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A novel human gene ZNF415 with five isoforms inhibits AP-1- and p53-mediated transcriptional activity ☆

Yingduan Cheng [1], Yuequn Wang [1], Yanmei Li, Yun Deng, Junjian Hu, Xiaoyang Mo, Ning Li, Yongqing Li, Na Luo, Wuzhou Yuan, Jing Xiao, Chuanbing Zhu, Xiushan Wu *, Mingyao Liu *

The Center For Heart Development, Key Lab of MOE for Development Biology and Protein Chemistry, College of Life Sciences, Hunan Normal University, Changsha, 410081 Hunan, PR China

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Abstract

The zinc finger proteins are the single largest class of transcription factors in human genome. Previous studies revealed that zinc finger proteins are involved in transcriptional activation and regulation of apoptosis, etc. Alternative splicing emerges as a major mechanism of generating protein diversity and many zinc finger proteins reported have isoforms. In this article, we identify and characterize five isoforms of a novel zinc finger gene named ZNF415; these five isoforms were named ZNF415-1 to ZNF415-5. The five isoforms display different subcellular localization and are expressed at different levels in both embryonic and adult tissues. Furthermore, the splicing variants of ZNF415 display different transcriptional activity. Except for ZNF415-1, overexpression of the other ZNF415 isoforms in COS-7 cells inhibits the transcriptional activities of AP-1 and p53, suggesting that the ZNF415 protein may be involved in AP-1- and p53-mediated transcriptional activity.

Keywords: Zinc finger protein; KRAB A box; Isoform; Expression pattern

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 [1–3]. Zinc finger proteins probably constitute the largest individual family of such nucleic acid-binding proteins [4]. A zinc finger is a conserved motif of 28 amino acids, which is often repeated within a protein [5] and may be involved in DNA–protein or protein–protein interactions [6]. 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 [7]. In addition to zinc finger regions, most of these proteins also contain a regulatory domain, the Krüppel-associated box (KRAB). The KRAB domain consists of an A box and a B box encoded by two separate exons [8]. Alternative splicing of A box and B box has been reported [8,9]. Alternative splicing has emerged as a major mechanism for expanding and regulating the repertoire of gene function. Alternative domain splicing in KRAB domain disrupts the protein interaction domain [10]. This kind of domain disruption has apparently been employed in evolution in addition to create useful new functions [11].

In this study, we identified a novel zinc finger protein named ZNF415, from human fetal cDNA library. Five isoforms of ZNF415 have been cloned. ZNF415-1 encodes 11 different C2H2 type zinc fingers, the other isoforms contain a linker region that normally joins the KRAB box to the

☆ *Abbreviations:* AP-1, activation protein 1; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; KRAB, Krüppel-associated box; MAPK, mitogen-activated protein kinase; ZFPs, zinc finger proteins.

* Corresponding authors. Fax: +86 731 8615078.
E-mail addresses: xiushanwu@yahoo.com (X. Wu), mliu@ibt.tamhsc.edu (M. Liu).

[1] These authors contributed equally to this work.

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region coding for the 11 zinc fingers, and finally ZNF415-5 also contains a KRAB A box. These five isoforms display different expression levels in 6-month-old embryos and in adult tissues. ZNF415-1 and ZNF415 have the highest expression in both embryo and adult, while ZNF415-3 has the lowest level in the tissues examined. ZNF415-2 and ZNF415-4 show intermediate levels. The ZNF415-1 protein is localized in the nucleus while the other isoforms are localized in both the nucleus and cytoplasm. Five isoforms fused to the GAL4 DNA-binding display different transcription activity suggesting that splicing may influence the function of ZNF415 isoforms.

All the isoforms except ZNF415-1 can inhibit AP-1 and p53 activity. AP-1 is a dimeric transcription factor which is mainly composed of either a Jun-Jun homodimer or a Jun-Fos heterodimer. AP-1 regulates the expression of multiple genes essential for cell proliferation, cell cycle control, apoptosis, differentiation, and tumorigenesis [12]. The p53 pathway is composed of hundreds of genes and their products that respond to a wide variety of stress signals. These responses to stress include apoptosis, cellular senescence or cell cycle arrest [13]. Thus, by regulating activity of these transcription factors, ZNF415 might also play a role in cell cycle control or apoptosis.

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. Briefly, 5 μg mRNA was purified from 500 μg total RNA using Rapid mRNA purification Kit (AMRESCO). Reverse transcription reactions were performed with the purified embryonic heart mRNA and oligo(dT)-RA primer according to 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 [14].

Full-length ZNF415 cDNA cloning and bioinformatics analysis. A search of the human EST database with the conserved Cys2/His2 type zinc finger motifs was performed through a combined BLAST search as previously described [15]. The sequence obtained was subjected to human homology searching against expressed sequence tag (EST) database using Blastn (http://www.ncbi.nlm.nih.gov/blast). We searched the ZNF415 consensus sequence and identified six overlapping human expressed sequence tags (ESTs) (BX414947, AL565661, BX414948, BX411247, BX431463, and AL534796) corresponding to a novel gene. PCR was performed with one pair of degenerate oligonucleotide primers in order to identify whether these ESTs belong to the same novel gene. ZNF415 sequence of open-reading frame (ORF) was confirmed by PCR amplification with a pair of primers (ORF1 and ORF2, Table 1) based on CA423205 and BX484548. The PCR products were cloned into pMD18-T-vector (Takara) and sequenced with 3770 DNA Sequencer (ABI PRISM). Jellyfish 1.4 was used to find ORF and the deduced translated product. Sequence analysis was performed using the DNAStar program and BLAST program from NCBI. Blastn program was used to identify the cytological locus of genes and to look for exons and introns. BLASTn and Pfam 9.0 were used to analyze the genomic structure and the protein domain, respectively. The homologues of ZNF415 were found with BLASTp. The CLUSTAL method was chosen to correct the distances for multiple substitutions at a single site. GenBank Accession Numbers of previously known members of the Krüppel family of C2H2-type zinc finger protein and novel C2H2 type zinc finger gene sequences used for this analysis are: NP_060770 (ZNF83); NP_115973 (ZNF347); NP_009080 (ZNF184); NP_003420 (ZNF85); NP_003416 (ZNF45); NP_003421 (ZNF91); NP_666016 (ZNF23); NP_009084 (ZNF208); NP_008889 (ZNF16); NP_057528 (ZNF226); NP_033559 (ZFP29, mouse); NP_076478 (AJ18 protein, *Rattus*); and AAP35086 (ZNF415).

Table 1
Oligonucleotide primers

Name	Sequence
ORF1	5′-CGGCGTCGAGCCATTGACTTCCAA-3′
ORF2	5′-AGCCTCTGCCACACAGTTAGGTGTA-3′
Rs	5′-CCTGGGTTACCTTCCTGTTG-3′
Ra	5′-GAAGTCCTGTTGCTGTTGCTG-3′
Zs	5′-GTGGCAGAGGCTTCATTTAGG-3′
Za	5′-GAGGCAGGAGAATCGTTTGAA-3′
SPs	5′-ATGAGGAAGAAACCCAGAA-3′
SPa	5′-TCCCTGAAGCAAAACTCT-3′
1S	5′-GAAAGGATCCAAATGTGATCTGTG-3′
2-3S	5′-GAGGATCCGAAATGCCTGAACTCTAC-3′
4S	5′-AGGGATCCCCTATGTGGGAGCAC-3′
5S	5′-AGGGATCCGGAATGGCTTTTACTCAG-3′
415A	5′-ACGGATCCATATTAATTTCTTTTATAAGG-3′
Gs	5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′
Ga	5′-CATGTGGGCCATGAGGTCCACCAC-3′

RACE analysis. A rapid amplification of cDNA ends (RACE) technique was performed using mRNA from human fetal heart. The 5′ upstream sequence and 3′ downstream sequence of ZNF415 were amplified by RACE PCR using the SMART cDNA Amplification Kit (TaKaRa Biotechnology) according to manufacturer's protocols [16]. The gene-specific primers used for 5′ RACE and 3′ RACE PCR were Rs /Ra and Zs/Za (Table 1). The heart cDNA was used as template. All the PCR products were then cloned into pMD18-T vector and sequenced.

RNA isolation and RT-PCR. Human tissues from therapeutically aborted fetuses were obtained under the approval of Health Center of Changsha Women and Children Hospital, People's Republic of China, with the consent of the patients and the regulation of university policy. Total RNA was isolated from various tissues (heart, brain, skeletal muscle, liver, spleen, small intestine, lung, and kidney of early human 6-month-old embryos and heart, atria, cerebra, cerebel, skeletal muscle, liver, and lung of adult). Total RNA (5 μg), primed with oligo(dT), was reverse-transcribed into cDNA using the first-strand cDNA synthesis kit (Ferments) according to the manufacturer's instructions. cDNA amplification reactions were performed with SPs and SPa, and TaKaRa Ex Taq, GAPDH was used as a control with Gs and Ga. In order to distinguish the isoforms exactly, the sample was run in a 6% polyacrylamide gel with 1× TBE buffer on a vertical electrophoresis system [17], The results were visualized by silver staining and the PCR products were purified and sequenced.

Plasmid construction. The following plasmids were constructed and used for mammalian cell transfections. To generate a fusion protein between the five isoforms of ZNF415 and Red fluorescent protein (DsRed), pDsRed-monomer-c1, the DNA fragment containing the ZNF415 isoform coding sequence was amplified from pMD18-T-ZNF415 (1, 2, 3, 4, 5) with primers 415A and 1S, 2–3S, 4S, 5S (ZNF415-2 and ZNF415-3 share the same start site, Table 1), respectively. All the primers contain *Bam*HI site. The amplified DNA fragments were subcloned to pDsRed-monomer-c1 (Clontech) with *Bam*HI site. The same DNA fragments were subcloned to pCMV-BD (Clontech) for GAL4-ZNF415 fusion protein and pCMV-Tag2B (Stratagene) including the *Bam*HI site.

Cell culture, transient transfection, and subcellular localization analysis. COS-7 cells used in all studies were maintained and passaged according to standard methods in DMEM (Dulbecco's modified Eagle's medium; Gibco-BRL) supplemented with 10% fetal calf serum (FCS) in a humidified atmosphere of 95% air and 5% CO_2. Cells were transfected with pDsRed-monomer-c1-ZNF415 (1, 2, 3, 4, 5) using Lipofect-AMINE (Invitrogen) according to described methods [18]. Forty-eight

hours after transfection, cells were fixed with 4% paraformaldehyde for 15 min and washed with PBS three times. Then the nucleus was stained with Hoechst. Subcellular localization of the pDsRed-monomer-c1-ZNF415 fusion protein was detected using a Nikon inverted fluorescence microscope.

Transient expression reporter gene assay. pCMV-BD-ZNF415 (each of the isoforms) or pCMV-BD was transiently co-transfected into COS-7 cells along with the pL8G5-Luc reporter and pLexA-VP-16 using LipofectAMINE as described above. Forty-eight hours later, the luciferase activity assay was performed according to the protocols of Stratagene [19]. Relative luciferase activity was normalized for transfection efficiency by co-transfection with pCMV-lacZ and spectrophotometric analysis. Each experiment was performed in triplicate and each assay was repeated at least three times. The means of the data from three individual transfected wells are presented after normalization for β-galactosidase activity.

COS-7 cells were co-transfected with AP-1-Luc, and pCMV-Tag2B-ZNF415 isoforms or pCMV-Tag2B vector to investigate the effect of ZNF415 isoforms on the transcriptional activity of AP-1. To examine the effects of ZNF415 isoforms on the transcriptional activity of p53, cells were co-transfected with p53-luc and pCMV-Tag2B-ZNF415 isoforms or pCMV-Tag2B vector. Forty-eight hours later, the luciferase activity assay was performed according to the protocols of Stratagene. Relative luciferase activity was normalized as described above.

Results and discussion

Molecular characterization and evolutional conservation of the human ZNF415

In an effort to understand the role of the new C2H2 type zinc finger, we screened the human EST database with the conserved Cys2/His2 type zinc finger motif. The partial cDNA sequence of this new gene was assembled from ESTs including BX414947, AL565661, BX414948, BX411247, BX431463, and AL534796. We designed specific primers ORF1/ORF2 based on the EST sequence and PCR was performed using heart cDNA library as the template. Among the PCR products, we identified five isoforms of the new ZNF415 gene. Full-length cDNA of the five isoforms was obtained using RACE. The splicing of five isoforms changes the proposed initiating ATGs in all five (ZNF415-2 and ZNF415-3 encode at the same start codon). We named the five isoforms ZNF415-1, ZNF415-2, ZNF415-3, ZNF415-4, and ZNF415-5. Human genomic BLAST showed that ZNF415 maps to chromosome 19q13.41.

The ZNF415-1 cDNA is 2160 bp long, with a putative ORF from nucleotides 657 to 1634 bp. It encodes a 326-amino acid (aa) polypeptide that has a molecular weight of approximately 37.2 kDa. ZNF415-2 is 2497 bp long, coding for 567 aa with a predicted molecular mass of about 64.6 kDa. ZNF415-3 is 2507 bp and can encode a 603 aa protein which weighs 68.9 kDa. ZNF415-4 is 2609 bp and encodes a 542 aa, 62.6 kDa protein while ZNF415-5 is 2298 bp and can encode a 555 aa, 64.1 kDa protein. The full-length sequences were submitted to GenBank and given Accession Nos. DQ925695 (ZNF415-1), DQ925696 (ZNF415-2), DQ925697 (ZNF415-3), DQ925698 (ZNF415-4), and AY283600 (ZNF415-5).

We analyzed the putative protein sequence by Pfam and found that ZNF415-5 contains the KRAB A box, and 11 different C2H2 type zinc fingers joined by a linker region, ZNF415-2, 3, and 4 lack the KRAB A box as does ZNF415-1, which also has lost the linker region. The splicing patterns are shown in Fig. 1A. The alignments of DNA sequence around the splicing exons can be seen in Supplementary data Fig. 1.

Phylogenetic tree analysis of amino acid sequences deduced from ZNF415 DNA sequences was 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 members of the Krüppel family of C2H2-type zinc finger protein and novel C2H2 type zinc finger gene sequences used for this analysis are NP_060770 (ZNF83); NP_115973 (ZNF347); NP_009080 (ZNF184); NP_003420 (ZNF85); NP_003416 (ZNF45); NP_003421 (ZNF91); NP_666016 (ZNF23); NP_009084 (ZNF208); NP_008889 (ZNF16); NP_057528 (ZNF226); NP_033559 (ZFP29, Mus); NP_076478 (AJ18 protein, *Rattus*); and AAP35086 (ZNF415) (Supplementary data Fig. 2). The results show that ZNF415 is one of the conserved proteins during evolution. The most closely related protein of ZNF415 is ZNF91.

Expression pattern of the five isoforms

In order to display the tissue distribution of the five isoforms of ZNF415, RT-PCR was performed on total RNA isolated from embryonic and adult tissues. The sizes of the products are as follows: ZNF415-1 183 bp, ZNF415-2 402 bp, and ZNF415-3 510 bp, ZNF415-4 525 bp, ZNF415-5 304 bp. All five isoforms are expressed in both embryonic and adult tissues but exhibit different levels of expression. ZNF415-1 and ZNF415 are high in both embryo and adult, ZNF415-2 and 4 both have a moderate level of expression, while ZNF415-3 has the lowest level in the tissues examined (Fig. 1B and 1C). This suggests that the five isoforms may perform different roles.

Subcellular localization of the ZNF415 protein

To examine the subcellular localization of the ZNF415 isoforms, the pDsRed-monomer-c1-ZNF415 (1, 2, 3, 4, 5) was transfected into COS-7 cells. Forty-eight hours after transfection, the cells were visualized with an epifluorescence microscope after labeling nuclei with Hoechst. The combined image of pDsRed-monomer-c1-ZNF415 labeled isoforms and the Hoechst-stained DNA show both nuclear and cytoplasm expression for the ZNF415-2, -3, -4, and -5 isoforms, however, the ZNF415-1 isoform is only found in the nucleus (Fig. 2). The different subcellular localization is likely to be caused by the splicing event since the ZNF415-1 isoform is the only isoform that lacks the KRAB A box and linker region. As long as the linker region is present

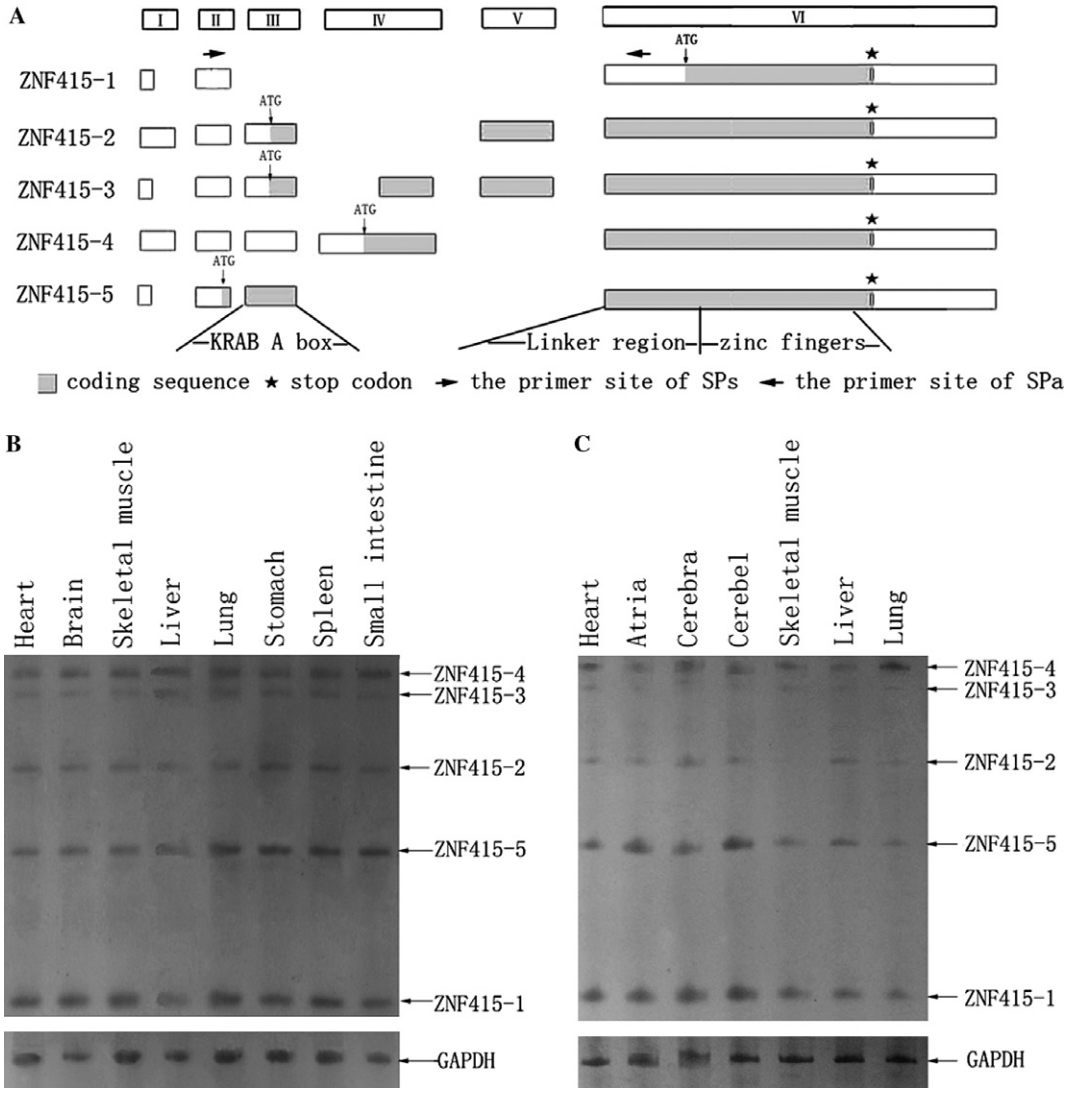

Fig. 1. (A) Schematic representation of ZNF415 isoforms. Boxes designate exons and gray areas reflect open-reading frames. The KRAB box is encoded in exon III while the linker region and zinc fingers are encoded in exon VI. The black arrows represent the location of the primers SPs and SPa. The five isoforms have different start sites but share the same stop codon. ZNF415-1 lacks exons III, IV, and V. ZNF415-2 lacks exon IV. ZNF415-3 has exon VI but a portion is spliced out. ZNF415-4 lacks exon V, but like ZNF415-2, has a longer exonI than the others. ZNF415-5 lacks both exons IV andV. (B,C) Expression analysis of human ZNF415 isoforms by RT-PCR amplification in tissues of 6 month embryo (B) and adult (C). We found that all the five isoforms were expressed in the tissues examined but at different levels. ZNF415-1 and Znf415-5 were high in embryo and adult, ZNF415-4 had an intermediate level, while ZNF415-2 is low in embryo and adult, ZNF415-3 has the lowest level in the tissues examined. GAPDH was used as a control.

the protein localizes to the cytoplasm and the absence of the KRAB A box does not affect nuclear localization. The different subcellular localizations suggest that the ZNF415 isoforms may have different cellular roles.

ZNF415 isoforms show different transcription activity

To examine the potential function of ZNF415, we examined the transcriptional activity of ZNF415. A series of full-length fusion proteins were made of each of the ZNF415 isoforms that included the DNA-binding domain (BD) of the yeast transcription factor GAL-4 under the control of a CMV promoter. We examined the transcriptional activity of ZNF415 isoforms by co-transfecting of the COS-7 cells with pCMV-BD-ZNF415 isoforms and the reporter, pL8G5-Luc. In this system, the five isoforms of ZNF415 are able to activate transcriptional activity of the reporter gene but the activities were different among the various isoforms (Fig. 3A and 3B). These suggest that alternative splicing may affect the transcriptional activation.

ZNF415 suppresses the transcriptional activities of AP-1 and p53

To investigate the role of ZNF415 in cell signal transduction, we performed pathway-specific reporter gene assays to measure the modulation of AP-1 by ZNF415

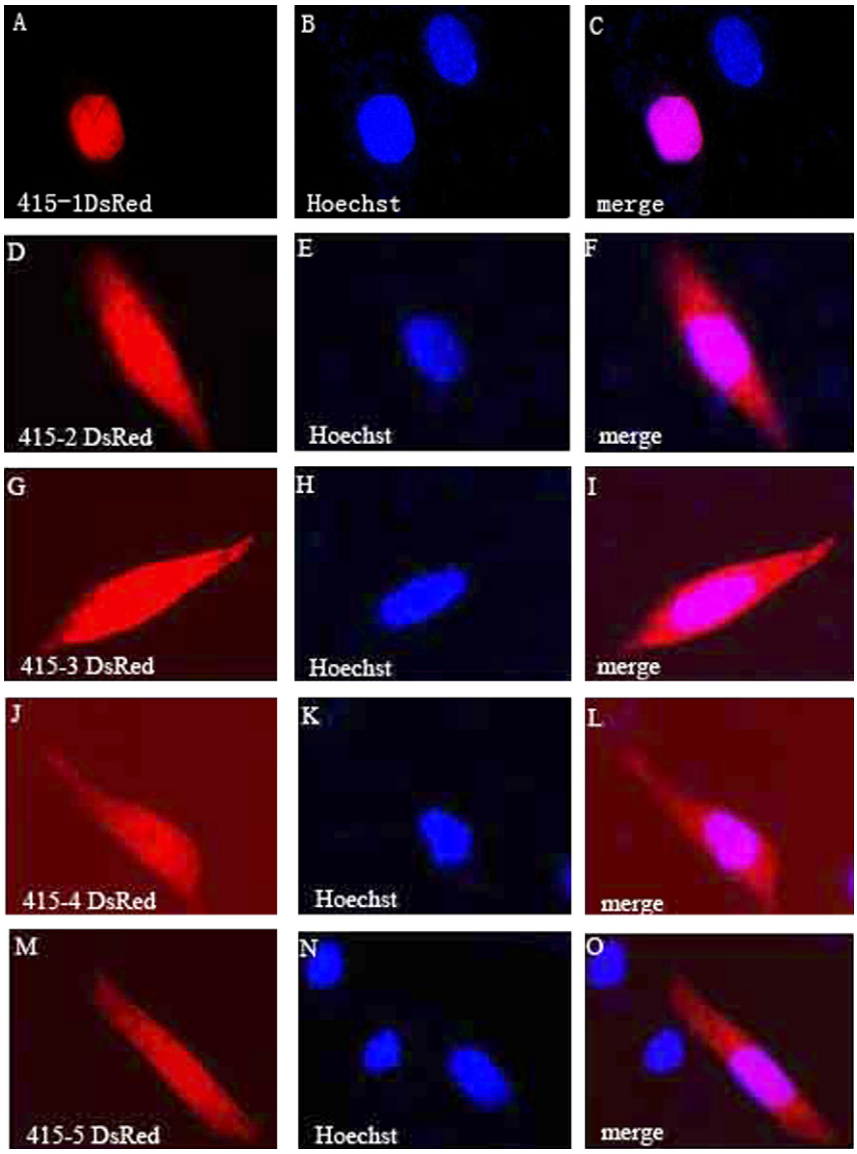

Fig. 2. Expression and cellular localization of five isoforms of ZNF415 in COS7 cells. ZNF415-1 located in nucleus and ZNF415-2, -3, -4, and -5 show a nuclear and cytoplasmic expression. Red represented DsRed fluorescence (A, D, G, J, and M) of ZNF415-1, -2, -3, -4, and -5, blue (B, E, H, K, and N) represent Hoechst-stained cell nuclei of ZNF415-1, -2, -3, -4, and -5, images (C, F, I, L, and O) present overlapping of red and blue fluorescence. All three panels of a row have the same field of view.

isoforms in COS7 cells. With the exception of ZNF415-1, all of the other isoforms suppressed AP-1-luciferase activity by approximately sixfold (Fig. 3C). We then tested the effect of ZNF415 on the activity of p53 and found that expression of ZNF415 can significantly inhibit p53 activity, again with the single exception of ZNF415-1 (Fig. 3D).

We have identified and characterized five isoforms of a novel human zinc finger protein. RT-PCR analysis indicates that the isoforms are expressed at different levels in human embryonic and adult tissues. From the present results we know that ZNF415-1 encodes 11 different C2H2 type zinc fingers, other isoforms contain the linker motif in addition to the 11 zinc fingers, and ZNF415-5 also contains a KRAB A box. The five isoforms show different transcriptional activity which may be caused by differential splicing. ZNF415-1 is different from the other isoforms in cellular localization and in their effects on signaling pathways. ZNF415-2 to ZNF415-5 show both nuclear and cytoplasm expression whereas ZNF415-1 is found only in nucleus. Finally, with the exception of ZNF415-1, overexpression of the ZNF415 isoforms inhibits the transcriptional activities of AP-1 and p53.

AP-1 and p53 are nuclear transcription factors, they are both regulated post-translationally by stress signaling pathways, they can induce apoptosis in response to genotoxic agents, and activate extrinsic and mitochondrial death pathways components. p53 can activate the c-Jun NH_2-terminal kinase (JNK) pathway to drive apoptosis [20] and AP-1 has been linked to modulation of p53 pathways to explain the role of AP-1 in cell survival [21]. The

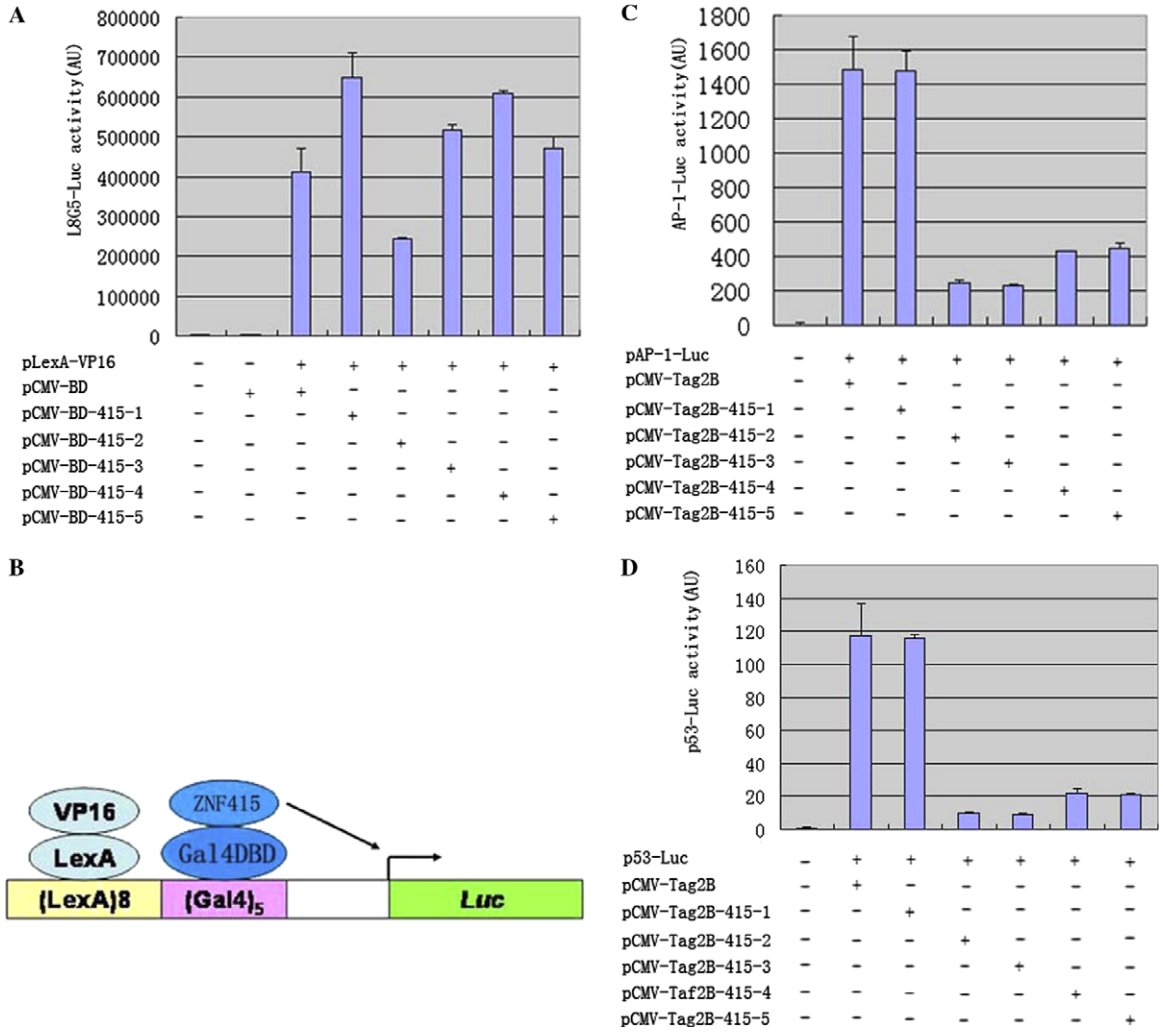

Fig. 3. (A,B) The isoforms show different transcriptional activities. Forty-eight hours after transient transfection, the luciferase activity assay was performed. The data are means of three repeats in a single transfection experiment after normalization for β-galactosidase activity. Each experiment was repeated at least three times. pCMV-BD-ZNF415 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. (C,D) Overexpression of ZNF415 four isoforms suppresses transcriptional activities of AP-1 and p53. (C) Inhibition of AP-1-Luc transcriptional activity by the overexpression of ZNF415 four isoforms; (D) inhibition of p53-Luc transcriptional activity by the overexpression of ZNF415 isoforms. COS-7 cells transfected with individual reporter plasmid and the corresponding plasmids are shown in the figures. Relative luciferase activity was normalized as described above.

interactions between p53 and AP-1 are complex but our results suggest that ZNF415 is involved in regulating both the AP-1 and p53 pathways and thus it may play a role in affecting cell fate and the linker region of ZNF415 may play a regulatory role in these effects. Taken together our results indicate that ZNF415 may be involved in cell cycle control and/or apoptosis and differential splicing may alter the effects of this gene.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.09.161.

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