Cell. Mol. Life Sci. (2013) 70:3947–3958
DOI 10.1007/s00018-013-1359-4

Cellular and Molecular Life Sciences

RESEARCH ARTICLE

Novel activity of KRAB domain that functions to reinforce nuclear localization of KRAB-containing zinc finger proteins by interacting with KAP1

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Received: 20 November 2012 / Revised: 22 April 2013 / Accepted: 29 April 2013 / Published online: 12 May 2013

Abstract Previously, we found that two isoforms of the *ZNF268* gene (ZNF268a and ZNF268b2, with and without the KRAB domain, respectively) might play distinct roles in normal epithelia and in cervical cancer. Here we further investigated that KRAB domain defined the function disparity in part by reinforcing nuclear localization of ZNF268a. We found that the A-box of KRAB alone retained major specific nuclear localization activity. In contrast, the B-box alone did not have nuclear localization activity but enhanced it significantly. Consistent with the critical function of the A-box, each mutation of six conserved residues (V9, V11, F13, E16, E17 and W18) in the A-box dramatically impaired nuclear localization activity. Furthermore, the unique nuclear localization activity of KRAB was verified in seven additional KRAB-containing zinc finger proteins (KRAB-ZFPs), suggesting that it is a universal feature of KRAB-ZFPs. Finally, KRAB exerted its unique nuclear localization activity by interacting with the RBCC domain of its corepressor KAP1. Our results have revealed a novel mechanism by which the KRAB domain reinforces nuclear localization of KRAB-ZFPs by interacting with KAP1. Our study also suggests that loss of the KRAB domain in KRAB-ZFPs due to aberrant alternative splicing might contribute to carcinogenesis.

Keywords KRAB-ZFP · Nuclear localization · KAP1

Introduction

Zinc finger proteins containing the *Krüppel*-associated box (KRAB-ZFP) are the largest single family of transcriptional regulators in mammals [1]. They bind target DNA sequences through the zinc finger domain. The KRAB domain further recruits corepressor KAP1 and other chromatin-remodeling proteins such as Mi2α, SETDB1, and HP1 to create a heterochromatic microenvironment, which ultimately leads to transcription repression. Nuclear localization signals (NLS) have been mapped to the zinc finger domain in many zinc finger proteins [2–4], consistent with the finding that the NLS and DNA binding domains in nucleic acid-binding proteins often overlap [5].

The KRAB domain at the amino terminus of KRAB-ZFPs is known to be a transcription repressor domain. It typically contains an A-box and a B-box, of which the A-box is highly conserved and plays a key role in repression while the B-box is less conserved and plays an auxiliary role [6]. Based on the conservation of KRAB, KRAB-ZFP proteins can be further classified into three subfamilies: KRAB (A) containing only the classical

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Electronic supplementary material The online version of this article (doi:10.1007/s00018-013-1359-4) contains supplementary material, which is available to authorized users.

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A-box, KRAB (AB) with both a classical A-box and a B-box, and KRAB (Ab) with a classical A-box and a highly divergent B-box [7]. Another obvious feature of KRAB is that it is found only in tetrapod vertebrate genomes [8], suggesting that KRAB-ZFP proteins evolved in the development of higher organisms [9]. Mechanistically, the KRAB domain has been shown to function as a transcriptional repressor by interacting with its corepressor KAP1 [10, 11].

We have recently shown that two isoforms of human ZNF268 likely play distinct roles in normal epithelia and in cancers. The ZNF268a contains the KRAB domain as well as 24 zinc fingers and is expressed at high levels in normal epithelia. On the other hand, ZNF268b2 has the 24 zinc fingers but lacks KRAB and its overexpression contributes to cervical cancer by enhancing NF-κB signaling [12]. To investigate the mechanism underlying this functional divergence, we characterized the molecular properties of these two isoforms. Interestingly, we observed that the presence of the KRAB domain in ZNF268a led to the exclusive nuclear localization of ZNF268a, in contrast to a diffused distribution pattern in both the cytoplasm and the nucleus of ZNF268b2, suggesting that KRAB may function to reinforce the nuclear localization of ZNF268a in addition to its transcription repressor activity. Our further analysis revealed that KRAB indeed could function to mediate nuclear localization with the activity in the A-box, and the KRAB domain functions through interaction with KAP1. Our study thus not only identified a novel nuclear localization pathway for KRAB-ZFP proteins, but also provided a potential mechanism by which the loss of KRAB in KRAB-ZFP due to dysregulation of alternative splicing may contribute to carcinogenesis.

Materials and methods

Plasmid constructs

pEGFP-N1 (Clontech) was mutated at both the Kozak sequence and initial ATG codon to produce pEGFP-M1 for the study of fusion protein expression and subcellular localization [13]. ZNF268a and ZNF268b2 coding regions were amplified by PCR from pCMV-ZNF268a, digested with *Bgl*II and *Sal*I and cloned into the corresponding sites of pEGFP-M1. Various KRAB domains and the A-box and B-box were cloned into the pEGFP-M1 vector via PCR. DNA sequences for SV40 NLS (CGGGPKKKRKVED) and KRAB–S (DVFVDFTWEEW) were synthesized, annealed, and then ligated into EGFP-M1 to generate the SV40 NLS-GFP and KRAB-S-GFP constructs, respectively. ZNF268 KRAB, KOX1 KRAB and ZNF300 KRAB were also cloned into pGEX-6p-1 (GE Healthcare) to express GST fusion proteins. FLAG-tagged KAP1 expression plasmid was a kind gift from Dr. Chuangui Wang [14] and the RBCC and 420–835 amino acid region of KAP1 were subcloned into pCMV-8tag-8 (Stratagene). Mutation of specific amino acids of KRAB was performed by PCR-directed mutagenesis.

Cell culture and transfection

Mouse macrophage RAW264.7 cells, rat glioma C6 cells, HeLa cells, monkey kidney fibroblast COS-7 cells, human embryonic kidney 293T cells, and human colorectal carcinoma HCT116 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum and 1 % (w/v) each of penicillin and streptomycin in a humidified atmosphere at 37 °C containing 5 % (v/v) CO_2. The day before transfection, cells were seeded on tissue culture plates or coverslips. Transfections were performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Confocal analyses

Cultured cells were fixed with 4 % paraformaldehyde for 20 min at room temperature, and permeabilized with 0.5 % Triton X-100 for 20 min at room temperature. Subsequently, the slides were incubated with primary antibody anti-FLAG M2 (Sigma-Aldrich) or anti-KAP1 (Cell Signaling Technology) in a solution of 1 % BSA and 0.05 % Triton X-100 overnight, followed by incubation with secondary antibody (tetramethyl rhodamine isothiocyanate-conjugated; Pierce) for 1 h at 37 °C. The slides were washed and the nuclei stained with DAPI. For cells expressing GFP proteins, sample treatment was performed as described above without antibody incubation. Fluorescent images were obtained using FV1000 configuration with a BX61 microscope (Olympus). Nuclear localization activity was measured by counting GFP-positive cells with only nuclear GFP. The percentages of cells with only nuclear GFP were calculated and are presented as means ± SD. For each experiment, no fewer than 200 GFP-positive cells were counted. All confocal analyses were performed three times with similar results. Representative photographs or histograms from three independent experiments are shown. Student's *t* test (two-tailed, unpaired) was used for statistical analysis of three independent experiments ($*p < 0.05$, $**p < 0.01$).

Cytoplasmic and nuclear protein extraction

Cytoplasmic and nuclear extracts were prepared with reagents according to the manufacturer's instructions (Boster, China). HeLa cells were collected and incubated in five

volumes of cytoplasmic extraction reagent for 30 min on ice, followed by centrifugation at 12,000 rpm at 4 °C. Cytoplasmic proteins were obtained from the supernatant. The pellets were incubated with two volumes of nuclear extraction reagent for 30 min, with vigorous rotation every 5 min, and then centrifuged at 12,000 rpm at 4 °C. Nuclear proteins were obtained from the resulting supernatant.

Western blotting

Cells were collected and lysed in RIPA buffer on ice for 15 min, followed by centrifugation at 12,000 rpm at 4 °C for 10 min. Supernatants were transferred to another new tube, boiled with an equal volume of SDS-PAGE 2× loading buffer, and then western blotting was performed according to standard procedures. p65 and PARP antibodies were purchased from Cell Signaling Technology. The SD antibody detects total ZNF268 proteins ZNF268a and ZNF268b2, the two predominant products of the ZNF268 gene, as previously described [15].

GST pull-down

GST and GST-KRAB fusion proteins were prepared as described previously [16]. Precleared HeLa cell lysates were incubated with 20 μg GST proteins and 50 μl of a 50 % slurry of glutathione-Sepharose 4B beads (GE Healthcare) overnight at 4 °C with gentle rotation. Beads were washed three times with GST lysis buffer (20 mM Tris-Cl, pH 8.0, 200 mM NaCl, 1 mM EDTA, pH 8.0, 0.5 % Nonidet P-40, 2 μg/μl aprotinin, 1 μg/μl leupeptin, 0.7 μg/ml pepstatin and 25 μg/ml phenylmethylsulfonyl fluoride). Then the KAP1 protein pulled down by the GST fusion proteins was eluted with SDS-PAGE 2× sample buffer and analyzed by western blotting. The GST fusion proteins were detected by coomassie blue staining.

Molecular modeling

The structures of ZNF268 KRAB were modeled using the KRAB domain of RIKEN (PDB code 1V65) as the template. DeepView/Swiss-PdbViewer (version 4.0.4) software was used to generate the three-dimensional model and analyze the tertiary structure.

Results

KRAB domain bears nuclear localization activity

The human ZNF268 gene encodes two proteins. The full-length ZNF268a contains the KRAB domain and 24 zinc fingers while ZNF268b2 has 24 zinc fingers but lacks KRAB. Previously, we observed that ZNF268b2 but not ZNF268a interacted with IKKs to activate NF-κB signaling and contributed to tumorigenesis of cervical cancer [12]. To investigate the underlying mechanism, we examined the subcellular localization of ZNF268 gene products. We fractionated cytosolic and nuclear extracts from HeLa cells and detected ZNF268a and ZNF268b2 by western blotting. As expected, p65 was found to be present only in the cytosolic fraction, whereas nuclear protein PARP was found only in the nuclear fraction (Fig. 1a). Interestingly, ZNF268a (with KRAB) was only detected in the nuclear fraction, while ZNF268b2 (without KRAB) was distributed in both the nuclear and the cytosolic fractions (Fig. 1a). To visualize the subcellular localization of the proteins of interest, we used the GFP fusion protein technique. We fused ZNF268a and ZNF268b2 to the N-terminus of GFP (ZNF268a-GFP and ZNF268b2-GFP). Consistently, ZNF268a-GFP was only localized in the nucleus, whereas ZNF268b2–GFP exhibited both a nuclear and a cytoplasmic distribution. The control GFP protein alone exhibited a diffused distribution pattern and was detected in both the nucleus and the cytoplasm (Fig. 1c, d). These observations suggest that the KRAB domain may show additional nuclear localization activity.

To further test if KRAB can mediate nuclear localization, we next fused the KRAB domain of ZNF268a or KRAB A-box or B-box to GFP protein and confirmed their expression (Fig. 1b, Supplementary Fig. 1a). As expected, about 90 % of cells expressing the KRAB-GFP fusion protein (KRAB) exhibited exclusive nuclear localization of the fusion protein (Fig. 1c, d) and this nuclear localization activity mainly resided in the A-box since over 40 % of the A-box GFP-expressing cells (A-box) had exclusive nuclear localization while no B-box GFP protein (B-box) exhibited exclusive nuclear localization (Fig. 1c, d). The nuclear localization activity of ZNF268 KRAB was also confirmed in other cell types such as RAW264.7 cells, C6 cells, COS-7 cells, 293T cells, and HCT 116 cells (Supplementary Fig. 2). These observations suggest that KRAB may function to mediate nuclear localization.

To exclude the possibility that the diffused distribution pattern of ZNFb2 may be due to exposure of a nuclear export sequence (NES) but not loss of KRAB, we inhibited nuclear export activity by using the nuclear export inhibitor leptomycin B (LMB) and checked the distribution pattern of ZNF268b2-GFP. We used influenza A virus nuclear export protein (NEP) as a control. NEP-GFP fusion protein localized in cytoplasm due to its strong NES as previously reported [17]. LMB treatment significantly increased nuclear localization of NEP-GFP fusion protein (Fig. 1e). However, we did not observe a significant change in ZNF268b2-GFP distribution (Fig. 1e). This finding

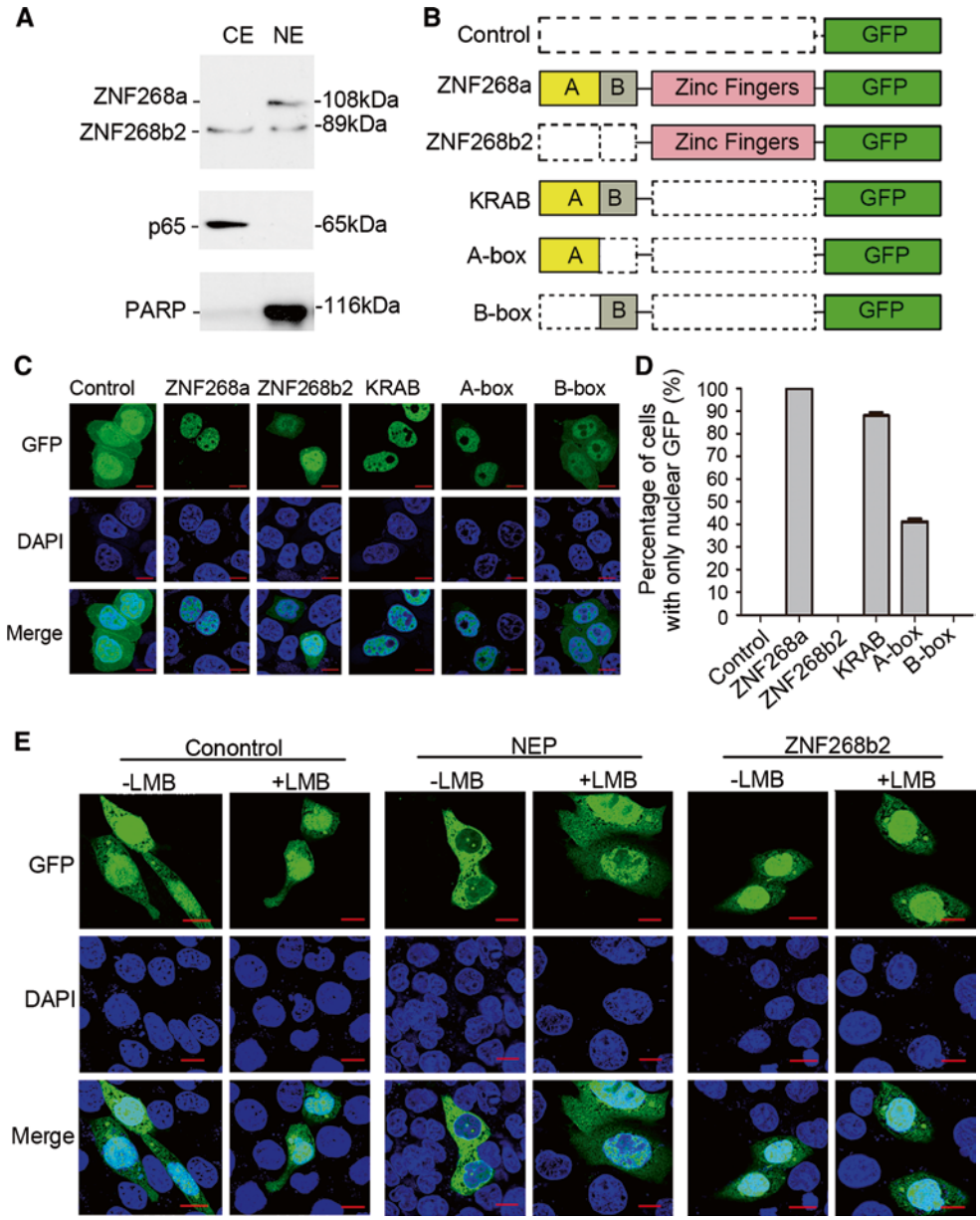

Fig. 1 The KRAB domain of ZNF268 possesses nuclear localization activity. **a** Cytoplasmic (*CE*) and nuclear (*NE*) extracts derived from HeLa cells were isolated and subjected to western blot analysis. p65 and PARP were used as internal quality controls for the fractionation of cytoplasmic and nuclear proteins, respectively. **b** Schematic illustration of five GFP fusion proteins with ZNF268a, ZNF268b2, KRAB, A-box, B-box, respectively. GFP alone served as control. **c** HeLa cells transfected with GFP alone (*Control*) or five GFP fusion protein expression constructs as indicated were subjected to confocal analysis to determine the localization of the fusion proteins (GFP) and stained with DAPI to locate the nucleus. Photographs from two fluorescent fields were also merged to show any colocalization (*Merge*). **d** Cells with only nuclear GFP fusion protein in **c** were counted under a fluorescent microscope. The data presented are the percentage of cells with only nuclear GFP in GFP-positive cells (means ± SD from three independent experiments). **e** Cells transfected with GFP alone (*Control*) or GFP fusion proteins (*NEP* and *ZNF268b2*) expression constructs were treated with (+) or without (−) nuclear export inhibitor leptomycin B (*LMB*) and subjected to confocal analysis to determine the localization of the fusion proteins (*GFP*) and stained with DAPI to locate the nucleus. Photographs from two fluorescent fields were also merged to show any colocalization (*Merge*). *Scale bars* 10 μm

supports the view that cytoplasm localization of ZNF268b2 is due to loss of KRAB.

Taken together, our findings suggest that ZNF268 KRAB shows novel nuclear localization activity that may reinforce nuclear localization of ZNF268. The A-box plays a major role in nuclear localization activity, which is significantly enhanced by the presence of the B-box, although the latter alone had no nuclear localization activity.

A number of conserved amino acids are critical for the nuclear localization activity of KRAB

To determine the mechanism of KRAB-mediated nuclear localization, we first identified conserved amino acids in KRAB-ZFPs by aligning eight amino acid sequences of KRAB domains. The A-box exhibited a high degree of conservation while the B-box was less conserved (Fig. 2a). We then carried out alanine-scanning mutagenesis to identify the specific residue critical for the nuclear localization activity of KRAB. Various mutant forms of KRAB were fused to GFP and their expression was confirmed (Fig. 2b, Supplementary Fig. 1b). Most substitutions had little effect on the nuclear localization activity of the KRAB domain (Fig. 2b, Supplementary Fig. 3). Notably, mutations of six conserved amino acid positions (V9A, V11A, F13A, E16A, E17A and W18A) in the A-box were found to significantly decrease the activity (Fig. 2b). Mutations in the other conserved sites from L21 to N36 had little effect on the nuclear localization activity. In the B-box, all single mutations tested failed to affect its activity, consistent with the auxiliary role of the B-box in nuclear localization activity and the less conserved nature of the B-box. Similarly, double or triple mutants (D8A-V9A or E16/17-W18A) in the A-box

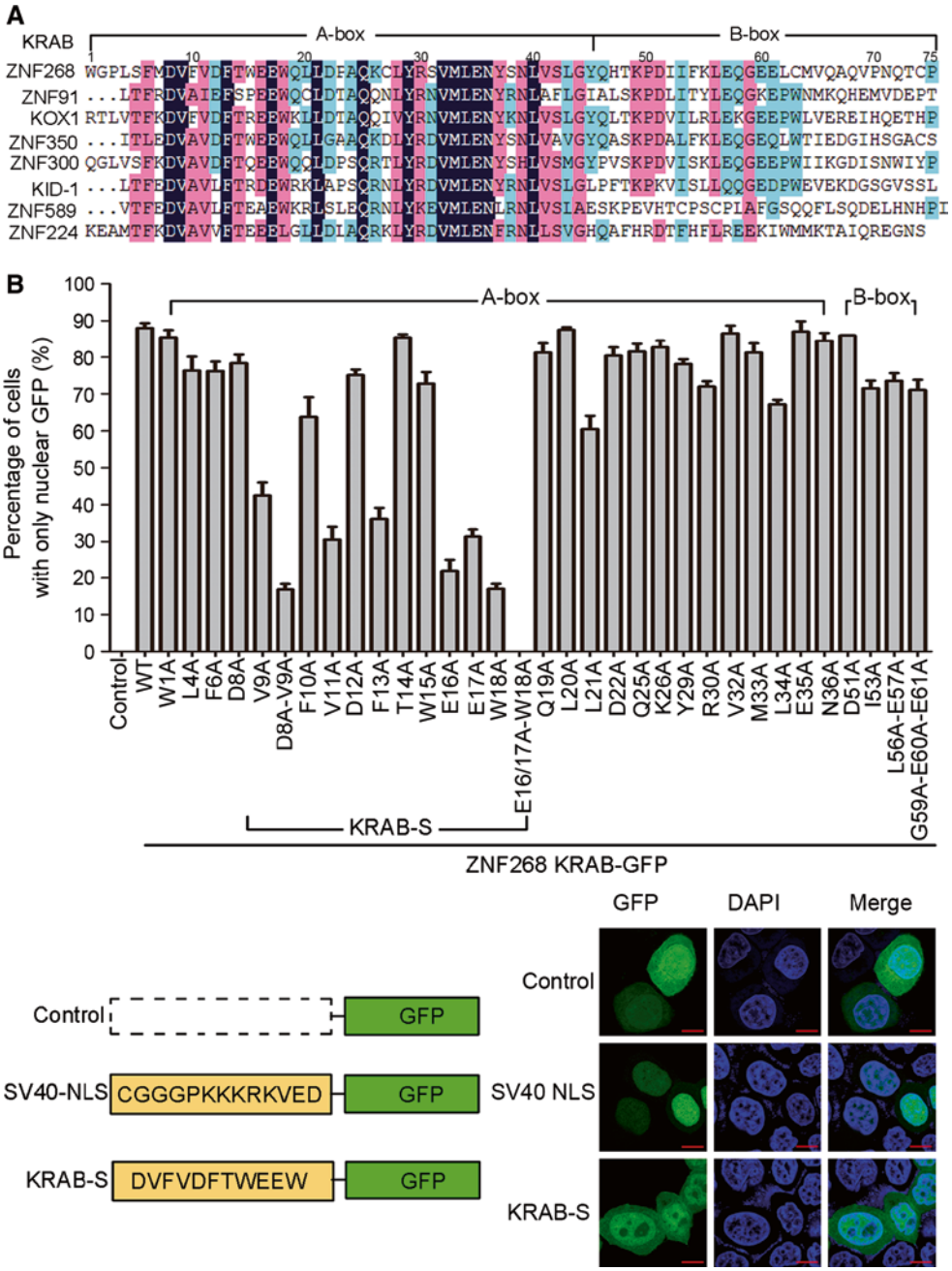

Fig. 2 A number of conserved residues in the A-box are critical for the nuclear localization activity of ZNF268 KRAB. **a** Alignment of the amino acid sequences of KRAB domains from eight human ZNFs revealed conserved residues. The *black boxes*, *red boxes* and *blue boxes* indicate a 100 %, more than 75 % and more than 50 % homology levels, respectively. The highly conserved residues are located in the *A-box* and not the *B-box*. **b** Mutations of the conserved residues in the A-box reduce nuclear localization activity of the KRAB. WT or mutant KRAB from ZNF268 as indicated was fused to GFP. Plasmids expressing GFP alone (*Control*) or various ZNF268 KRAB-GFP fusion proteins were transfected into HeLa cells for confocal analysis. The percentages of cells with exclusively nuclear GFP were calculated and the results are presented as means ± SD from three independent experiments. **c** A short peptide covering the conserved residues alone has no nuclear localization activity. *Left* schematic illustration of three GFP constructs expressing GFP alone, SV40-NLS-GFP, or KRAB-S-GFP (with KRAB-S being the peptide from D8 to W18 in KRAB). *Right* representative images showing the nuclear localization of GFP alone or SV40-NLS-GFP, or KRAB-S-GFP. *Scale bars* 10 μm

drastically impaired the activity, while double or triple mutants in the B-box (L56A-E57A or G59A-E60A-E61A) still had comparable nuclear localization activity to that of wild-type (WT) KRAB. Thus, some of the conserved amino acids in the A-box but none in the B-box are critical for the nuclear localization activity of KRAB, consistent with the findings from the A-box and B-box deletion studies.

The classical NLS contains a linear stretch of a basic amino acid sequence. We next tested whether the amino acid sequence spanning the key six conserved sites (V9A, V11A, F13A, E16A, E17A and W18A; designated as KRAB-S) had nuclear localization activity (Fig. 2b). Again we fused KRAB-S to GFP and confirmed its expression (Supplementary Fig. 1b). In contrast to the positive control of SV40 NLS showing exclusive nuclear localization when fused to GFP, no obvious nuclear enrichment of KRAB-S-GFP was detected (Fig. 2c), suggesting that the nuclear localization activity of KRAB is not determined by the linear stretch of sequence; rather, its structural integrity may be critical.

The nuclear localization activity of KRAB is a conserved feature of KRAB-ZFPs

The sequence similarity between the A-box and the conserved key amino acid residues important for the nuclear localization activity of ZNF268 KRAB suggest functional conservation of KRAB nuclear localization activity among different zinc finger proteins (Fig. 2). To test this possibility, we fused KRAB domains from seven other zinc finger proteins to GFP and confirmed their expression (Supplementary Fig. 1c). As predicted, all KRABs showed nuclear localization activity to varying degrees, with ZNF268 and ZNF224 KRAB having the strongest and the weakest activities, respectively (Fig. 3a, Supplementary Fig. 4a). To further characterize the nuclear localization function of these different KRABs, we selected KOX1 KRAB and ZNF300 KRAB and expressed each domain or mutant form as GFP proteins in HeLa cells (Supplementary Fig. 1c). Similar to that of ZNF268 KRAB, the nuclear localization activity of KRAB-GFP from ZNF300 and KOX1 resided mainly in the A-box and mutations of the key conserved residues (E16, E17 and W18) impaired their activities. Double and triple substitutions of D8 plus V9 (D8A-V9A) or E16, E17 plus F18 (E16/17A-F18A) for Ala completely abolished the activity (Fig. 3b, c; Supplementary Fig. 4b, c). These results suggest that nuclear localization activity of KRAB is a conserved feature of KRAB-ZFPs.

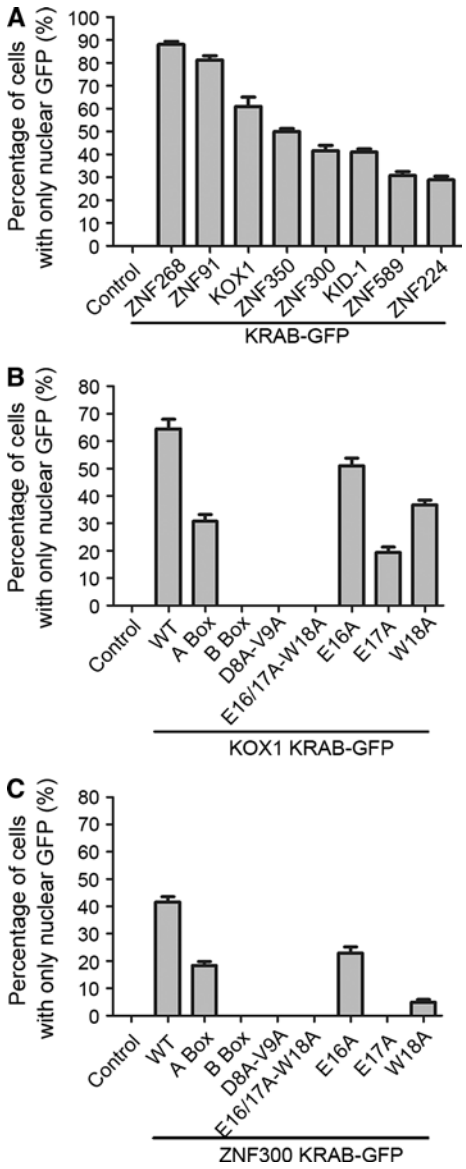

Fig. 3 The nuclear localization activity of the KRAB domain is a conserved feature of KRAB-ZFPs. **a** KRAB domains derived from different genes as indicated were fused to GFP (KRAB-GFP). The plasmids expressing GFP or various KRAB-GFP proteins were transfected into HeLa cells for confocal analysis to measure nuclear localization activity (*Control* GFP alone). **b** WT or mutant KOX1 KRAB as indicated was fused to GFP (KOX1 KRAB-GFP). Plasmids expressing GFP or various KOX1 KRAB-GFP proteins were transfected into HeLa cells for confocal analysis. **c** WT or mutant ZNF300 KRAB as indicated was fused to GFP (ZNF300 KRAB-GFP). Plasmids expressing GFP or various ZNF300 KRAB-GFP proteins were transfected into HeLa cells for confocal analysis. The percentages of cells with exclusively nuclear GFP were calculated and the results are presented as the means ± SD from three independent experiments

KRAB domain exerts nuclear localization function by interacting with KAP1

As discussed above, the KRAB domain lacks a classic linear NLS sequence that contains a stretch of basic amino acids. A piggy-back mechanism has been proposed for proteins lacking the classic NLS [18, 19]. Thus we examined whether the nuclear localization activity of KRAB is mediated by its major partner, the corepressor KAP1 [11]. KAP1

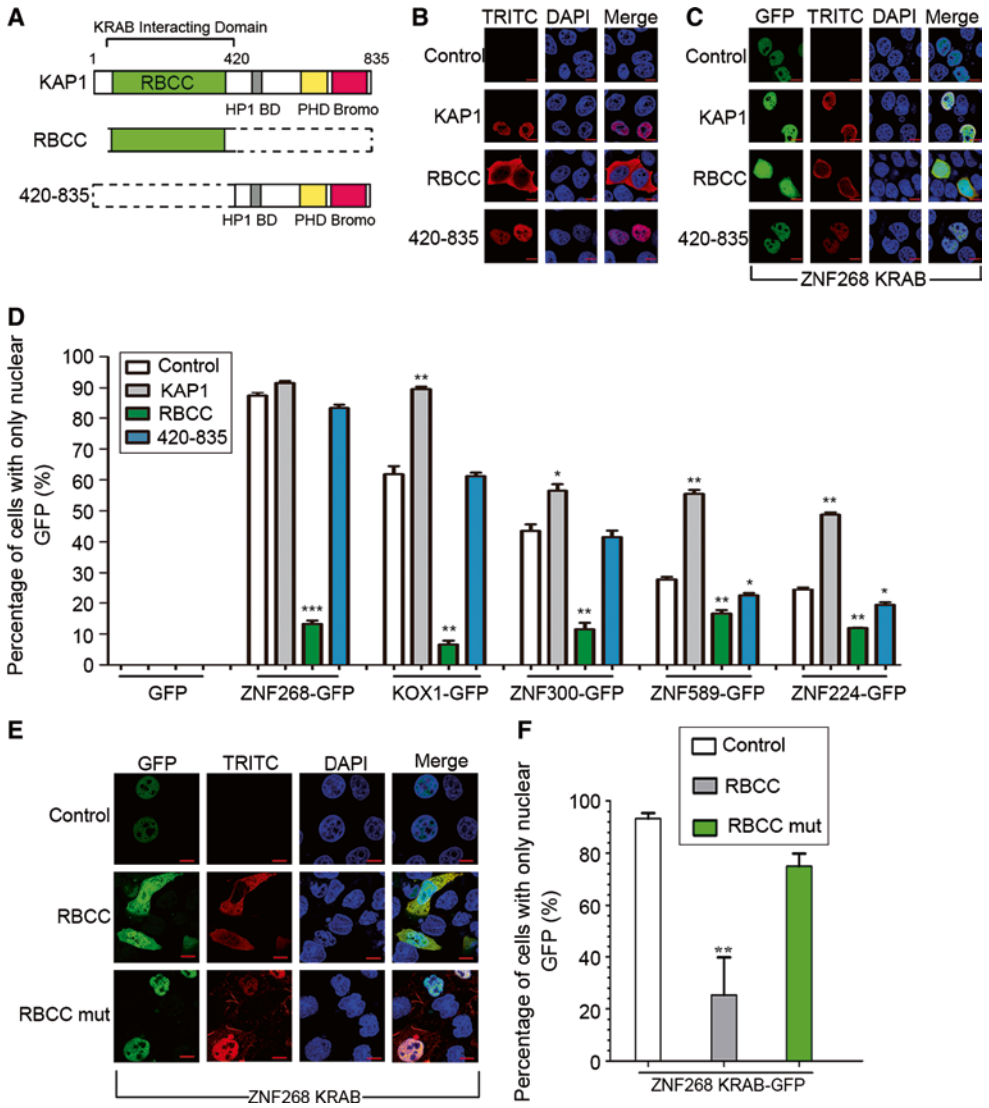

Fig. 4 KAP1 mediates nuclear localization activity of KRAB. **a** Schematic illustration of human KAP1 (*numbers* indicate the amino acid positions; *RBCC* a tripartite domain consisting of a Ring finger, two B-box zinc fingers and a coiled coil; *HP1 BD* HP1 binding domain; *PHD* plant homeodomain; *Bromo* bromo domain). Two deletion mutants of KAP1, the RBCC domain and C-terminal amino acids 420–835, were fused to a FLAG tag at the C-terminus for subcellular localization analysis. **b** Control vector or plasmids expressing full-length of FLAG-KAP1, FLAG-RBCC domain (*RBCC*), or FLAG-420-835 amino acid region of KAP1 were transfected into HeLa cells. The cells were stained with an anti-FLAG antibody followed by incubation with a TRITC-conjugated second antibody for confocal analysis. *Scale bars* 10 μm. **c** Different KRABs derived from different ZNFs as indicated were fused to GFP. Plasmids expressing GFP or different KRAB-GFP fusion proteins were transfected into HeLa cells in combination with full-length KAP1 (*KAP1*), RBCC domain of KAP1 (*RBCC*), or the 420–835 region of KAP1 (*420-835*); *Control* GFP alone. The nuclear localization of KRAB-GFP fusion protein was analyzed 24 h after transfection. Representative confocal images for the cotransfections of KRAB-GFP and KAP1, RBCC or 420–835 are shown. **d** The percentages of cells with exclusively nuclear GFP were calculated and the results are presented as the means ± SD from the statistical analysis of the findings in **c**. **e** ZNF268 KRAB-GFP fusion proteins or GFP alone were transfected into HeLa cells (*Control* GFP alone, *RBCC* RBCC domain of KAP1, *RBCC mut* mutant RBCC domain). The nuclear localization of KRAB-GFP fusion protein was analyzed 24 h after transfection. Representative confocal images for cotransfections of KRAB-GFP fusion proteins or GFP alone (*Control*) are shown. **f** The percentages of cells with exclusively nuclear GFP were calculated and the results presented are the means ± SD from the statistical analysis of the findings in **e**. $*p < 0.05$; $**p < 0.01$

protein contains several domains including RBCC, HP1 BD, PHD, and Bromo domains (Fig. 4a). KRAB is known to interact with KAP1 through the RBCC domain [16]. We thus expressed full-length KAP1, the RBCC domain of KAP1, or the C-terminus of KAP1 (sequence from 420 to 835 amino acids, denoted as 420–835) as FLAG-tagged proteins (Supplementary Fig. 1d). We observed that the RBCC domain alone localized in the cytoplasm while

the 420–835 was exclusively in the nucleus, just like full-length KAP1 (Fig. 4b). Cytoplasmic localization of RBCC is not due to exposure of NES since LMB treatment did not promote RBCC nuclear localization (data not shown). Interestingly, coexpression of RBCC with four KRAB-GFP fusion proteins (ZNF268, KOX1, ZNF300, ZNF589) significantly decreased their nuclear localization percentage (Fig. 4c, d). Although coexpression of full-length KAP1 only slightly increased nuclear localization of ZNF 268 KRAB compared to ZNF268 KRAB–GFP alone, KAP1 overexpression did significantly increase nuclear localization of three other KRAB-GFPs. We believed that this was due to the initially high nuclear localization percentage for ZNF268 KRAB (about 90 %). It was very difficult to increase it further. On the other hand, overexpression of 420–835 had only a minimal effect on nuclear localization of all four KRAB-GFPs (Fig. 4c, d). These results suggest that KRAB may exert nuclear localization activity by interacting with KAP1 through the RBCC domain. KAP1 may thus be a universal partner on which KRAB-ZFPs can piggy-back into the nucleus.

To further investigate the importance of the interaction between KRAB and RBCC in KRAB nuclear localization, we mutated two residues C65 and C68 to alanine (C65/68/A) in the RBCC domain to abolish the interaction [16]. As expected, ZNF268 KRAB-GFP alone localized exclusively in the nucleus and coexpression of RBCC significantly reduced the percentage of cells with only nuclear GFP. However, RBCC mut (C65/68/A) lost its function and only slightly affected nuclear localization of ZNF268 KRAB-GFP (Fig. 4e, f). These results suggest that the interaction between KAP1 and KRAB through the RBCC domain plays an important role in nuclear localization of KRAB-ZFPs.

To determine whether the direct interaction between KRAB and KAP1 is essential for the nuclear localization

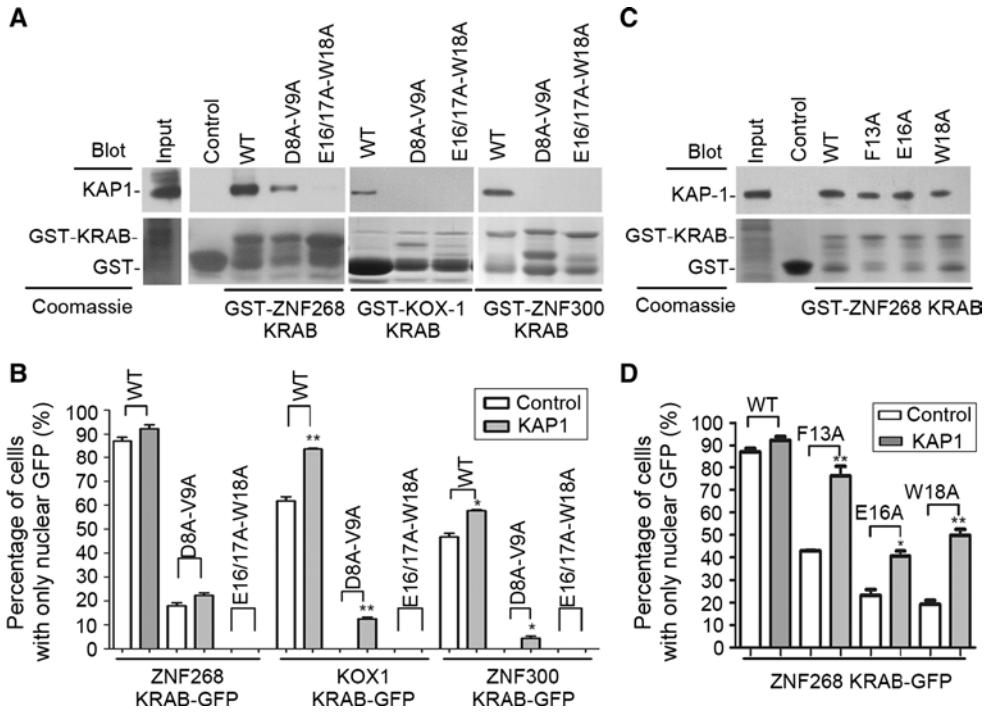

Fig. 5 The nuclear localization activity of KRAB correlates with its ability to interact with KAP1. **a** WT or mutant KRABs as indicated (D8A-D9A, E16/17A-W18A) from ZNF268, KOX1 or ZNF300 genes were fused to GST. The fusion proteins were expressed in bacteria, purified, and incubated with whole HeLa cell lysates. The KAP1 protein pulled down by various GST-KRAB fusion proteins was measured by western blotting with KAP1 antibody. GST alone was used as a control. GST-KRAB proteins were detected by coomassie blue staining. **b** WT or mutant KRABs as indicated (D8A-D9A, E16/17A-W18A) from ZNF268, KOX1 or ZNF300 genes were fused to GFP. Plasmids expressing various WT or mutant KRAB-GFP were transfected into HeLa cells in combination with KAP1 (*gray columns*) or Control (*white columns*) vector as indicated. The effect of KAP1 overexpression on nuclear localization of various -GFP was determined by confocal analysis. **c** WT or various ZNF268 KRAB mutants with single residue mutation as indicated (F13A, E16A, W18A) were fused to GST for the GST pull-down assay as described in **a**. **d** WT or various ZNF268 KRAB mutants with a single residue mutation as indicated (F13A, E16A, W18A) were fused to GFP. Plasmids expressing various WT or mutant KRAB-GFP were transfected into HeLa cells in combination with KAP1 (*gray columns*) or Control (*white columns*) vector as indicated. The effect of KAP1 overexpression on nuclear localization of various KRAB-GFP was determined by confocal analysis as described above. The percentages of cells with exclusively nuclear GFP were calculated and the results are presented as means ± SD. $^{*}p < 0.05$; $^{**}p < 0.01$

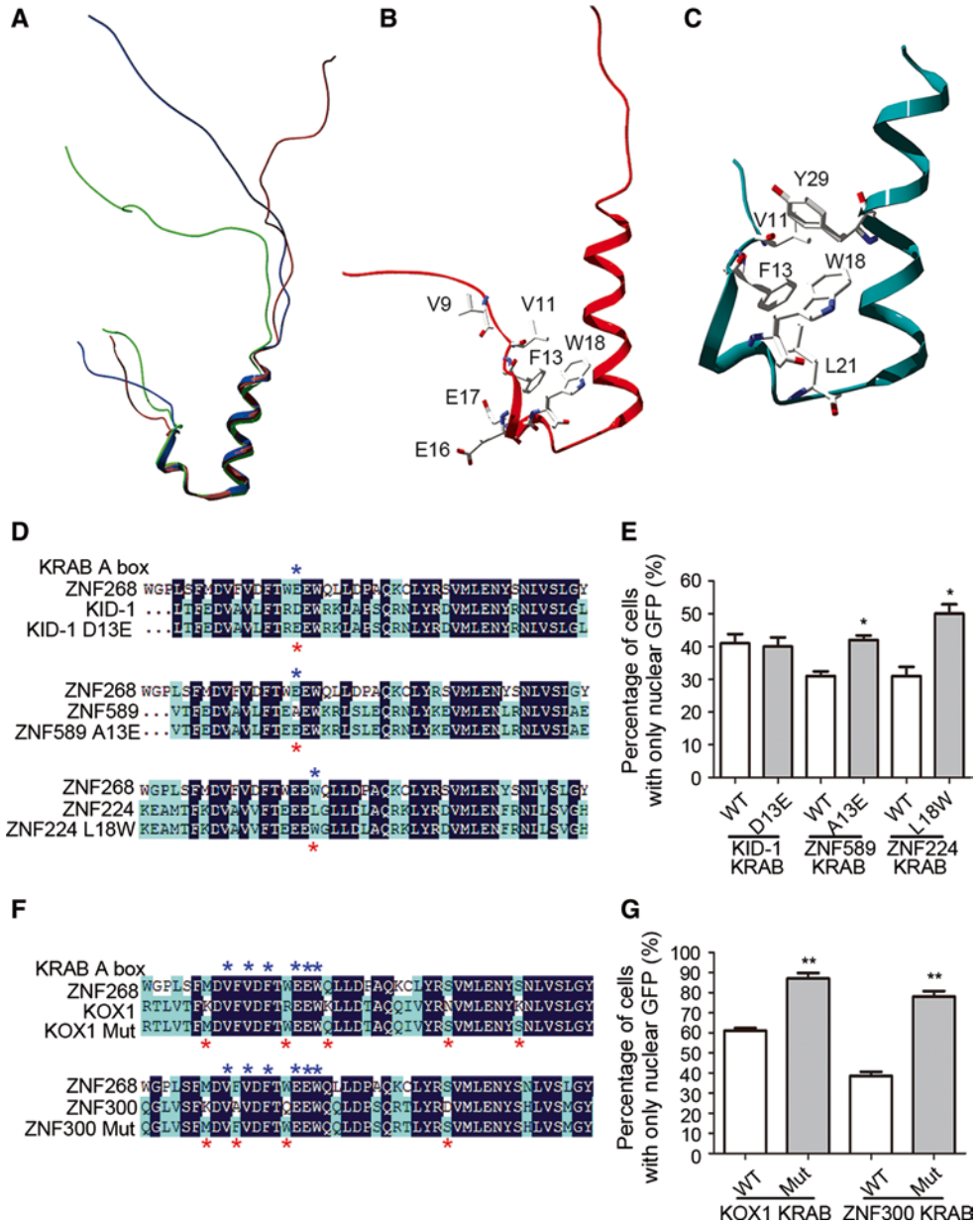

Fig. 6 Conserved residues that are critical for nuclear localization activity of KRAB are located in a stable core helix structure. **a** The ZNF268 structure was modeled using the different conformations of RIKEN KRAB domain as templates (PDB code 1V65). The overlapping of representative conformations (*green*, *red* and *blue*, respectively) of ZNF268 KRAB reveals a stable core structure with two helixes and flexible structures in the N-terminus and C-terminus. **b** Enlarged view of the conformation in *red* shows the distribution of six conserved residues that were essential for the nuclear localization activity of ZNF268 KRAB. **c** Predicted intramolecular hydrophobic interactions of the indicated amino acid residues in ZNF268 KRAB that help to maintain a hydrophobic environment. **d** Sequence alignment reveals that the amino acids of E13 and W18 of ZNF268 in KID-1, ZNF589, and ZNF224 are less conserved. The amino acids of these less conserved sites are designated by *blue asterisks* in ZNF268. The amino acids in KID-1, ZNF589, and ZNF224 (*red asterisks*) at these sites were mutated back to the equivalent amino acids of ZNF268 to create D13E, A13E, and L18W mutants respectively. **e** WT or mutant KRABs (D13E, A13E, L18W) from KID-1, ZNF589 or ZNF224 were fused to GFP. Plasmids expressing WT or mutant KRAB-GFP were transfected into HeLa cells for confocal analysis of GFP fusion protein nuclear localization. **f** Sequence alignment reveals that the amino acids near the conserved sites in KOX-1 and ZNF300 are less conserved. The key sites are designated by *blue asterisks*, and the adjacent sites of KOX1 and ZNF300 KRAB were mutated back to the equivalent amino acids of ZNF268 KRAB (*red asterisks*) designated as KOX1-Mut and ZNF300-Mut respectively. **g** WT or mutant (Mut) KRABs from KOX-1 or ZNF300 was fused to GFP. Plasmids expressing WT or various mutant KRAB–GFPs were transfected into HeLa cells for confocal analysis. The percentages of cells with exclusively nuclear GFP were calculated and the results are presented as means ± SD. $*p < 0.05$; $**p < 0.01$

activity of KRAB, we first prepared GST fusion proteins with WT or mutant forms of KRAB from ZNF268, KOX1, or ZNF300 and used the fusion proteins in a pull-down assay. As control all WT GST-KRAB fusion proteins were able to interact with KAP1, and KAP1 overexpression increased KRAB-GFP nuclear localization, which is especially significant for KOX1 and ZNF300 because of their initially low levels (Fig. 5a, b). Impressively, double mutation (D8A-V9A) in two residues D8 and V9, which have been shown to be important for the interaction with KAP1 [11], led to residual interaction between ZNF268 KRAB and KAP1, and nondetectable levels of interaction between KAP1 and KRAB from KOX1 or ZNF300. Consistent with impaired interaction, D8A-V9A mutant KRAB of ZNF268 only retained about 20 % nuclear localization activity and coexpression of WT KAP1 failed to significantly increase this (Fig. 5b). Accordingly, D8A-V9A mutant KRAB of KOX1 and ZNF300 completely lost their nuclear localization ability. Although KAP1 appeared to increase nuclear localization of the D8A-V9A mutant KRAB of KOX1 and ZNF300, the percentage was still less than that of the D8A-V9A mutant KRAB of ZNF268 and far less than that of their corresponding WT (about 5–10 % in mutants vs. 60–90 % in WT). Unlike the double/triple substitutions, single residue substitutions such as F13A, E16A and W18A only marginally decreased the interaction of ZNF268 KRAB with KAP1 (Fig. 5c). All such mutants retained significant (about 20–40 %) nuclear localization activity and KAP1 overexpression dramatically enhanced their nuclear localization (Fig. 5c, d).

Taken together, these results suggest that the interaction between KRAB and RBCC of KAP1 involves multiple conserved amino acids and that the nuclear localization activities of different KRAB domains are correlated with their ability to interact with KAP1.

A conserved tertiary structure is critical for nuclear localization activity of KRAB

Failure to detect a short linear sequence for nuclear localization activity of KRAB suggests that a three-dimensional structure may be important. Thus we performed a structure modeling experiment. Interestingly, we found that a stable two-helix structure existed in the A-box and that the N- or C-terminus displayed considerable flexibility (Fig. 6a). Importantly, six conserved residues that were essential for nuclear localization activity were all located in this helical region (Fig. 6b). In particular, residues V9, E16 and E17 were located on the surface for possible interactions with other proteins such as KAP1 (Fig. 6b), and the other three key residues V11, F13 and W18 were mainly in the interior for strong hydrophobic interactions with L21 and Y29 residues (Fig. 6c). These interactions likely play a critical role in maintaining the hydrophobic environment in the core of KRAB that is important for the nuclear localization activity of KRAB. Thus the presence of the conserved residues critical for nuclear localization activity in the region of a stable core two-helix structure supports the idea that the conserved stable core structure is essential for the nuclear localization activity of KRAB. In support of this, changing the less conserved residues in ZNF589 and ZNF224 KRAB (A13E for ZNF589 and L18W for ZNF224) to the corresponding residues in ZNF268 KRAB increased the activities, while a similar change in KID-1 KRAB involving substitution of a very similar acidic residue (D13E) had little effect on its activity (Fig. 6d, e). In addition, we observed a significant increase in the nuclear localization activity of ZNF300 KRAB and KOX1 KRAB when several apparently noncritical residues were mutated to the equivalent amino acids in ZNF268 (Fig. 6f, g). Thus, a conserved tertiary structure may be required for nuclear localization activity of KRAB.

Discussion

The KRAB domain consists of about 75 amino acids and is a well-established potent transcriptional repression module [9]. In this study, we revealed a novel feature of KRAB as an enhancer of nuclear localization of KRAB-ZFPs. This novel function is likely critical for the physiological and pathological roles of KRAB-ZFPs and offers a novel mechanism by which alternative splicing of KRAB-ZFPs participates in carcinogenesis.

Our studies revealed a novel function of KRAB that reinforces the nuclear localization of KRAB-ZFPs. Although the ZNF268 KRAB domain exhibited strong nuclear localization activity, we could not identify a typical NLS in KRAB, which is rich in basic amino acids such as the monopartite NLS in SV40 large T-antigen protein [20] and the bipartite NLS in nucleoplasmin [21]. NLSs are able to interact with the nuclear import machinery such as importin α/β. To determine whether KRAB can interact with the nuclear import machinery, we performed pull-down assays and coimmunoprecipitation. We did not detect any importin α/β that was pulled down by GST-KRAB fusion proteins. However, we did observe coimmunoprecipitation of KRAB-GFP when FLAG-tagged importin α/β was immunoprecipitated by FLAG antibody (Supplementary Fig. 5). These observations suggest that KRAB may not directly interact with importin α/β. It is very possible that KRAB-ZFPs may interact with the nuclear import machinery through intermediary proteins.

KAP1 may mediate nuclear localization of KRAB by bridging between the KRAB-ZFP and the nuclear import machinery. KAP1 is known to be an important corepressor

of KRAB-ZFPs [11]. Our results showed that KAP1 also played an important role in the nuclear localization activity of KRAB. However, KAP1 does not bear typical NLS either. It may function in mediating nuclear localization by interacting with HP1, which is known to have a typical NLS [22]. Interestingly, when we knocked down KAP1, we failed to observe any significant decrease in KRAB-GFP nuclear localization (Supplementary Fig. 6). It is possible that multiple proteins, including the closely related KRAB-interacting proteins, may also bind to the KRAB domain and compensate KAP1 for nuclear localization of KRAB.

Many KRAB-ZFPs, including ZF268, have been implicated in tumor development and progression. Our findings suggest that altered subcellular distribution of KRAB-ZFP due to loss of KRAB through dysregulated alternative splicing may contribute to carcinogenesis. Aberrant localization of proteins has been shown to contribute to human diseases, such as metabolic, cardiovascular and neurodegenerative diseases and cancer [23]. We have shown recently that overexpression of ZNF268b2 but not ZNF268a contributes to cervical cancer by enhancing NF-κB signaling [12]. Since ZNF268b2 is present in both the cytoplasm and nucleus while ZNF268a is exclusively nuclear, ZNF268b2 but not ZNF268a is able to enhance NF-κB signaling, thus affecting cancer development. On the other hand, this novel mechanism does not exclude the possibility that ZNF268b2 in the nucleus fails to repress target genes that contribute to cervical carcinogenesis.

The nuclear localization function of KRAB suggests that it may serve as a novel vector for nuclear delivery. Due to its potent activity, KRAB may be used to carry therapeutic peptides to the nucleus. In particular, its ability to bind KAP1 may enable the transport of drugs to specific target genes or regions. KAP1 is a critical regulator of normal development and differentiation [22]. KRAB-ZFPs recruit KAP1 to specific genome loci and KAP1 in turn recruits chromatin-remodeling proteins such as Mi2α, SETDB1 and HP1 to create a heterochromatic microenvironment for gene regulation [24, 25]. Aberrant expression of KAP1 has been implicated in the development of various cancers [26–28], and the development of anti-KAP1 drugs for anticancer therapy has been proposed [29]. It is conceivable that KRAB may serve as such a candidate for anti-KAP1 drugs by utilizing its interaction with KAP1 and the nuclear localization activity.

In conclusion, our study has revealed that KRAB functions to reinforce nuclear localization of KRAB-ZFPs via its interaction with the co-repressor KAP1. These findings should help us understand the function of zinc finger proteins in human development and disease.

Acknowledgments This work was supported by a National High Technology Research and Development Program of China (863 Program; grant no. 2006AA02A306 to W. Li), National Natural Science Foundation of China (grant nos. 30871245 and 31271511 to M. Guo, 81070406 to Z. Huang), and a Ph.D. Programs Foundation of Ministry of Education of China (grant no. 20110141110016 to Z. Huang), New Century Excellent Talents in University (NCET) of Ministry of Education of China (grant no. NCET-12-0422 to Z. Huang), Scientific Research Foundation for Returned Scholars of Ministry of Education of China (to Z. Huang).

Conflict of interest The authors declare no conflict of interest.

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