

# CMP-N-acetylneuraminic acid synthetase interacts with fragile X related protein 1

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**Abstract.** Fragile X mental retardation protein (FMRP), fragile X related 1 protein (FXR1P) and FXR2P are the members of the FMR protein family. These proteins contain two KH domains and a RGG box, which are characteristic of RNA binding proteins. The absence of FMRP, causes fragile X syndrome (FXS), the leading cause of hereditary mental retardation. FXR1P is expressed throughout the body and important for normal muscle development, and its absence causes cardiac abnormality. To investigate the functions of FXR1P, a screen was performed to identify FXR1P-interacting proteins and determine the biological effect of the interaction. The current study identified CMP-N-acetylneuraminic acid synthetase (CMAS) as an interacting protein using the yeast two-hybrid system, and the interaction between FXR1P and CMAS was validated in yeast using a  $\beta$ -galactosidase assay and growth studies with selective media. Furthermore, co-immunoprecipitation was used to analyze the FXR1P/CMAS association and immunofluorescence microscopy was performed to detect expression and intracellular localization of the proteins. The results of the current study indicated that FXR1P and CMAS interact, and colocalize in the cytoplasm and the nucleus of HEK293T and HeLa cells. Accordingly, a fragile X related 1 (*FXR1*) gene overexpression vector was constructed to investigate the effect of *FXR1* overexpression on the level of monosialotetrahexosyl-ganglioside 1 (GM1). The results of the current study suggested that FXR1P is a tissue-specific regulator of GM1 levels in SH-SY5Y cells, but not in HEK293T cells. Taken together, the results initially indicate that FXR1P interacts with CMAS, and that FXR1P may enhance the activation of sialic acid via interaction with CMAS, and increase GM1 levels to affect the

development of the nervous system, thus providing evidence for further research into the pathogenesis of FXS.

## Introduction

Fragile X syndrome (FXS) is the most common inherited developmental disorder. The disorder results from defects in the fragile X mental retardation 1 (*FMRI*) gene, and lack of *FMRI* expression caused by hyper-expansion and methylation of CGG repeats in the first exon of this gene (1-3). The FMR protein family has three highly homologous members. The fragile X mental retardation protein (FMRP) is coded by the *FMRI* gene. Two other members are the fragile X related protein 1 (FXR1P) (4) and FXR2P (5), which are coded by the fragile X related 1 (*FXR1*) gene and *FXR2*, respectively. These proteins possess two KH domains and a RGG box, which suggests that they are involved in intracellular RNA transport and translation regulation (6-10). They also contain a nuclear localization signal and a nuclear export signal, which suggests that they can shuttle between the nucleus and the cytoplasm (11).

FMRP exhibits similar sequence identity (>60%) to FXR1P/FXR2P, and also contains two KH domains and RGG box (12). The absence of FMRP is the cause of FXS, however, it is not established whether FXR1P and FXR2P are associated with FXS pathology or phenotype. Additionally, it is not established whether FXR1P and FXR2P may compensate for the absence of FMRP. Furthermore, no significant neuropathological abnormalities were demonstrated in fetal brain tissue from FXS with FXR1P and FXR2P are normally expressed (13). FMRP, FXR1P and FXR2P can interact with themselves and each other (5,14,15), however, their distributions demonstrated individual expression patterns in certain mouse and human tissues (16,17), which indicates that each protein also may function autonomously (18).

FXR1P is expressed throughout the body, particularly in muscle and heart tissue. There are seven FXR1P isoforms in mouse (19,20), including three muscle-specific isoforms (20) and one cardiac-specific isoform (21). FXR1P normal expression is essential for postnatal viability. A previous study demonstrated that inactivation of FXR1P in mice caused the death of neonates shortly after birth, which was likely due to abnormal development of the myocardial or respiratory muscle (20). In *Xenopus*, FXR1P regulates the development

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of somites, eyes and cranial cartilage, however, reduction of FXR1P disrupted the rotation and segmentation of somitic myotomal cells and hindered normal myogenesis (22). In a previous study in zebrafish, inactivation of FXR1P caused abnormalities in striated muscle and severe cardiomyopathy, and led to heart failure in embryos (23). In humans, altered expression of muscle-specific isoforms of FXR1P has been previously implicated in facioscapulohumeral muscular dystrophy (24). Additionally, a previous study demonstrated that inactivation of FXR1P enhanced the expression level of tumor necrosis factor- $\alpha$  in mouse macrophages (25). Collectively, these previous studies demonstrated that FXR1P has an important regulatory function in the development of normal and cardiac muscle (26,27).

## Materials and methods

**Strains and cell lines.** The *Escherichia coli* (*E.coli*) strains, DH5 $\alpha$  and TOP10 (China Center for Type Culture Collection, Wuhan, China), were cultured in LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.0; Oxoid; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C. The *Saccharomyces cerevisiae* strains, Y187 and AH109, were cultured at 30°C in yeast extract peptone dextrose (YPD) medium (2% tryptone, 1% yeast extract, and 2% glucose; Oxoid; Thermo Fisher Scientific, Inc.) or SC medium (0.67% yeast nitrogen base without amino acids, 2% glucose) supplemented with an amino acid mixture without the indicated amino acids for selection. HeLa and HEK293T cells (Academy of Military Medical Sciences, Beijing, China) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal calf serum (Zhejiang Tianhang Biotechnology Co., Ltd., Hangzhou, China) in a humidified 5% CO<sub>2</sub> incubator at 37°C. SH-SY5Y cells were cultured in DMEM:nutrient mixture F12 supplemented with 10% fetal calf serum in a humidified 5% CO<sub>2</sub> incubator at 37°C.

**Plasmids.** For two-hybrid screening, the full open reading frame of the human *FXR1* (GenBank NM\_005087.3) gene was subcloned into the pGBKT7 vector (28) (Clontech Laboratories, Inc., Mountainview, CA, USA) to construct pGBKT7-*FXR1* (Table I), which was used as the bait plasmid. In the co-immunoprecipitation assay, the full open reading frames of the human *FXR1* gene and human CMP-N-acetylneuraminic acid synthetase (*CMAS*) gene (GenBank NM\_018686.4) were subcloned into the pCMV-HA vector (28) (Clontech Laboratories, Inc.) and pCMV-Myc vector (28) (Clontech Laboratories, Inc.) to construct the recombinant vectors, pCMV-HA-*FXR1* and pCMV-Myc-*CMAS*. In the colocalization assay, the full open reading frame of the human *FXR1* gene and human *CMAS* gene were subcloned into the pEGFP-N1 vector (28) (Clontech Laboratories, Inc.) and pDsRed-Monomer-N1 vector (28) (Clontech Laboratories, Inc.), respectively, to construct pEGFP-N1-*FXR1* and pDsRed-Monomer-N1-*CMAS* (Table I). Additionally, the full open reading frame of the human *FXR1* gene was inserted into the pcDNA3.1(-) vector (29) (Clontech Laboratories, Inc.) to generate the recombinant vector, pcDNA3.1(-)-*FXR1* (Table I), which was transfected into SH-SY5Y cells using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific,

Inc.) and biological effect of the interaction between FXR1P and CMAS was detected.

**Yeast two-hybrid screening.** All procedures were performed out according to the yeast two-hybrid system manufacturer's instructions (Clontech Laboratories, Inc.). The yeast strain AH109 (Clontech Laboratories, Inc.) was first transformed with the pGBKT7-*FXR1* recombinant plasmid, which was used as the bait to screen the library. The Human Fetal Brain Matchmaker cDNA library (Clontech Laboratories, Inc.) cloned into the vector pGADT7 was transformed into the yeast strain Y187, which was used as prey for two-hybrid screening (30). The yeast strain AH109-pGBKT7-*FXR1* was mated with Y187 pre-transformed with the cDNA library and then cultured for 20 h in YPD adenine medium supplemented with kanamycin to produce diploid zygotes. The products were plated on leucine, tryptophan and histidine-free SD medium (SD/LTH) and incubated for 3 days at 30°C. After 3 days, colonies on individual SD/LTH plates were counted and the total number of transformants was calculated. To verify putative interactions between bait protein and prey proteins from the cDNA libraries, the colonies present on SD/LTH medium were collected and assayed for  $\beta$ -galactosidase ( $\beta$ -gal) activity (31). The  $\beta$ -gal activity assay was conducted using a colony-lift filter assay according to the manufacturer's instructions (Clontech Laboratories, Inc.). Subsequently, the library from the positive colonies (positive growth in SD/LTH media and positive  $\beta$ -gal activity) were extracted and digested with *Hind* III using a Qiagen Plasmid Midi and Maxi kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. Plasmid fragment sizes were analyzed by 1% agarose gel electrophoresis to segregate colonies. Plasmid DNA purified from the positive yeast colonies was used to transform *E.coli* TOP10. Finally, the identity of the positive clones was determined by sequencing and Basic Local Alignment Search Tool searches.

**Validation of the interaction between CMAS and FXR1 using the yeast two-hybrid system.** The purified bait plasmid pGBKT7-*FXR1* was transformed into the AH109 strain and was then cultured on tryptophan-free SD medium (SD/T) plates for toxicity and autonomous activation assays (32). The recombinant vector pGADT7-*CMAS* was transformed into the yeast strain Y187 and then plated on SD medium lacking leucine (SD/L) (33) (Oxoid; Thermo Fisher Scientific, Inc.). Subsequently, AH109-pGBKT7-*FXR1* was mated to Y187-pGADT7-*CMAS*. The transformants were cultured in yeast peptone dextrose adenine medium for 20 h and then plated on the SD/LTH. The interaction between CMAS and FXR1P was confirmed following colony formation on the plates and  $\beta$ -gal assays.

**Co-immunoprecipitation assay.** HEK293T cells were maintained in DMEM medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin. At 70% confluency, HEK293T cells were seeded into 6-well plates with basal serum and antibiotic-free DMEM. According to manufacturer's protocols, the plasmids pCMV-HA-*FXR1* and pCMV-Myc-*CMAS* were co-transfected into HEK293 cells with Lipofectamine 2000 (Invitrogen;

Table I. Plasmids used in the current study.

Plasmid name	Vector	Insert(s)	Insertion site	Primers (5'-3')
pGBKT7-FXR1	pGBKT7	FXR1	<i>EcoR I</i>	ctagaattcatggcggacgtgacgggtgctagaatt cttatgaaacaccattc
pCMV-HA-FXR1	pCMV-HA	FXR1	<i>EcoR I, XhoI</i>	tatgaattcggatggcggagctgacgggtggaggcg ctcagttatgaaacaccattcaggac
pCMV-Myc-CMAS	pCMV-Myc	BTF	<i>EcoR I, XhoI</i>	gtagaattccgatggactcgggtggagcgtctcag ctatTTTTggcatgaat
pEGFP-N1-FXR1	pEGFP-N1	FXR1	<i>Xho I, EcoR I</i>	tatctcgagctatggcggagctgacgggtggaggcg gaattcttatgaaacaccattcaggac
pDsRed-Monomer-N1-CMAS	pDsRed-Monomer	BTF	<i>Xho I, EcoR I</i>	tatctcgagctatggactcgggtggaggcggaattc ctatTTTTggcatgaat
pcDNA3.1(-)-FXR1	pcDNA3.1(-)	FXR1	<i>EcoR I, XhoI</i>	tatctcgagctatggcggagctgacgggtggaggcg gaattcttatgaaacaccattcaggac

Thermo Fisher Scientific, Inc.), and pCMV-HA-FXR1 and pCMV-Myc-CMAS were transfected as controls. After 5 h, the cells were cultured with DMEM containing 10% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin. After 48 h, the transfected cells were collected, and then lysed with RIPA lysis buffer containing protease inhibitor. The cell lysates were harvested and mixed with 2  $\mu$ g anti-hemagglutinin (HA; CW0092; Beijing ComWin Biotech Co., Ltd., Beijing, China) or anti-*v*-myc avian myelocytomatosis viral oncogene homolog (Myc; CW0088; Beijing ComWin Biotech Co., Ltd.) antibody and placed on a shaker for overnight at 4°C. Subsequently, protein A/G agarose beads were added and then rocked at 4°C for 4 h. The pulled-down proteins were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride membranes. The membranes were blocked with dried skim milk for 2 h at room temperature and the blot was probed with Myc monoclonal antibody (1:1,000) overnight at 4°C. The membranes were then incubated for 2 h at 4°C with horseradish peroxidase-conjugated goat anti-mouse antibody (1:1,000; CW0102; Beijing ComWin Biotech Co., Ltd.). Bands were visualized using an ECL Western Blotting Detection System (Shanghai Majorbio Bio-Pharm Technology Co., Ltd., Shanghai, China) and imaged (34).

**Subcellular colocalization of FXR1P and CMAS.** HEK293T and HeLa cells were maintained in DMEM medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin. At 70% confluency, HEK293T and HeLa cells were plated in laser scanning confocal petri dish with basal serum- and antibiotic-free DMEM. The plasmids pEGFP-N1-FXR1 and pDsRed-Monomer-N1-CMAS were single-transfected and co-transfected into HEK293T cells and HeLa cells using Lipofectamine 2000 (35). After 5 h, the medium was then replaced with complete DMEM containing 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin. After 48 h, the transfected cells were collected and then washed twice with phosphate-buffered saline. Subsequently, 4% paraformaldehyde was used to fix the transfected cells and

the nuclei were stained with 4',6-diamidino-2-phenylindole (Beyotime Institute of Biotechnology, Nantong, China) for 10 min. A laser confocal microscope (Carl Zeiss AG, Oberkochen, Germany) was used to observe the localization and colocalization of CMAS and FXR1P in the cells.

**Monosialotetrahexosylganglioside 1 (GM1) enzyme-linked immunosorbent assay (ELISA).** HEK293T and SH-SY5Y cells were maintained in basal DMEM and DMEM/F12, respectively, lacking FBS and antibiotics. HEK293T and SH-SY5Y cells were divided into normal cell, empty vector and FXR1 overexpression group cells. The empty vector group and FXR1 overexpression group cells were transfected with the empty plasmid pcDNA3.1(-) and recombinant vector pcDNA3.1 (-)-FXR1, respectively, using Lipofectamine 2000 (36). Subsequently, cells from each group were collected and lysed. Lysates were centrifuged to remove cell debris (400 x g, 5 min, 4°C), and ELISA was performed by a human anti-GM1 ELISA kit (YM-L0587; Shanghai YuanMu Biological Technology Co., Ltd., Shanghai, China) according to the manufacturer's protocol. Measurements in all groups were repeated six times.

**Statistical analysis.** Data are presented as the mean  $\pm$  standard deviation. The association among three groups was assessed by analysis of variance using SPSS software, version 19.0 (IBM SPSS, Armonk, NY, USA).  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**FXR1P-interacting protein screening using the yeast two-hybrid system.** The bait plasmid pGBKT7-FXR1 was constructed with complete human FXR1 and the prey plasmids pGADT7-cDNA were constructed with the complete Human Fetal Brain Matchmaker cDNA library. The results of sequencing analysis demonstrated that the inserts were in-frame and the restriction sites were correct. Subsequently, AH109-pGBKT7-FXR1 with Y187-pGADT7-cDNA

transformants were mated on SD/LTH medium and  $\beta$ -gal assays were performed. In total, 10 positive colonies were formed on the SD/LTH medium and exhibited positive results in the  $\beta$ -gal assay. Following sequencing and analysis, a colony that produced a 434-amino acid protein with high identity with human CMAS (99% identity) was identified and used in the subsequent experiments.

*Validation of the interaction between FXR1P and CMAS using the yeast two-hybrid system.* To validate the interaction between the bait protein and prey protein from the cDNA library, Y187-pGADT7-cDNA colonies and AH109-pGBKT7-FXR1 were mated again and cultured on SD/LTH medium plates. Prey plasmid was recovered as described previously from colonies that continued to grow after 5 days on SD/LTH medium. Subsequently, the  $\beta$ -gal assay demonstrated a positive result. Additionally, no  $\beta$ -gal activity was observed in the yeast strain transformed with only the bait plasmid (pGBKT7-FXR1).

*Directing verification of interaction between FXR1P and CMAS by co-immunoprecipitation.* To verify the direct interaction between FXR1P and CMAS, two recombinant vectors, pCMV-HA-FXR1 and pCMV-Myc-CMAS, were constructed and the results of sequencing analysis suggested that the inserts were in-frame and the restriction sites were correct. Subsequently, pCMV-Myc-CMAS was co-transfected with pCMV-HA-FXR1 into HEK293T cells, and cell extracts were immunoprecipitated using the polyclonal anti-HA antibody or anti-Myc antibody, and protein A/G agarose beads. Following precipitation with anti-HA antibody, CMAS-Myc was detected using anti-Myc antibody and FXR1P-HA using anti-HA antibody. Furthermore, following precipitation with the anti-Myc antibody, FXR1P-HA was detected by anti-HA antibody (Fig. 1) and CMAS-Myc was detected by anti-Myc antibody (Fig. 1). Additionally, a negative control was performed using IgG (Fig. 1). The results indicated that the demonstrated interaction between FXR1P and CMAS is not because of nonspecific binding with IgG.

*FXR1P colocalizes with CMAS in HEK293T and HeLa cells.* To measure the intracellular interaction between FXR1P and CMAS, two recombinant eukaryotic expression vectors, pEGFP-N1-FXR1 and pDsRed-N1-CMAS, were constructed. pEGFP-N1-FXR1 contained a gene segment of FXR1 and pDsRed-N1-CMAS contained a gene segment of CMAS. All recombinant constructs were sequence-verified. HEK293T and HeLa cells were transfected with the constructs, and the expression and subcellular distribution of these proteins were analyzed by laser confocal microscopy.

HEK293T and HeLa cells transfected with pEGFP-N1-FXR1 exhibited strong cytoplasmic fluorescence, whereas transfection with pDsRed-N1-CMAS produced fluorescence in the nucleus (Fig. 2). Thus, the fusions to the N-terminus of EGFP or DsRed did not affect the fluorescence properties of the native protein, allowing the fusion protein to be correctly localized *in vivo*.

To evaluate the colocalization of FXR1P and CMAS, pEGFP-N1-FXR1 and pDsRed-N1-CMAS plasmids were transiently cotransfected into HEK293T and HeLa cells (Fig. 3). Transfection with pEGFP-N1-FXR1 produced green

fluorescence, predominantly in the cytoplasm, whereas pDsRed-N1-CMAS transfection predominantly produced red fluorescence in the nucleus and less in the cytoplasm. The two polypeptides (pEGFP-N1-FXR1 and pDsRed-N1-CMAS) maintained intracellular localization, with yellow fluorescence demonstrating FXR1P and CMAS colocalization in the cytoplasm and the edge of the nucleus (Fig. 3). Typically, these genes are expressed in different parts of the cell, with no fluorescence overlap, but co-overexpression leads to the formation of oligomers in the cytoplasmic and nucleus, producing yellow fluorescence.

*Overexpression of FXR1 increases the concentration of GM1 in SH-SY5Y cells.* The current study demonstrated that FXR1P interacts with CMAS. CMAS catalyzes the synthesis of CMP-sialic acid, which is an activated form of sialic and the raw material of ganglioside synthesis. Accordingly, CMAS activity has been demonstrated to be indirectly associated with amount of the sialic acid converted to GM1 in cells. To estimate the effect of FXR1P overexpression on CMAS activity, recombinant vector pcDNA (-) 3.1-FXR1 was constructed and then the concentration of GM1 was measured in SH-SY5Y and HEK293T cells transfected with pcDNA3.1 (-)-FXR1. ELISAs demonstrated that the concentration of GM1 was significantly increased in SH-SY5Y cells transfected with recombinant vector compared with untransfected cells ( $P=0.016$ ; Table II; Fig. 4A), whereas there was no significant difference between the empty vector-transfected SH-SY5Y cells compared with untransfected cells (Table II; Fig. 4A). However, there was no significant difference in GM1 concentration among the three groups of HEK293 cells (Fig. 4B). These results demonstrated that overexpression of FXR1 increases the concentration of GM1 in SH-SY5Y cells by promoting CMAS activity, but not in HEK293T cells (Table II; Fig. 4B).

## Discussion

FMRP and FXR1P members of the FMR protein family. It has been previously demonstrated that FMRP is highly expressed in neurons (37) and involved in the development of dendritic spines, and the plasticity and structural remodeling of synapses (38,39). Deficiency of FMRP can cause mental retardation and impaired memory and learning. It was previously demonstrated that FXR1P and FMRP are co-expressed in certain tissues and regulated other proteins at the translational level (40). A previous study demonstrated that when FXR1 is expressed normally in the brain tissue of patients with FXS, there were no significant neuropathological abnormalities, which indicated that FXR1P may rescue the function of FMRP (41). Currently, it remains unclear whether the mechanism of FXS is associated with FXR1P, but a variety of protein and factors are likely to be involved in this process. Thus, the study of protein and factors that interact with FXR1P will aid the clarification of the function of FXR1P in the pathogenesis of FXS.

Various methods, including co-immunoprecipitation, the yeast two-hybrid system, surface plasmon resonance and pull-down technology, are used to investigate interactions between proteins. The current study screened proteins using the yeast two-hybrid system, and demonstrated that CMAS

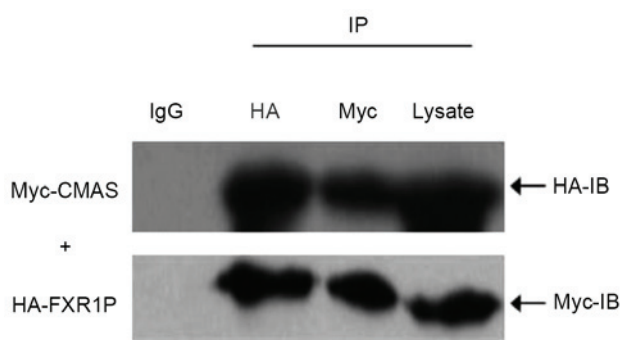


Figure 1. Co-IP of FXR1P and CMAS in HEK293T cells. Cells were transfected with pCMV-HA-FXR1 and pCMV-Myc-CNS, then lysates were subjected IP using anti-HA or anti-Myc mouse monoclonal antibodies, normal mouse IgG used as a negative control. The IP components of the assay and whole cell lysate were examined by western blot analysis with anti-HA and anti-Myc antibodies. IP, immunoprecipitation; IgG, immunoglobulin G; HA, hemagglutinin tag; Myc, *v*-myc avian myelocytomatosis viral oncogene homolog; CMAS, CMP-N-acetylneuraminic acid synthetase; FXR1P, fragile X related 1; IB, immunoblot.

interacted with FXR1P. However, the two-hybrid system tends to identify a high proportion of false positives (protein hits that are unlikely to associate *in vivo*) (42). Thus, the results were validated using immunoprecipitation and cellular colocalization analysis in subsequent experiments.

Co-immunoprecipitation is a powerful and simple method to detect the interaction between proteins. Its principle is that certain binding proteins are preserved intact when cells are lysed under non-denaturing conditions. Based on the specificity between the antibody and the antigen, a protein of interest (protein X) can be immunoprecipitated with a targeted antibody, then protein Y, which binds with protein X, can be also precipitated. In the present study, the interaction between FXR1P and CMAS was investigated using this method. SDS-PAGE and western blotting demonstrated that CMAS was also precipitated when FXR1P was pulled-down by a specific antibody from the total proteins of cells co-transfected with pCMV-Myc-CMAS and pCMV-HA-FXR1 (Fig. 1). This demonstrated an interaction between FXR1P and CMAS. However, co-immunoprecipitation does not fully explain the specific interaction between two proteins *in vivo*, because the interaction may not be direct and may be an indirect interaction through other proteins (43). Thus, the result of co-immunoprecipitation was validated by colocalization analysis of CMAS and FXR1P cells. The areas where the proteins interact in cells were demonstrated by laser confocal microscopy.

For intracellular colocalization experiments, HEK-293T cells and HeLa cells were transfected with an EGFP-FXR1 fusion vector and DsRed-CMAS fusion vector. This demonstrated that FXR1P and CMAS were respectively located in the cytoplasm and nuclei. However, when the cells were co-transfected with both the fluorescent protein fusion vectors, the merge signal was observed in the cytoplasm and nucleus. The interaction between the two proteins was confirmed by co-immunoprecipitation *in vitro*, and the overlapping fluorescence signal indicated there was an interaction between FXR1P and CMAS in live mammalian cells.

CMAS is an enzyme that can catalyze the synthesis of sialic acids, which are a family of nine-carbon sugars

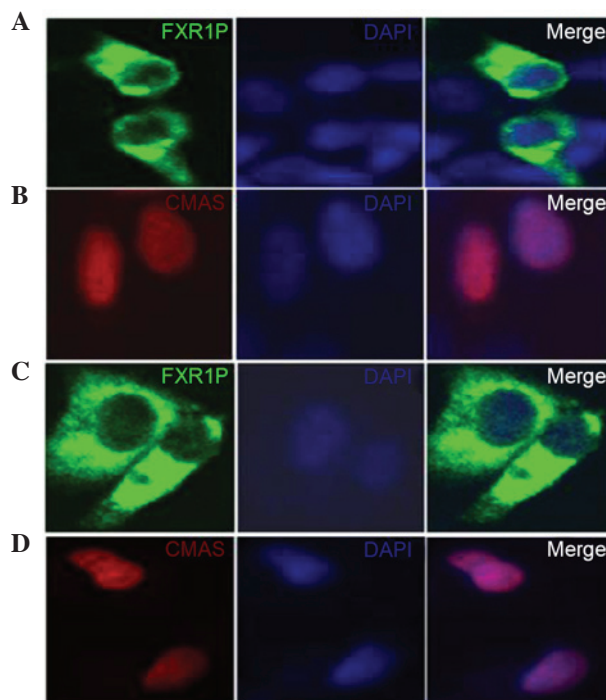


Figure 2. Expression and localization of FXR1P and CMAS in HEK293T cells and HeLa cells. HEK293T cells respectively transfected with (A) pEGFP-N1-FXR1 and (B) pDsRed-N1-CMAS. FXR1P is located in the cytoplasm. CMAS is located in the nucleus. HeLa cells respectively transfected with (C) pEGFP-N1-FXR1 and (D) pDsRed-N1-CMAS. Cells were visualized by fluorescence microscopy. FXR1P is also located in the cytoplasm and CMAS in the nucleus. The nuclei were stained with DAPI. Magnification,  $\times 100$ . FXR1P, fragile X related 1; CMAS, CMP-N-acetylneuraminic acid synthetase; DAPI, 4',6'-diamidino-2-phenylindole.

on cell surface glycoproteins and glycolipids important for determining the structure and function of various animal tissues, including cell-cell communications and immune responses (44). Sialylation occurs in two stages; activation of sialic acid by the enzyme CMAS and transfer to the target molecule to generate various GMs (45,46). The current study demonstrated that overexpression of *FXR1* increased the concentration of GM1 in SH-SY5Y cells, which may be caused by increased CMAS activity. However, this effect was limited to SH-SY5Y cells, and the GM1 concentration in HEK293T cells was not changed by *FXR1* overexpression. It is well established that gangliosides promote the occurrence, growth and differentiation of nerve in the nervous system, however, HEK293T are human embryonic kidney cells. Thus, it is speculated that the regulation of GM1 level is tissue-specific.

Previous investigation has demonstrated that the level of sialic acid is very high in the brain and important for various cellular activities (47), including cell adhesion and migration, neurite growth, neuron differentiation and synapse formation. Additionally, it has been previously demonstrated that high levels of sialic acid increased the concentration of gangliosides and promoted the development of cognitive ability (48). For some patients with neurological diseases, including Alzheimer's disease and schizophrenia, the levels of GM1 is decreased in the blood and the brain (49,50).

In conclusion, FXR1P may enhance the activation of sialic acid via interaction with CMAS, and increase the GM1 levels

Table II. GM1 concentration in two cell lines under conditions of different expression level of *FXR1*.

	Normal group	Empty vector group	Overexpression of FXR1 group
SH-SY5Y	13.938±0.126	13.786±0.231	18.245±0.241 <sup>a</sup>
HEK293T	10.213±0.084	10.975±0.187	12.138±0.382

<sup>a</sup>P<0.05 vs. normal group, n=6. GM1, monosialotetrahexosylganglioside; FXR1, fragile X related 1.

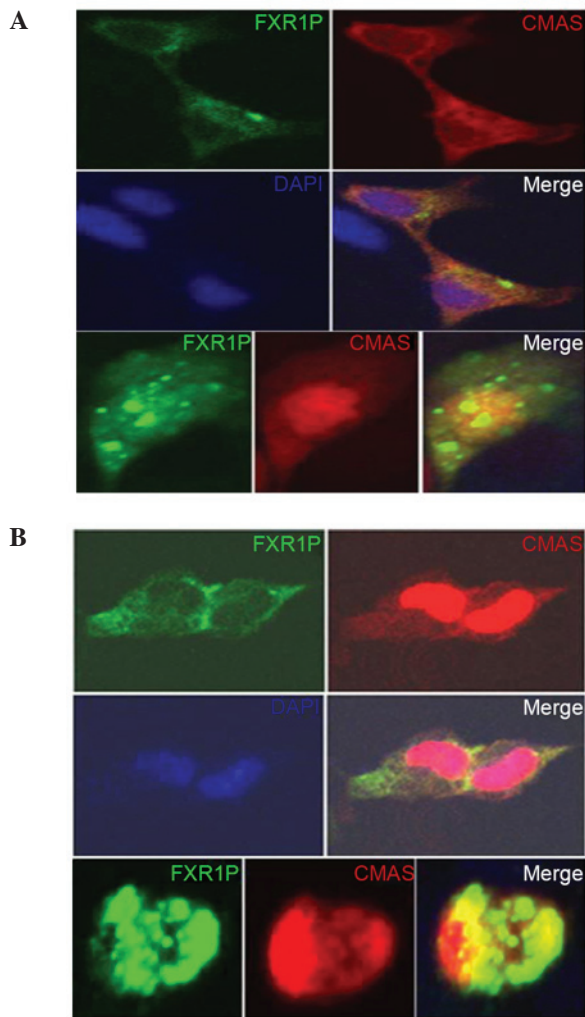


Figure 3. Colocalization of FXR1P and CMAS in HEK293T and HeLa cells. (A) HEK293T and (B) HeLa cells were transiently transfected with pEGFP-N1-FXR1 and pDsRed-N1-CMAS, then visualized by fluorescence microscopy. FXR1P (green) is visible in the cytoplasm, CMAS (red) is visible mostly in the nucleus, with less in the cytoplasm. Positive colocalization (yellow) is visible in the cytoplasm and around the nucleus. Yellow indicated the colocalization of pEGFP-FXR1P with pDsRed-CMAS. The nuclei (blue) were stained by DAPI. Magnification, x100. FXR1P, fragile X related 1; CMAS, CMP-N-acetylneuraminic acid synthetase; DAPI, 4',6-diamidino-2-phenylindole.

to affect the development of the nervous system. The results of the present study indicate that FXR1P interacts with CMAS *in vivo*, and that FXR1P and CMAS can co-localize in the cytoplasm around the nucleus. Additionally, FXR1P is able to enhance CMAS activity to increase the concentration of GM1 in SH-SY5Y cells. Taken with the results of a previous

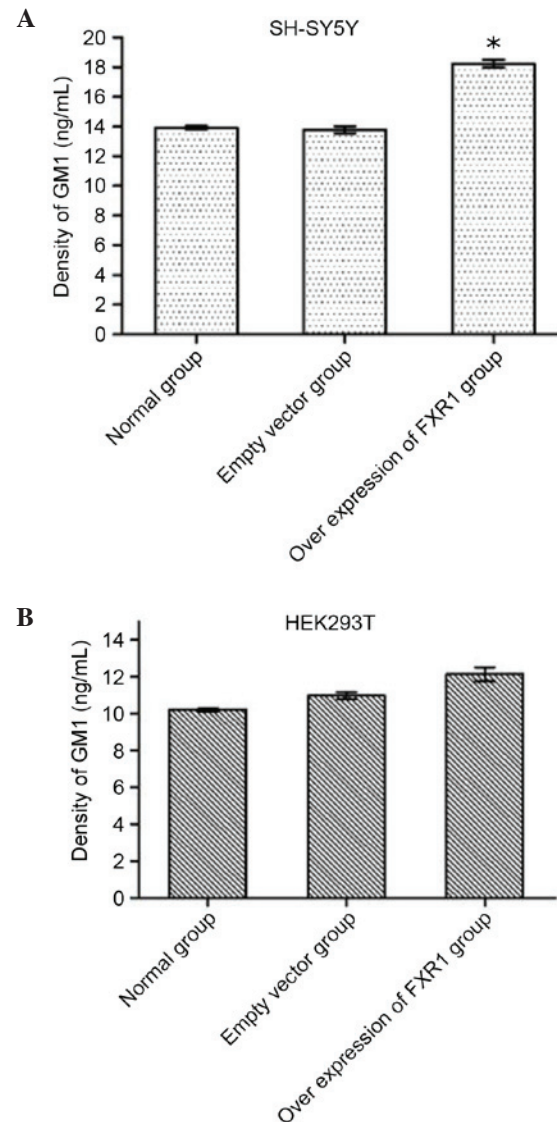


Figure 4. Effect of different expression levels of the *FXR1* gene on the concentration of GM1. (A) SH-SY5Y cells transfected with pcDNA3.1(-)-FXR1, (B) HEK293T cells transfected with pcDNA3.1(-)-FXR1, then lysed and enzyme-linked immunosorbent assay was performed. The GM1 concentration was detected by ND 2000c trace UV spectrophotometer. In SH-SY5Y cells, the GM1 concentration was not significantly increased when comparing the empty vector group with normal group, but there was a significant increase in the FXR1 gene overexpression group compared with the normal group (n=6). \*P<0.05 vs. normal group. There were no significant differences in GM1 concentration in HEK293T cells among all three groups (P>0.05, n=6). GM1, monosialotetrahexosylganglioside; FXR1P, fragile X related 1.

study (51), the present study suggests that FXR1P serves an important role in the development of the brain and nervous system.

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