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A novel human KRAB-related zinc finger gene *ZNF425* inhibits mitogen-activated protein kinase signaling pathway

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Zinc finger (ZNF) proteins play a critical role in cell growth, proliferation, apoptosis, and intracellular signal transduction. In this paper, we cloned and characterized a novel human KRAB-related zinc finger gene, *ZNF425*, which encodes a protein of 752 amino acids. *ZNF425* is strongly expressed in the three month old human embryos and then is almost undetectable in six month old embryos and in adult tissues. An EGFP-ZNF425 fusion protein can be found in both the nucleus and the cytoplasm. *ZNF425* appears to act as a transcription repressor. Over-expression of *ZNF425* inhibits the transcriptional activities of SRE, AP-1, and SRF. Deletion analysis indicates that the C2H2 domain is the main region responsible for the repression. Our results suggest that the *ZNF425* gene is a new transcriptional inhibitor that functions in the MAPK signaling pathway. [BMB reports 2011; 44(1): 58-63]

INTRODUCTION

Zinc finger (ZNF) genes are one of the largest gene families which play important roles in a variety of cellular functions, including cell growth, proliferation, apoptosis, and intracellular signal transduction (1). ZNF proteins contain a number of ZNF domains, which are frequently arranged in groups or clusters of tandem repeats (2). Although there are 20 different types of ZNF domains, the most common is the Cys2-His2 (C2H2) class (3). There are about 700 genes in the human genome that contain C2H2 motifs (4). Members of the ZNF subfamily that contains this motif have spatially conserved cysteines (C) and histidines (H), which coordinate a Zn^{2+} and cause the intervening amino acid chain to loop out and form the secondary finger-like structure. Each ZNF specifically binds three to five base pairs in the major groove of DNA and makes non-specific interactions in the minor groove, explaining its ability to stimulate or repress gene expression (5). Among the ZNF genes about one-third contain an N-terminal domain known as the KRAB domain, which acts as a powerful transcriptional repressor even when fused to a heterologous DNA-binding domain (DBD) in a variety of systems (6). Some ZNF proteins have been implicated as critical cardiac-specific transcription factors that regulate cardiac development and growth (7). However, the functions of many of these cardiac-specific ZNF proteins are still largely unknown.

The mitogen-activated protein kinases (MAPKs) are well known as transducing enzymes in mammals that play crucial roles in cell proliferation, cell differentiation, and cell cycle regulation (8). MAPKs include three kinases, MAPK kinase kinase (RAF), MAPK kinase (MEK), and MAPK (ERK1/2) (8) that sequentially phosphorylate and activate each other. One of the most studied targets of the MAPK signaling pathway are transcription factors, including zinc finger containing transcription factors, which regulate transcription immediate-early gene expression (9) in response to environmental stimuli. Jun, Fos, and ATF-2 form DNA-binding homo- or heterodimers with AP-1 components. Activation of AP-1 involves the direct phosphorylation/dephosphorylation of AP-1 components as well as the phosphorylation and activation of transcription factors that induce elevated expression of c-jun or c-fos (8). The MAPKs also phosphorylate their specific endpoint targets such as Elk-1 and SRE, which form a ternary complex together with SRF to induce expression of c-fos and other early response genes (9).

With the aim of identifying new transcriptional factors involved in heart development, we cloned a novel KRAB/C2H2-type zinc-finger gene named *ZNF425* from human heart cDNA library. *ZNF425* is evolutionarily conserved across species and encodes a predicted protein of 752 amino acids with a conserved KRAB domain followed by 19 C_2H_2 type zinc finger motifs. An EGFP-ZNF425 fusion protein was detected mainly in the nucleus, supporting its function as a transcriptional regulator. Reporter gene assays showed that *ZNF425* could repress transcriptional activities of the SRE, AP-1, and SRF. These results suggest that *ZNF425* protein may act as a negative regulator in MAPK signaling pathway to mediate cellular functions.

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RESULTS

Molecular characterization and sequence analysis of the human *ZNF425*

We cloned a novel gene that was named *ZNF425* as approved by HUGO nomenclature committee. *ZNF425* consists of an open reading frame (ORF) of 2259 bp extending from the first ATG codon at nucleotide 74 to a termination codon TAA at 2332. The ORF contains four exons and three introns (Supplement Table 1), with a typical signal sequence AATAAA and a PolyA tail (Supplement Fig. 1A). The exon-intron boundaries conform to the consensus splicing signals, with a gt and an ag dinucleotide at the 5'-donor and 3'-acceptor site, respectively.

The deduced *ZNF425* protein has 752 amino acids, with a chemical formula $C_{3818}H_{6028}N_{1168}O_{1088}S_{63}$ and a calculated molecular mass of 88.7 kDa (Supplement Fig. 1A). The theoretical pI of *ZNF425* protein is 9.43, which suggests that it may be a basic protein. Further studies indicate that *ZNF425* contains an N-terminal krüppel-associated box (KRAB) domain (amino acids 9-69) and 19 C_2H_2 zinc finger motifs that extend to the end of the protein sequence (Supplement Fig. 1B). Sequence comparison of the KRAB box among its orthologues indicated that the KRAB box of *ZNF425* may be a highly conserved one. About 50% of the total 61 amino acids of *ZNF425* KRAB box are conserved sequences (Fig. 1A). In the polygenetic tree, human *ZNF425* is closely related to *M. musculus* ZNF26 (Fig. 1B). Sequence analysis and database comparison indicated that *ZNF425* was a classical krüppel zinc finger with 21 highly conserved C_2H_2 zinc finger domains in tandem arrays (Fig. 1C).

Expression of the *ZNF425* mRNA and subcellular localization of GFP-ZNF425 fusion protein.

Northern blot analysis detected a 3.2 kb *ZNF425* mRNA transcript in 30 day embryos. However, no obvious signals were detected among the tissues tested at the 60th day embryo and at adult stage (Fig. 2A, B).

To examine the subcellular localization of *ZNF425*, the

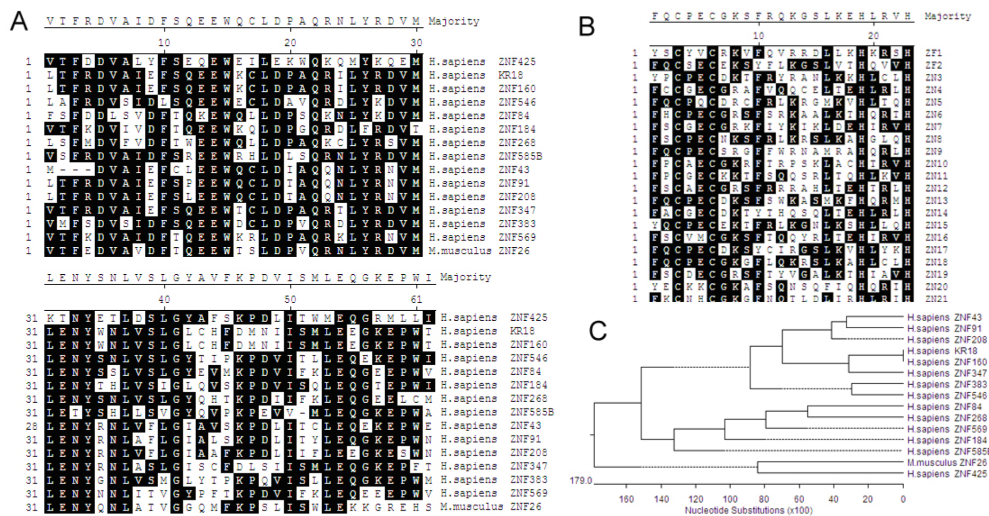

Fig. 1. *ZNF425* is conserved during evolution. (A) Amino acid sequence alignment of multiple KRAB domains. (B) Amino acid comparison of 21 zinc finger motifs within *ZNF425*. (C) Polygenetic tree analysis of amino acid sequence of *ZNF425* and its homologues.

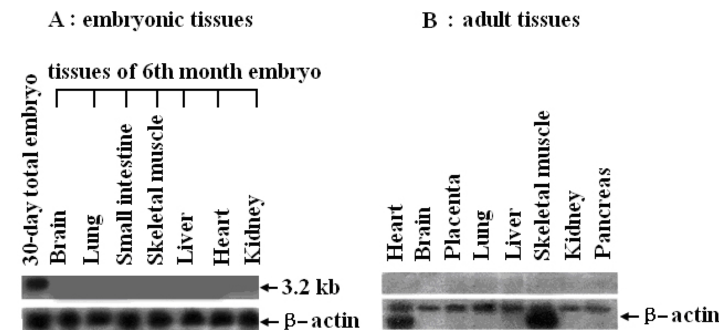

Fig. 2. Northern blot analysis of ZNF425. (A) A single strongly expressed mRNA band of 3.2 kb was observed in 30-day total embryo. (B) No obvious hybridization signals were detected in the tissues tested at late embryo and adult stages.

pEGFP-N1-ZNF425 (ORF) plasmid was transfected into COS-7 cells. Forty-eight hours after transfection, cells transfected with pEGFP-N1-ZNF425 show GFP fluorescence that colocalizes with DAPI indicating a nuclear localization (Fig. 3A, B). The combined image (Fig. 3C) shows that some *ZNF425* protein can also be found in the cytoplasm of the cells.

Transcriptional activation analysis of *ZNF425*

Reporter gene assays showed that the GAL4-ZNF425 fusion protein, driven by a CMV promoter, inhibited luciferase activity by 94.5%, and when co-transfected with pLexA-VP16, it inhibited luciferase activity by 89.9% (Fig. 4A), suggesting *ZNF425* is a transcriptional inhibitor.

A series of truncated GAL4-BD-*ZNF425* fusion proteins were co-transfected with the pL8G5-luc plasmid and pLexA-VP1. Compared to the repressive activities of the full-length *ZNF425*, which inhibited the VP16 activated luciferase activity by 53.5%, the ZNF motif alone significantly inhibited the VP16 activated luciferase activity by 75.2% (Fig. 4B). The linker region inhibited the activity 11.0% (Fig. 4B). Despite the different transcription activities among various protein domains, the results suggest that the ZNF motifs of *ZNF425* represent the basal repression domain, and may play direct roles in transcriptional repression.

ZNF425 represses SRE and AP-1-mediated transcriptional activation

To examine the effect of *ZNF425* protein in the MAPK signal pathways, expression plasmids (pCMV-Tag2B-ZNF425 or pCMV-Tag2B) were co-transfected with pSRE-Luc and pAp-1-Luc in COS-7 cells. The results indicated that expression with the *ZNF425* containing plasmid reduced the endogenous SRE-luciferase activity by 84.4% (Fig. 4C), and also strongly inhibited the transcriptional activity of AP-1 by 89.1% (Fig. 4D). These results show that *ZNF425* may function in the MAPK signaling pathway by inhibiting the activities of SRE and AP-1.

ZNF425 also represses SRF-mediated transcriptional activation

To further examine the transcriptional regulatory activity of *ZNF425*, we performed reporter gene assays to measure the modulation of SRF by the full-length and truncated portions of *ZNF425* in COS-7 cells. Compared to full-length *ZNF425* which reduces the endogenous SRF-luciferase activity by 94.1%, the ZNF motif alone reduces activity by 99.8%, and the linker region reduces activity by 97.7% (Fig. 4E). These results suggested that *ZNF425* can inhibit the activities of SRF, and the ZNF motifs and the link may represent the potent repression domain.

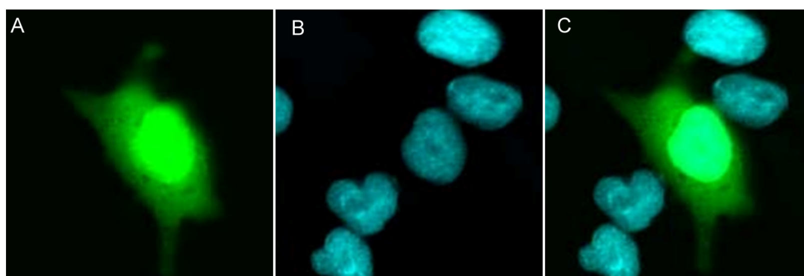

Fig. 3. Subcellular localization of *ZNF425* protein in COS-7 cells. (A) EGFP-*ZNF425* protein is expressed in the cell nucleus. (B) The nuclei of cells stained with DAPI. (C) The combined image of (A) and (B) showing subcellular localization of *ZNF425*.

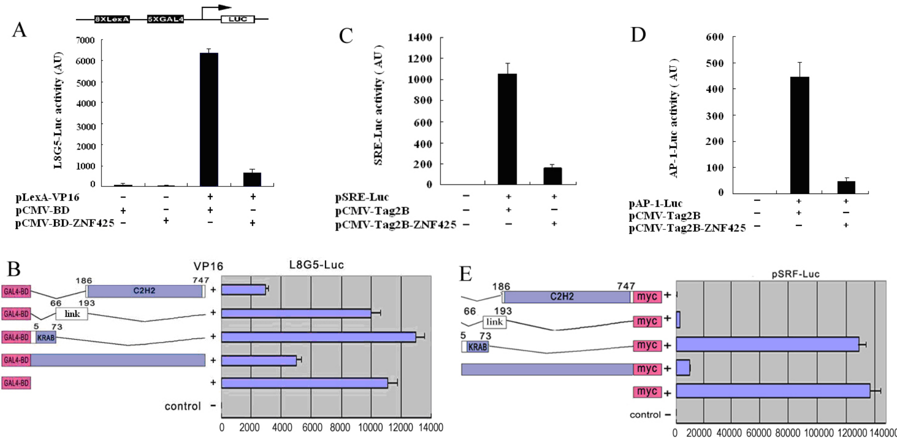

Fig. 4. Luciferase reporter assay of *ZNF425* in COS-7 cells. (A) The transcriptional activity analysis of *ZNF425* fusion proteins. (B) The transcriptional activity analysis of full- length and truncated *ZNF425* fusion proteins. (C) Co- transfection of pCMVTag2B-*ZNF425* and pSRE-Luc suppresses SRE activation in the reporter assay. (D) Inhibition of AP-1 transcriptional activity by expression of *ZNF425*. (E) Analysis of the transcriptional activity to SRF of full-length and truncated *ZNF425* fusion proteins.

DISCUSSION

In mammals, zinc-finger proteins probably constitute the largest individual family of such nucleic acid-binding proteins (10). The KRAB domain is present in the aminoterminal regions of more than one-third of Kruppel-class C2H2 zinc-finger proteins and is highly conserved from yeast to human (11). Although more than one hundred members of the KRAB/C2H2 zinc-finger protein family have been described, little is known of their biological function (12). Increasingly, studies have implicated ZNF proteins in embryonic development and disease (1, 13-18). For example, the Kruppel-class C2H2 zinc-finger protein ZNF382 has been shown to play a role in myogenesis (13, Wu, unpublished) and in tumor suppression through heterochromatin-mediated silencing (17). The promyelocytic leukemia zinc finger gene, which is an important regulator of cell growth, death, and differentiation, may play a critical role in promyelocytic leukemia (19). The multitype U-shaped functions of zinc-finger proteins in heart cells of Drosophila embryo have been reported (7). Some ZNF proteins, such as *ZNF480* (14), *ZNF569* (15) and *ZNF418* (16), have been shown to be involved in cellular functions via the MAPK signaling pathway.

In the present study, we isolated a novel Kruppel-class C2H2 zinc-finger protein ZNF425 containing 19 C2H2-type zinc fingers and a KRAB box from a heart cDNA library. We found that *ZNF425* is strongly expressed in very early stage embryos (at about 30-days) after which its expression is dramatically decreased to undetectable levels by Northern blot. RT-PCR analysis (E-analysis), which is more sensitive than Northern blot, showed that *ZNF425* has a higher expression in heart than in other tissues (not shown). The first indication of human heart development is around day 16-19, the first heartbeat occurs at 21 to 22 days, and the heart divides into 4-chambered heart between weeks 4 and 7, indicating that the critical period of development is from day 20 to day 50 after fertilization (20, 21). Therefore, the timing of *ZNF425* expression suggests that it might be involved in the early development of human heart.

The MAPK pathways are major components of pathways involved in embryogenesis including cell differentiation, cell proliferation, and cell death. One of the most explored functions of MAPK signaling is the regulation of gene expression by direct or indirect phosphorylation and subsequent activation of transcription factors (22). MAPK pathways are also involved in multiple cellular processes through phosphorylation of their specific targets such as ElK-1 and SRE, which forms a ternary complex to induce expression of c-fos and other early response genes. The c-fos products heterodimerize with c-Jun proteins to form AP-1 complexes. Activation of AP-1 involves the direct phosphorylation/dephosphorylation of AP-1 components as well as the phosphorylation and activation of transcription factors that induce elevated expression of c-Jun or c-fos. The *ZNF425* protein appears to function in the MAPK pathway by strongly suppressing SRE and AP-1 transcription activities. These results suggest that ZNF425 might be a strong negative regulator of the mitogen-activated protein kinase signaling pathways.

C2H2-type zinc finger proteins containing KRAB domains function commonly as transcriptional repressors when tethered to the template DNA by a DNA-binding domain and are also involved in protein-protein interactions (23). Our observations show that *ZNF425* strongly suppresses SRF transcription activities. SRF may play an important role in both heart development and disease. SRF mutant embryos show defects in both the cardiac compact layer and in the trabeculations, in addition several critical cardiac transcriptional factors were down regulated in these embryos (24). The absence of SRF in cardiomyocytes and smooth muscle cells leads to ultrastructural defects in contractile/cytoskeletal assembly (25). The DNA-binding activity of AP-1/c-Jun was found to be dramatically increased in failing hearts, an increase that was not observed in compensatory cardiac hypertrophy (26). SRE is one of the several cis elements which mediate c-fos induction and is recognized by a dimer of SRF that recruits the monomeric ternary complex factors. SRE is related to SRF through monomeric ternary complex factors, whose members include Elk-1, SAP-1, or SAP-2 (27). Thus our data suggest that ZNF425 could be involved in heart development and disease as a negative regulator of SRF.

In a summary, our results suggest that *ZNF425* is a novel human KRAB-related zinc finger gene and is strongly expressed in the human embryos of three months. *ZNF425* could be a strong transcription repressor in mitogen-activated protein kinase (MAPK) signaling pathway to mediate cellular functions and it has a potential role in regulating some essential cellular processes of heart development. Further study to elucidate its special expression pattern at early heart development stages and functional studies will contribute to the understanding of its role in human heart development and cardiovascular diseases.

MATERIALS AND METHODS

RNA isolation and Northern blot hybridization

Total RNA was extracted from total embryos (gestation 20 to 24 weeks) and various adult tissues using standard methods (28). The embryonic tissues were prepared as described in previous studies (14). Adult human tissue Northern blots (Clontech, USA) and embryonic tissue blots were hybridized with cDNA probes of *ZNF425* labeled with [α-^{32}P] dCTP using a Random Primer Labeling Kit (TaKaRa). The membranes were then exposed to X-ray films at 80°C for several days, and probed with radiolabeled β-actin cDNA (Clontech), USA as an indicator of mRNA loading. The use of human embryos in this study was approved by the Ethic Committee of Hunan Normal University and Changsha Women and Children Hospital.

Construction of cDNA library of human embryo heart
Total RNA from 6 month month embryonic hearts was pretreated with DNase I (RNase free) to eliminate DNA contamination. Reverse transcription reactions were performed with the purified embryonic heart mRNA (5 ug) and oligo (dT)- RA primers according to cDNA Synthesis kit (TaKaRa, Dalian, China). After Cassette Adaptor Ligation reactions using cDNA PCR Library Kit (TaKaRa, Dalian, China), cDNA amplification reactions were performed with RA primer, CA primer, and TaKaRa *Ex Taq* (TaKaRa, Dalian, China).

Full-length ZNF425 cDNA cloning and bioinformatics analysis
The consensus sequence of the conserved Cys2/His2 type zinc finger motifs was used to search human EST database through a combined BLAST search as previously described (18). PCR was performed with one pair of primers P1/P2 (Supplement Table 2) on a PCR SPRINT reactor (Thermo Hybaid, USA). The amplification products were separated on agarose gels and the bands were cloned into pUCm-T vector (TaKaRa, Dalian, China). The transformants were randomly chosen and sequenced with 250 DNA Sequencer (ABI PRISM, USA). The 5' upstream and 3' downstream fragments were amplified using the SMART cDNA Amplification Kit (TaKaRa, Dalian, China) using gene specific primers for 3'-RACE and 5'-RACE (Supplement Table 2). The open-reading frame sequence of *ZNF425* was confirmed by PCR amplification with a pair of primers (sORF and aORF) and retested by another pair of primers (sORFout and aORFout) (Supplement Table 2). All the PCR products were confirmed by sequencing and then assembled to complete the full-length cDNA of *ZNF425*. BLASTn program was used to search a human EST database to identify the cytological locus of genes and to look for exons and introns. The homologs of *ZNF425* were found with BLASTp, and the sequence alignment and phylogenetic tree analysis were performed with MegAlign program (BGI Life Tech, China)

Cell culture and subcellular localization analysis
COS-7 cells were maintained and passaged in DMEM according to standard methods (Dulbeccos modified Eagles medium, Gibco-BRL) and were transfected with pEGFP-N1-*ZNF425* using LipofectAMINE (Invitrogen, USA) according to the method described previously (18). 48 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 ZNF425-EGFP fusion proteins was detected using fluorescence microscopy Olympus IX71 (Olympus, Japan).

Transcriptional reporter gene assays
To understand the potential transcriptional regulatory activity of *ZNF425*, the full length pCMV-BD-ZNF425 and truncated fragments were transfected into COS-7 cells along with L8G5-Luciferase and pLexA-VP16 using LipofectAMINE as described previously (18). To examine the effects of *ZNF425* on the transcriptional activity of SRE, AP-1 and SRF, COS-7 cells were also co-transfected with the expression plasmids pCMV-Tag2B-*ZNF425*, pSRE-Luc, pAP-1-Luc, pCMV-Tag2B or the pSRF-Luc vector. All the luciferase activity assays were performed according to the protocols of Stratagene at 48 hours following transfection. Relative luciferase activity was normalized for transfection efficiency by co- transfection with pCMV-lacZ. Each experiment was performed in triplicate and each assay was repeated at least three times. The mean of the data from three individual transfected wells are presented after normalization for β-galactosidase activity.

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