatase 6 (*DUSP6*) is a member of the mitogen-activated protein kinases (MAPKs) that participates in the crosstalk between the two signaling systems of MEK/ERK and mTOR. By interacting with multiple nodes of MAPK and PI3K/AKT signaling pathways (including the mTOR complex), DUSP6 regulates cellular growth, proliferation, metabolism and participates in pathological processes of cancer and cognitive impairment. However, whether there is an interaction between FXR1P and DUSP6 and the effects of DUSP6 on the growth of SK-N-SH cells remains elusive. As demonstrated by our results, FXR1P was identified in the cytoplasm and nucleus of SK-N-SH cells co-localized with DUSP6, which might have regulated ERK1/2 signaling pathways in SK-N-SH cells. To a certain extent, FXR1P may reverse the negative regulation of ERK1/2 by DUSP6. Moreover, we discovered that not only does DUSP6 inhibit proliferation, but it also promotes the apoptosis of SK-N-SH cells.

Keywords: Apoptosis, Co-localization, ERK1/2, Nervous system, SK-N-SH

FXR1, *FMR1*, and Fragile X-related gene 2 (*FXR2*) comprise a family of homologous genes involved in post-transcriptional mRNA regulation[1,2]. The protein products of these genes (FXR1P, FMRP, FXR2P) share the exact RNA-binding structural domains (two KH domains and the RGG box) as well as nuclear localization signals (NLS) and nuclear export signals (NES). This particular structure enables them to shuttle between the cytoplasm and the nucleus[3]. FXR1P often regulates neurological disorders by mediating signaling pathways or its downstream molecules[4], *e.g.*, FXR1P interacts with glycogen synthase kinase 3β (GSK3β) in schizophrenia, affecting mood stability and amygdala activity[5].

Dual-specificity phosphatises (DUSPs) are dual-specific protein phosphatases that can specifically dephosphorylate phosphotyrosine and phosphothreonine within MAP kinases, thus offering a transcriptional system for the functional loss of selected activities of MAP kinase[6]. The atypical expression level of DUSP6 can trigger the dysfunction of the MAPK signaling pathway[7,8]. Since MAPK is a pivotal factor in signal transduction and cellular response, once the pathway is activated by external stimuli, it is followed by a series of reactions in ERK double phosphorylation (Tyr site and Thr Site). The ERK is subsequently transported to the nucleus, resulting in the activation of other downstream target genes. Besides, the N-terminal domain of DUSP6 contains two types of serines, namely Ser159 and Ser197[9], and the PI3K/mTOR pathway can phosphorylate and degrade DUSP6 through Ser15[10]. Moreover, DUSP6 has been widely reported in various tumors[11,12], proliferation[13], differentiation[14], and apoptosis[15,16], It has also evoked interest in neurological diseases, such as Alzheimer's disease (AD), where persistent hypermethylation of AD-associated DNA on the Dusp6 promoter inhibits its transcription, which provokes p-ERK1/2 dysregulation and disturbs the homeostasis of microglia, ultimately participating in the pathogenesis of AD[17].

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Furthermore, DUSP6 is associated with the crosstalk between MEK/ERK and mTOR signaling systems and plays a significant role in neurological diseases, proliferation, differentiation, and apoptosis[18]. On the other hand, FXR1P is an RNA binding protein (RBP) that interacts with target mRNAs and proteins[19]. Our previous study revealed that FXR1P could affect the expression level of the *Dusp6* mRNA, however, the mechanism of FXR1P and DUSP6 is yet to be defined. Based on this, we hypothesized that FXR1P may interact with DUSP6 and participate in the MAPK signaling pathway by regulating the expression level of Dusp6, while DUSP6 may affect the growth of SK-N-SH cells. These researches will be conducive to revealing the functions of *FXR1* and contribute to furthering our knowledge in the pathogenesis of FXS.

Materials and Methods

Cell line, bacterial strain, vectors and *recombinant plasmid*

SK-N-SH cell line, Escherichia coli strain DH5α, pDsRed2-N1, pCMV-Flag, pcDNA3.1(+), pCMV-HA-*FXR1*, pEGFP-N1-*FXR1* and pcDNA3.1(-)-*FXR1* plasmids were collected from the lab stock. pDsRed2-N1-*Dusp6*, pCMV-Flag-*Dusp6* and pcDNA3.1(+)-*Dusp6* recombinant plasmids were generated, and the details are illustrated in (Table 1). Escherichia coli strain DH5α was used for cloning.

Cell culture and transfection

SK-N-SH cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, USA) containing 10% FBS and 1% penicillin/streptomycin at 37°C under 5% CO_2 in a humidified atmosphere. Appropriate plasmids (4 μg per 35 mM dish) were transfected into the cells using the Lipofectamine™ 2000 Transfection Reagent (Invitrogen, USA), as per the manufacturer's protocol. After transfecting for 48 h, the cells were used in the experiments outlined below.

Bioinformatics analysis

Interactive prediction between proteins

The STRING database was used to predict possible associations between FXR1P and DUSP6. The two proteins were bound in tandem to identify shared nodes.

Prediction of DUSP6 candidate target genes

BioGRID (https://thebiogrid.org/), Hitpredict (https://oxfordindex.oup.com/view/10.1093/nar/gkq897), Genemania (http://genemania.org/), STRING (https://string-db.org/)[20], InBioMap (https: //www.intomics.com/inbio/map/#home) and IntAct (https://www.ebi.ac.uk/intact/) are six online prediction programs used to predict probable targets for DUSP6. Our method involved mixing different software to exploit the strengths of each software to compensate for the weaknesses of every single software so that the most likely candidate targets for DUSP6 could be distinguished.

Confocal microscopy

The transfected cells were first fixed with 1 mL 4% paraformaldehyde for 20 min at 4°C. Subsequently, 500 μL 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI, Beyotime, Shanghai, China) was added for 5 min at room temperature (25°C) in the dark. High-resolution images were taken on the Zeiss LSM 980 (Zeiss, Oberkochen, Germany) and processed with the ImageJ software[21].

Immunoprecipitation

SK-N-SH cells were co-transfected with HA-FXR1P and Flag-DUSP6. 48 h post-transfection, the cells were lysed in RIPA buffer containing PMSF and incubated with anti-HA-Tag mouse monoclonal antibody, anti-Flag-Tag mouse monoclonal antibody (1:500, CWBIO, Beijing, China) and protein A/G agarose beads（Santa Cruz Biotechnology, CA, USA）at 4°C overnight. The immunoprecipitated proteins were separated by SDS-PAGE gels and detected by anti-HA and anti-Flag antibodies.

Table 1 — Detailed information on recombinant plasmids

Recombinant Plasmid name	Vector name	Insert(s)	Insertion site	Primers (5'-3')
pDsRed2-N1-*Dusp6*	pDsRed2-N1	*Dusp6*	Hind III, Kpn I	F:5′-CCGGACTCTCAGATCTCGAGCTCAAGCTTATGATAGATACGCTCAGACCCGTGCCCTTCGC-3′ R:5′-GACCGGTGGATCCCGGGCCCGCGGTACCCGCGTAGATTGCAGAGAGTCCACCTG -3′
pCMV-Flag-*Dusp6*	pCMV-Flag	*Dusp6*	Hind III, Kpn I	F:5′-CCCAAGCTTATGATAGATACGCTCAGACCCGTG-3′ R:5′-GGGGTACCCTACGTAGATTGCAGAGAGTCCACCTG-3′
pcDNA3.1(+)-*Dusp6*	pcDNA3.1(+)	*Dusp6*	Bam HI, Spe I	F:5′-CGCGGATCCATGATAGATACGCTCAGA-3' R；5′-TAGACTAGTTCACGTAGATTGCAGAGA-3′

RNA extraction and quantitative Real-Time PCR (qRT-PCR)

qRT-PCR was carried out as previously described[22]. In short, the total RNA was extracted from the SK-N-SH cell lines using Trizol (Invitrogen, Carlsbad, CA, USA). It was then reversely transcribed into cDNA with the PrimeScript™ RT reagent Kit (Takara, Dalian, China). The mRNA levels were quantified using the SYBR® Fast qPCR Mix on the Real-Time PCR System (Agilent, Germany). The relative mRNA expression was calculated by the $2^{-\Delta\Delta CT}$ method with GAPDH as an internal standard. Primer sequences for mRNA targets are provided in Supplemental (Table 1).

Western blotting

Total proteins from the SK-N-SH cells were lysed with RIPA (Beyotime, Shanghai, China) in the presence of PMSF, as previously reported[23]. The proteins were subjected to SDS-PAGE gel and then transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was shaken with a blocking buffer containing 5% non-fat milk in 1xTBST for 2 h at room temperature and then incubated with the primary antibody at 4°C overnight. Next, the membranes were washed three times with 1xTBST and incubated for 1 h at room temperature with the secondary antibody. The BeyoECL Plus kit (Beyotime, Shanghai, China) and the automatic chemiluminescence imaging analysis system (Tanon, Shanghai, China) were utilized to visualize the proteins. GAPDH was utilized as an internal control. Primary antibodies and dilution rates applied are as follows: p-ERK1/2 (1:1000, Abcam, Cambridge, UK), ERK1/2 (1:1000, Abcam, Cambridge, UK). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (CWBIO, Beijing, China) was added as a secondary antibody at a dilution ratio of 1:2000.

Cell viability assay

The viability of cells was analyzed by the cell counting kit-8 (CCK-8) assay[24]. SK-N-SH cells were cultured for 36h after transfection on a 96-well plate at a density of 5×10^3/well, and afterwards, 10 μL CCK-8 solution was added to each well and incubated at 37°C for 2 h. Cells in each plate were assayed at different exposure times (0 h, 24 h, 48 h, 72 h, 96 h) and the absorbance values were measured at 450 nm by a multifunctional microplate reader (Cytation 3, Biotek, Vermont, USA).

Cell apoptosis assay

Flow cytometry, Hoechst 33342 staining and Wright-Giemsa staining were used to assess the SK-N-SH cell apoptosis. The Annexin V-FITC/ propidium iodide (PI) staining assay kit (4A Biotech, Beijing, China) was used to determine apoptotic rates[25]. In short, the SK-N-SH cells were plated onto 6-well microplates at a density of 5×10^5 cells/mL, and after 5.5 h, the medium was altered and incubated for 48 h. Cells were harvested after incubation with trypsin, supplemented with 5 μL Annexin V-FITC, followed by the addition of 10μL propidium iodide (PI), which was then mixed and incubated for 5 min at room temperature in the dark. The cells were measured by flow cytometry (BD Biosciences, San Jose, CA) and evaluated with the Flow Jo software (Tree Star, Ashland, OR).

SK-N-SH cells were cultured at a density of around 1×10^5 cells/mL on cell culture dishes for 48 h and stained with Hoechst 33342. They were then washed 3 times with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde at 4°C for 20 min. The cells were finally stained with 400 μL Hoechst 33342 reagent (Beyotime, Shanghai, China) for 15 min at room temperature. Lastly, the cells were observed and photographed under a fluorescent microscope (Nikon, Japan).

After transfection and incubation for 48 h, the SK-N-SH cells (1×105 cells/mL) cells were subjected to Wright-Giemsa staining. Thereupon, 4% paraformaldehyde was fixed at 4°C for 30 min. Subsequently, 1 mL Wright-Giemsa (Solarbio, Beijing, China) was added for 30 min at room temperature. The morphology of the cells was observed under a light microscope (Nikon, Japan), and images were captured with a digital camera (Olympus, Japan).

Statistical analysis

All assay results obtained are representative of at least three experiments, and the data were expressed as means ± standard deviation (SD). The student's *t*-test was used to assess differences between the mean values. Results with *P*-values less than 0.05 were considered statistically significant.

Results

Regulation of DUSP6 and FXR1P on the potential target genes of DUSP6

We employed six online predicting programs (BioGRID, Hitpredict, Genemania, STRING, InBioMap, and IntAct) to predict potential target genes for DUSP6. In total, seven potential target genes were selected for subsequent experiments based on the five or six programs that predicted them.

Furthermore, the qRT-PCR analysis indicated that when transfected with pcDNA3.1 (+)-*Dusp6*, the mRNA expression level of *Dusp6* increased whereas those of *ERK1, ERK2, TRKA, SRPK1,* and *TEX11* decreased ($P< 0.05$), and that there was no significant difference in the mRNA expression levels of *PI3K* and *mTOR* when compared to pcDNA3.1(+) (Fig. 1A & B). Even after being transfected with pcDNA3.1(-)-*FXR1*, the mRNA expression levels of *FXR1, ERK1, ERK2*, *PI3K*, and *TRKA* increased while the *mTOR* mRNA expression level did not show significant changes (Fig. 1A & C). However, when co-transfected with pcDNA3.1(+)-*Dusp6* and pcDNA3.1 (-)-*FXR1*, there was a significant decrease in the mRNA expression levels of *PI3K* and *mTOR* while those of *ERK1*, *ERK2,* and TRKA did not show any significant changes. (Fig. 1D)

FXR1P partially reverses the negative regulation of ERK1/2 by DUSP6 in SK-N-SH cells

We selected *ERK1* and *ERK2*, the target genes of DUSP6, for western blot. The results of the Western blot assay revealed that when the expression levels of DUSP6 increased, not only did the protein expression levels of ERK1/2 decrease, but that of p-ERK1/2 also decreased (Fig. 2A); when the FXR1P expression level increased, the protein expression levels of ERK1/2 and p-ERK1/2 increased as well (Fig. 2B); when the expression levels of FXR1P and DUSP6 simultaneously increased, the protein expression level of ERK1/2 and p-ERK1/2 did not significantly change (Fig. 2C).

FXR1P interacts with DUSP6 in SK-N-SH cells

Fluorescence microscopy, confocal microscopy, and immunoprecipitation were used to evaluate the interaction between FXR1P and DUSP6. Fluorescence microscopy showed a high transfection efficiency (~90%) of pDRed2-N1 at 50 nM concentration in SK-N-SH cells (Fig. 3A). pDsRed2-N1-*Dusp6* and pEGFP-N1-*FXR1* were then transfected into SK-N-SH cells, respectively, and a single transfection of pDsRed2-N1-*Dusp6* or pEGFP−N1-*FXR1* was discovered to be primarily confined in the cytoplasm (Fig. 3B & C). On the other hand, co-transfected pDsRed2-N1-*Dusp6* and pEGFP-N1-*FXR1* superimposed green-red fluorescence into yellow fluorescence at the same position in the cytoplasm and nucleus (Fig. 3D). Additionally, and in accordance with this observation, DUSP6 and FXR1P in SK-N-SH cells co-localized in the cytoplasm and nucleus (Fig. 3E). More importantly, our immunoprecipitation assay showed that FXR1P and DUSP6 could bind to each other (Fig. 3F). These

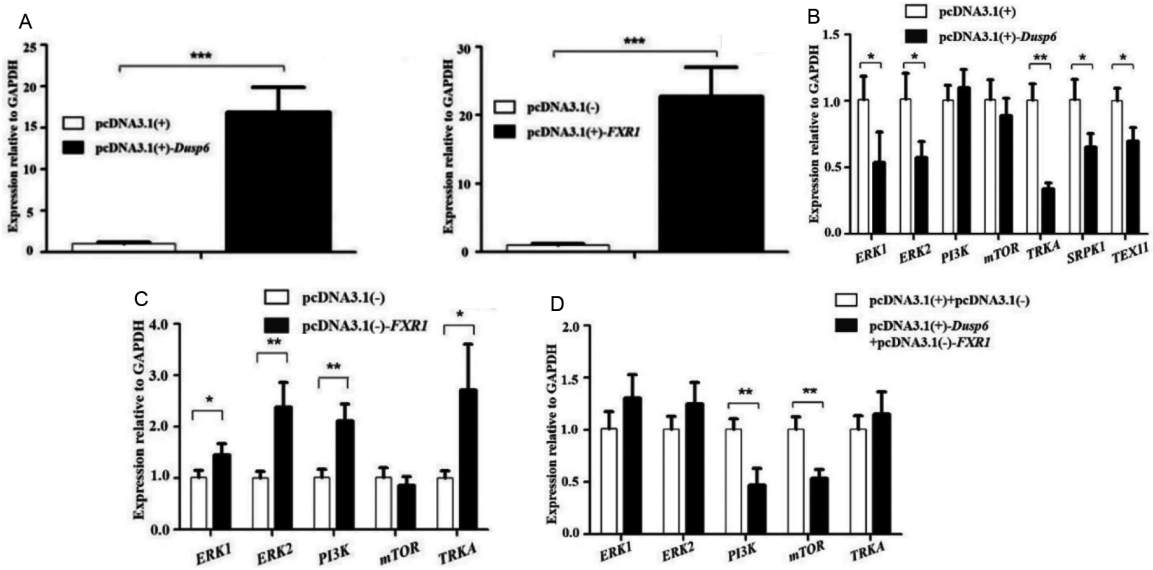

Fig. 1 — Regulatory effects of DUSP6 and FXR1P on DUSP6 target genes. (A) The expression of *Dusp6* (left) and *FXR1* (right) mRNA were significantly up-regulated after transfection; (B) SK-N-SH cells were transfected with pcDNA3.1(+)-*Dusp6* and the mRNA expression levels of seven potential target genes were detected by qRT-PCR; (C) SK-N-SH cells were transfected with pcDNA3.1(-)-*FXR1* and the mRNA expression levels of seven potential target genes were examined by qRT-PCR; and (D) SK-N-SH cells were transfected with pcDNA3.1(+)-*Dusp6* and pcDNA3.1(-)-*FXR1* and the mRNA expression levels of seven potential target genes were determined by qRT-PCR. Data are expressed as the means ± SD. The student's *t*-test was used to calculate statistical significance: *P* values, *<0.05, ** <0.01, ***<0.001

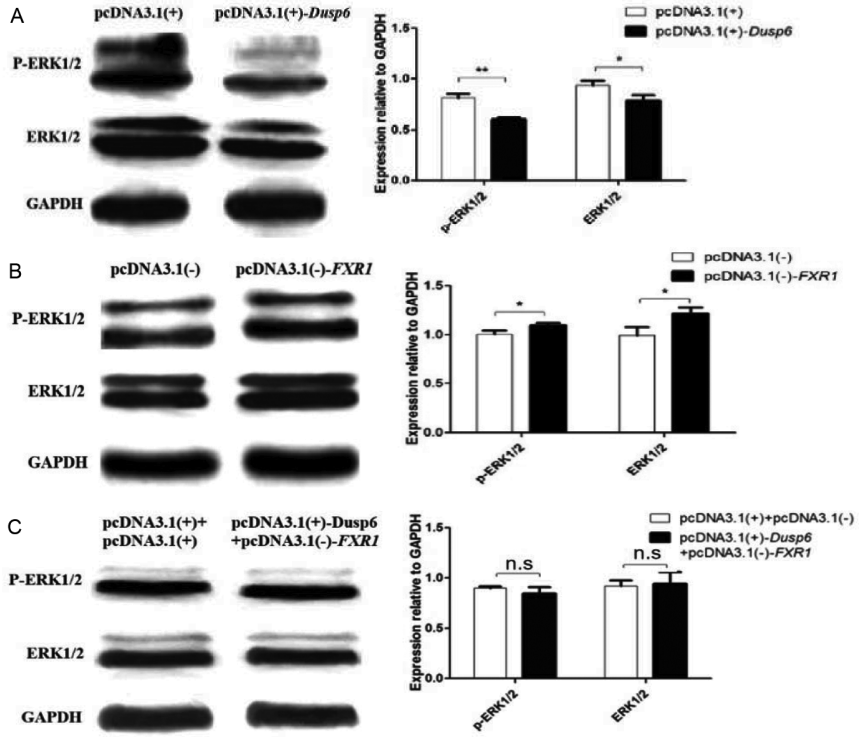

Fig. 2 — Effects of DUSP6 and FXR1P on ERK1/2 and p-ERK1/2 protein levels. (A) SK-N-SH cells were transfected with pcDNA3.1(+)-*Dusp6* and the protein levels of ERK1/2 and p-ERK1/2 were detected by western blot; (B) SK-N-SH cells were transfected with pcDNA3.1(-)-*FXR1* and the protein levels of ERK1/2 and p-ERK1/2 were determined by western blot; and (C) SK-N-SH cells were transfected with pcDNA3.1(-)-*FXR1* and pcDNA3.1(+)-*Dusp6* and the protein levels of ERK1/2 and p-ERK1/2 were analyzed by western blot. Data are expressed as means ± SD. The student's *t*-test was used to calculate statistical significance: *P* values, *<0.05, **<0.01, n.s. = not significant

results suggest that FXR1P and DUSP6 interact in SK-N-SH cells and are co-localized in the cytoplasm and nucleus.

DUSP6 inhibits proliferation and promotes apoptosis in SK-N-SH cells

We used CCK8, flow cytometry, Hoechst 33342 staining, and Wright-Giemsa staining to detect the effects of DUSP6 on the growth of SK-N-SH cells. To determine the effects of DUSP6 on the proliferation of SK-N-SH cells, we used the CCK8 assay to evaluate cell viability at 0 h, 24 h, 48 h, 72 h, and 96 h. The results indicated that DUSP6 inhibited the proliferation of SK-N-SH cells, which was significant at 48 h and 72 h. (Fig. 4A). Meanwhile, SK-N-SH cells transfected with pcDNA3.1(+)-Dusp6 were quantified at 48 h by flow cytometry. The results showed that DUSP6 inhibited proliferation and promoted the apoptosis of SK-N-SH cells (Fig. 4B), while the results detected in Wright-Giemsa staining and Hoechst 33342 staining were consistent with those from the flow cytometry analysis. (Fig. 4C & D)

Discussion

RBPs, the primary regulators of co-transcription and post-transcription, control and coordinate each stage of the RNA life cycle[26]. As part of the fragile X protein family, the RNA binding protein FXR1P, along with FMRP and FXR2P, are involved in the transportation, translation, and degradation of mRNAs. However, the molecular mechanisms and pathogenesis of FXS, as well as the role of FXR1P in the nervous system, are still unclear. The lack of FMRP prompts raised protein products, bringing about upgraded signaling in various intracellular pathways, including extracellular signal-regulated kinase (ERK)[27], mammalian target of rapamycin (mTOR)[28,29], glycogen synthase kinase 3 beta (GSK3β), phosphatidylinositol 3-kinase (PI3K)[26] and metabotropic glutamate receptor 5 (mGLuR5) pathways[30], which are responsible for FXS patients' molecular mechanisms underlying behavioral and cognitive deficits in FXS patients. Past studies of FXS have demonstrated that the expression levels of ERK1/2 and PI3k were both upregulated and

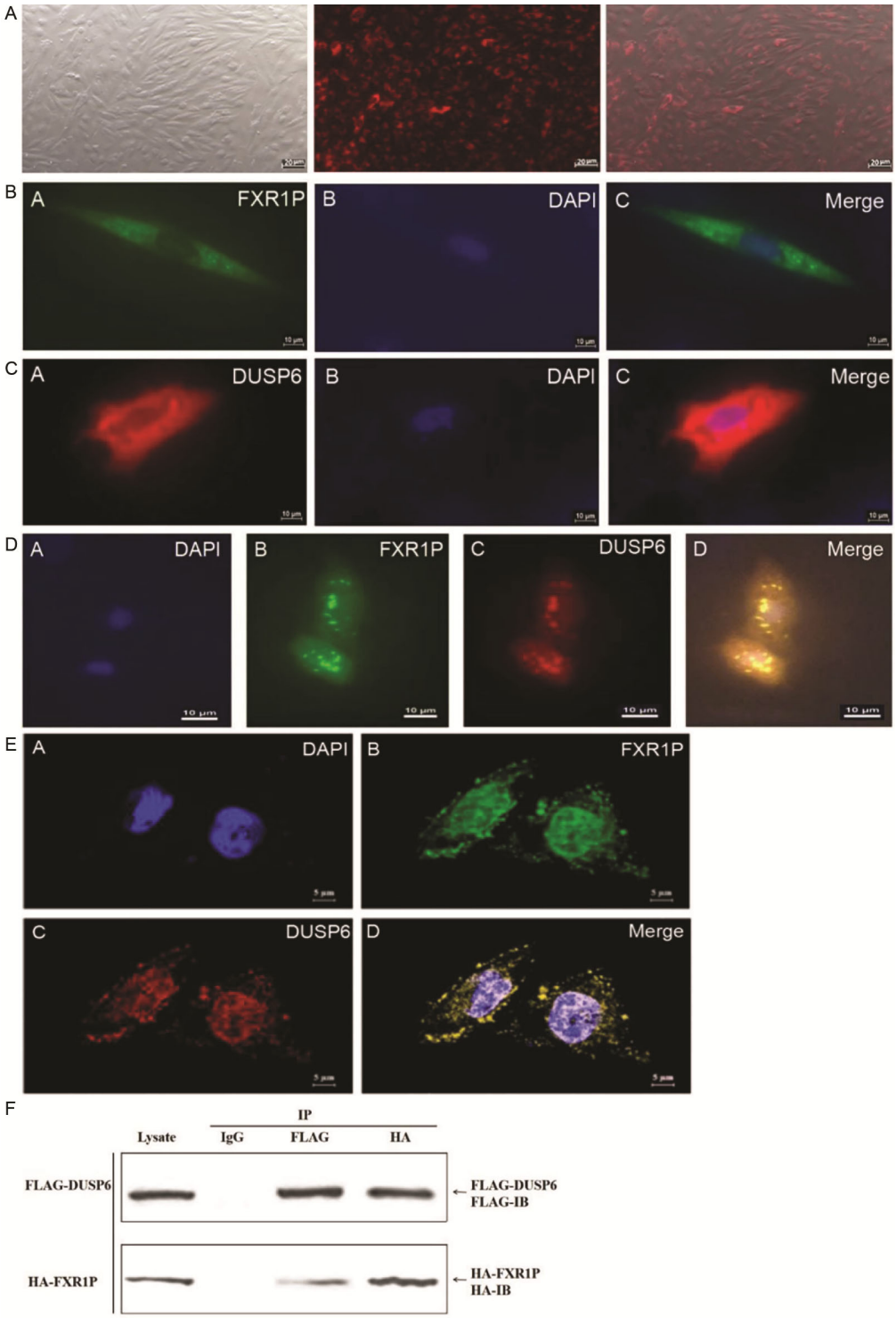

Fig. 3 — Interaction between FXR1P and DUSP6 in SK-N-SH cells. (A) SH-N-SH cell transfection efficiency test (10×) (Light: Bright; Middle: Dark; Right: Merge); (B) Localization of FXR1P in SK-N-SH cells by fluorescence microscopy (40×); (C) Localization of DUSP6 in SK-N-SH cells by fluorescence microscopy (40×); (D) Localization of DUSP6 and FXR1P in SK-N-SH cells by fluorescence microscopy (40×); (E) Localization of DUSP6 and FXR1P in SK-N-SH cells by confocal (60× Oil glass); and (F) Immunoprecipitation showing the interaction between FXR1P and DUSP6 in SK-N-SH cells

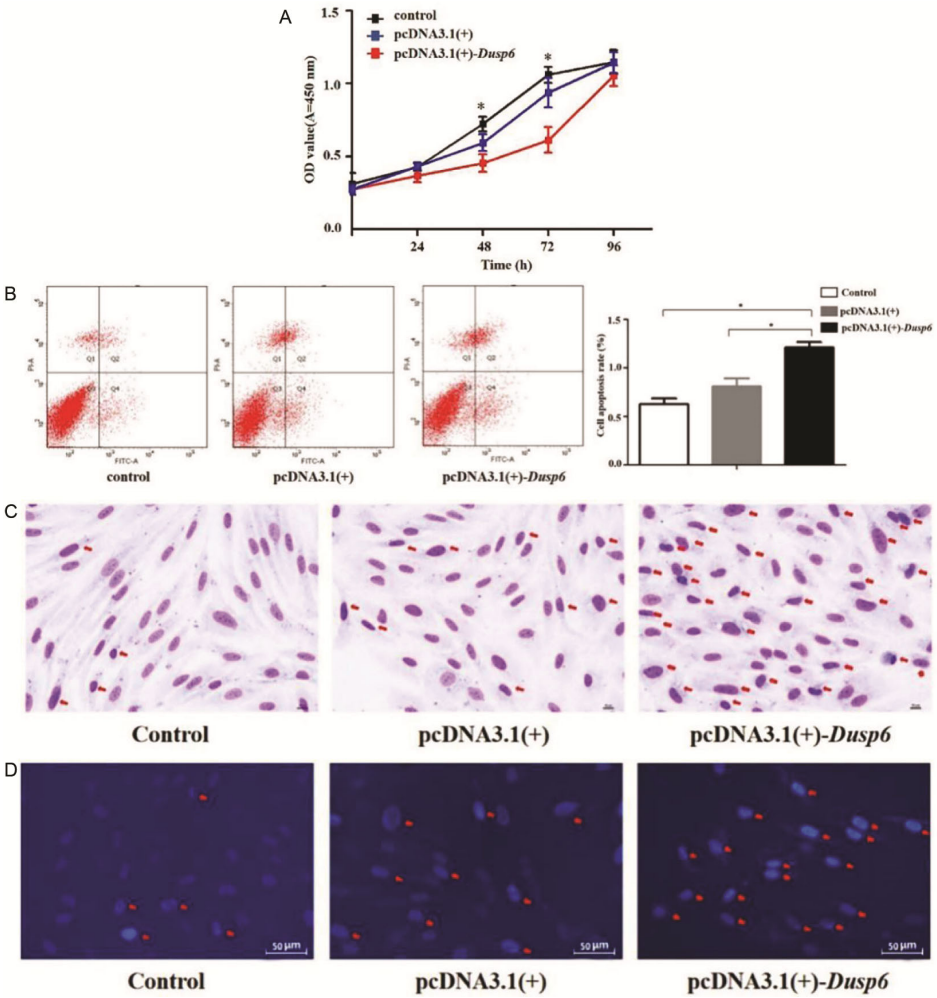

Fig. 4 — DUSP6 promoted apoptosis in SK-N-SH cells. (A) The growth curve of SK-N-SH cells when transfected with pcDNA3.1(+)-*Dusp6* at 0 h, 24 h, 48 h, 72 h, and 96 h; (B) Apoptosis was analyzed with Annexin-V/ PI staining assay; (C) Apoptosis was determined by Wright-Giemsa staining assay (20X); and (D) Apoptosis was analyzed with Hoechst 33342 staining assay (20X). Data are expressed as means ± SD. The student's *t*-test was used to calculate statistical significance: *P* values, * <0.05

down-regulated[31,32]. Interestingly, ERK phosphatase DUSP6 may constitute a new branch point between the two principal signal transduction pathways induced by the growth factor MEK/ERK pathway and PI3K/mTOR pathway[33]. Results from immune-precipitation and confocal microscopy suggested that FXR1P interacts with DUSP6 in SK-N-SH cells, whereby both co-localize in the cytoplasm and nucleus clarifying the function of FXR1P in the pathogenesis of FXS.

DUSP6, a member of MAPK, inactivates its target kinase within MAP kinase by dephosphorylating phosphoserine/threonine and phosphotyrosine residues, thereby inactivating ERK1/2[34,35]. Meanwhile, it has been identified as a negative regulator of the RAS-ERK pathway during vertebral development. In fact, in our study, when *Dusp6* was highly expressed in SK-N-SH cell lines, the mRNA expression levels of *ERK1*, *ERK2*, *TRKA*, *SRPK1*, and *TEX11* decreased, and similarly, the protein levels of ERK1/2 and p-ERK1/2 also decreased; when *FXR1* was highly expressed in SK-N-SH cell lines, the mRNA expression levels of *ERK1*, *ERK2*, *PI3K*, and *TRKA* mRNA expression levels were up-regulated, and the protein levels of ERK1/2 and p-ERK1/2 were elevated as well; when in *Dusp6* and *FXR1* were simultaneously highly expressed, the mRNA expression levels of *ERK1*, *ERK2* and *TRKA* did not change significantly, and neither did the protein levels of ERK1/2 and p-ERK1/2. These results imply that FXR1P may regulate MEK/ERK cell signaling pathways in SK-N-SH cells by interacting with DUSP6, and FXR1P may partially

reverse the negative regulation of ERK1/2 by DUSP6 in SK-N-SH cells.

DUSP6 is universally expressed and reported in multiple tumors, proliferation, differentiation, apoptosis, and homeostasis[36]. Inter- and intracellular communication distinctly requires the MEK/ERK pathway to regulate cellular activities such as growth, survival, differentiation, and apoptosis[37-39]. Hence, the dysregulation of the MEK/ERK pathway may disrupt cellular activities, resulting in developmental abnormalities, cancer, and cognitive impairment[40]. Herein, results of flow cytometry, Hoechst 33342 staining, Wright-Giemsa staining, and CCK8 demonstrated that DUSP6 inhibited cell proliferation and promoted apoptosis in SK-N-SH cells. One of the primary downstream targets of ERK signaling is E26(ETS)2, a transcriptional factor that binds to the *Dusp6* promoter, resulting in the upregulation of *Dusp6*[41]. The Ets2 transgenic murine experiment determined that Ets2 is correlated with the p53 promoter region[42,43], which may play a pivotal role in inducing apoptosis within SK-N-SH cells by DUSP6.

Conclusion

Our results revealed that FXR1P and DUSP6 interacted in SK-N-SH cells, and both were co-localized in the cytoplasm and nucleus of the cells. Furthermore, the overexpression of *Dusp6* promoted apoptosis and inhibited cell proliferation in SK-N-SH cells. Meanwhile, FXR1P regulated the ERK1/2 signaling pathways in SK-N-SH cells by interacting with DUSP6. At the same time, FXR1P may also reverse the negative regulation of ERK1/2 through DUSP6 in SK-N-SH cells to a certain extent.

Conflict of interest

All authors declare no conflict of interest.

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