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A novel human zinc finger protein ZNF540 interacts with MVP and inhibits transcriptional activities of the ERK signal pathway ☆

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Abstract

Mitogen-activated protein kinases (MAPKs) are evolutionarily conserved enzymes in cell signal transduction. Previous studies revealed that zinc finger proteins are involved in the regulation of the MAPK signaling pathways. Here we report the identification and characterization of a novel human zinc finger protein, ZNF540. The cDNA of *ZNF540* is 3.3 kb, encoding 660 amino acids in the nucleus and the cytoplasm. Northern blot analysis indicates that *ZNF540* is expressed in most of the fetal tissues. Overexpression of FLAG-ZNF540 in COS-7 cells represses the transcriptional activities of SRE and ELK-1, which can be relieved by siRNA. MVP, one of MAPK scaffold proteins, is identified as a potential ZNF540-binding protein. This interaction is detected by a yeast two-hybrid assay, reporter gene assays, and co-immunoprecipitation. Taken together, these results suggest that ZNF540 may act as a transcriptional repressor in MAPK signaling pathway to mediate cellular functions.

Keywords: *ZNF540*; Transcriptional suppressor; RNAi; MVP; Interacting protein; MAPK signaling pathway

Transcriptional regulation of gene expression is mediated primarily by sequence-specific DNA-binding transcription factors that are composed of a DNA-binding domain and one or more separable effector domains [1–3]. Zinc finger gene family belongs to one of the biggest families of transcription factors and can be divided into many subclasses based on the number and type of zinc fingers they contain [4]. The family of Krüppel-like proteins is one of the largest families of zinc finger proteins. These proteins contain two or more C_2H_2-type zinc fingers that are separated by a conserved consensus sequence, T/SGEKPY/FX. It has been estimated that the human genome contains 564–706 C_2H_2 type zinc finger genes [5,6]. The C_2H_2 zinc finger motif corresponds to the consensus sequence: $CX_{2,4}CX_3FX_5LX_2HX_{3,4}H$ where X is any amino acid; the cysteines and histidines are conserved and coordinating the zinc [7,8]. The C_2H_2 type zinc finger motif is initially found in the TFIII, a transcription factor of *Xenopus* and subsequently in the Krüppel of *Drosophila* [9]. Since then, this motif has been found in many proteins with transcriptional regulatory functions such as transcription factor Sp1, yeast regulatory gene GAL4 [10], the Wilms' tumor suppressor gene (WT1) [11], and Gli-3 [12]. Most C_2H_2 zinc finger proteins can bind to specific DNA sequences and are involved in the transcriptional regulation of gene expression [7,8]. Members of the Krüppel-like zinc finger family function as activators or repressors of

☆ *Abbreviations:* DAB, 3,3-diaminobenzidine tetrahydrochloride; DMEM, Dulbecco's modified Eagle's medium; DAPI, 4′,6′-diamidino-2-phenylindole hydrochloride; MAPK, mitogen-activated protein kinase; MAPKK, MKK or MEK, MAPK kinase; MAPKKK or MEKK, a MAPKK kinase or MEK kinase; SRE, serum response element.

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gene transcription, and therefore regulate embryonic development as well as a variety of physiological processes in the adult [13–15].

With the aim of identifying genes involved in human heart development and diseases, we isolated a novel KRAB/C_2H_2-type zinc finger gene named ZNF540 from heart cDNA library in this study. ZNF540 encodes a zinc finger protein with 17 different C_2H_2 type zinc fingers and a KRAB-A box. Northern blot analysis indicates that ZNF540 is expressed in most of 6-month fetal tissues. Overexpression of pCMV-tag2A-ZNF540 in the COS-7 cells inhibits the transcriptional activities of ERK, which can be relieved by siRNA. MVP, one of MAPK scaffold proteins, is identified as a potential ZNF540-binding protein. Together, the results suggest that ZNF540 protein may act as a negative regulator in MAPK signaling pathway to mediate cellular functions.

Materials and methods

RNA isolation, construction of cDNA Library of human embryo heart. Total RNA was isolated from various tissues of 20-week human fetuses using improved method of extracting total RNA with phenol-chloroform as described previously [16]. mRNA preparation of human embryo heart and reverse transcription reaction were performed using a cDNA PCR Library Kit and cDNA Synthesis Kit according to manufacturer's protocol (TaKaRa Co). Briefly, 5 μg mRNA was purified from 500 μg total RNA using Rapid mRNATM purification Kit (AMRESCO). Reverse transcription reactions were performed with the purified embryonic heart mRNA and oligo(dT)-RA primer according cDNA Synthesis Kit protocol. After Cassette Adaptor Ligation reactions using cDNA PCR Library Kit, cDNA amplification reactions were performed with RA primer, CA primer, and TaKaRa Ex Taq.

Full-length ZNF540 cDNA cloning and bioinformatics analysis. PCR was performed on a PCRSPRINT reactor (Thermo Hybrid) with one pair of degenerated oligonucleotide primers P1 (Table 1) corresponding to a highly conserved connecting sequence (TGEKPYX) and conserved C_2H_2 zinc finger sequence (CRECGKAF) of Krüppel-like type zinc finger genes. A PCR was performed using the heart cDNA library as the template according to standard procedures. The amplification products were separated by agarose gel and the bands were cloned into pMD18-T vector (TaKaRa). The transformants were randomly chosen and sequenced with 3771 DNA Sequencer (ABI PRISM) according to manufacturer's procedures. The sequence obtained was subjected to human homology searching against expressed sequence tag (EST) database using Blastn. To confirm the cDNA sequence from the database, one pair of gene specific primers A1 (Table 1) was designed based on the sequences of a contig from ESTs for PCR. The heart cDNA library was used as template. The band was excised, cloned into pMD18-T vector (TaKaRa), and sequenced. 5′ RACE and 3′ RACE were performed using SMART RACE cDNA Amplification Kit (Clontech). Gene-specific primers were designed using Primer Premier 5.0 for 5′-RACE (5′-GSP1 and 5′-GSP2) and 3′-RACE (3′-GSP1 and 3′-GSP2) reactions, respectively. The products were then cloned into pMD18-T vector and sequenced. Sequence analysis was performed using the DNASTAR program and BLAST program from NCBI. Pfam 9.0 was used to analyze genomic structure and the protein domain, respectively.

Sequence comparisons and phylogenetic tree analysis. Comparison of ZNF540 and its homologues was performed using the MegAlign program of DNASTAR. Phylogenetic tree analysis of amino acid sequences deduced from *ZNF540* DNA sequences was also performed using the MegAlign program of DNASTAR. The clustal method was chosen to correct the distances for multiple substitutions at a single site. GenBank accession numbers of previously known and novel C_2H_2 type zinc finger gene sequences used for these analyses are BAA80870 (*Aeropyrum pernix* K1), AAD33066 (*Zea mays*, TRM protein), AAB06876 (*Mus musculus*, zfp60), XP_867624 (*Canis familiaris*), CAH92890 (*Pongo pygmaeus*), CAA74933 (*Homo sapiens*, APM-1 protein), and ZNF540 (*H. sapiens*).

Northern blot analysis. The *ZNF540* cDNA was labeled with [α-^{32}P]dCTP by using a Random Primer Labeling Kit (TaKaRa). An adult human Northern™ blot containing mRNA from variety of adult tissues purchased from CLONTECH Company was hybridized sequentially to the radiolabeled *ZNF540* cDNA probe and β-actin cDNA probe (CLONTECH). Hybridization was carried out with 5× SSC, 5× Denhardt, 10% Dextran sulfate, and denatured human DNA, at 65 °C overnight. After hybridization, the blots were washed three times at 65 °C in 2× SSC containing 0.1% SDS for 5 min and twice in 0.1× SSC and 0.1% SDS at 65 °C for 15 min, and then subjected to autoradiography at −80 °C. The

Table 1
Sets of specific oligonucleotide primers

Primer	Orientation	Nucleotide sequences
P1	Sense	5′-ACIGG(C/A)GAGAAGCC(T/A)TTCCA(A/G)TGT-3′
	Antisense	5′-GAAIC(A/T)CTT(G/A)CCGCATTCGTT(A/G)CA-3′
A1	Sense	5′-AGAATAATCCTGCGGAAGACTGAGC-3′
	Antisense	5′-TCCACTACCATAAACTTCCATCCCG-3′
5′-GSP1	Antisense	5′-GCAACGGTCCGAACCTCAT-3′
5′-GSP2	Antisense	5′-AGATACCCCACCAAACCCA-3′
3′-GSP1	Sense	5′-GCTCGAGTCTTGTGAGTGTAAAACC-3′
3′-GSP2	Sense	5′-ATGAATGTCCTTTTCTGAAGATAAT-3′
A2	Sense	5′-GCTCGAGTCTTGTGAGTGTAAAACC-3
	Antisense	5′-TCTGCAGTACATTATGAGTTTTCTG-3′
A3	Sense	5′-TCTGCAGTTCTTGTGAGTGTAAAAC-3′
	Antisense	5′-TCTGCAGAAATTACATTATGAGTTT-3′
A4	Sense	5′-TCTGCAGTTCTTGTGAGTGTAAAAC-3′
	Antisense	5′-ACTGCAGTTCTGCCACTCATTTCTA-3
A5	Sense	5′-GA CTGCAG TAAAACTCTTCGTCTGA-3′
	Antisense	5′-GCTGCAGTGTGTAAAAAAACATCAA-3′
A6	Sense	5′-GACTGCAGCTTGTGAGTGTAAAACCATG-3′
	Antisense	5′-TCTCTGCAGAGAGCATGACATGGAAACC-3′
P_{MVP}	Sense	5′-GGAATTCGACCATGGTGGTCAGCTA-3′
	Antisense	5′-GTCTAGAACAGGCACCACGTGGTTG-3′

blots were stripped by incubated for 10 min in 0.1× SSC and 0.5% SDS at 95 °C. The membranes were reprobed with radiolabeled β-actin cDNA as an indicator of mRNA loading.

Plasmid construction. The following plasmids were constructed and used for mammalian cell transfections. *Escherichia coli* DH5α was used as recipient for all transformations. (i) Construction of pEGFP-N1-ZNF540 with primers A2: the coding region of *ZNF540* was subcloned into the *Xho*I and *Pst*I sites of pEGFP-N1 vector in-frame with the CAG code instead of the TAA stop code in the *ZNF540* coding sequence. (ii) Construction of pGAL4-ZNF540 and two pGAL4-ZNF540 mutants (deletion fragments): the DNA fragment containing the coding region of *ZNF540* was amplified with primers A3 containing *Pst*I restrictions, respectively (Table 1). The amplified DNA fragment was subcloned into pMD18-T vector, after cleaved with *Pst*I from pMD18T-ZNF540, the DNA fragment was subcloned into expression vector pCMV-BD that contained the coding region of the GAL4 DNA-binding domain (DBD) to create an expression plasmid named pCMV-BD-ZNF540, which expresses a fusion protein with GAL4 DBD and ZNF540. Two deletion fragments were amplified by PCR from the pMD18T-ZNF540 (287–2917 bp) plasmid with primers A4 and A5 for pCMV-BD-ZNF540 (1–124 aa) (Abbr: pGAL-KRAB) and pCMV-BD-ZNF540 (107–660 aa) (Abbr: pGAL-C_2H_2), respectively. All of the primers contain *PstI* restrictions. (iii) Construction of pCMV-tag2A-ZNF540 (Abbr: pFLAG-ZNF540) and two pCMV-tag2A-ZNF540 mutants (deletion fragments): the expression plasmid for FLAG epitope tagged ZNF540, pCMV-tag2A-ZNF540, was constructed by inserting *ZNF*540 ORF downstream of the FLAG epitope sequence (MDYKDDDDK) in a pCMV-tag2A expression vector. pCMV-tag2A-ZNF540 (1–124 aa) (Abbr: pFLAG-KRAB) and pCMV-tag2A-ZNF540 (107–660 aa) (Abbr: pFLAG-C_2H_2) were constructed similarly to pGAL-KRAB and pGAL-C_2H_2, respectively, instead of pCMV-BD vector with pCMV-tag2A vector. All of the recombination plasmids constructed were cleaved with enzymes and the inserted fragments of each clone were sequenced. (iv) Construction of pGBKT7-ZNF540: with primers A6, the coding region of ZNF540 was subcloned into the *Pst*I sites of pGBKT-7 vector in-frame without the TAA stop code in the ZNF540 coding sequence. (v) Construction of pcDNA3.1/myc-His-MVP (452–894 aa) (Abbr: pMyc-MVP): the part coding region of MVP was amplified with primers P_{MVP} using pACT2-MVP as the template. Then the fragment was subcloned into the *Eco*RI and *Xba*I sites of pCDNA3.1/myc-His B MCS vector (Invitrogen) in-frame with the MVP coding sequence. All of the recombination plasmids constructed were cleaved with enzymes and the inserted fragment of each clone was sequenced.

Yeast two-hybrid assays. Competent yeast AH109 cells were transformed with the bait plasmid pGBKT7-ZNF540 and selected on medium lacking *Trp*. The resulting transformants were isolated and used to screen a human heart cDNA library following the Matchmaker Two-Hybrid System protocol. Transformants were selected on media lacking *Trp*, *Leu*, *His*, and *Ade*. After 3–4 days, positive colonies were picked and further assessed for β-galactosidase activity by colony-lift filter assay. The pACT-derivative plasmids containing positive cDNA clones were then isolated and retransformed into the yeast strain containing the bait plasmid for confirmation of true positives. Positive cDNA clones were sequenced. Network BLAST searches were conducted using the NCBI online service.

Cell culture and subcellular localization analysis. COS-7 cells were cultured in DMEM (Gibco-BRL) which was supplemented with 10% fetal calf serum (FCS) in a humidified atmosphere of 95% air and 5% CO_2. Sixteen to twenty hours prior to transfection, growing cells were seeded (10^5 cells/ml) on sterile microscope cover-glasses placed in a 35 mm Petri dish. To investigate the subcellular localization of ZNF540, COS-7 cells were transfected with pEGFP-N1-ZNF540 using LipofectAMINE (Invitrogen) according to the method described before [17]. Forty-eight hours after transfection, cells were fixed with 4% paraformaldehyde for 15 min and nuclear fractions were stained with 4′,6′-diamidino-2-phenylindole hydrochloride (DAPI). Subcellular localization of the ZNF540-EGFP fusion proteins was detected using fluorescence microscopy.

Transient transfection and reporter gene assays. For luciferase assays, using Lipofect AMINE (Invitrogen) reagent according to the same method [16], COS-7 cells were co-transfected with 2 μg pSRE and 2 μg pCMV-tag2A-ZNF540 (or pMyc-MVP), or 2 μg pCMV-tag2A (or pcDNA3.1/myc-His B MCS) vector to investigate the effect of ZNF540 on the transcriptional activity of SRE-Luc. For ELK-1luciferase assay, pFR-luc 2 μg, pFC-MEKK 2 μg, and pGAL4-ELK-1 2 μg were co-transfected. Forty-eight hours later, cells were harvested and the luciferase activity assay was performed according to the protocols of Stratagene. Each experiment was performed in triplicate and each assay was repeated at least three times. The means of the data from three individually transfected wells are presented after normalization for β-galactosidase.

RNAi analysis. A pSUPER.retro.puro vector-based system was used to deliver siRNA into COS-7 cells. A pair of oligonucleotides was designed by the RNAi program (http://www.openbiosystems.com/RNAi). The sequences are s1: 5′-GATCTCGGTTTGTTATCCAGGCATTTCTCTAG AATGCCTGGATAACAAACCG-3′ and s2: 5′-TCGAGCGGTTTGT TATCCAGGCATTCTAGAGAAATGCCTGGATAACAAACCG-3′. The oligos contain a unique 19-nt sequence derived from ZNF540 for suppression. The forward and reverse oligos were annealed and cloned into the unique *Bgl*II and *Xho*I sites of pSUPER.retro.puro vector. The luciferase activity assay for pSRE-Luc, pCMV-Tag2A-ZNF540, and pSUPER-ZNF540 was performed according to the protocols described above.

Co-immunoprecipitation assay. COS-7 cells were transfected with FLAG-ZNF540 and Myc-MVP expression plasmids and harvested after 24 h for immunoprecipitation, and then Western blot analyses were performed. The cells were lysed in immunoprecipitation cell lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-Glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, and 1 mM PMSF) and incubated overnight at 4 °C with antibody against Myc or FLAG (Santa Cruz Biotechnology), respectively. The proteins were immunoprecipitated with protein-A/G (Santa Cruz Biotechnology). After 12 h incubation, beads were washed three times with PBS buffer, subjected to SDS–PAGE, and then electrotransferred to nitrocellulose membranes. The blot was incubated for 1 h at 4 °C in blocking buffer (10 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween 20, and 5% nonfat dried milk) and then at 4 °C overnight with anti-FLAG or anti-Myc antibody. Detection was performed using the DAB and H_2O_2 after incubation with a peroxidase-conjugated secondary antibody.

Results

Identification and sequence analysis of ZNF540

In an effort to understand the role of KRAB/C_2H_2 zinc finger transcription factors in heart development and in cell signaling pathway, we used oligonucleotide primers P1sen and P1ant (Table 1) based on conserved sequences of Krüppel-like gene family to drive homologue PCR amplification. The PCR was performed using the heart cDNA library as the template and amplification products were cloned into pMD18-T vector and sequenced. The sequences obtained were subjected to human homology searching against expressed sequence tag (EST) database using Blastn. An EST DN993065 was found to match the novel cDNA clone. A number of ESTs representing the same novel gene were identified in a further search. The partial cDNA sequence of this novel gene was assembled from ESTs, including DB450305, BX409998, DA064998, CJ465482, BF575073, and DB548268. To confirm the cDNA sequence from the database, one pair of gene-specific primers A1sen and A1ant (Table 1) was designed for PCR based on the sequences of partial cDNA. The heart cDNA library was used as template and a 2630 bp PCR product was

obtained and confirmed to be the cDNA sequence of this new gene.

To obtain the full-length cDNA, we performed 5′-rapid amplification of cDNA ends (5′-RACE) and 3′-RACE and obtained a 330 bp DNA for 5′-RACE fragment and a 460-bp 3′-RACE fragment. Analysis of these two cDNAs confirmed that they were cDNA fragments from the novel gene. The full-length new KRAB/C_2H_2 zinc finger gene was named *ZNF540*.

The *ZNF540* gene consists of an open reading frame (ORF) of 1983 bp extending from the first ATG code at nucleotide 333 to a termination TAA at 2315 (Fig. 1A). The deduced ZNF540 protein has 660 amino acids (Fig. 1A) with a calculated molecular mass of 77.09 kDa. *ZNF540* is assigned to human chromosome 19q13.13 and spans approximately 62.8 kb in the human genome, which contains five exons and four introns. A summary of the various sizes of the exons and introns and the sequence of the splice junctions is shown in Table 2. The exon–intron boundaries conform to the consensus splicing signals, where there are a gt and an ag dinucleotide at the 5′-donor and 3′-acceptor site, respectively. Analysis of ZNF540 protein using the SMART program indicates that ZNF540 contains an N-terminal Krüppel-associated box (KRAB) domain (amino acids 7–77) and 17 C_2H_2 zinc finger motifs that extend to the end of the protein sequence (Fig. 1B).

A search of published DNA databases for sequences similar to *ZNF540* demonstrated that they had varying degrees of similarity to a number of previously identified C_2H_2-type zinc finger proteins. The alignment of amino acid sequences in the N-terminus (KRAB domain) of ZNF540 indicates that the KRAB box of ZNF540 belongs to the classical KRAB-A box. It consists of about 43 amino acid residues and shows high homology to the KRAB-A box of other zinc finger proteins (Fig. 2A). The two conserved motifs in the KRAB box of ZNF540, DV (at position 10–11) and MLE (at position 35–37, Fig. 2A), are important for repression and interaction with TIF1h (also named KAP-1, KRIP-1) [18]. These sequence analysis and database comparison indicate that the predicted protein contains 17 different C_2H_2 zinc finger domains in tandem arrays, characteristic of transcription factor proteins of this family. In addition, each finger motif conforms closely to the consensus sequence of C_2H_2 and is connected by highly conserved H/C linkers (T/S)GE (K/R)PYX, which strongly suggests a role in DNA binding. The conserved Cys and His residues in C_2H_2 zinc finger are bound to a tetrahedrally coordinated zinc ion, resulting in organization of the key amino acid residues into a structure capable of binding to target DNA. These also suggest that *ZNF540* encodes a DNA binding protein with transcriptional regulatory property. Furthermore, we analyzed the evolutionary relationship among different zinc finger proteins with phylogenetic tree analysis (Fig. 2B), showing that ZNF540 is one of the conserved proteins during evolution. The most closely related protein of ZNF540 is CAH92890 (*P. pygmaeus*).

Expression of the ZNF540 mRNA and subcellular localization of ZNF540-EGFP fusion protein

To characterize the expression distribution of *ZNF540* gene, Northern blot was performed using *ZNF540* cDNA as the probe. A single *ZNF540* mRNA transcript was detected in multiple fetal tissues, such as gut, heart, brain, muscle, lung, testis, and liver. The expression of *ZNF540* showed a high level in liver and a lower level in muscle (Fig. 3). The *ZNF540* transcript was not detected in prostate and kidney.

To examine the subcellular localization of ZNF540, pEGFP-N1-ZNF540 plasmid was introduced into COS-7 cells by transient transfection. Forty-eight hours after the transfection, the localization of the fusion protein (ZNF540-EGFP) was visualized with epifluorescence microscope after labeling with DAPI for nuclei. Cells transfected with pEGFP-N1-ZNF540 showed a nuclear and cytoplasm fluorescence pattern (Fig. 4A) and DAPI binds to DNA (Fig. 4B). The combined image (Fig. 4C) shows that ZNF540 protein exists in the nuclei and cytoplasms of the cells.

ZNF540 is a transcriptional inhibitor

Previous studies have indicated that some members of the Krüppel-like family of transcription factors consist of multiple domains that function in transcriptional activation or inhibition of protein-protein interaction and DNA binding [17,19–21]. To examine the potential function of ZNF540 in transcriptional regulation, we constructed a fusion protein of ZNF540 with the DNA-binding domain (DBD) of the yeast transcription factor GAL4 under the driving of a CMV promoter, the pCMV-BD-ZNF540, and then co-transfected COS-7 cells with pCMV-BD-ZNF540 and the reporter, pL8G5-Luc, and pLexA-VP-16, respectively (Fig. 5). The results suggest that ZNF540 is a potent transcriptional inhibitor.

To map the transcriptional repression domain of ZNF540, we tested the ability of the truncated GAL4-ZNF540 fusion proteins to regulate the activity of the GAL4 site-driven luciferase reporter gene. As shown in Fig. 5, both of GAL-KRAB and GAL-C_2H_2 showed transcriptional repressor activity. However, GAL-KRAB exhibited a stronger effect. These results indicate that the KRAB domain is a main repressive region for the transcriptional repressor activity of ZNF540.

ZNF540 represses SRE and ELK-1-mediated transcriptional activation

Mitogen-activated protein kinase (MAP) signal transduction pathways are the most widespread mechanisms of eukaryotic cell regulation [22]. As an important nuclear effector of MAPK signaling pathway, the c-*fos* SRE forms a ternary complex together with serum response factor (SRF) and ETS proteins. To examine the effect of

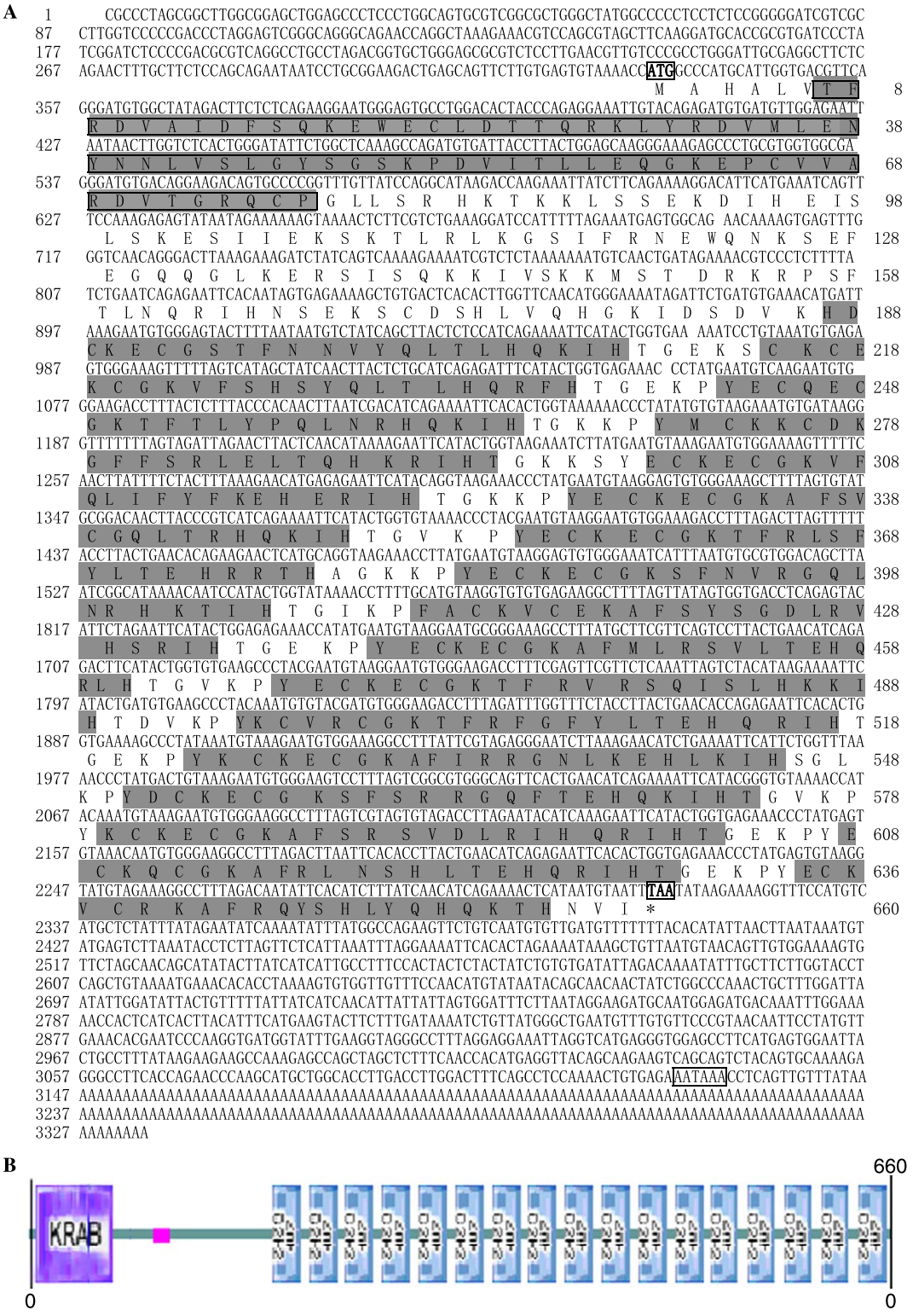

Fig. 1. (A) Nucleotide sequence and deduced protein sequence of the human *ZNF540* gene. *ZNF540* encodes a polypeptide of 660 amino acids. The initiation ATG and termination TAA codons are boxed. Amino acids are identified by their one-letter code. The KRAB box is boxed and the five zinc finger regions are marked. Both nucleotides and amino acids are numbered at the left side and the right side of each line, respectively. The putative polyadenylation signal sequence AATAAA is boxed. Asterisk (*) indicates the location of termination codon. (B) The domain structure of ZNF540 protein. KRAB box is at N-terminal of the sequence and 17 zinc fingers in the C-terminal of the sequence.

ZNF540 in this pathway, we performed reporter gene assays to measure the modulation of SRE and ELK-1 by ZNF540 in the cells. COS-7 cells were co-transfected with the expression plasmids pCMV-tag2A-ZNF540, pSRE-Luc, or pFC-MEKK, GAL4-Elk1, and pFR-Luc. pSRE-Luc and pFR-Luc encoded for luciferase are controlled

Table 2
Genomic structure of the *ZNF540* gene

Exon number	Exon size (bp)	Splice donor	Intron size (bp)	Splice acceptor
I	260	**gt**aggtaccgcc	47959	tcctctaactc**ag**
II	81	**gt**aaggtcacct	1233	ttcttgtaagc**ag**
III	127	**gt**aaggtcacct	1256	ttcttgtaagc**ag**
IV	96	**gt**gagttgagag	10407	tgttttctttc**ag**
V	2583			

Summary of the size of the exons and introns of the *ZNF540* gene based on comparison of the cDNA and the genomic sequence. Invariant nucleotides (ag/gt) are in boldface type.

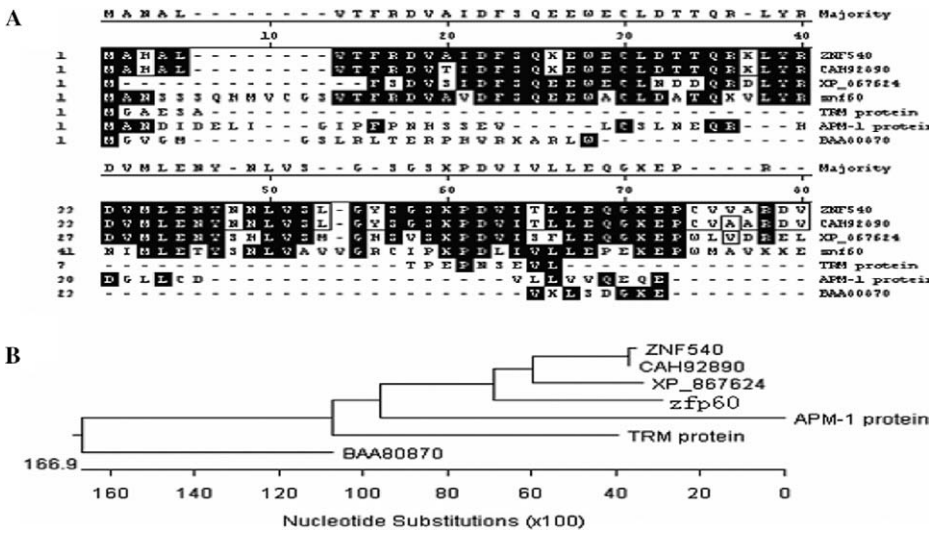

Fig. 2. ZNF540 is conserved during evolution. (A) Amino acid sequence alignment of ZNF540. Comparison of the amino acid sequences of ZNF540 and its homologues (CAH92890 *Pongo pygmaeus*, XP_867624 *Canis familiaris*, znf60 *Mus musculus*, TRM protein *Zea mays*, and BAA80870 *Aeropyrum pernix* K1) indicates that ZNF540 is conserved during evolution. (B) The proposed evolutionary tree of the zinc finger family of proteins.

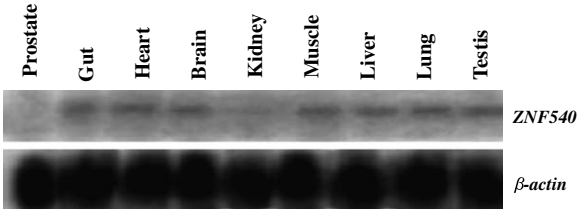

Fig. 3. Northern blot analysis of *ZNF540* in human 6-month fetal tissues. The cDNA of *ZNF540* was labeled with [α-^{32}P]dCTP and used as the probe. The same membrane was also hybridized with β-actin to normalize for loading differences.

by SRE and Elk-1, respectively. As shown in Fig. 6A, expression of ZNF540 significantly inhibits the SRE-luciferase activity. We then tested the effect of ZNF540 on the transcriptional activity of Elk-1. As observed in the Elk-1-luciferase assays, we found that the expression of ZNF540 inhibits the transcriptional activity of Elk-1 (Fig. 6B).

To demonstrate whether the KRAB motif in ZNF540 functions as a potent main repression domain, two truncated ZNF540 fusion proteins were also examined for pathway-specific reporter gene assays. As shown in Fig. 6A and B, expression of FLAG-KRAB inhibited transcriptional

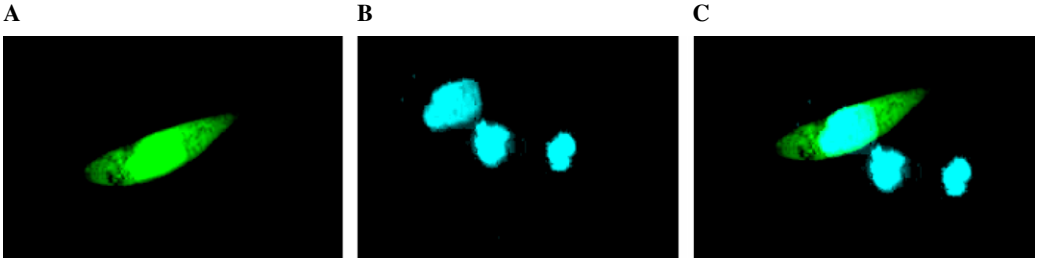

Fig. 4. ZNF540 is expressed in cell nucleus and in cytoplasm (A) COS-7 cells expressing ZNF540-GFP were detected in cell nucleus and in cytoplasm. (B) The nucleus of cells stained with DAPI. (C) The combined image of (A) and (B).

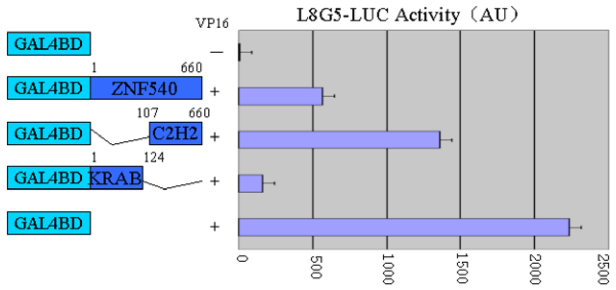

Fig. 5. ZNF540 is a transcriptional suppressor. Forty-eight hours after transient transfection, the luciferase activity assay was performed. The data are means of three repeats in a single transfection experiment after normalization for β-galactosidase activity. Each experiment was repeated at least three times. pCMV-BD-ZNF540 or pCMV-BD is transiently co-transfected into COS-7 cells along with the pL8G5-Luc reporter and pLexA-VP16 as indicated in the figure.

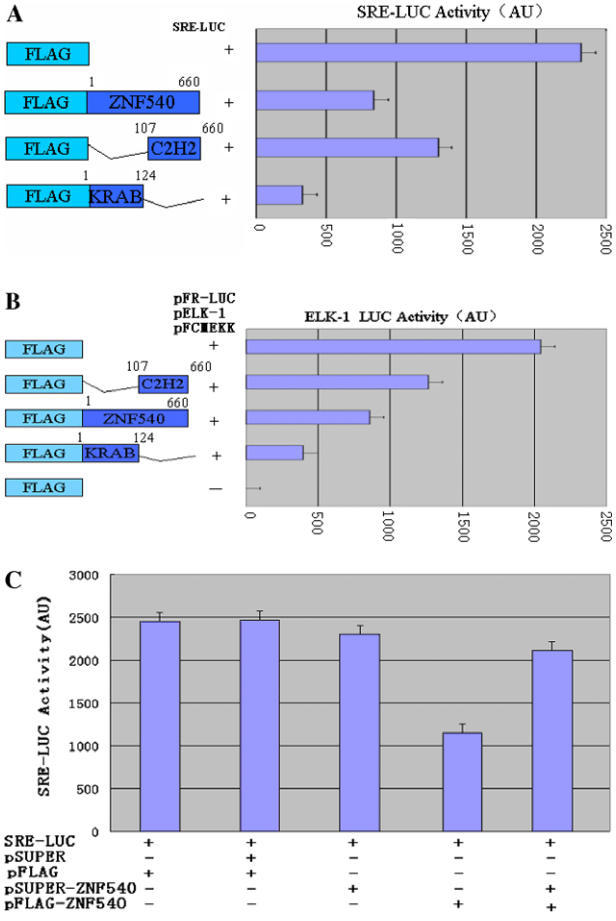

Fig. 6. (A) Inhibition of SRE-Luc transcriptional activity by the overexpression of ZNF540. (B) Inhibition of Elk-1-Luc transcriptional activity by the overexpression of ZNF540. COS-7 cells transfected with reporter plasmid and the corresponding plasmids are shown in the figures. Forty-eight hours later, the luciferase activity assays were performed. The data are means of three repeats in a single transfection experiment after normalization for β-galactosidase activity. Each experiment was performed at least three times.

activity of SRE and Elk-1, and FLAG-C_2H_2 showed a slightly repressive effect on the transcriptional activities of SRE and Elk-1. Taken together, our results suggest that the KRAB domain of ZNF540 represents a mainly repressive domain in transcriptional regulation.

To further prove the function of ZNF540, ZNF540 was silenced by siRNA in COS-7 cells over-expressing this gene. The results show that the SRE and Elk-1 luciferase activity increased when co-transfected with RNAi plasmid, suggesting that siRNA targeting ZNF540 abolished the transcriptional suppression of ZNF540 (Fig. 6C). Taken together, our results suggest that ZNF540 may act as a transcriptional repressor and suppresses MAPK signaling pathway to mediate cellular functions.

Identification of MVP as a ZNF540 interacting protein by yeast two-hybrid screen

In order to explore the functions of ZNF540 in cells, we tried to isolate some interacting proteins whose functions have been known by a yeast two-hybrid screen. A human adult heart cDNA library fused to the Gal4 activation domain was screened using Gal4-BD-ZNF540 fused the Gal4 DNA-binding domain as the bait. Yeast colonies, which grew on media lacking Trp, Leu, His, and Ade, and turned blue in β-galactosidase filter assay, were picked and further confirmed by retransforming the recovered plasmids into the yeast strain containing the bait construct.

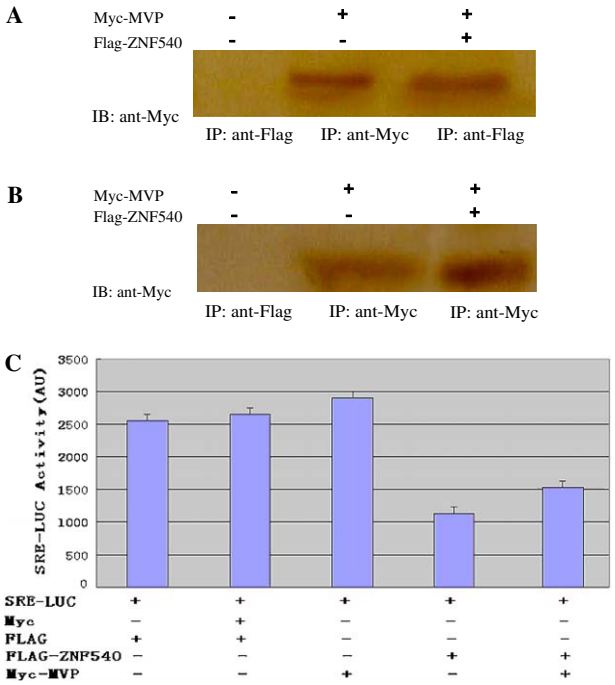

Fig. 7. ZNF540 and MVP associate in eukaryonic cells. Immunoprecipitation analysis was performed in COS-7 cells transfected with FLAG-ZNF540 and Myc-MVP expression plasmids. (A) Precipitated by anti-FLAG beads and detected with anti-Myc antibody. (B) Precipitated by anti-FLAG beads and detected with anti-FLAG antibody. Controls are designed by using extracts from nontransfected COS-7 cells or cells transfected with FLAG-ZNF540 or Myc-MVP expression plasmids alone. (C) Inhibition of SRE-Luc transcriptional activity by the co-overexpression of ZNF540 and MVP.

One positive clone encoded a part of Major Vault Protein (MVP) was isolated.

ZNF540 interacts with MVP in COS-7 cells

To demonstrate the interaction between ZNF540 and MVP in eukaryotic cells, we performed co-immunoprecipitation assay. Western blotting revealed that MVP was co-precipitated with anti-FLAG antibody, and ZNF540 was co-precipitated with anti-Myc antibody (Fig. 7A). Moreover, the transcriptional suppression of ZNF540 on the SRE luciferase activity was weakened when FLAG-ZNF540 and Myc-MVP were co-transfected compared with single transfection of FLAG-ZNF540 or Myc-MVP (Fig. 7B). These results indicate that ZNF540 may interact with MVP in eukaryotic cells.

Discussion

With the aim of identifying genes involved in human heart development and diseases, we isolated a novel gene *ZNF540* encoding a zinc finger protein with 17 C_2H_2-type zinc fingers and a KRAB-A box from heart cDNA library. C_2H_2-type zinc finger proteins were found as transcriptional regulators to bind to specific DNA sequences and to be involved in the transcriptional regulation of gene expression involving in embryonic development as well as a variety of physiological processes in the adult. Recently, studies focused on the C_2H_2-type zinc finger genes have suggested their extensive involvement in development, gene regulation, diseases, and embryogenesis. For example, the hypoalphalipoproteinemia susceptibility gene, *ZNF202*, encodes a transcriptional repressor that binds to elements found in genes involved in lipid metabolism [23]; *ZNF215*, a Beckwith–Wiedemann syndrome-associated gene [24]; and *ZNF213*, which is linked with familial Mediterranean fever [25]. Although animal models, especially the mouse, have provided us with much of our knowledge of early mammalian development, gene regulation, and diseases, there are vital differences between mouse and human. It is therefore very meaningful to systematically search for more C_2H_2-type zinc finger genes that are relative to gene regulation, diseases, and heart development of human being. The KRAB domain in KRAB/C_2H_2-type zinc finger is an evolutionarily conserved domain of about 75 amino acids that are subdivided into an A box and a B box, usually coded for by separate exons. The A box is present in every KRAB domain, while the B box is not always included [26]. Approximately one-third of all C_2H_2-type zinc finger genes in the human genome are estimated to contain the KRAB domain [26]. The KRAB domain has been shown to function as a repressor of transcription through protein–protein interaction [27–29]. In this study, we report the identification of *ZNF540*, a novel zinc finger transcription factor, functioning as an inhibitor of transcription.

Mitogen-activated protein kinase (MAP) signal transduction pathways are the most widespread mechanisms of eukaryotic cell regulation. MAPK pathways consist of four major groupings and numerous related proteins that constitute interrelated signal transduction cascades activated by stimuli such as growth factors, stress, cytokines, and inflammation [30]. All eukaryotic cells possess multiple MAPK pathways, each of which is preferentially recruited by distinct sets of stimuli, thereby allowing the cell to respond coordinately to multiple divergent inputs. Mammalian MAPK pathways can be activated by a wide variety of different stimuli acting through diverse receptor families, including hormones and growth factors. The biological effects of MAPKs are mediated by downstream phosphorylation substrates, which in the nucleus are often transcription factors. MAPK pathways are involved in multiple cellular processes through phosphorylating their specific endpoint targets such as Elk-1 and SRE, which forms a ternary complex together with SRF to induce expression of c-fos and other early response genes. SRE is one of the several cis-elements which mediate c-fos induction and is recognized by a dimer of the serum response factor (SRF) that recruits the monomeric ternary complex factors (TCFs), whose members include Elk-1, SAP-1 or SAP-2 [31].

Using transient transfection and reporter assays, here we have shown that ZNF540 has a transcriptional inhibit function and represses transcriptional activities of SRE and Elk-1. Moreover, we identified MVP as a protein that interacted with ZNF540 by yeast two-hybrid and co-immunoprecipitation. Kolli and his colleagues (2004) have demostrated that MVP interacts with the activated form of the extracellular-regulated kinases (Erks) in response to EGF and tyrosyl-phosphorylated MVP forms a constitutive complex with SHP-2 and the Erks. Their experiments showed that MVP, like other MAPK scaffold proteins, facilitates EGF-dependent transcriptional activation and is required for cell survival [32]. Hence, we suggest that ZNF540 is a new zinc finger protein that interacts with MVP and inhibits Elk-1 and SRE-mediated transcriptional activities in MKAP signaling pathways.

Taken together, we have identified and characterized a novel human KRAB/C_2H_2 zinc finger transcription factor. Northern blot analysis indicates that *ZNF540* was expressed in most of the human fetal tissues. Our results suggest that ZNF540 may act as a transcriptional repressor in MAPK signaling pathway to mediate cellular functions.

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