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Supplementary Information for:

An Intracellular Nanobody Targeting T4SS Effector Inhibits *Ehrlichia* Infection

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This PDF file includes:

Tables S1 to S2
Figures S1 to S5

Supplementary Tables

Table S1. Etf-1 homologs in members of the genera *Ehrlichia* and *Anaplasma*

Species	NCBI accession number	Similarity (% aa)	E-Value
***Ehrlichia* species**[1]			
***E. muris* subsp. *muris* AS145**	WP_024071892.1	79	$1E^{-166}$
***E. muris* subsp. *eauclairensis* Wisconsin**	WP_045804476.1	76	$1E^{-130}$
***Ehrlichia* sp. HF**	WP_044195177.1	77	$2E^{-155}$
***E. minasensis* UFMG-EV**	WP_045170612.1	75	$1E^{-159}$
***E. canis* Jake**	WP_102574816.1	74	$7E^{-157}$
***E. ruminantium* Welgevonden**	WP_065433648.1	63	$4E^{-100}$
***Anaplasma* species**[2]			
***A. platys* Mili1**	WP_169193309.1	52	$1E^{-56}$
***A. centrale* Israel**	WP_012880860.1	57	$4E^{-36}$
***A. ovis* Haibei**	WP_075138831.1	55	$1E^{-31}$
***A. marginale* Florida**	WP_010269731.1	56	$3E^{-31}$

[1] *E. chaffeensis* Arkansas Etf-1 protein (WP_011452831.1, 380 aa) was used to perform a BLASTP search against the NCBI RefSeq protein database among representative *Ehrlichia* species.

[2] *Anaplasma phagocytophilum* encodes Ats-1 protein (ABD43383.2, 376 aa), which is 41% similar to *E. chaffeensis* Arkansas Etf-1 protein (E-value: $6E^{-13}$) and was used to perform a BLASTP search against the NCBI RefSeq protein database among representative *Anaplasma* species.

Table S2. Primer sequences for cloning truncated and mutated Etf-1 and Nbs [1]

Etf-1 AA Residues	Primer sequence (5′–3′)
Cloning truncations of Etf-1 into pET33b(+) for expression in *E. coli* BL21(DE3)	
1–380	F: GGAATTCCATATGGAAAACCTGTATTTTCAGGGCATGCTTACTTTCTTAAAG R: CGCGGATCCTTATTCATGCCATTCAATTTTCTGCGCTTCAAAAATATCGTTCA GGCCTCTTGCATGTAC
26–380	F: GGAATTCCATATGGAAAACCTGTATTTTCAGGGCCATCAAGAAAGCGTAGGT R: CGCGGATCCTTATTCATGCCATTCAATTTTCTGCGCTTCAAAAATATCGTTCA GGCCTCTTGCATGTAC
51–380	F: GGAATTCCATATGGAAAACCTGTATTTTCAGGGCAAACACTTTGACTTAAGA R: CGCGGATCCTTATTCATGCCATTCAATTTTCTGCGCTTCAAAAATATCGTTCA GGCCTCTTGCATGTAC
80–380	F: GGAATTCCATATGGAAAACCTGTATTTTCAGGGCGAAGTAGCTCTGAAAGTA R: CGCGGATCCTTATTCATGCCATTCAATTTTCTGCGCTTCAAAAATATCGTTC AGGCCTCTTGCATGTAC
113–380	F: GGAATTCCATATGGAAAACCTGTATTTTCAGGGCACTAAAAAAGATACCTTA R: CGCGGATCCTTATTCATGCCATTCAATTTTCTGCGCTTCAAAAATATCGTTCA GGCCTCTTGCATGTAC
Cloning truncations and point-mutations of codon-optimized Etf-1 into pEGFP-N1 for expression in mammalian cells	
1–380	F: AGTGCTAGC**CGCCACCATGG**TGCTGACCTTCCTGAAG R: CAAGAATTCGTCTGGCATGCACCTTTCC
1–306	F: AGTGCTAGC**CGCCACCATGG**TGCTGACCTTCCTGA R: CAAGAATTCGGGTCCGAGAATAAGGCATA
ΔCoil1 [2]	Etf-1-Opt-F: AGTGCTAGC**CGCCACCATGG**TGCTGACCTTCCTGAAG ΔCoil1-R: CCGGGAGTGCTGTTCACTGT GAACTCCTGGGTGGCTGACA; ΔCoil1-F: TGTCAGCCACCCAGGAGTTC ACAGTGAACAGCACTCCCGG Etf-1-Opt-R: CAAGAATTCGTCTGGCATGCACCTTTCC
1–380 (K6,7A)	F: AGTGCTAGC**CGCCACCATGG**TGCTGACCTTCCTG***GCCGCT***GGCGCCAACG R: CAAGAATTCGTCTGGCATGCACCTTTCC
21–380	F: AGTGCTAGC**CGCCACCATGG**TGACCACAAGCAAGCTGCCTC R: CAAGAATTCGTCTGGCATGCACCTTTCC
21–380 (K23A)	F: AGTGCTAGC**CGCCACCATGG**TGACCACAAGC***GCT***CTGCCTC R: CAAGAATTCGTCTGGCATGCACCTTTCC
23–380	F: AGTGCTAGC**CGCCACCATGG**TGAAGCTGCCTCACCAGGAGTC R: CAAGAATTCGTCTGGCATGCACCTTTCC
25–380	F: AGTGCTAGC**CGCCACCATGG**TGCCTCACCAGGAGTCCGTG R: CAAGAATTCGTCTGGCATGCACCTTTCC

[1] F, forward primer; R, reverse primer; underlined, restriction enzyme sites; bold, Kozak sequences for mammalian cell expression; bold and italicized, point mutations.

[2] The first-step PCR amplification used primer pairs Etf-1-Opt-F/ΔCoil-1R and ΔCoil-1F/Etf-1-Opt-R, and both first-step PCR products were combined and further amplified by the second-step PCR using primer pairs Etf-1-Opt-F and Etf-1-Opt-R.

Cloning anti–Etf-1 Nbs into pEGFP-C1 for expression in mammalian cells	
Full length	F: CTAGCTAGCGCTACCGGT**CGCCACCATGG**CCCAGGTGCAGCTGCAG R: CCGGAATTCTTATCAGGAACCGTAGTCCGGAAC

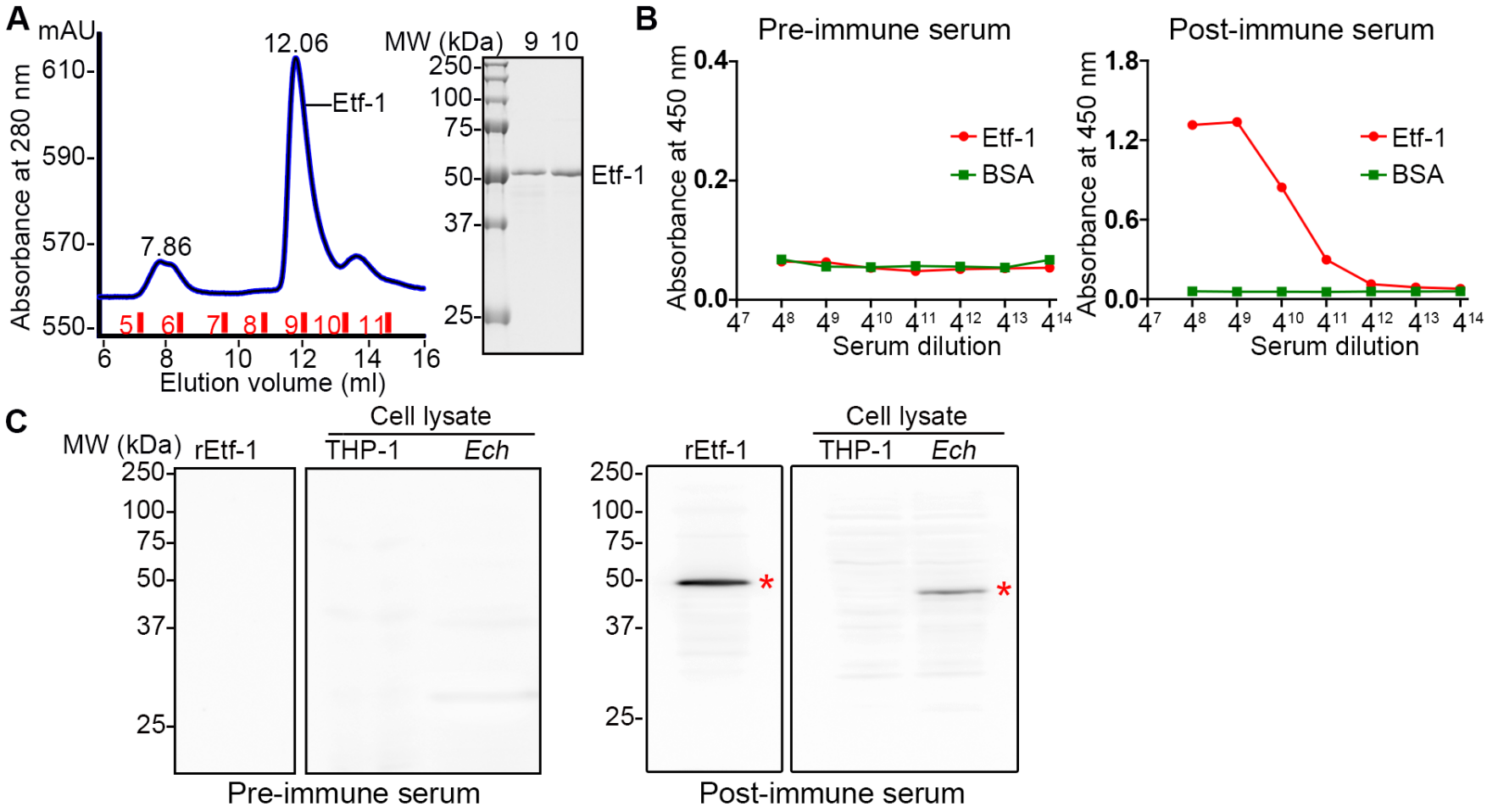

FIG S1. Llama immunization with rEtf-1. (A) The recombinant full-length Etf-1 protein (rEtf-1, 380 aa) was purified by gel filtration. The indicated peak fractions (fractions 9 and 10) were analyzed by SDS-PAGE and Coomassie blue staining. (B) Etf-1-specific antibody response of pre- and post-immune serum was analyzed by ELISA, and BSA was used as a negative control. Sera samples were prepared as four-fold serial dilutions. (C) Western blot analysis of the llama pre- and post-immune sera against recombinant and native Etf-1. *Ech*, cell lysate of *E. chaffeensis*-infected THP-1 cells at 3 dpi; THP-1, uninfected THP-1 cell lysate. Red asterisks, recombinant and native Etf-1. Llama sera were pre-adsorbed using uninfected THP-1 cell lysates to reduce non-specific interacting bands.

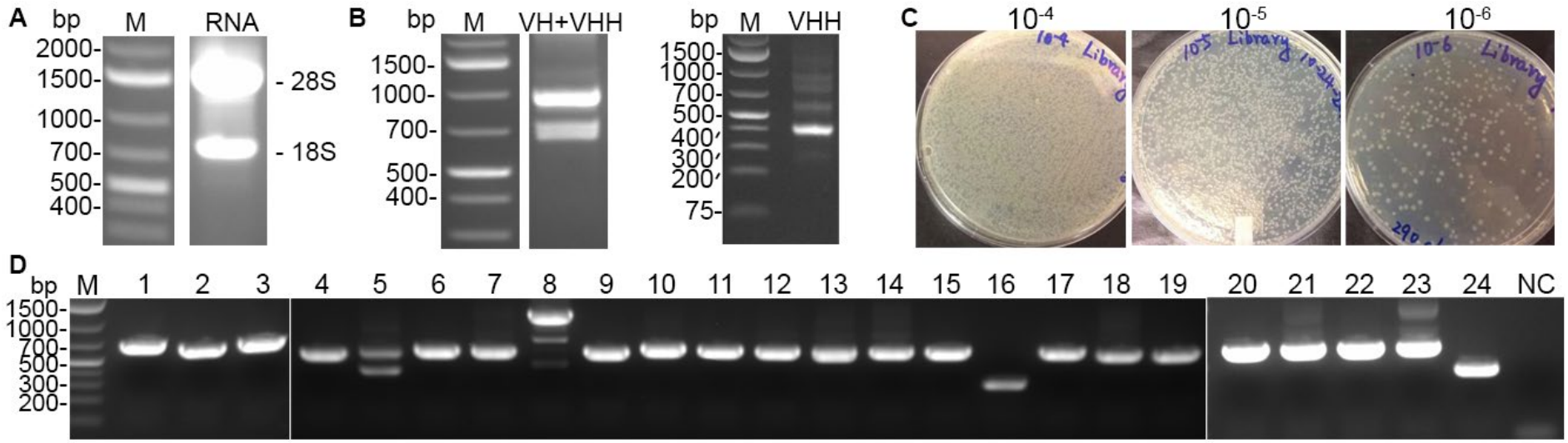

FIG S2. Cloning of anti–Etf-1 Nbs from the llama immune library. (A) Agarose gel electrophoresis of total RNA purified from PBLs of the Etf-1 protein–immunized llama. M, molecular weight marker (GeneRuler 1 kb Plus DNA Ladder). (B) Agarose gel electrophoresis of the variable domains of all immunoglobulin heavy chains (VHs and VHHs) amplified from cDNA (VH + VHH, left), and Nb-encoding genes re-amplified from the gel-purified VH + VHH PCR products (VHH, right). (C) Library size determination by serial dilutions shown as colonies on LB-agar plates (290 clones at 10^{-6} dilution). (D) Colony PCR for assessing the percentage of clones in the library with the proper insert. Twenty-four isolated colonies were randomly picked from the transformants, and DNA was extracted from each. The amplicon size for clones with a Nb gene insert is ~700 bp. Transformants without a Nb gene insert have a smaller amplicon size (~350 bp).

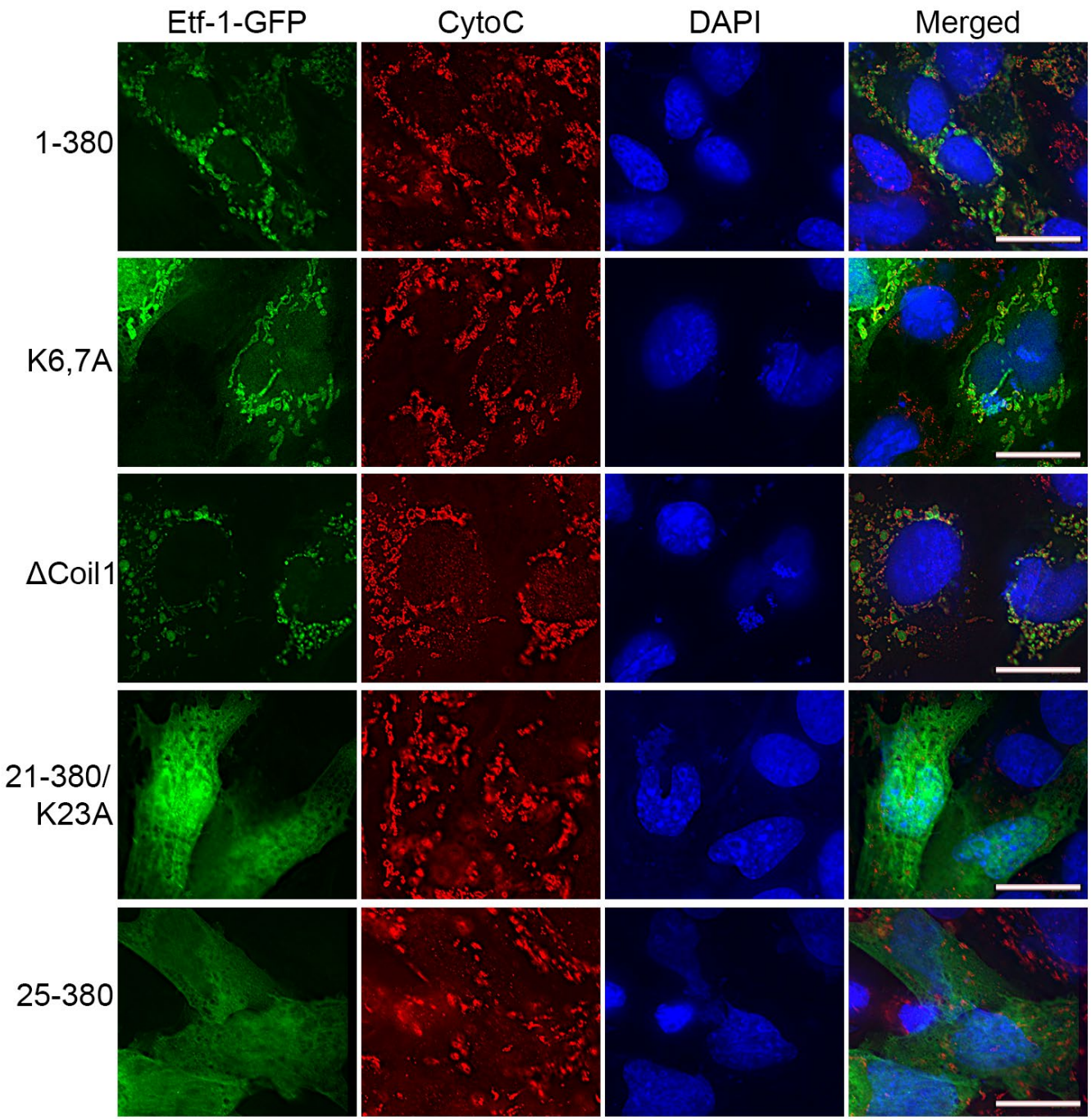

FIG S3. K23 within the N-terminal 24 aa of Etf-1 is critical for mitochondrial targeting. Immunofluorescence labeling of RF/6A cells transfected with truncated, internal deletion, or point mutations of Etf-1–GFP (as described in Fig. 3). Cells were labeled with mouse monoclonal anti–cytochrome *c* (CytoC; red). Representative images show the presence (full-length, 1-380 aa; K6,7A; and ΔCoil1) and absence (21-380/K23A and 25–380 aa) of Etf-1 colocalization with mitochondria. Colocalization was quantified and is presented in Fig. 3. Scale bars: 15 μm.

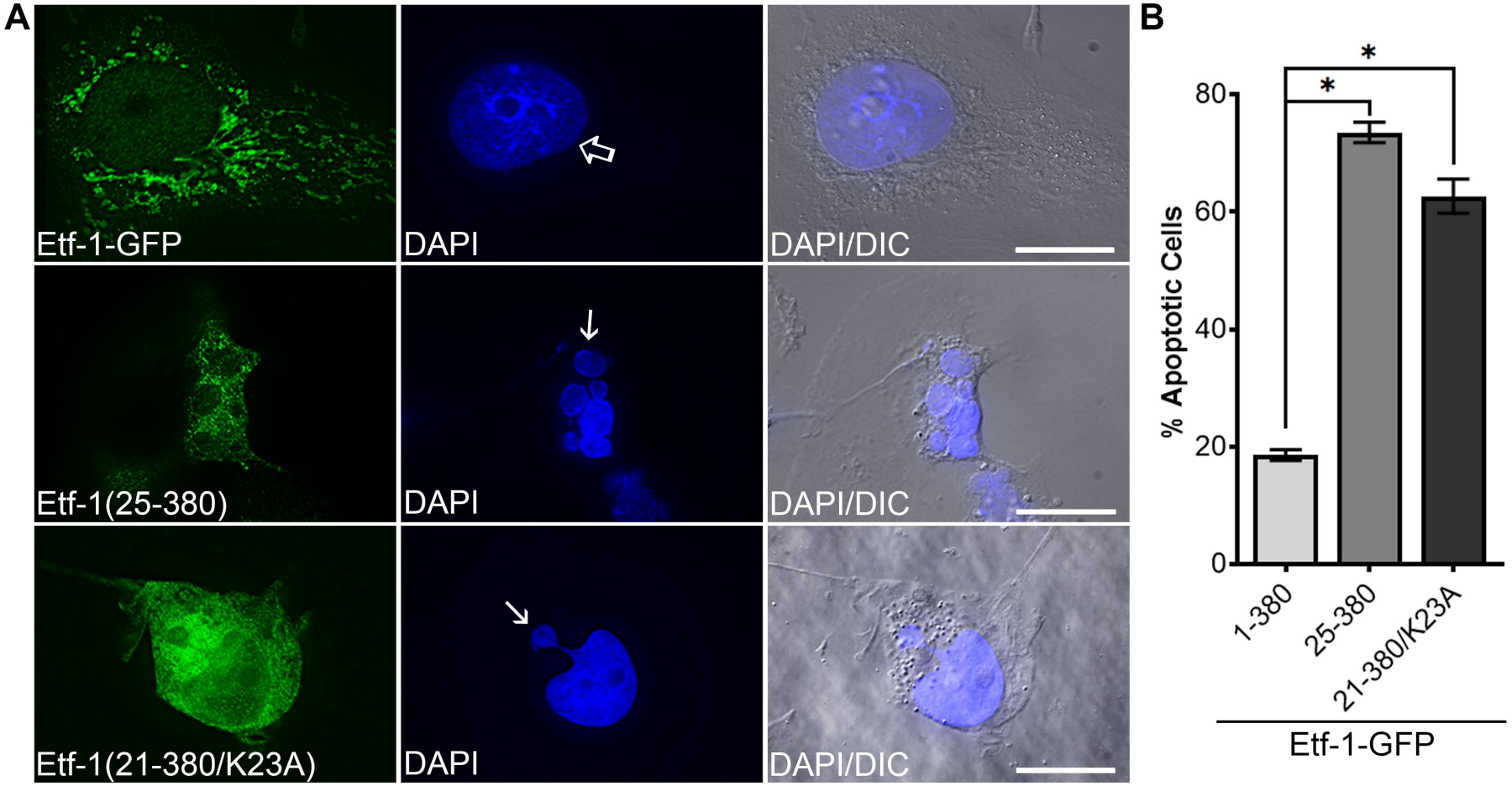

FIG S4. K23 within the N-terminal 24 aa of Etf-1 is critical for inhibition of etoposide-induced apoptosis.
(A) RF/6A cells were transfected with plasmids expressing Etf-1-GFP (full length, 1-380 aa), Etf-1(21-380/K23A)-GFP, or Etf-1(25-380)-GFP. At 28 h pt, cells were treated with 100 μM etoposide for 41 h. Cells were fixed by 4% PFA, and the host cell nuclei were stained with DAPI. Immunofluorescence labeling of RF/6A cells. DIC, differential interference contrast; arrows: apoptotic nuclei; open arrow: normal nuclei. Scale bar, 10 μm. (B) Quantification of apoptosis (nuclear fragmentation) in 100 cells expressing transfected genes. Data are represented as the mean ± standard deviation (n = 3). * $P < 0.05$ by one-way ANOVA.

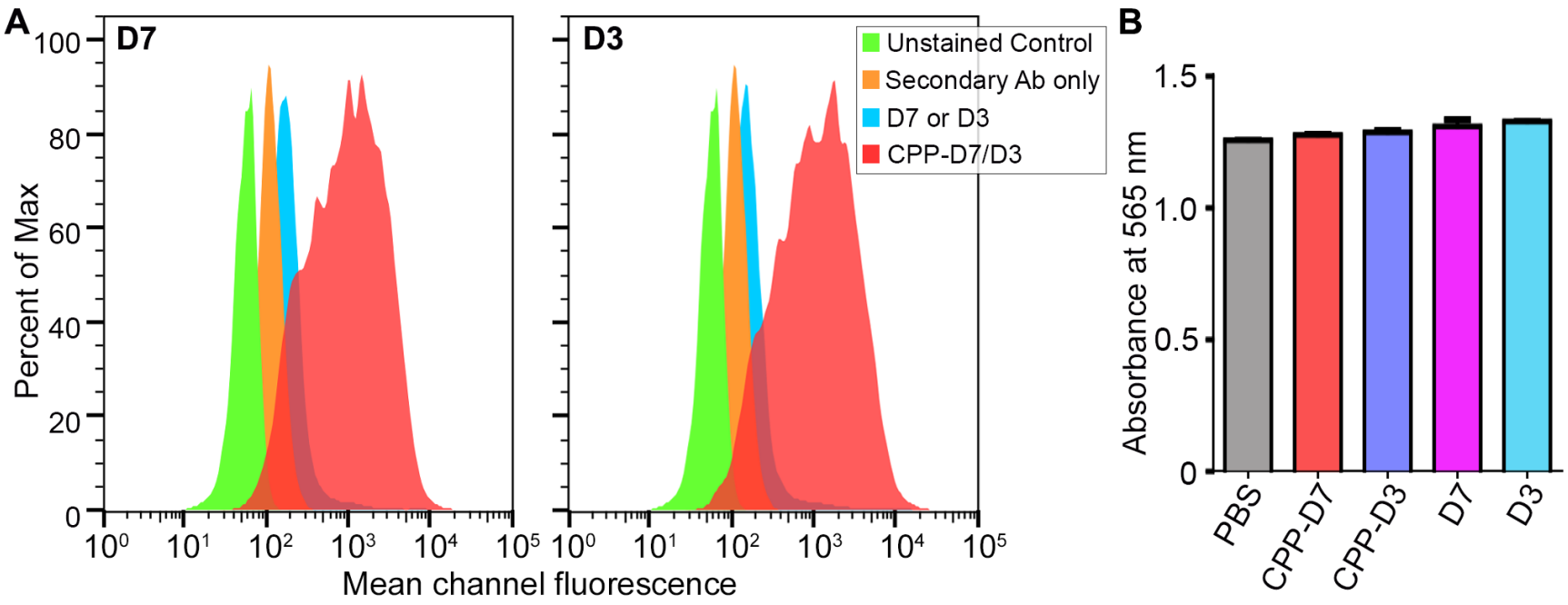

Fig. S5. CPP-Nbs have high intracellular uptake efficiency without any cytotoxicity. (A) Flow cytometry histograms showing intracellular levels of CPP-Nbs and of Nbs alone. There was a substantial increase in the fluorescence of cells incubated with CPP-Nb as compared with that of cells incubated with Nb or PBS with/without secondary antibody labeling. (B) Cytotoxicity of CPP-Nbs, Nbs, and PBS was measured using the MTT assay. Values are represented as the mean ± standard deviation (*n* = 3).