

## ZNF552, a novel human KRAB/C2H2 zinc finger protein, inhibits AP-1- and SRE-mediated transcriptional activity

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**In this study, we report the identification and characterization of a novel C2H2 zinc finger protein, ZNF552, from a human embryonic heart cDNA library. ZNF552 is composed of three exons and two introns and maps to chromosome 19q13.43. The cDNA of ZNF552 is 2.3 kb, encoding 407 amino acids with an amino-terminal KRAB domain and seven carboxyl-terminal C2H2 zinc finger motifs in the nucleus and cytoplasm. Northern blotting analysis indicated that a 2.3 kb transcript specific for ZNF552 was expressed in liver, lung, spleen, testis and kidney, especially with a higher level in the lung and testis in human adult tissues. Reporter gene assays showed that ZNF552 was a transcriptional repressor, and overexpression of ZNF552 in the COS-7 cells inhibited the transcriptional activities of AP-1 and SRE, which could be relieved through RNAi analysis. Deletion studies showed that the KRAB domain of ZNF552 may be involved in this inhibition. [BMB reports 2010; 43(3): 193-198]**

### INTRODUCTION

Transcription factors regulate important cellular processes, such as cell-lineage determination, cell growth, and differentiation via the temporal or spatial gene expression of specific cell type genes (1, 2). 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 that play an important role in activating or repressing initiation of transcription (3, 4). Zinc finger proteins (ZFPs) are involved in the binding of transcription factors to their cognate DNA recognition site, resulting in the specific activation or repression of gene expression during cell differentiation and development (5). Zinc finger proteins probably constitute the largest in-

dividual family of such nucleic acid-binding proteins and can be divided into many subclasses based on the number and type of zinc-fingers they contain (6). The family of Kruppel-like proteins is one of the largest families of zinc-finger proteins. These proteins contain two or more C2H2-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 C2H2-type zinc-finger genes (7, 8). In addition to zinc finger regions, most of these proteins also contain a regulatory domain, the Kruppel-associated box (KRAB). The KRAB domain consists of an A box and a B box encoded by two separate exons (9). Members of the Kruppel-like zinc finger family can function as activators and/or repressors of gene transcription and regulate embryonic development as well as a variety of physiological processes in the adult (10). Recently, studies focusing on C2H2 type zinc finger genes have suggested their unique involvement in the regulation of embryogenesis (11, 12) and in a variety of diseases (13).

In this study, we report the identification and characterization of a novel C2H2 zinc finger protein, ZNF552, from a human embryonic heart cDNA library. ZNF552 is composed of three exons and two introns and maps to chromosome 19q13.43. The complete sequence of the ZNF552 cDNA is 2,352 bp in length and contains a putative open reading frame (ORF) of 1,224 nucleotides, which encodes a putative protein of 407 amino acid protein with an amino-terminal KRAB domain and seven carboxyl-terminal C2H2 zinc finger motifs. Northern blotting analysis indicated that a 2.3 kb transcript specific for ZNF552 was expressed in liver, lung, spleen, testis, kidney, and especially at a higher level in the lung and testis in human adult tissues. ZNF552 protein was located both in the nucleus and cytoplasm when it was overexpressed in cultured cells. Reporter gene assays showed that ZNF552 was a transcriptional repressor, and overexpression of ZNF552 in the COS-7 cells inhibited the transcriptional activities of AP-1 and SRE which could be relieved through RNAi analysis. Deletion studies showed that the KRAB domain of ZNF552 may be involved in this inhibition. These results clearly indicate that ZNF552 is a novel member of the zinc finger transcription factor family.

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Received 15 September 2009, Accepted 5 October 2009

**Keywords:** C2H2, KRAB, RNAi, Signal pathway, ZNF552

## RESULTS

### Molecular characterization and evolutionary conservation of the human ZNF552

In an effort to understand the role of the new C2H2 type zinc finger proteins, we cloned a 2352 bp full length cDNA sequence of a predicted novel human gene named ZNF552 as approved by HUGO Nomenclature Committee using the Genscan program and polymerase chain reaction (PCR) technology. The full-length ZNF552 gene (Supplement Fig. 1A) consists of an open reading frame of 1221 bp extending from the first ATG code at nucleotide 171 to a TGA stop code at 1394. The deduced ZNF552 protein has 407 amino acids with a theoretical molecular mass of 46 kDa. Furthermore, SMART analysis results indicated that there exist a KRAB domain at the NH<sub>2</sub>-terminus and seven C2H2 motifs in the COOH terminus (Supplement Fig. 1B). Comparison of the ZNF552 sequence with the genomic sequence showed that the ZNF552 gene maps to chromosome 19q13.43. The ZNF552 gene consists of three exons and two introns (Supplement Table 1).

BLAST searches using the sequence of ZNF552 identified closely related sequences in *Homo sapiens* (NP\_079038), *Rattus norvegicus* (XP\_233568.4), *Gallus gallus* (XP\_001235202.1), *Pan troglodytes* (XP\_524426.1), *Xenopus* (NP\_001016767), *Canis familiaris* (XP\_541447.2), *Danio rerio* (XP\_693963.1) and *Mus musculus* (NP\_033583.2). Sequence comparison of the KRAB among its orthologues indicated that the degree of conservation in this domain is modest (Supplement Fig. 2A). We then tried to analyze the evolutionary relationship between the ZNF552 protein and the other zinc-finger proteins with phylogenetic tree analysis (Supplement Fig. 2B). The most closely related protein of ZNF552 is XP\_524426.1. Further sequence comparison found that the zinc fingers in these proteins are less homologous, suggesting that ZNF552 gene is a less conserved member of the zinc-finger family.

### Expression of the ZNF552 mRNA and subcellular localization of EGFP-ZNF552 fusion protein

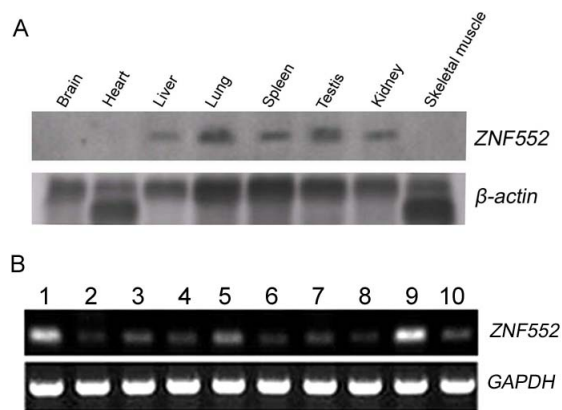
To characterize the transcript of ZNF552 gene with respect to its size and expression distribution, the expression of the ZNF552 gene was detected in eight different adult human tissues using Northern blot analysis. A single expressed mRNA band of 2.3kb was observed strongly in adult testis and lung; moderately in liver, spleen and kidney; no hybridization signal was detected in other tissues (Fig. 1A). The expression of ZNF552 in multiple embryo tissues was examined by RT-PCR and the results showed that the ZNF552 gene was expressed in all kinds of tissues tested, with a higher level in heart and testis (Fig. 1B).

To determine EGFP-ZNF552 fusion protein expression in mammalian cells, a pEGFP-C1-ZNF552 fusion plasmid was constructed and transiently introduced into COS-7 cells by liposome transfection. Under fluorescence microscope, the green fluorescence produced by pEGFP-C1-ZNF552 was detected in

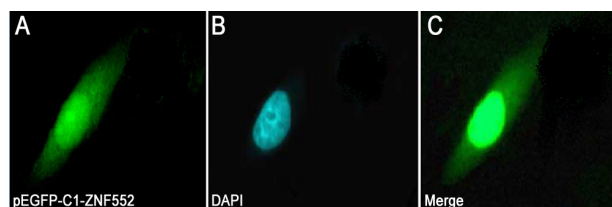
the nucleus and cytoplasm of COS-7 cells 24 h post-transfection (Fig. 2A) and DAPI binds to DNA (Fig. 2B). The combined image (Fig. 2C) showed that ZNF552 encoded protein existed predominantly in the nuclei of the cells.

### ZNF552 is a transcriptional repressor and suppresses AP-1 and SRE-mediated transcriptional activation

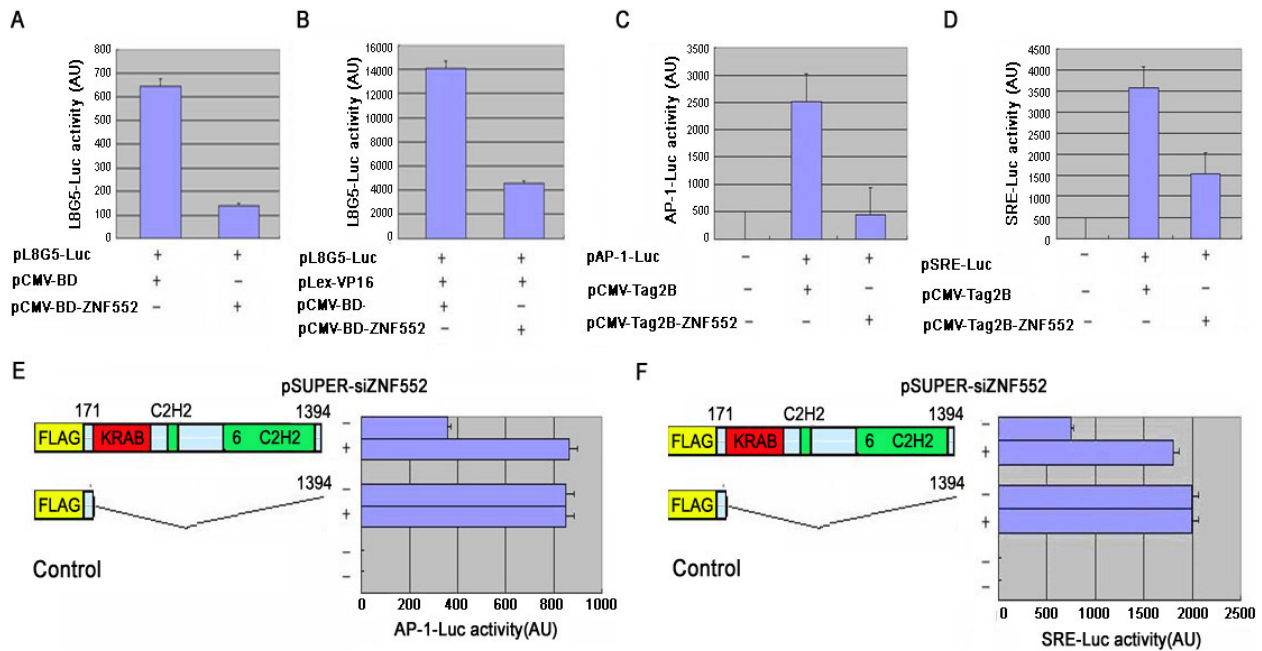
To examine the potential transcriptional activity of ZNF552, we used a system in which a luciferase reporter gene was activated by a fusion protein of the LexA DNA-binding domain fused to the potent activation domain of the viral co-activator protein, VP16 (LexAVP16) (15). As shown in Fig. 3B, co-transfection of GAL4-ZNF552 fusion protein with Vp16 strongly suppressed luciferase expression by approximately 60% (Fig. 3B), suggesting that ZNF552 is a potent transcriptional repressor.



**Fig. 1.** (A) Northern blot analysis of ZNF552 in eight adult human tissues. A single strongly expressed mRNA band of 2.4 kb was observed strongly in adult testis and lung; moderately in liver, spleen and kidney; no hybridization signal was detected in other tissues.  $\beta$ -Actin was used as a control for the equivalent amount of loaded mRNA (total RNA) in each other. (B) RT-PCR analysis of ZNF552 gene expression in various human embryo tissues. 1, heart; 2, skeletal muscle; 3, brain; 4, lung; 5, liver; 6, kidney; 7, spleen; 8, intestine; 9, testis; 10, bladder.



**Fig. 2.** Cellular localization of the ZNF552 protein in COS-7 cells. (A) EGFP-ZNF552 fusion protein is localized in the nucleus and the cytoplasm when transfected into the COS-7 cells. (B) The nuclei of the cells was stained with DAPI. (C) The combined image of (A) and (B), showing that ZNF552 encoded protein exists predominantly in the nuclei of the cells.



**Fig. 3.** ZNF552 is a transcription repressor. (A) pCMV-BD-ZNF552 or pCMV-BD is transiently co-transfected into COS-7 cells along with the pL8G5-Luc reporter. (B) pCMV-BD-ZNF552 or pCMV-BD is transiently co-transfected into COS-7 cells along with the pL8G5-Luc reporter and pLexA-VP16. (C, D) pCMV-Tag2B-ZNF552 or pCMV-Tag2B is transiently co-transfected into COS-7 cells along with the pAP-1-Luc reporter and pSRE-Luc reporter respectively. (E, F) pSUPER-siZNF552 and pCMV-Tag2B-ZNF552 (or pCMV-Tag2B) is transiently co-transfected into COS-7 cells along with the pAP-1-Luc reporter and pSRE-Luc reporter respectively. as indicated in the figure. Forty-eight hours later, the luciferase activity assay was performed. The data are means of three repeats in a single experiment after normalization for galactosidase activity. Each experiment was repeated at least three times.

To investigate the role of ZNF552 in cell signal transduction, we examined the effect of ZNF552 on this specific cell signaling pathway using pathway-specific reporter gene assays to measure the modulation of SRE and AP-1 by ZNF552 in the cell. Expression of ZNF552 significantly suppresses the AP-1-luciferase activity by approximately 72% (Fig. 3C) and SRE transcription by 58% (Fig. 3D). To verify that the transcriptional repression we observed was due to the activity of ZNF552 protein, we used RNA interference to block the translation of ZNF552. COS-7 cells were transfected with pSUPER-SiZNF552, together with pAP-1-Luc (or pSRE-Luc) and pCMV-Tag2C-NF552. As shown in Fig. 3E and 3F, pSUPER-SiZNF552 blocked the suppression by ZNF552. Taken together, our results suggest that ZNF552 may potentially participates in the transcriptional repression involved in the MAPK signaling pathway in cells.

### ZNF552 contains a transcriptional suppression domain—the KRAB motif

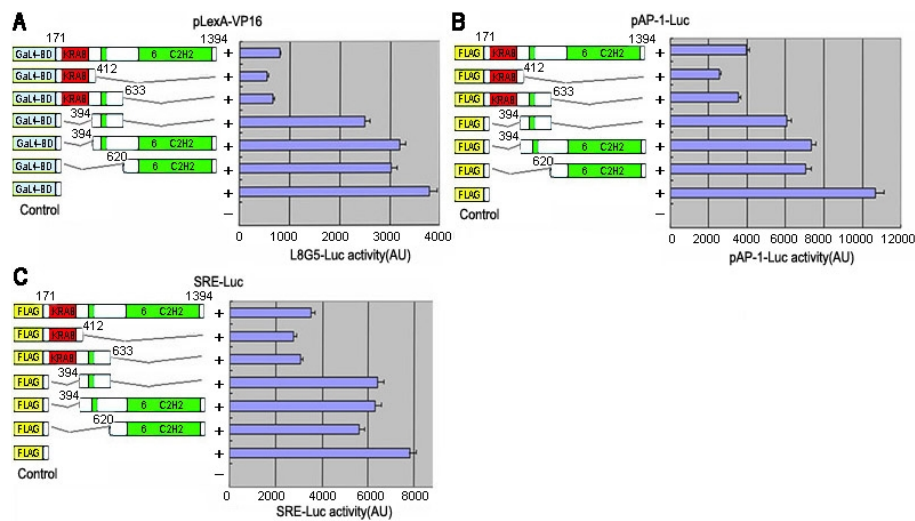
To further identify potential transcriptional regulatory domains in ZNF552, we constructed five truncated GAL4-ZNF552 fusion proteins: pCMV-BD-KRAB (1-80 aa), pCMV-BD-KRAB-1C2H2 (1-154 aa), pCMV-BD-1C2H2 (131-154 aa), pCMV-BD-1C2H2-6C2H2 (131-407 aa) and pCMV-BD-6C2H2 (154-407 aa) (shown in Fig. 4A). Reporter gene assays suggested that the KRAB domain

seems to be a transcriptional repressor as pCMV-BD-KRAB and pCMV-BD-KRAB-1C2H2 reduced luciferase activity by approximately 86%. While pCMV-BD-1C2H2, pCMV-BD-1C2H2-6C2H2 and pCMV-BD-6C2H2 suppressed activity by approximately 15-34% (Fig. 4A). These results indicated that both the KRAB domain and the C2H2 motif contribute to the transcriptional repression by ZNF552, the KRAB domain may play predominantly role in ZNF552.

In order to investigate the role of the KRAB domain of ZNF552 in MAPK-mediated transcriptional regulation, five truncated FLAG-ZNF552 fusion proteins were constructed and reporter gene assays were performed using pAP-1-Luciferase and SRE-luciferase reporters respectively. The results showed that the KRAB domain significantly reduced AP-1 transcriptional activity by approximately 76% (Fig. 4B) and SRE transcription by 64% (Fig. 4C).

### DISCUSSION

In this study, we report the identification of ZNF552, a novel zinc finger transcription factor never reported previously. Using simple modular architecture research tool (SMART) analysis we found that ZNF552 protein contains a KRAB box and 7 C2H2 zinc fingers motifs (Supplement Fig. 1B). The KRAB domain in



**Fig. 4.** Luciferase reporter assay of full-length and five truncated ZNF552 fusion proteins. (A) pCMV-BD-ZNF552, pCMV-BD-KRAB, pCMV-BD-KRAB-1C2H2, pCMV-BD-1C2H2, pCMV-BD-1C2H2-6C2H2 and pCMV-BD-6C2H2 is transiently co-transfected into COS-7 cells along with the pL8G5-Luc reporter respectively. (B, C) pCMV-Tag2B-ZNF552, pCMV-Tag2B-KRAB, pCMV-Tag2B-KRAB-1C2H2, pCMV-Tag2B-1C2H2, pCMV-Tag2B-1C2H2-6C2H2 and pCMV-Tag2B-6C2H2 is transiently co-transfected into COS-7 cells along with the pAP-1-Luc reporter (B) or pSRE-Luc reporter (C) respectively. Forty-eight hours later, the luciferase activity assay was performed. The data are means of three repeats in a single experiment after normalization for galactosidase activity. Each experiment was repeated at least three times.

KRAB/C2H2 type zinc finger proteins are subdivided into an A box and a B box, usually coded for by separate exons (15). The KRAB domain region spanning amino acids 14-67 of ZNF552 shows homology to other zinc finger proteins, such as ZNF418, ZNF417, ZNF486, and ZNF487 (data not shown). The KRAB-A box of ZNF552 consists of about 41 amino acid residues, including a conserved motif: DV (at position 5-6), which have been shown to be important for repression and interaction with TIF1h (also named KAP-1, KRIP-1) (16, 17). Seven C2H2 zinc finger motifs of ZNF552 extend to the C-terminal portion of the protein sequence (Supplement Fig. 1A). Each finger motif conforms closely to the consensus sequence X2CX2CXKXFX4HL R(S)HX3H. The conserved Cys and His residues in C2H2 zinc finger are bound to a tetrahedrally coordinated zinc ion. Six zinc finger units at the C-terminus are connected by highly conserved H/C linkers TGE(K/R)P (Supplement Fig. 1A), suggesting a role in DNA binding (18). Taken together, these results suggest that ZNF552 belongs to the KRAB/C2H2 subfamily of zinc finger proteins.

Approximately one-third of C2H2 type zinc finger genes in the human genome are estimated to contain the KRAB domain (15). The KRAB domain in KRAB/C2H2 type zinc finger is an evolutionarily conserved domain of about 75 amino acids. Sequence comparison of the KRAB indicates that the degree of conservation in this domain is modest among vertebrate members (Supplement Fig. 2A). Further sequence comparison shows that the C2H2 zinc fingers in these proteins are less homologous, suggesting that ZNF552 is a less conserved member of the zinc-finger family. Although not detected in adult human heart tissue by Northern blotting, the RT-PCR results showed that ZNF552 was expressed in embryo heart with a higher level, suggesting that ZNF552 may play a role in human early heart development.

A lot of studies have shown that zinc finger protein family members are transcriptional intermediary factors, most of them are corepressors, and few of them are coactivators (10). Our experiments showed that full-length ZNF552 inhibited transcrip-

tional activity when fused to the GAL4 DBD. MAPKs are important signal transducing enzymes that are involved in multiple cellular living processes through phosphorylating their specific endpoint targets, such as AP-1 and SRE, which compose a ternary complex together with SRF to induce expression of c-fos and other early growth response genes that control the transition from quiescence to proliferation (19). Reporter assays showed that overexpression of ZNF552 in the cell negatively regulates transcriptional function by significantly inhibiting the transcriptional activities of AP-1 and SRE, suggesting that ZNF552 is a new zinc-finger protein that potentially participates in the transcriptional repression of AP-1 and SRE activities, which can be mediated by MAPK pathways.

KRAB box-containing zinc finger proteins in most of studies have been shown to act as potent transcriptional repressors that are dependent on DNA-binding (17). Previous studies have indicated that some members of the Kruppel-like family of transcription factors consist of multiple domains that function in transcriptional activation, inhibition of activation, protein-protein interaction, DNA binding and transcriptional repression. For example, the KRAB domain has been shown to function as a repressor of transcription through protein-protein interaction (19-21). In order to further identify potential novel transcriptional regulatory domains in ZNF552, we tested the ability of the truncated GAL4-ZNF552 fusion proteins to regulate the activity of the GAL4 site-driven luciferase reporter gene. Full-length ZNF552 (1-407 aa) fused to the GAL4 DBD repressed the reporter gene strongly (proximately 78%). Both amino acids 1-80 which contain portion of KRAB box and amino acids 1-195 containing KRAB domain and the first C2H2 motif repressed the LexA-VP16-luciferase activity even stronger (by approximately 86%) (Fig. 4A). C2H2 motif portion repressed the reporter gene modestly (by approximately 15-34%). Pathway-specific reporter gene assays were performed to measure the transcription of AP-1 and SRE by the truncated FLAG-ZNF552 fusion proteins in COS-7 cells,

using pAP-1-Luc and SRE-luciferase reporters respectively (Fig. 4B and C). The results showed that the KRAB domain repressed transcriptional activities mediated by MAPK signaling pathways in the cell. Our results suggest that the conserved KRAB domain in ZNF552 protein may predominantly be a transcriptional repression domain and consistent with the traditional viewpoint that KRAB domain was identified as a repressor.

Taken together, we have identified and characterized a novel human KRAB/C2H2 zinc finger transcription factor. Northern blot analysis indicated that ZNF552 was expressed strongly in adult testis and lung; moderately in liver, spleen and kidney. Overexpression of ZNF552 repressed the transcriptional activities of SRE and AP-1, suggesting that ZNF552 may act as a negative transcriptional regulator in MAPK signaling pathways.

## MATERIALS AND METHODS

### Construction of cDNA library of human embryo heart

The total RNA from 20-week-human embryo heart was extracted using standard methods. The RNA was pretreated with DNase I (RNase free) to eliminate DNA contamination. mRNA preparation and reverse transcription reaction were performed using a cDNA PCR Library Kit and cDNA Synthesis Kit according to manufacturer's protocol (TaKaRa) described previously (22).

### Full-length ZNF552 cDNA cloning and bioinformatics analysis

BLASTn program (<http://www.ncbi.nlm.nih.gov/blast>) was used to search human EST database. The full-length cDNA sequence of a predicted novel human gene was obtained from these ESTs by splicing on an EST annotation machine at IFOM ([http://bio.ifom-firc.it/EST\\_MACHINE/index.html](http://bio.ifom-firc.it/EST_MACHINE/index.html)) and using the Genscan program (<http://genes.mit.edu/GENSCAN>). The primers ZNF552-F and ZNF552-R (Supplement Table 2) were designed according to the above full-length cDNA sequence and used in polymerase chain reaction (PCR) assay with the heart cDNA library as the template according to standard procedures to confirm the open reading frame (ORF). Amplification was carried out at 94°C, 4 min; 94°C, 30 s; 60°C, 30 s; and 72°C, 2 min for 30 cycles; then 72°C, 10 min. These PCR products were confirmed by sequencing. Analysis of ZNF552 sequence was performed by DNASTar software. SMART (<http://smart.embl-heidelberg.de/>) was used to analyze motifs. Both BLAST program at NCBI and CLUSTAL program were performed to analyze similarities for nucleotides and proteins (14).

### Northern blot analysis

To confirm the tissue distribution of the new gene mRNA, Northern blot analysis was performed using the PCR amplified 761 bp segment of the ZNF552 cDNA as a probe. The ZNF552 cDNA was labeled in the presence of a digoxigenin labeling system according to the manufacturer's protocol (Roche, Basel, Switzerland). Hybridization of the probe to the MTN-membrane-bound RNA was performed following the manufacturer's instructions (Clontech, San Jose, USA). Equal loading was con-

formed using the  $\beta$ -actin gene probe as a control.

### Identification of ZNF552 mRNA in human embryo tissues by RT-PCR

Total RNAs from multiple human embryo tissues (heart, skeletal muscle, brain, lung, liver, kidney, spleen, intestine, testis and bladder.) were extracted using an RNA isolation kit (Invitrogen, Carlsbad, USA). cDNA was synthesized according to the instructions (Promega, Madison, USA) and was used as a template in the PCR amplification with the primer pair ZNF552-F1/ZNF552-R3 (Supplement Table 2) and DNA polymerase (Fermentas, MBI, Lithuanian). RT-PCR product was separated on 2.0% agarose gel. GAPDH was amplified as the control.

**Plasmid construction:** The L8G5-Luc, pSRE-Luc and pAP-1-Luc constructs used were generated previously in the lab (22). An EGFP-C1-ZNF552 expression plasmid was constructed by inserting ZNF552 cDNA which had cloned into pUCm-T vector into pEGFP-C1 (Clontech, San Jose, USA) vector at the downstream of EGFP with HindIII and Sall sites. To generate a fusion protein of ZNF552 with GAL4 or FLAG tag, the ZNF552 ORF was amplified by PCR with primers ZNF552-S1/ZNF552-A1 (Supplement Table 2) and then subcloned in-frame into the BamHI and Sall site of the pCMV-BD or pCMV-Tag2B, respectively. To generate a fusion protein of KRAB, KRAB -1C2H2, 1C2H2, 1C2H2-6C2H2 and 6C2H2 fragments with GAL4 or FLAG tag, the five fragments were amplified by PCR with primers ZNF552-S1/ZNF552-A2, ZNF552-S1/ZNF552-A3, ZNF552-S2/ZNF552-A3, ZNF552-S2/ZNF552-A1 and ZNF552-S3/ZNF552-A1 (Supplement Table 2), respectively, and then subcloned to the BamHI and Sall sites of the pCMV-BD and pCMV-Tag2B.

**Cell culture, transient transfection, and subcellular localization analysis:** COS-7 cells used in all studies were maintained and passaged according to standard methods described previously (14, 22), and transfected with pEGFP-C1-ZNF552 plasmid DNA using Lipofectamine 2000 (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. Forty-eight hours after the transfection, the localization of the fusion protein (EGFP-ZNF552) was visualized with epifluorescence microscope after labeling with DAPI for nuclei. Observation of fluorescence of the EGFP-ZNF552 fusion protein was performed with a Nikon fluorescence microscope. The COS-7 cells transfected with pEGFP-C1 vector were used as a control.

### Transient expression reporter gene assay and deletion analysis

COS-7 cells were transfected using lipofectamine as described above. pCMV-BD-ZNF552 and other truncated GAL4-ZNF552 fusion constructs were co-transfected into COS-7 cells along with the pL8G5-Luc reporter respectively, or pCMV-Tag2B-ZNF552 and other truncated FLAG-ZNF552 fusion constructs were co-transfected into COS-7 cells along with the pAP-1-Luc or pSRF-Luc respectively. The luciferase activity assay was performed 48 h later according to the methods described previously (14).



### 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 were SiZNF552-F: 5'-GATCCCCCTTCTGTGTGGTGTGAATTTCTAGAGAATTCACACCACACAGAAGGTTTTC-3' and SiZNF552-R: 5'-TCGAGAAAACCTTCTGTGTGGTGTGAATTTCTAGAAATTCACACCACACAGAAGGGGG-3'. The oligos were annealed and cloned according to the instructions (OligoEngine). Luciferase activity assay was performed in MCF-7 cells transfected with pAP1-Luciferase (or pSRE-Luciferase), pCMV-Tag2B-ZNF552, and pSUPER-SiZNF552.

### Acknowledgements

We are grateful to all members of the Center for Heart Development, College of Life Sciences in Hunan Normal University for their excellent technical assistance and encouragement. This study was supported in part by the National Natural Science Foundation of China (No. 30930054, 30671137, 30671054, 30671053, 30671171, 30670274, 30771170, 30771146, 30871340, 30871417, 30970425, 30971663), National Basic Research Program of China (2005CB522505), program for excellent talents in Hunan Normal University (No.ET30904), and the Foundation of Hunan Province (05J2007, 06JJ4120).

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