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# ZNF424, a novel human KRAB/C2H2 zinc finger protein, suppresses NFAT and p21 pathway

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**Zinc finger-containing transcription factors are the largest single family of transcriptional regulators in mammals, which play an essential role in cell differentiation, cell proliferation, apoptosis, and neoplastic transformation. Here we have cloned a novel KRAB-related zinc finger gene, ZNF424, encoding a protein of 555aa. ZNF424 gene consisted of 4 exons and 3 introns, and mapped to chromosome 19p13.3. ZNF424 gene was ubiquitously expressed in human embryo tissues by Northern blot analysis. ZNF424 is conserved across species in evolution. Using a GFP-labeled ZNF424 protein, we demonstrate that ZNF424 localizes mostly in the nucleus. Transcriptional activity assays shows ZNF424 suppresses transcriptional activity of L8G5-luciferase. Overexpression of ZNF424 in HEK-293 cells inhibited the transcriptional activity of NFAT and p21, which may be silenced by siRNA. The results suggest that ZNF424 protein may act as a transcriptional repressor that suppresses NFAT and p21 pathway to mediate cellular functions. [BMB reports 2010; 43(3): 212-218]**

## INTRODUCTION

Zinc-finger proteins containing the Krüppel-associated box (KRAB-containing proteins) were discovered in 1991 (1). This group of proteins is the largest single family of transcriptional regulators in mammals. The C2H2 domain is the most common motif in various ZNF proteins. This motif frequently occurs in tandem repeats and is defined by two cysteine and two histidine residues coordinating a zinc ion (2). The KRAB domain is found at the amino terminus of nearly one third of all zinc-finger proteins (3), which is a transcriptional repression module, thus KRAB-associated ZNF proteins function as potent transcriptional repressors (4). The biochemical functions of KRAB-containing proteins are thought to be critical to their cellular roles, which include cell differentiation, cell proliferation, apoptosis, and neoplastic transformation (5).

Nuclear factor of activated T-cells (NFAT) was first identified as a transcription factor in activated T cells (6). NFAT plays a vital role in regulating the production of a large number of growth factors, cytokines and cell-cell interaction molecules essential for the morphogenesis, development and function of many cell types and organs. Activated NFAT can regulate transcription through binding its own cognate DNA binding site. The cyclin-dependent kinase inhibitor, p21 gene, is activated by NFAT by a different mechanism, with NFAT activating the p21 promoter by acting as a co-activator for the transcription factors Sp1 and Sp3. The p21 gene, mainly regulated at the transcriptional level, plays a crucial role in mediating growth arrest when cells are exposed to DNA-damaging agents (7). Over-expression of p21 results in G1-, G2-, or S-phase arrest upon exposure to DNA-damaging agents (8, 9). Whereas induction of p21 predominantly leads to cell cycle arrest, repression of p21 may have a variety of outcomes depending on the cellular context (10).

With the aim of identifying transcriptional factor genes involved in NFAT and p21 signaling pathway, we identified ZNF424, a novel KZNF family member, which encodes a zinc finger protein containing 13 tandem repeated C2H2 type zinc fingers and a KRAB box. When overexpressed in COS-7 cells, EGFP-ZNF424 fusion protein was detected mostly in the nucleus. The GAL4-BD-ZNF424 fusion protein shows transcriptional suppressor activity by luciferase assay, and this activity can be attributed to different domains. Reporter gene assays show that ZNF424 inhibits the transcriptional activities of NFAT and p21 in HEK-293 cell lines and inactivation of ZNF424 by siRNA abolished the inhibition. These results suggest that ZNF424 may mediate cellular activities by acting as a transcriptional repressor in the NFAT-p21 pathway.

## RESULTS AND DISCUSSION

### Identification and sequence analysis of ZNF424

The KRAB-type zinc-finger proteins constitute the largest single-family transcriptional regulators in mammals (11). To spe-

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cifically study the function of the zinc-finger proteins, we cloned a novel gene, named ZNF424 (EF534355) as approved by HUGO Nomenclature Committee. To confirm the cDNA sequences identified from the database, a pairs of primers (P1 and P2, Supplement Table 1) based on the sequences of four overlapping ESTs (CN314434, BU903009, BM459174 and BM473599) were used to carry out standard PCR using the human embryonic heart cDNA library as template according to previously described (12). The PCR product fragment was obtained and confirmed to be the cDNA sequences of ZNF424.

Alignment between the cDNA sequence and human genome indicates that ZNF424 is identical to the genomic sequence of AC119403 on chromosome 19p13.3, spanning approximately 17.57 kb and organized into four exons. A summary of the various sizes of the exons and introns and the sequence of the splice junctions is shown in Supplement Table 2. 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 ZNF424 gene consists of an open-reading frame (ORF) of 1668 bp expending from atg codon at nucleotide 149 a tga codon at 1,816 (Supplement Fig. 1A). The deduced human ZNF424 protein consists of 555 amino acids and has a predicted relative molecular weight of 64.4 kDa, and an estimated isoelectric point of 8.88. The predicted amino acid sequence of ZNF424 contains an N-terminal Krüppel-type-associated box (KRAB) domain (aa 4-71), and a series of 13 C2H2 zinc-finger motifs that extend to the end of the protein sequence (aa 140-162, 196-218, 224-246, 252-274, 280-302, 308-330, 336-358, 364-386, 392-414, 420-442, 448-470, 476-498 and 504-526) (Supplement Fig. 1A, B).

## ZNF424 is conserved during evolution

A search of published DNA databases for sequences similar to that of ZNF424 demonstrated that they had varying degrees of similarity to a number of previously identified KRAB/C2H2-type zinc-finger proteins. We then analyzed the evolutionary relationship between the ZNF424 protein and other zinc finger proteins with a phylogenetic tree analysis (Supplement Fig. 2A). Sequence alignment of these proteins demonstrates that ZNF424 is one of the more conserved proteins during evolution.

The alignment of amino acid sequences between the N-terminus (KRAB domain) of ZNF424 indicates that KRAB box of ZNF424 belongs to the classical KRAB-A box. The KRAB-A box of ZNF424 consists of about 40 amino acid residues and shows high homology to the KRAB-A box of other zinc-finger proteins (Supplement Fig. 2B), including two conserved motifs, DV (at position 5-7) and MLE (at position 33-35) which have been shown to be important for repression and interaction with TIF1β (13) (also named KAP-1, KRIP-1). TIF1βhas been found to interact with HP-1-like proteins such as M31, M32, hHp1α, and hHP1γ, thus changing the structure of the chromatin and inhibiting transcription (14).

In addition, these sequence analysis and database comparison indicate that the predicted protein contains 13 different C2H2 zinc-finger domains in tandem arrays, characteristic of transcription factor proteins of this family, as shown in Supplement Fig. 2C, each finger motif conforms closely to the consensus sequence $CX_2CX_3FX_5LX_2HX_3H$, where X represents any amino acid, strongly suggesting a role in DNA binding. These features of ZNF424 strongly suggest that it encodes a DNA-binding protein with transcriptional repressive properties.

## The ZNF424 mRNA is expressed in multiple tissues

To characterize the transcript of ZNF424 with respect to its size and expression distribution, fetal multiple tissue Northern blots were performed using ZNF424 cDNA as the probe. A 2.0-kb transcript of ZNF424 was detected in heart, skeletal muscles, liver, kidney, brain, gut and lung, while in gut and lung with a higher level (Fig. 1). The β-actin mRNA was used as a control in all tissues. The results indicate that ZNF424 is expressed in multiple human tissues.

## Subcellular localization of ZNF424 protein

Many KRAB/C2H2 type ZFPs have been demonstrated to localize in the nucleus and to act as transcription repressors (15). To determine whether ZNF424 is localized in the nucleus, we constructed plasmid pEGFP-N1-ZNF424. The pEGFP-N1-ZNF424 was transfected into COS-7cells, and 48h after the transfection, the cells were visualized with fluorescence microscope after labeled with DAPI for nucleus (Fig. 2B). ZNF424-EGFP protein is found in both the nucleus and the cytoplasm when overexpressed in COS-7 cells (Fig. 2A). The combined image shows that the majority of ZNF424-EGFP fluorescence localizes to the nucleus in COS-7 cells (Fig. 2C).

## ZNF424 functions as a potential transcriptional repressor

Although ZNF424 protein belongs to zinc-finger protein family, the potential role of ZNF424 is not clear. To examine the potential function of ZNF424, we examined transcriptional activity of ZNF424 by using a luciferase reporter gene (pL8G5-Luc) system. The luciferase reporter exhibited a basal level of

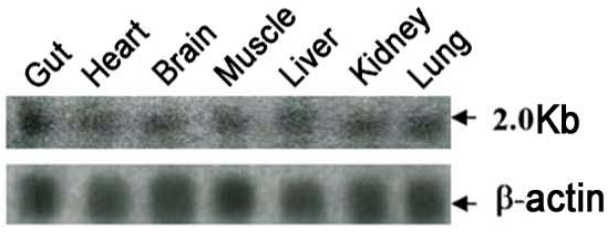


**Fig. 1.** Expression of *ZNF424* in human 17- week fetal tissues analyzed by Northern blot. The RNA filters were hybridized with a $\alpha$-$^{32}$P randomly labeled ZNF424 cDNA probe. Hybridization with β-actin was used to normalize loading amounts. A band at 2.0 kilobases (kb) was detected.

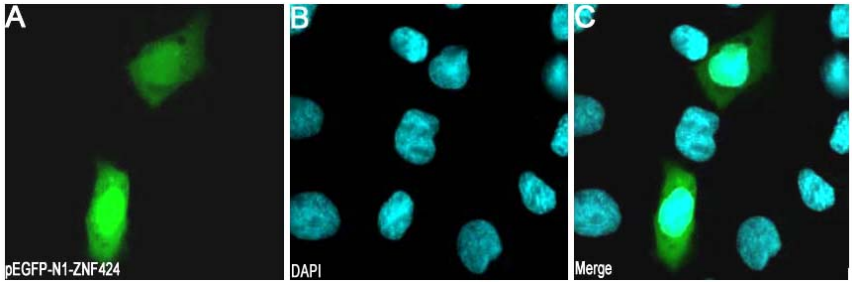


**Fig. 2.** Subcellular localization of ZNF424 protein in COS-7 cells. EGFP-ZNF424 is localized in both the nucleus and the cytoplasm and mostly in cell nucleus of COS-7 cells. (A) EGFP-ZNF424 protein is expressed in COS-7 cells. (B) The nucleus of the cells was stained with DAPI. (C) The combined image has (A) and (B) showing subcellular localization of ZNF424 when overexpressed in COS-7 cells.

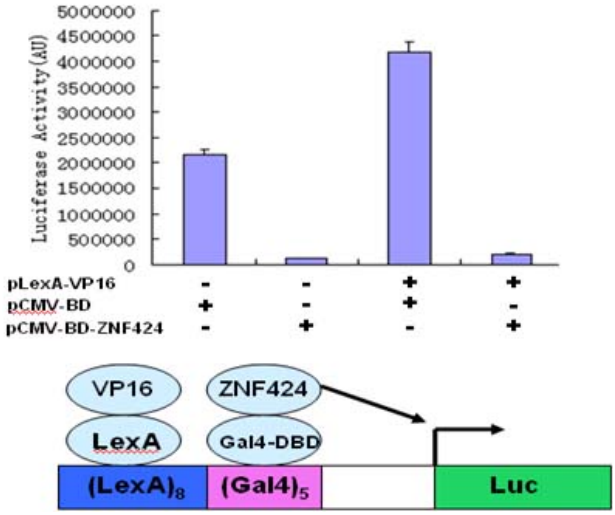


**Fig. 3.** ZNF424 is a transcription repressor. pCMV-BD-ZNF424 or pCMV-BD is transiently co-transfected into HEK-293 cells along with the pL8G5-Luc reporter and pLexA-VP16 as indicated in the figure. Forty eight hours later, the luciferase activity assay was performed. The data are means of three repeats in a single experiment after normalization for β-galactosidase activity. Each experiment was repeated at least three times.

transcriptional activity when transfected with the control plasmid pCMV-BD encoding the GAL4-DBD alone. LexA-VP16, which can directly activate the expression of the luciferase reporter gene, was used as a positive control (16). When co-transfecting with pL8G5-luc plasmid, the GAL4-ZNF424 fusion protein inhibited luciferase activity by approximately 30% (Fig. 3). While co-transfecting with pLexA-VP16, the GAL4-ZNF424 fusion protein inhibited the VP16 activated luciferase activity by approximately 95% (Fig. 3). This result suggests that ZNF424 functions as a negative regulator of transcription.

### ZNF424 suppresses NFAT and p21- mediated transcriptional activation

Zinc finger proteins play important roles in a variety of cellular functions including cell proliferation, cell apoptosis, and cell signal transduction pathways. We performed pathway-specific reporter gene assays to measure the effect on different pathways (such as SRF, NFκB, NFAT and p21) by ZNF424 in the HEK-293 cells. We found ZNF424 suppressed NFAT and p21 pathway more obviously than others. As shown in Fig. 4A, full-length ZNF424 reduced NFAT transcriptional activity by ~62%. Furthermore, using p21-Luc, expression of ZNF424 inhibited the transcriptional activity of p21 by ~56% (Fig. 4B). The results show that the NFAT and p21 luciferase activity increased when co-transfected with RNAi plasmid, suggesting siRNA targeting ZNF424 abolished the transcriptional suppression of ZNF424 (Fig. 4A, B). Taken together, our results suggest that ZNF424 regulates components involved in the NFAT and p21 signaling pathway in cells.

To identify the key regulatory motifs in ZNF424 protein, we generated three truncated ZNF424 protein constructs, pCMV-tag2B-KRAB (amino acids 1-74 with KRAB motif), pCMV-tag2B-LINK (amino acids 68-148 with LINK motif) and pCMV-tag2B-ZNF(amino acids 135-539 with 13 zinc fingers motif), as shown in Fig. 4C. In comparison to pCMV-tag2B control, reduced reporter activation was observed in both pCMV-tag2B-KRAB and pCMV-tag2B-ZNF fusion protein constructs (Fig. 4C). When co-transfected with three truncated ZNF424 protein constructs and pNFAT-luc plasmid, respectively, the ZNF motif of ZNF424 inhibited the luciferase activity by ~43%, and the KRAB motif of ZNF424 inhibited the luciferase activity by ~41% (Fig. 4C). Furthermore, using p21-luc, the ZNF motif of ZNF424 inhibited the luciferase activity by ~44% (Fig. 4D). The results above suggest the ZNF motif of ZNF424 represses NFAT and p21 pathway.

In addition, overexpressing full-length and truncated ZNF424 fusion protein also represses both NFκB and SRE pathway, but the effect was much weaker than that on NFAT and p21 pathway (Supplement Fig. 3A, B).

Members of the KRAB-containing protein family bind DNA through their C2H2 zinc-finger domains (17), and the KRAB domain functions as a strong transcriptional repressor domain. Previous studies show that both the KRAB and ZNF motifs represent potent repression domains such as Apak, a KZNF (KRAB-type zinc-finger) family member, whose KRAB domain and zinc fingers are required for inhibition of p53 activity (18). While other studies shown that the KRAB motif represents basal repressive domains in transcriptional regulation, such as

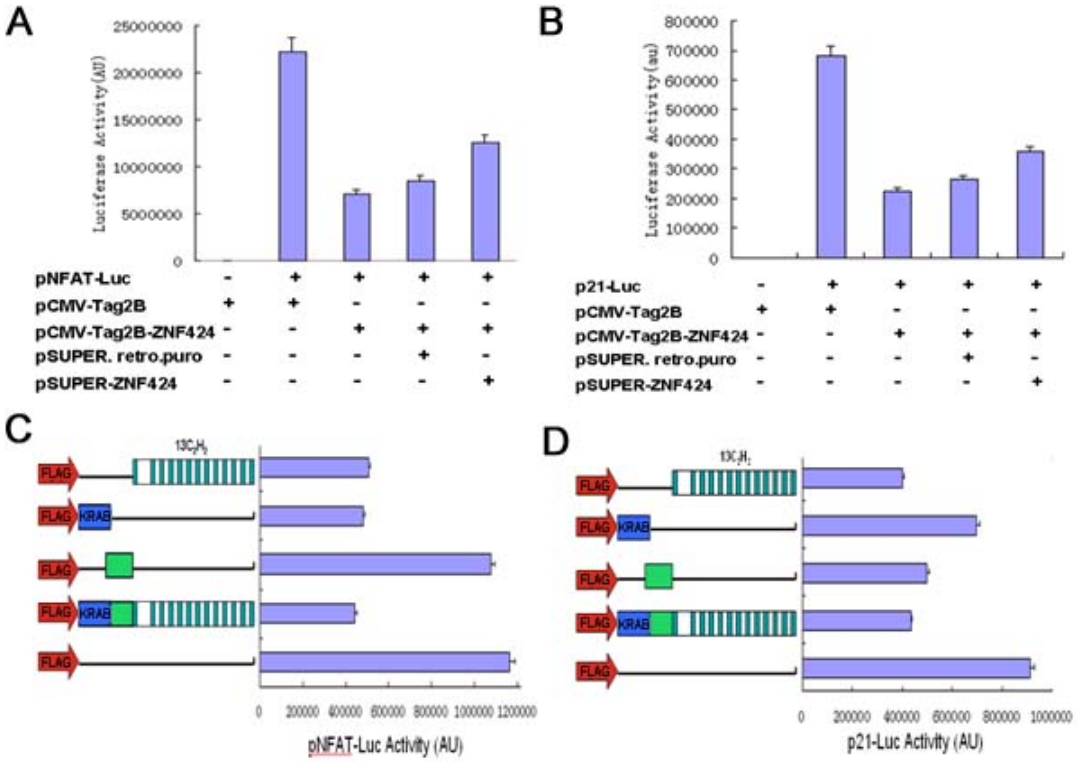


**Fig. 4.** Overexpression of ZNF424 suppresses transcriptional activities of NFAT and p21. (A) ZNF424 suppresses NFAT- mediated transcriptional activation in HEK- 293. ZNF424 suppresses NFAT transcriptional activity, which was abated by siRNA knockdown of ZNF424 in ZNF424 overexpression cells. (B) ZNF424 suppresses p21-mediated transcriptional activity in HEK-293. ZNF424 suppresses p21 transcriptional activity, which was abated by siRNA knockdown of ZNF424 in ZNF424 overexpression cells. (C, D), The transcriptional activity analysis of full-length and truncated ZNF424 fusion proteins (pCMV-tag2B-ZNF424, pCMV- tag2B-KRAB, pCMV-tag2B-LINK, pCMV-tag2B-ZNF) on both NFAT pathway (C) and p21 pathway (D) in HEK-293 cells. Overexpressing full-length and truncated ZNF424 fusion protein (pCMV-tag2B- ZNF424, pCMV-tag2B-KRAB, pCMV-tag2B-LINK, pCMV-tag2B-ZNF) in HEK-293 represses p21-Luciferase. HEK-293 cells were transfected with reporter plasmid and the corresponding plasmids were shown in the figures. Forty-eight hours later, the luciferase activity assay was performed. The data are means of three repeats in a single experiment after normalization for b-galactosidase activity.

ZNF333 (19). Our studies with truncated ZNF424 protein agree that the ZNF motifs of ZNF424 represent basal repressive domains in transcriptional regulation.

The C2H2 zinc finger motifs found in the KRAB-containing proteins and other zinc-finger proteins are defined by the presence of the consensus sequence ϕ-X-Cys-$X_{(2-4)}$-Cys-$X_3$-ϕ-$X_5$-ϕ$X_2$-His-$X_{(3,4)}$-His, where X represents any amino acid and ϕ represents a hydrophobic residue. The two cysteine and two histidine residues coordinate a zinc ion and fold the domain into a finger-like projection that can interact with DNA. Previous studies strongly suggest that each of these motifs can contact three to four nucleotides (20). However, it had not been investigated fully whether these zinc fingers bind DNA in a sequence-specific manner or function in transcriptional regulation outside of an artificial GAL4-based transcriptional assay. Until recently, some studies suggested that wild-type KRAB-containing proteins are indeed transcriptional repressors that use most of their collection of zinc fingers to bind to DNA (4). Our laboratory has provided evidence that a novel zinc finger protein, ZNF424, may regulate the NFAT-p21 signaling pathway, and the zinc finger motifs may represent basal repressive domains.

## CONCLUSIONS

In summary, we have cloned a novel human KRAB-containing krüppel-like zinc finger gene, ZNF424. The deduced protein is composed of 555 amino acids with a highly conserved KRAB-A domain at its amino terminus and 13 tandem repeated zinc fingers at its carboxyl terminus. Sequence analysis reveals that ZNF424 displays structural homology with other previously described KRAB/C2H2 zinc finger proteins. ZNF424 is expressed in multiple human tissues. ZNF424 protein is localized primarily in the nucleus and acts as a transcriptional repressor. Overexpression of ZNF424 inhibits the transcriptional activities of NFAT and p21. Studies with truncated ZNF424 protein show

that the ZNF motifs of ZNF424 represent basal repressive domains in transcriptional regulation. Our studies suggested that ZNF424 may function as a negative transcriptional regulator in NFAT-p21 signaling pathways.

## MATERIALS AND METHODS

### Construction of cDNA library of human embryonic heart

The 20-week human embryonic heart cDNA library was constructed as reported previously (21). Briefly, 5 μg mRNA was purified from 500 μg total human embryonic heart 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 (TaKaRa). After Cassette Adaptor Ligation reactions using cDNA PCR Library Kit, cDNA amplification reactions were performed with RA primer, CA primer and Ex Taq (TaKaRa).

### Cloning and bioinformatics analysis

The consensus sequence of KRAB region was used to search human EST database with BLAST program (http://www.ncbi.nlm.nih.gov/BLAST) as previously described (22). A pairs of gene-specific primers were designed using Primer Premier 5.0 for PCR based on the sequences of a contig from ESTs (primers P1 and P2, Supplement Table 1). Jellysh1.4 was used to find the open-reading frame (ORF). Then, the coding sequence was cloned from human heart library with a pair of primers (PORF1 and PORF2, Supplement Table1). All the PCR products were then cloned into pMD18T-vector (Sagon) and sequencing with 377 DNA Sequencer (ABIPRISM).

Primary sequence analysis for ZNF424 was performed in BLAST (www.ncbi.nlm.nih.gov/BLAST/). Blastn program was used to identify the cytological locus of gene and to look for exons and introns. Secondary protein structure predictions were performed using the web tools SMART (http://smart.embl-heidelberg.de/).

### RNA isolation and northern blot hybridization

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 according to the university policies. A multiple embryonic tissues membrane was prepared as described in previous studies (23). The 80 days embryo multiple tissue membrane were hybridized with cDNA probe of ZNF 424 and β-actin cDNA probe (Clontech). The method of Northern blot was described in previous studies (24).

**Phylogenetic tree analysis:** Phylogenetic tree analysis of amino acid sequences deduced from ZNF424 cDNA sequences was performed using the Sequence Manipulation Suit software. The GenBank accession numbers for previously known members of C2H2-type zinc finger proteins used in this analysis are ZNF490(NP_065765), ZNF708(NP_067092), ZNF555(NP_690004), ZNF699(NP_940937), ZNF77(NP_067040), ZNF564(NP_659413), ZNF136(NP_003428), ZNF709(NP_689814), ZNF14(NP_066358), ZNF433(NP_001073880), ZNF791(NP_699189) and ZNF424. All of these genes originate from Homo sapiens.

**Plasmid construction:** The following plasmids were constructed and used for mammalian cell transfections. All inserted fragments were obtained by PCR amplification using the primers listed in Table 1.

The ZNF424 ORF was subcloned into the *Sal*I and *Kpn*I sites of pEGFP-N1 vector in-frame with the TGG codon instead of the TGA stop codon. The full-length ZNF424 was subcloned into the eukaryotic expression vector pCMV-tag2B at *Sal*I sites using primers P3 and P4 (Supplement Table 1).

To identify the key regulatory motifs in ZNF424 protein, we generated three truncated ZNF424 protein constructs, pCMV-tag2B-KRAB (amino acids 1-74 with KRAB motif), pCMV-tag2B-LINK (amino acids 68-148 with LINK motif) and pCMV-tag2B-ZNF (amino acids 135-539 with 13 zinc fingers motif) using three pairs of primers (P6/P7, P8/P9, P10/P11, Supplement Table 1). After confirmation by sequencing, all eukaryotic expression plasmids were extracted and purified for transfection using Plasmid Maxiprep Kit (OMEGA).

### Cell culture and subcellular localization analysis

COS-7 cells and HEK-293 cells used in all studies were maintained and passaged according to standard methods described previously (25). Cells were and transfected with pEGFP-N1-ZNF424 using lipofectamine (Invitrogen) according to the company's protocol. Subcellular localization of the pEGFP-N1-ZNF424 fusion protein was detected 48 h following transfection by fluorescence microscopy (Nikon, E400).

### Transcriptional reporter gene assays

HEK-293 cells were co-transfected with L8G5-Luciferase and pCMV-BD-ZNF424 to investigate the effect of ZNF424 on transcriptional activity. To investigate the effect of ZNF424 on the transcriptional activity of p21 and NFAT, cells were co-transfected with p21-Luc (or pNFAT-Luc) and pCMV-Tag2B-ZNF 424 or other truncated FLAG-ZNF424 fusion constructs, respectively. The luciferase activity assay was performed 48 h later according to the methods described previously (26).

**RNAi analysis:** A pSUPER.retro.puro vector-based system was used to deliver siRNA into HEK-293 cells. A pair of oligo nucleotides was designed by the RNAi program (http://www.openbiosystems. com/RNAi). The sequences were S1: 5'-GATCCCCGGACAGATTCTGTACACATATTCTAGAGATATGTGTACAGAATCTGTCCTTTT-3', S2:5'-GATCCCCGCGTTTAATGGGTTCGCAAGTTCTAGAGACTTGCGAACCCATTAAACGCTTTT -3'.

The oligos were annealed and cloned according to the instructions (OligoEngine). The luciferase activity assay for pNFAT-Luc (p21-Luc), pCMV-Tag2B-ZNF424, and pSUPER-ZNF424, or pSUPER.retro.puro vector was performed according to the protocols described above.

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