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Basic amino acid residues located in the N-terminal region of BEND3 are essential for its nuclear localization

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ABSTRACT

BEN domain-containing protein 3 (BEND3) has recently been reported to function as a heterochromatin-associated protein in transcriptional repression in the nucleus. BEND3 should have nuclear localization signals (NLSs) to localize to the nucleus in light of its molecular weight, which is higher than that allowed to pass through nuclear pore complexes. We here analyzed the subcellular localization of deletion/site-directed mutants of human BEND3 by an immunofluorescence assay in an attempt to identify the amino acids essential for its nuclear localization. We found that three basic amino acid residues located in the N-terminal region of BEND3 ($BEND3_{56-58}$, KRK) are essential, suggesting that these residues play a role as a functional NLS. These results provide valuable information for progressing research on BEND3.

1. Introduction

BEN domain-containing protein 3 (BEND3, also known as KIAA1553) contains four BEN domains that mediate DNA-protein or protein–protein interactions [1–3]. A recent study has demonstrated that BEND3 associates with heterochromatin and is involved in transcriptional repression, possibly by altering the chromatin structure through its interactions with histone deacetylases (HDACs) and Sall4 [3,4]. Consistent with these findings, Fujita et al. identified BEND3 as a telomere-binding molecule using their unique system called enChIP [5]. In both studies, overexpressed exogenous BEND3 mainly localized to the nucleus [3,5]. These findings strongly indicate that BEND3 functions as a DNA-binding protein in the nucleus.

Proteins that function in the nucleus have to migrate into the nucleus after being generated in the cytoplasm. Proteins smaller than 40 kDa can be imported into the nucleus through nuclear pore complexes through passive diffusion [6,7], whereas larger proteins are generally transported into the nucleus via a receptor-mediated mechanism [8]. Therefore, they are often required to possess nuclear localization signal(s) (NLSs) that are recognized by importin α/β [9]. Since the molecular weight of BEND3 is approximately 95 kDa, it appears to be impossible for it to pass through nuclear pore complexes passively. Therefore, BEND3 may contain NLSs, but its functional NLSs have not yet been determined.

In the present study, we attempted to identify the functional NLSs of BEND3. We prepared a series of deletion mutants or site-directed mutants of FLAG-tagged BEND3 and examined their subcellular localization by an immunofluorescence assay. We here showed that three basic amino acid residues located in the amino-terminal region of BEND3 are essential for its nuclear localization.

2. Materials and methods

2.1. Cell culture

HeLa cells (obtained from the ATCC) were maintained at 37 °C with 5% CO_2 in DMEM with 10% FCS, penicillin (100 U/ml), and streptomycin (0.1 mg/ml).

2.2. Plasmid preparation and transfection

The FLAG-BEND3 gene was amplified from a pCMV-FLAG-BEND3 plasmid [10] using the primers 5′-GTATATATGCTAGCATG GACTACAAGGACGACGATG-3′ and 5′-AATATAGCGGCCGCTCACTT CTCCACTTTCTTTGC-3′, and then subcloned into the NheI/NotI site of the pcDNA3.1 Hygro(−) vector (Invitrogen) (pcDNA3.1 Hygro(−)-FLAG-BEND3). The C-terminally truncated BEND3 genes were amplified from the plasmid using the following reverse primers:

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BEND3$_{1–490}$ 5′-AATATAGCGGCCGCTCATTCACTGCCCGCGTCCAGCC-3′, or BEND3$_{1–660}$ 5′-AATATAGCGGCCGCTCAGACCTTGCGCTG CTGCTGGTAG-3′. The PCR products were subcloned into the HindIII/NotI site of the pCMV-(DYKDDDDK)-N vector (Clontech). The N-terminally truncated FLAG-BEND3 genes were amplified from the pCMV-FLAG-BEND3 plasmid using 5′-GTATA-TATGCTAGCATGGACTACAAGGACGACGATGACAAGAAGCTTGTCAGC GATGGCCTGCTAGAC-3′ for BEND3$_{61–828}$, or 5′-GTATA-TATGCTAGCATGGACTACAAGGACGACGATGACAAGAAGCTTATGCG GAACCGTGAGAACAGCTC-3′ for BEND3$_{86–828}$ as a forward primer, and 5′-AATATAGCGGCCGCTCATTCACTGCCCGCGTCCAGCC-3′ as a reverse primer, respectively. The PCR products were subcloned into the NheI/XhoI site of the pcDNA3.1 Hygro(−)-FLAG-BEND3. Site-directed mutants were generated by overlap-extension PCR using the following primer sets: FLAG-BEND3 K56A 5′-CCATCGCTGAC-CAGCTGCTTTCGTGCGCTGGAGTCCTGCAGGGC-3′ and 5′-AAG-CAGCTGGTCAGCGATGG-3′, FLAG-BEND3 R57A 5′-CCATCGCTGACCAGCTGCTTTGCTTTGCTGGAGTCCTGCAGGG-3′ and 5′-AAGCAGCTGGTCAGCGATGG-3′, FLAG-BEND3 K58A 5′-GGCCATCGCTGACCAGCTGCGCTCGTTTGCTGGAGTCCTGCAG-3′ and 5′-CAGCTGGTCAGCGATGGCC-3′, respectively. The resulting plasmids were transfected into HeLa cells using Lipofectamine LTX and Plus reagent (Invitrogen) according to the manufacturer's protocol.

2.3. Fractionation and immunoblotting

Cells were washed with PBS, and subcellular fractionation was performed using NE-PER nuclear and cytoplasmic extraction reagents (Thermo Scientific) according to the manufacturer's protocol. The resultant cytoplasmic and nuclear fractions were separated by SDS-PAGE, and proteins were electrotransferred onto PVDF membranes (GE Healthcare). After blocking with Blocking One (Nacalai Tesque), the membrane was incubated overnight at 4 °C with antibodies against α-tubulin (both were purchased from Sigma), FLAG tag (Wako), or Lamin A/C (Cell Signaling Technology). The blots were developed using Immobilon western chemiluminescent HRP substrate (Millipore).

2.4. Immunofluorescence

HeLa cells on coverslips were transfected with FLAG-BEND3-or its various mutant-expression vectors. After 24 h, these cells were fixed with 4% paraformaldehyde/PBS for 15 min, permeabilized with 0.1% Triton X-100/PBS for 10 min, and then blocked with 1% BSA/PBS for 30 min at room temperature. The samples were stained with an FITC-conjugated anti-FLAG antibody (Sigma). Images were obtained with an LSM 710 confocal microscope (Carl Zeiss).

3. Results

3.1. FLAG-tagged BEND3 predominantly localized to the nucleus

Previous studies reported that exogenously expressed BEND3 fused with an artificial tag, such as T7, HA, or fluorescent protein, localized to the nucleus [3,5]. To confirm this, we first determined whether N-terminally FLAG-tagged BEND3 (FLAG-BEND3, Fig. 1A) expressed in HeLa cells localizes to the nucleus. Using an immunofluorescence analysis, we observed the nuclear localization of FLAG-BEND3 in HeLa cells (Fig. 1B). To further confirm this, we also fractionated HeLa cells into cytoplasmic and nuclear fractions, each of which was subjected to immunoblotting. The band corresponding to BEND3 was only detected in the nuclear lysates and not in the cytoplasmic fraction (Fig. 1C). These results demonstrate that exogenously expressed FLAG-BEND3 is located in the nucleus. Similar results were obtained when another cell line, the human T cell lymphoma, Jurkat was used (data not shown).

3.2. Three different sequences were predicted as potential NLSs by a prediction program

We next analyzed the amino acid sequence of full length BEND3 to predict NLSs using the PSORT II program. This analysis revealed that BEND3 had the following three potential NLSs within the molecule: BEND3$_{73–76}$ (KRRR), BEND3$_{522–528}$ (PGRRSKK), and BEND3$_{810–826}$ (RRPNRKKCDILKKAKKV) (Fig. 2A). The third one was

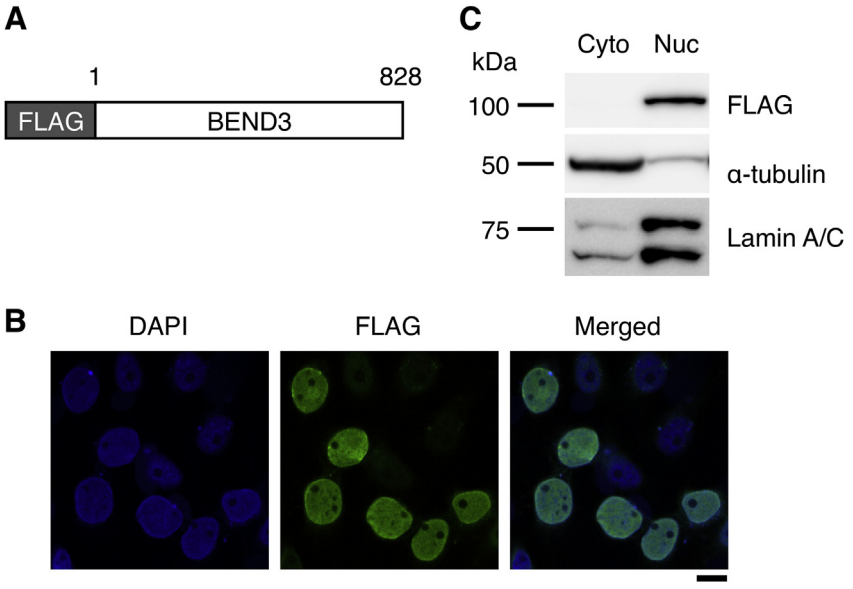

Fig. 1. BEND3 mainly localized to the nucleus. (A) Schematic representation of FLAG-tagged BEND3. (B) HeLa cells were transfected with an FLAG-BEND3 expression vector. Twenty-four hours later, these cells were stained with an anti-FLAG antibody (green). The nucleus was stained with DAPI (blue). Bar = 10 μm. (C) HeLa cells transfected with an FLAG-BEND3 expression vector were fractionated for cytoplasmic (Cyto) or nuclear (Nuc) lysates. Each fraction was separated by SDS-PAGE and immunoblotted with the indicated antibodies. α-tubulin and Lamin A/C were used as technical controls for cell fractionation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

predicted to be a bipartite NLS. We then compared these potential NLSs with those of other species such as *Pan troglodytes*, *Canis familiaris*, *Bos taurus*, *Mus musculus*, and *Xenopus* (Fig. 2B). All three sequences were highly conserved among the vertebrates, suggesting that one or several of them may function as NLS(s).

3.3. The N-terminal 60 amino acid region of BEND3 was required for nuclear localization

In order to verify the prediction result described above, we constructed a series of N- or C-terminally truncated mutants of FLAG-BEND3 (Fig. 3A) and transfected each of these into HeLa cells. After 24 h, we performed immunofluorescence with an anti-FLAG antibody, followed by confocal microscopic observations. As shown in Fig. 3B, both of the C-terminally truncated mutants, FLAG-BEND3$_{1–660}$ and FLAG-BEND3$_{1–490}$, localized to the nucleus as well as FLAG-BEND3 (Fig. 1B). In contrast, the N-terminally truncated mutant, FLAG-BEND3$_{86–828}$ predominantly localized to the cytoplasm. However, FLAG-BEND3$_{61–828}$, which was predicted to possess three potential NLSs by the PSORT II program, was not able to migrate to the nucleus, suggesting that these sequences are insufficient for the nuclear localization of BEND3. These results indicate that the N-terminal 60 amino acid region of BEND3 is required for its nuclear localization and may contain a functional NLS.

3.4. Basic amino acid residues located in the N-terminal 60 amino acid region of BEND3 were essential for its nuclear localization

We found an NLS-like motif that contained the basic amino acid residues, BEND3$_{56–58}$ (KRK) within the N-terminal 60 amino acid region of BEND3; however, it was not predicted to be an NLS by the prediction program (described in red-letters in Fig. 2A). The sequence was highly conserved among the vertebrates as well as the above-mentioned three potential NLSs (Fig. 2B). We performed site-directed mutagenesis analysis to confirm that the sequence located in the amino acids between 56 and 58 (KRK) of BEND3 is a part of an NLS. We constructed three kinds of single alanine-substitution mutants of FLAG-BEND3, Lys56Ala (K56A), Arg57Ala (R57A), and Lys58Ala (K58A). Immunofluorescence analysis revealed that the replacement of any of these residues to Ala abolished the nuclear localization of FLAG-BEND3 (Fig. 4A and B), which was consistent with earlier experiments obtained using truncated mutants (Fig. 3). Based on these results, we concluded that the basic amino acid residues, BEND3$_{56–58}$ (KRK), are essential for the nuclear localization of BEND3 and also that this sequence may function as an NLS.

4. Discussion

BEND3 has recently been described as a heterochromatin-associated protein, which indicates that it travels into the nucleus and then binds to DNA. Since BEND3 is likely to be a large protein, it should have NLSs within the molecule for its passive import into nuclear pores. We here identified the amino acids essential for the nuclear localization of human BEND3 using truncation and point mutation analyses. We found that amino acids 56–58 (KRK), located in the N-terminal region of BEND3, are required for its nuclear localization.

Although the PSORT II prediction program has been widely used to predict the functional domains of proteins including NLSs [11–15], the three potential NLSs within BEND3 predicted by the program in the present study were not essential for its nuclear localization. We eventually narrowed the region responsible for the nuclear localization of BEND3 using a series of N- or C-terminal deletion mutants and then identified key residues using site-directed mutants in immunofluorescence analyses. The amino acid sequence (KRK) itself is known to work as an NLS of the other nuclear protein, supporting our data in this study [16]. However, we could not exclude the possibility that the sequence is just involved in unmasking the other sequence(s) that plays a role of an authentic NLS. Further experimental support would be required for more accurate interpretation of the data. In any case, since the eventual key sequence we identified was not predicted to be an NLS, a combination of bioinformatic and molecular biological approaches may still be needed to determine the functional domains of proteins of interest.

Our results showed that a single amino acid substitution, K56A, R57A, and K58A, altered the localization of BEND3 from the nucleus to the cytoplasm, respectively. However, it currently remains unclear whether BEND3 variants that lack an NLS are present *in vivo*. In our preliminary study, a 5′RACE analysis suggested the presence of the splicing variant of BEND3 generated from exon 4 and exon 5 and lacking 85 N-terminal amino acids, even though it was not detected at the protein level. Such a differentially-localized BEND3 may interact with proteins other than HDACs and Sall4 or play different roles from intranuclear BEND3.

Although previous studies reported that BEND3 interacts with HDACs and Sall4 [3,4], to the best of our knowledge, BEND3-interacting proteins have not yet been identified by using BEND3 as a bait protein. In the case where BEND3$_{56–58}$ (KRK) functions as a classical NLS, BEND3 may interact with the importin-α protein family [17,18], and, if not, BEND3 itself may function as a nuclear transport factor similar to β-catenin, which functions as a transporter of the transcription factor lef-1 [19,20]. In any case, exploring

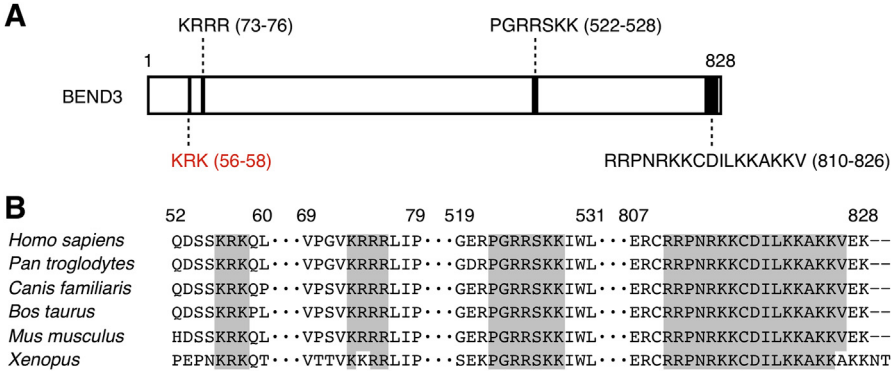

Fig. 2. Potential nuclear localization signals (NLSs) in the BEND3 protein. (A) Four putative NLSs were shown. Among them, one putative sequence (KRK), located between amino acids 56 and 58 (shown in red), was not predicted as an NLS by the PSORT II program (http://psort.hgc.jp/form2.html). (B) A sequence alignment of the four putative NLSs in human BEND3 with those of other vertebrates. Amino acids conserved within the NLSs are shaded gray. The number of residues corresponds to the human sequence. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

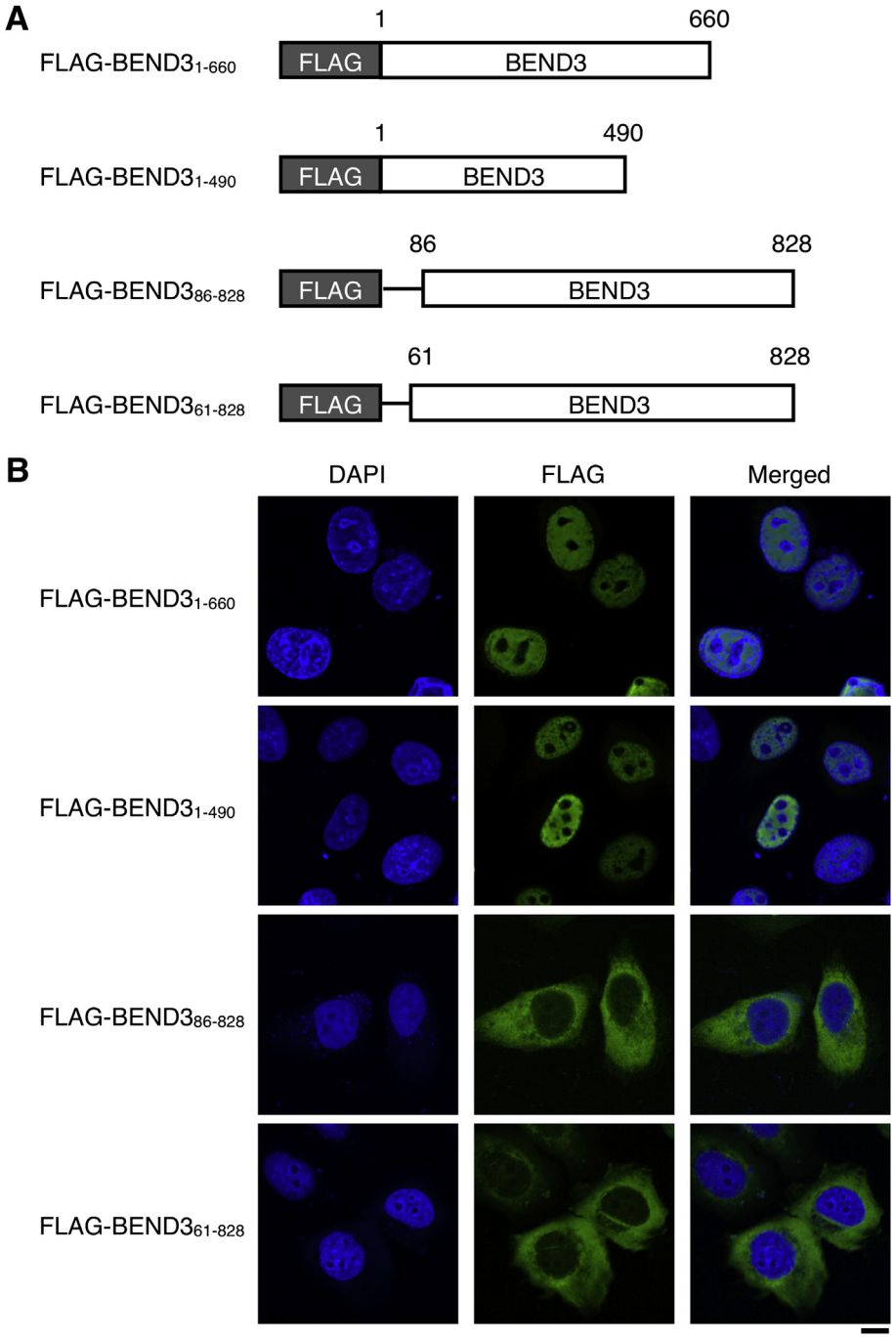

Fig. 3. The amino-terminal region of BEND3 was responsible for its nuclear localization. (A) Schematic representation of the truncated mutants of FLAG-tagged BEND3. (B) HeLa cells were transiently transfected with various truncated mutants of the FLAG-BEND3 expression vector described in (A). Twenty-four hours later, the cells were stained with an anti-FLAG antibody (green). The nucleus was stained with DAPI (blue). Bar = 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

novel BEND3-binding proteins will lead to a better understanding of not only the mechanism underlying nuclear localization, but also the biological function of BEND3.

The biological function of BEND3 has not yet been elucidated in detail. Furthermore, information regarding sites subjected to post-translational modifications, such as phosphorylation and sumoylation, is limited [3,21,22]. The accumulation of more information on BEND3 is important for promoting research on the protein. Studies aimed at determining functional domains, similar to the present study, or identifying novel functions *in vivo* will lead to a clearer understanding of BEND3 biology.

Conflict of interest

None.

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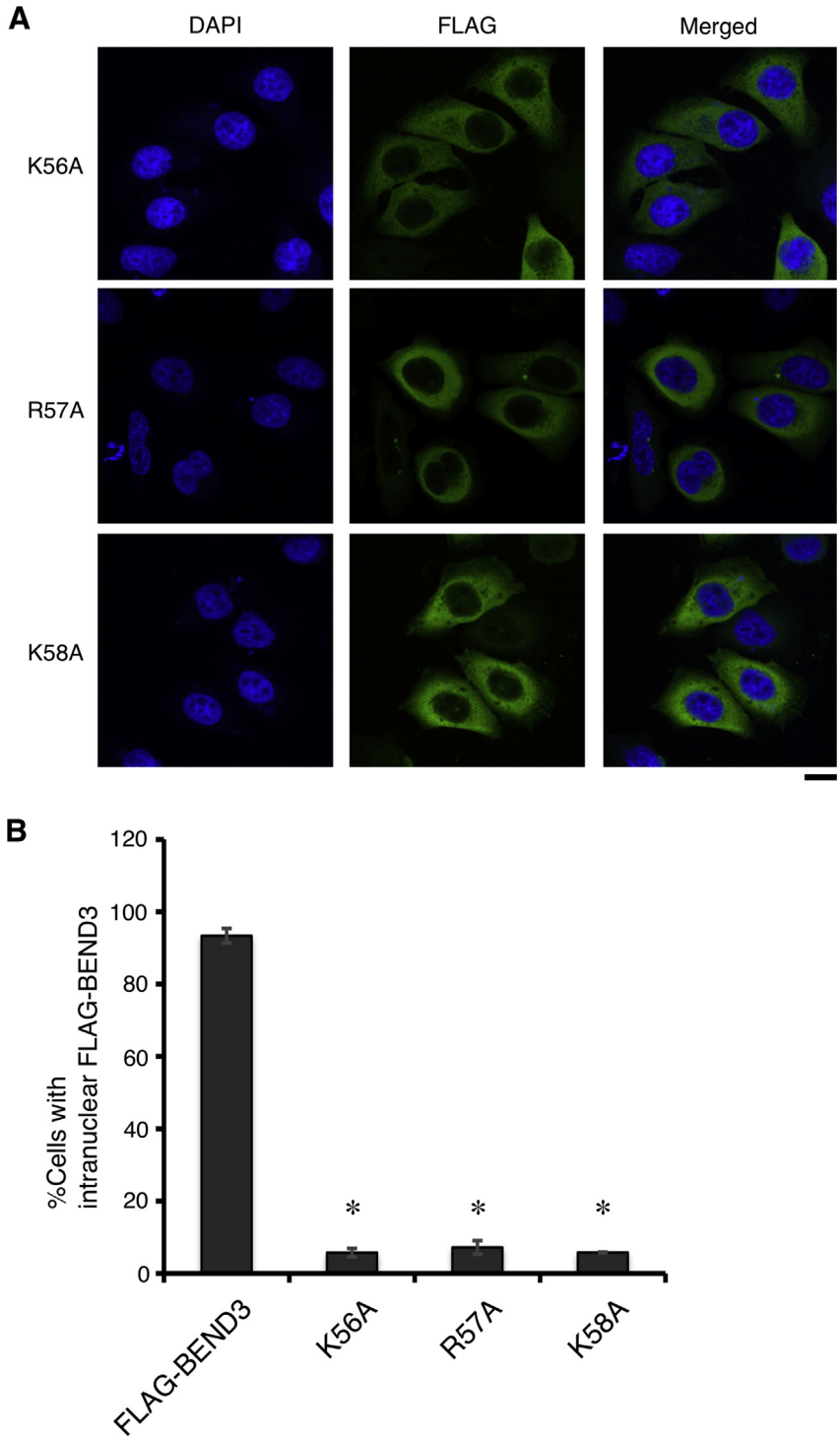

Fig. 4. Replacement of any of $BEND3_{56-58}$ (KRK) abolished the nuclear localization of FLAG-BEND3. (A) HeLa cells were transiently transfected with an FLAG-BEND3 mutant (K56A, R57A or K58A) expression vector. After 24 h, cells were stained with an anti-FLAG antibody (green). The nucleus was stained with DAPI (blue). Bar = 10 μm. (B) At least 200 transfected cells were counted in three independent experiments. The ratio of cells with intranuclear FLAG-BEND3 (See also Fig. 1B) or its mutants was quantified. Values are shown as the mean ± SD; n = 3. An asterisk denotes a significant difference according to the Student's *t*-test ($P < 0.0001$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Transparency document

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