

# NACA (Nascent-Polypeptide-Associated Complex $\alpha$ Subunit) Against Apoptosis in B Lymphoma Cell is Independent of $\beta$ Subunit (NACB)

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**Abstract:** We found depletion of NACA in two kinds of B lymphoma cell lines, Raji and Kapas, were able to induce apoptosis in this study. We also explored whether depletion of  $\beta$  subunit had the same effect, and we were interested in which domain of NACA was potentially responsible to this anti-apoptosis function. Lentivirus-based shRNA was used to deplete endogenous NACA or NACB. Those cells' viabilities were measured by Alamar-blue™ assay. Cell apoptosis was identified by molecular markers caspase9 and PARP, as well as cellular markers Annexin V and propidium iodide (PI) staining. NACA mutants were constructed by PCR site-directed mutagenesis and delivered into cells by Lentivirus. Immunofluorescence was used to investigate cellular distribution in 293FT cells. Our results demonstrated that the depletion of NACA, but not NACB, was able to induce apoptosis. Deletion of middle or C-term rather than N-term induced obvious apoptosis. The middle part of NACA was response to bind NACB and form a complex. Without middle part, NACA redistributed into nuclei. We conclude NACA against apoptosis is independent of  $\beta$  subunit. C-term of NACA, which is identified as ubiquitin binding domain, and may take important role in anti-apoptosis function.

**Keywords:** NACA, B-cell lymphoma, anti-apoptosis, depletion mutant.

## INTRODUCTION

NAC (nascent-polypeptide-associated complex) is a hetero-dimeric complex that was identified in higher eukaryotes as a ribosome-associated factor in close proximity to nascent chains [1, 2]. NAC containing  $\alpha$  and  $\beta$  subunit interacts with unfolded polypeptide chains independent of their amino acid sequences [2]. What ribosome-bound NAC does exactly *in vivo* is poorly understood and remains controversial. In addition, the individual subunits of NAC have been implicated in different processes related to transcriptional regulation.

Further studies revealed that NACA functions as a transcriptional co-activator in osteoblasts [3, 4] through an interaction with phosphorylated c-Jun, a member of the activator-protein-1 (AP-1) family. A recent study indicated that NACA also regulates the function of the adaptor protein Fas-associated death domain (FADD) [5]. These data suggest that NACA participates in several protein complexes by playing a role in proliferation, apoptosis or protein degradation, depending on the cellular context and stimuli. Recent study showed that hypoxia down-regulates NACA but not NACB, and NACA down-regulation in hypoxic cells or NACA ablation by NACA short-interfering RNA

(siRNA) leads to the initiation of ER stress responses, which result in caspase-dependent apoptosis [6].

In clinic, recent studies revealed NACA may also play a pivotal role in both the transcriptional and post-transcriptional regulation of genes associated with the formation of malignant gliomas [7], breast adenocarcinoma [8] and Juvenile myelomonocytic leukemia [9].

NACB alone directly binds to the ribosome and is sufficient to prevent ribosome binding to the endoplasmic reticulum membrane [10]. The function of NACA with or without NACB largely remains unknown. In this article, we have determined the anti-apoptosis function of NACA is NACB independent and NACA might associated with its C-terminal UBA domain.

NACA was found overexpressed in B-cell acute lymphoblastic leukemia by microarray screening, while the function of NACA in blood cancer is unclear [22]. In this study, we demonstrated that NACA has anti-apoptotic function in B-cell cancer cell line, and the function is independent of NACB.

## 1. MATERIALS AND METHODS

### 1.1. Reagents and Cell Culture

All enzymes have been purchased from Takara (Dalian, China). The antibodies have been purchased from Cell Signaling (MA, USA). Raji and Kapas cell lines were obtained from the American Type Culture

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Collection (ATCC, VA, USA) and maintained in RPMI1640 (Thermo Scientific, MA, USA). 293FT cells (Clontech Laboratories Inc., CA, USA) were maintained in DMEM (Thermo Scientific, MA, USA). Cell morphology was monitored using microscopy, and the cell morphological images were compared to the original images. Hypoxia at 1% O<sub>2</sub> was carried out at 37°C in 95% humidity and 5% CO<sub>2</sub>, 94% N<sub>2</sub> in an airtight anaerobic incubator (Thermo Scientific, MA, USA). Cell viability was measured with Alamar Blue assay (Invitrogen, CA, USA). For cell growth curve assay, 1X10<sup>3</sup> cells were seeded into each well of 96 well plates, and triplicated wells were set for each sample. Cell numbers were counted every day. Error bars were drawn from three independent experiments.

### 1.2. PCR Directed Mutagenesis

Human (or mouse) NACA full-length open reading frame and deletion mutants were constructed by PCR site-directed mutagenesis. All primers were listed in Table 1.

### 1.3. Lentivirus Construction, Packaging and Infection

Oligo nucleotides(GFP\_KD\_F: 5'-ccggAAGCTGAC CCTGAAGTTCATCctcgagGATGAACTTCAGGGTCAG

CTTtttt; GFP\_KD\_R: 5'-aattaaaaAAGCTGACCC TGAAGTTCATCctcgagGATGAACTTCAGGGTCAGCT T; NACA\_KD652\_F: 5'-CCGGCCTGCTTCAGATACTT ACATACTCGAGTATGTAAGTATCTGAAGCAGGTTTT T; NACA\_KD652\_R: 5'-AATTA AAAACCTGCTTCAGA TACTTACATACTCGAGTATGTAAGTATCTGAAGCAG G; NACA\_KD901\_F: 5'-CCGGGCCCTGAAGAACA CAGTAATCTCGAGATTACTGTTGTTCTTCAGGGCTT TTT; NACA\_KD901\_R: 5'-AATTA AAAAGCCCTGAAG AACACAGTAATCTCGAGATTACTGTTGTTCTTCAG GGC; NACB\_KD599\_F: 5'-CCGGTGAGTCAACTTCT GAAGATAACTCGAGTTATCTTCAGAAGTTGACTCAT TTTT; NACB\_KD599\_R: 5'-AATTA AAAATGAGTCAAC TTCTGAAGATAACTCGAGTTATCTTCAGAAGTTGAC TCA; NACB\_KD186\_F: 5'-CCGGCGCAGAAAGAAGA AGGTGGTTCTCGAGAACCACCTTCTTCTTTCTGCG TTTTT; NACB\_KD186\_R: 5'-AATTA AAAACGCAGAAA GAAGAAGTGGTTCTCGAGAACCACCTTCTTCTTT CTGCG) were commercially synthesized (Sangon, shanghai, China) and annealed, followed by insertion into AgeI/EcoRI double digested pLKO.1 lentivirus backbone. The purified lentivirus plasmids were introduced into 293FT cells with delta8.9 and VSVG plasmids. The media containing virus were collected after 72 hours and incubated with lymphoma cells for 12 hours. After 24 hours, puromycin was added into culture media to select infected cells for 3 days.

**Table 1: Oligos**

Name	Sequence
hNACA_Bam_F	5'-AAGGATCCATGCCCGGCGAAGCCAC-3'
hNACA_Eco_R	5'-GCCCGCAATTCTTACATTGTTAATTCCATAATCGC-3'
mNACA_Bam_F	5'-CTGGATCCAAAATGCCCGGTGAAGC-3'
mNACA_Eco_R	5'-GCGCTGCCGAATTCTTACATTGTTAATTCC-3'
mNACA_D1-58_F	5'-GCAGGATCCATCGATGAAGAACCTGTTAG-3'
mNACA_D35-80_F	5'-ACAGTGATGAGTCAATGTCCAACTGGG-3'
mNACA_D59-88_F	5'-AGCCGCAGCAGAGACAGGGGTTACGA-3'
mNACA_D81-128_F	5'-GTCTTCGACAGGTTGAAGATTTGTCTCAG-3'
mNACA_D89-144_F	5'-GGAAGCCAAGATTGTTCAAGTGAAGC-3'
mNACA_D129-179_F	5'-GCTGAGAAATTC AAAGACATAGA ACTGGTC-3'
mNACA_D35-80_R	5'-GACCCAGTTTGATGACTCATCACTGTCA G-3'
mNACA_D59-88_R	5'-TCGTAACCCCTGTCTCTGCTGCGGCTG-3'
mNACA_D81-128_R	5'-TGAGACAAATCTTCCATAGCCTTCTTGCCTT-3'
mNACA_D89-144_R	5'-GCTTCACCTTGAACAACTGTCAAGACCC-3'
mNACA_D129-179_R	5'-GACCAGTTCTATGTCAATCTTGGCTTCCCCA-3'
mNACA_D145-215_R	5'-TCGAATTCAGTTTTGAATTTCTCAGCAGCTGC-3'
mNACA_D180-215_R	5'-TCGAATTCCTTA ACTTCCACACCCGTCTC-3'

#### 1.4. Western Blot

Cells were scraped from culture dishes after PBS washing, and transferred into 1.5ml microtubes. RIPA buffer were added into tubes and mixed well, and then left on ice for 30 min. After centrifugation at 4°C, supernatants were transferred into new tubes. Cell lysates were mixed with loading buffer and boiled at 95°C for 10 min, and then loaded on 12% polyacrylamide gel. Western blot were performed following standard method with Bio-Rad mini system and ChemiDoc XRS<sup>+</sup> system (Bio-Rad, CA, USA). NACA antibody (Santa Cruz Biotechnology, Inc., CA, USA), Caspase9 antibody (Santa Cruz Biotechnology, Inc., CA, USA), cleaved PARP antibody (Cell Signaling, MA, USA),  $\beta$ -actin antibody (Sigma, Germany) was diluted at 1:500, 1:500, 1:1000 and 1:10000, respectively.

#### 1.5. Immunofluorescence Staining

293FT cells grown on coverslips were transfected with plasmids encoding mNACA ORF or deletion mutants. At 36 hours post-transfection, cells were washed in phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde for 15 min. After washing with PBS for 3 times, the cells were permeabilized with 0.5% Triton X-100 in PBS for 10 min and incubated in blocking buffer supplemented with 1%BSA for 1h at room temperature. The slides were incubated for 1h with anti-Flag (anti-rabbit, Sigma, 1:200), followed by a mixture of Fluorescein isothiocyanate (FITC)-conjugated secondary antibodies for 1h at room temperature. In order to preserve the fluorescence for a long time, the slides were covered with VECTASHIELD<sup>R</sup> Mounting medium with DAPI (Vector Laboratories) and coverslips. The stained sections were examined with an inverted fluorescence microscope (Nikon).

#### 1.6. Semi-Quantitative PCR

NACA or NACB mRNA levels were measured by semi-quantitative PCR with corresponding primers (NACA\_RT\_F: 5'-GAAACAGGTGTAGAAGTTAAGGACATTG; NACA\_RT\_R: 5'-ATTGTTAATTCCATAATCGC; NACB\_RT\_F: 5'-GACGCTGTCTCAACCTCCACCTC; NACB\_RT\_R: 5'-GCTGGGTAGCATTCTGTCA GCTG).

## 2. RESULTS

### 2.1. Depletion of NACA in B Lymphoma Cells Induces Cell Growth Inhibition

NACA was knocked down in B lymphoma cell Raji, by lentivirus-based method. The efficiency of two

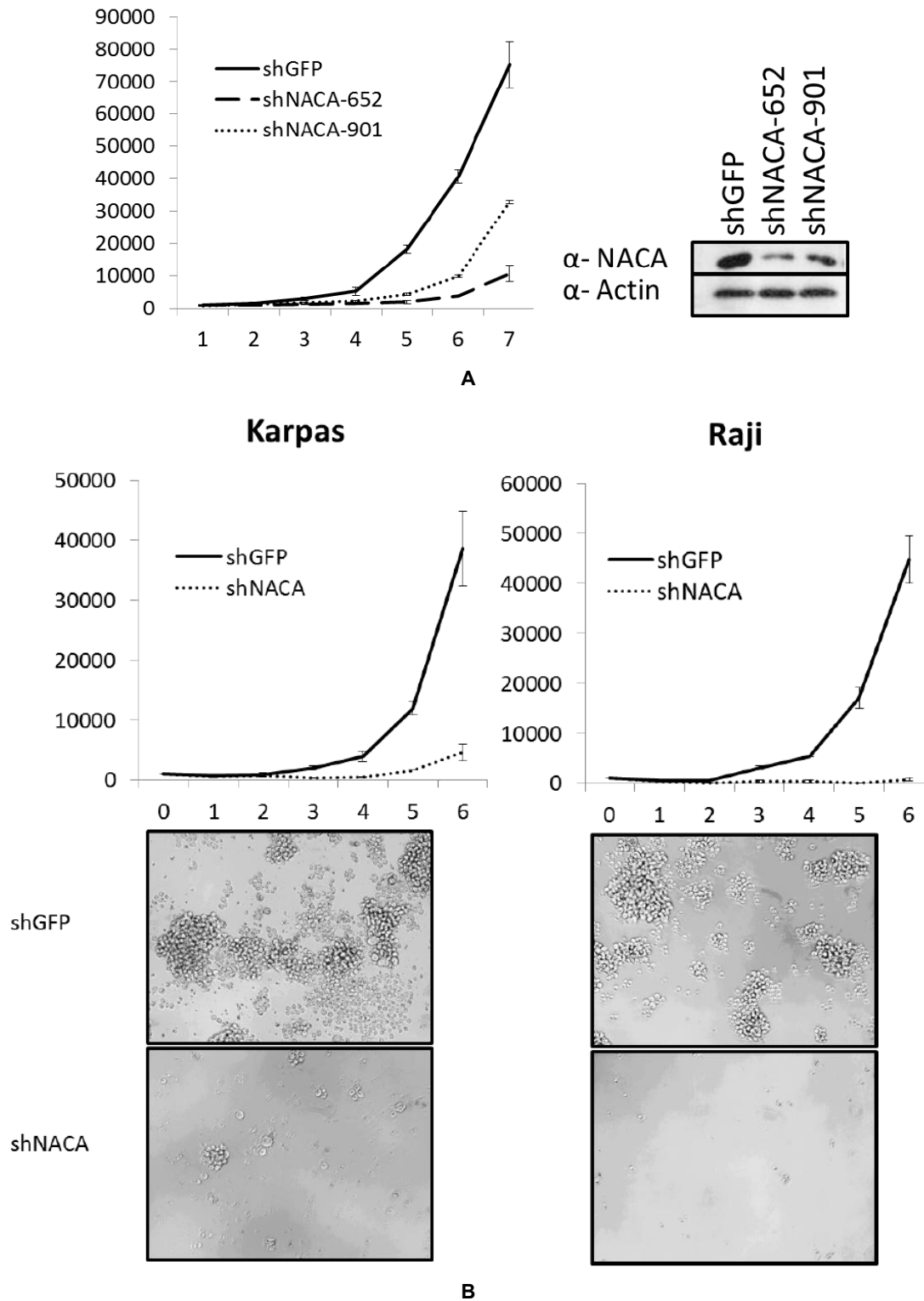
different shRNA sequences was investigated by Western blot. Both shRNAs were able to decrease NACA protein level over 70% (Figure 1A, right panel), and both cell lines decreased cell growth by silencing NACA in a similar manner. The sequence 652 demonstrated elevated depletion efficiency and decreased cell growth rate (Figure 1A, left panel). To avoid the cell line specific effect, we depleted NACA in Raji as well as Kapas by mixed shRNAs containing both shNACA-652 and shNACA-901. Both Kapas and Raji cells showed growth inhibition (Figure 1B, upper panel), and fewer cells survived under NACA depleted condition (Figure 1B, lower panel). Comparing single-target sequence, mixed shRNAs showed better effect on cell growth inhibition in Raji cell (Figure 1A and 1B). As such, we used mixed shRNAs in subsequent experiments.

### 2.2. The Depletion of NACA, but not NACB, Induces Cell Apoptosis

To clarify the mechanism of NACA depletion inhibiting cell growth, NACA depleted cells and control Raji cells were collected and investigated by two different methods. Western blot showed higher caspase 9 protein level and higher activity in shNACA cell. PARP, which is the substrate of caspase enzyme commonly used as apoptotic marker, showed clear cleaved band in shNACA cells (Figure 2A). We also used immune-fluorescence method to investigate annexin V, which would expose on apoptotic cell surface, and PI staining, which was excluded by living cells. shNACA cells showed higher annexin V and PI signals comparing to shGFP cells (Figure 2B). To investigate the NAC complex function in this apoptotic effect, we depleted beta subunit of NAC (Figure 2C, left panel). The depletion efficiency of NACB was similar with NACA depletion, while shNACB cells showed similar annexin V and PI signals comparing to shGFP cells (Figure 2B). Correspondingly, the growth curve of NACB depleted cells is similar with shGFP cells (Figure 2C, right panel).

### 2.3. Exogenously Express NACA Protein without C-Terminal Decreased Cell Growth

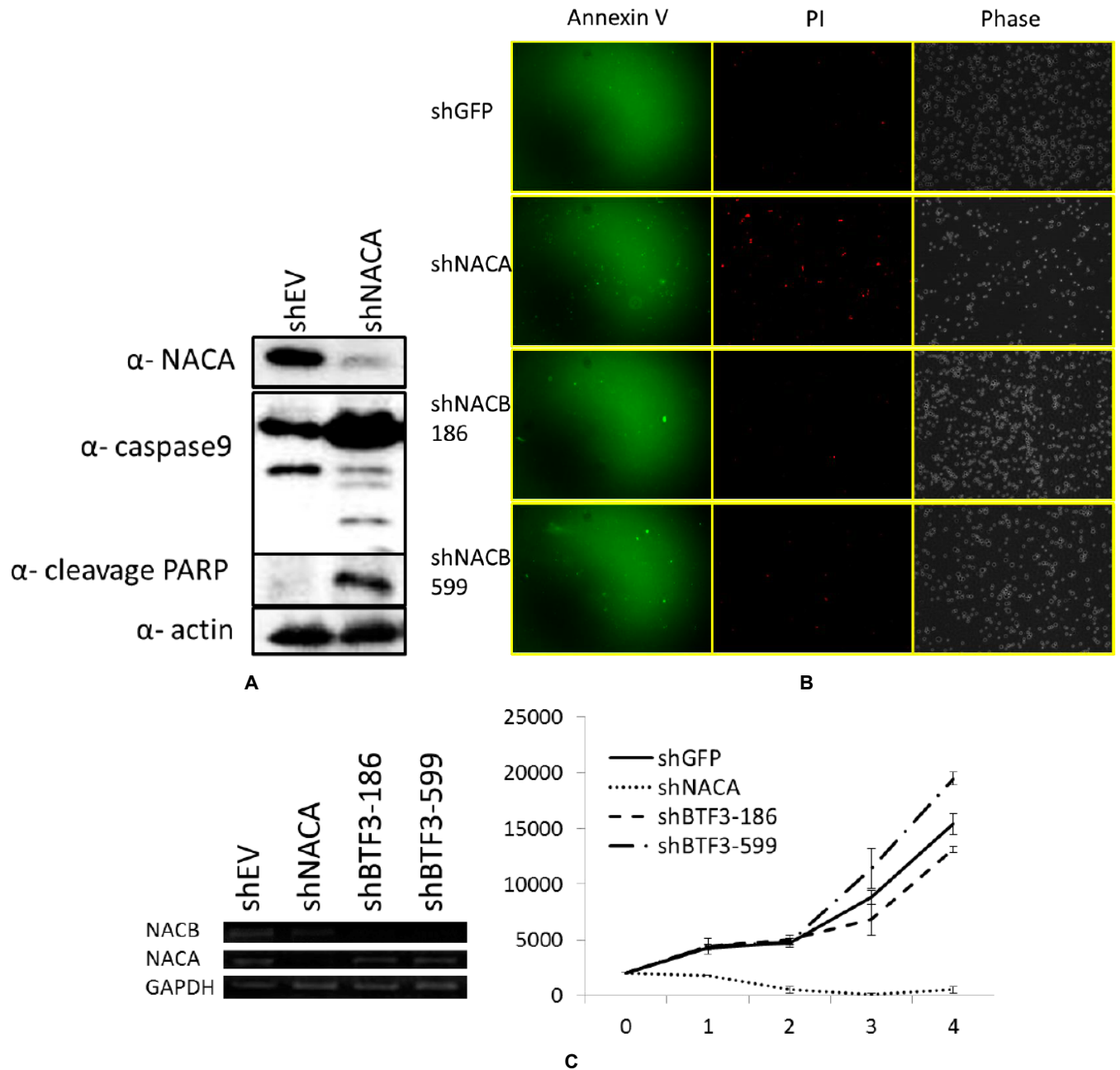
NACA has multiple functions based on cellular distribution and binding partner. To further investigate which region is apoptosis-associated, we constructed a series of single domain deletion mutants (Figure 3A). Flag tag was used as ectopic marker for western blot (Figure 3B). Cells, which expressed exogenous full NACA protein or mutants without amino acids 1-118,



**Figure 1: Depletion of NACA in B lymphoma cells induces cell growth inhibition.**

**A.** B lymphoma cell line Raji were individually infected with lentivirus containing the sequence encoding small hairpin RNA (shRNA) against NACA at different target sites. For Left panel, after selection, survive cells were collected and seeded into 96 well plates as 1000 cells/well. Cell number counting was conducted every day. Triple cases would be set for each sample, and error bar would be drawn from 3 independent experiments. For right panel, after selection, half mounts of cells were collected and subjected to western blot. The membrane was probed with NACA or actin antibody to investigate the efficiency of knocking down.

**B.** B lymphoma cell line Raji and Kapas were individually infected with mixed lentivirus containing the two different targets using in Figure 1A. Cell growth curve would be drawn by same way with Figure 1A.



**Figure 2: Depletion of NACA in Raji cells induces apoptotic cell death, while depletion NACB cannot.**

**A.** Raji cells were infected with mixed lentivirus containing the two different targets or shGFP. After selection, survive cells were collected and subjected to western blot. The antibodies against apoptotic marker caspase9 or cleaved PARP were used to probe membrane.

**B.** Raji cells depleted NACA or NACB were incubated with apoptotic assay solution containing green fluorescence conjugated Annexin V antibody and PI for 30 minutes, then were observed under fluorescence microscope.

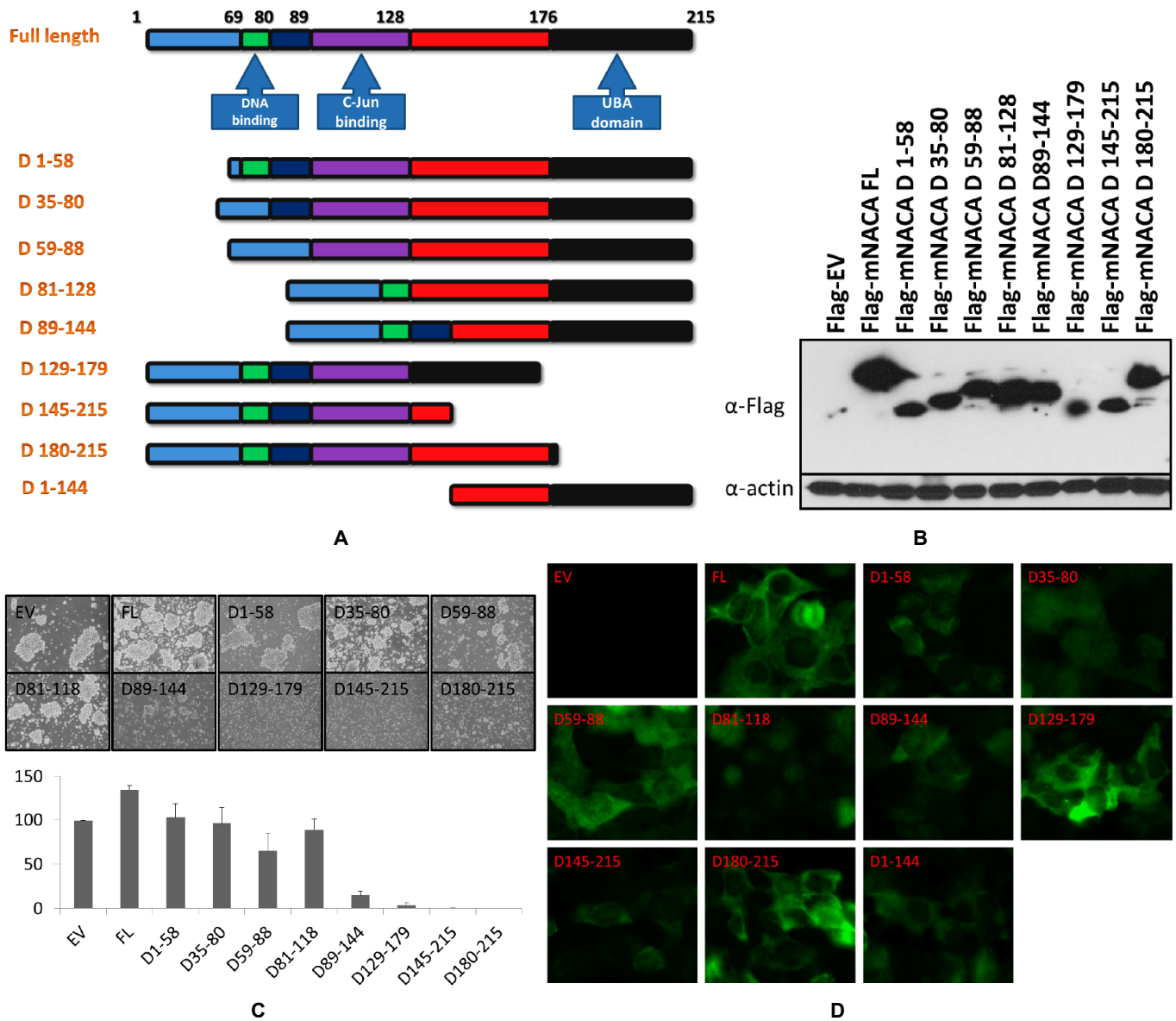
**C.** Left panel shows mRNA level of NACA or NACB in shGFP, shNACA or shNACB cells lines, GAPDH level was served as control. Right panel shows those cells' growth curve.

grew like control cells. While the mutants without amino acids 119-215 largely inhibited cell growth (Figure 3C).

#### 2.4. The Region 35-118 of NACA is Related to Cellular Distribution

NACA has different binding partner in cytoplasm or nuclei. Immunofluorescences were done to detect the

cellular distribution of NACA mutants. Full NACA protein showed majorly cytoplasm localization, as well as the mutants without amino acids 1-58, 89-144, 129-179, 145-125, 180-215 or 1-144. While three mutants, D35-80, D59-88 and D81-118, showed nuclei localization (Figure 4).



**Figure 3: C-terminal of NACA is important for its anti-apoptosis activity.**

**A.** The information of NACA conserved domains were shown at the top of graph, and all deletion mutants of NACA were followed.

**B.** Raji cells were infected with lentivirus expressing NACA full length ORF or deletion mutants. After selection, cells were collected and lyzed in RIPA buffer. Total proteins were subjected to western blot. Flag antibody was used to probe membrane to detect flag-tagged NACA protein.

**C.** Raji cells were infected with lentivirus and selected for 72 hours. All the stable cell lines expressed NACA full or deletion mutant proteins were seeded into 96 well plates as 1000 cells per well, medium were changed every day. After 5 days, cell numbers were counted. Error bars were drawn from 3 times independent experiments.

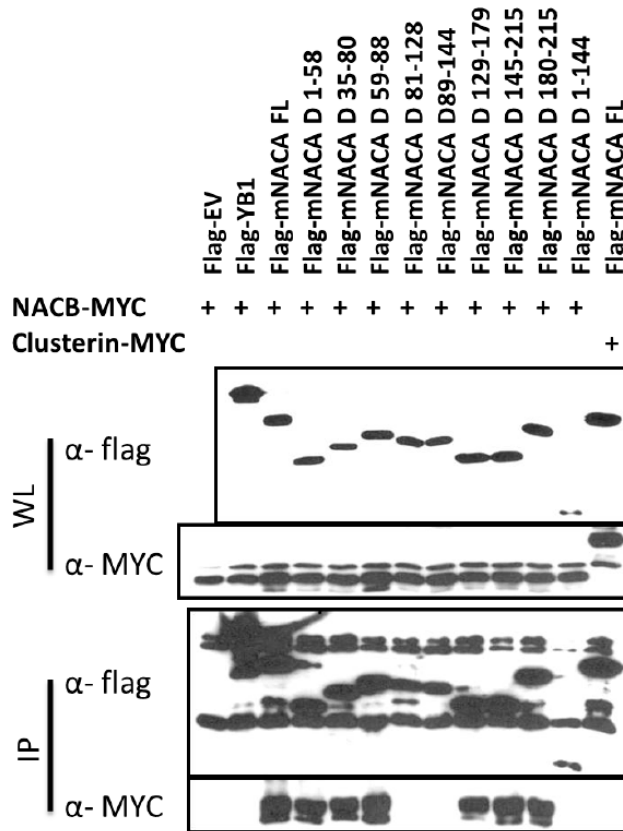
**2.5. The Amino Acids 81-144 of NACA are Response to Bind NACB**

Flag tagged NACA or deletion mutants co-expressed with MYC tagged NACB in 293FT cells. IP results showed that NACA cannot bind to protein clusterin, and NACB cannot bind to YB1. Both clusterin and YB1 would serve as negative control to eliminate the non-specific binding between two highly expressed exogenous proteins. NACA was able to bind NACB

even without N-terminal or C-terminal, but the binding affinity was completely lost when the middle part of NACA was deleted.

**3. DISCUSSION**

Nascent-polypeptide-associated complex was linked with ribosome and newly synthesized peptide over twenty years [2], while recent studies revealed



**Figure 4: NACA cellular distribution is regulated by middle part.** 293FT cells were transfected with flag tagged NACA full length ORF or deletion mutants. After 48 hours, cells were fixed and continued to immunofluorescence assay.

single-subunit of NAC has unique function that is independent of complex, such as apoptosis and differentiation [10]. NACA depletion has been reported to be an initiator of apoptosis under hypoxia in mouse brain [6]. Nonetheless, few evidence supported NACA function in cancer cell apoptosis. Based on bioinformatics analysis ([www.oncomine.org](http://www.oncomine.org)), NACA was significantly upregulated in multiple types of cancer, especially in B-cell leukemia. From the study comparing pre- and immature- B-lymphocyte with cancer cells from 18 cases of B-cell acute lymphoblastic leukemia patients, NACA overexpressed in all patients ( $p=1.67E-6$ ). To investigate the underlying mechanism of NACA elevated expression, we depleted NACA in B-cell lymphoma cell lines and found NACA was essential for Raji and Kapas cells survival.

NACA has dual function, dependent or independent of NAC complex. By dependent of NAC complex, NACA cooperates with NACB and protects newly synthesized peptides, or directs parts of those proteins to destination [11]. Recently, accumulating publication

showed that NACA had nuclei function as transcription co-effector with c-Jun or histone deacetylase to control osteocalcin genes' expression [12, 13]. Beside c-Jun, TBP was identified as binding partner of NACA in nucleus [4]. Interestingly, NACA was proved to play a role in apoptosis induced by hypoxia in mouse brain. A group reported NACA was able to bind FADD, and may contribute to cell apoptosis [5]. To clarify the role of NACA in apoptosis, we first checked cell status under NACB depletion, and found knocking NACB did not induce cell death in B lymphoma cells. This observation suggests NACB is not required for B lymphoma cell survival, and the anti-apoptosis function of NACB would be independent of NAC complex. Further investigation based on functional domains deletion showed amino acids 81-118 is a key region of NACA cytoplasmic localization as well as NACB binding. Thus, when those deletion mutants were introduced into Raji cells, C-term of NACA deletion mutants induce cell death but not AA81-118 mutant. That suggested the anti-apoptosis function of NACA might be not associated with nuclei distribution as well as NACB binding.

C-term of NACA harbors an UBA domain, which is one kind of ubiquitin binding domains that has been proved to bind ubiquitin chain. Interestingly, NACA itself has ubiquitin modification site [14-16]. Ubiquitin chain mediating protein degradation is important in cellular process including apoptosis [17-21]. It is possible that NACA binds to some proteins and mediates their degradation, then contributes to anti-apoptosis function.

In conclusion, NACA is highly overexpressed in B-cell leukemia patients, and is required for cancer cell survival. Transiently inhibiting NACA not NACB by shRNA induces B lymphoma cell apoptosis. The anti-apoptosis function of NACA is unassociated with nuclei distribution and NACB. Further study would be performed to investigate the mechanism on anti-apoptosis function of NACA, and FADD binding of NACA might be the potential candidate.

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## POTENTIAL CONFLICTS OF INTERESTS

The authors declare no conflict of interests.

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