

**m-Bop, a repressor protein essential for cardiogenesis, interacts
with skNAC, a heart- and muscle-specific transcription factor**

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Abstract

The m-Bop protein encoded by the mouse *Bop* gene is strongly expressed in heart and skeletal muscle, and recent studies with *Bop* knockout mice have demonstrated that m-Bop is essential for cardiogenesis *in vivo* and can act as a HDAC-dependent repressor *in vitro*. In the present studies, m-Bop was observed to interact with skNAC, a reported transcriptional activator specific to heart and skeletal muscle. The amino-terminal S region of m-Bop's split S-ET domain as well as its MYND domain were required for interaction with skNAC in both the two-hybrid system and in co-immunoprecipitation experiments from cultured mammalian cells. As shown previously for interaction of the MYND domain-containing transcriptional co-repressor, BS69, with several viral and cellular oncoproteins, a PXLXP motif in skNAC was required for interaction with m-Bop. Similar kinetics of induction and localization of m-Bop and skNAC during the induction of myogenesis in cultured C2C12 cells suggests a possible associated role for these proteins during this process.

Introduction

The control of skeletal and cardiac maturation is ordered in a complex cascade of transcriptional activation and repression. The MyoD and MEF2 families of transcription factors dictate important developmental events that result in the formation of mature skeletal muscle. The MyoD family is comprised of the basic helix-loop-helix (bHLH) proteins MyoD, Myf5, MRF4, and myogenin. These myogenic regulators form heterodimers with the ubiquitous bHLH E proteins that subsequently activate key elements needed for the myogenic program (1). The MEF2 family is defined by the MADS domain and consists of MEF2A, MEF2B, MEF2C, and MEF2D. Members of the MEF2 family are expressed in tissues besides skeletal muscle including cardiac tissue, neurons, and T cells (2). Together, the MyoD and MEF2 families cooperate directly and indirectly to transduce the requisite signals for proper skeletal muscle formation.

The discovery of chamber specific transcription factors, such as the Hand1 and Hand2 proteins, has revealed the complex nature of cardiac transcriptional regulation (3). The list of genes that correspond to specific cardiac defects continues to grow while the molecular nature of these defects remains largely elusive (4). To further understand the molecular underpinnings of cardiac development, it is critical to identify the relationships of transcriptional regulators shown to be important in cardiac morphogenesis.

The *Bop* gene encodes distinct proteins expressed in skeletal and cardiac muscle, as well as in cytotoxic T lymphocytes (CTLs). The Bop proteins found in skeletal muscle (m-Bop) and CTLs (t-Bop) are identical over 90% of their primary amino acid sequence, differing only at their extreme amino terminus (5). Bop contains the evolutionarily conserved MYND and SET domains found in transcriptional regulators linked to development, chromatin stability, and

cancer. The MYND domains in the transcriptional regulators ETO (MTG8) and BS69 function as protein-protein interaction domains (6,7,8,9). The MYND of ETO has been implicated in the recruitment of HDACs (6,7,8), and in BS69, the MYND domain is required for interaction with proteins bearing the PXLXP sequence (10). The SET domains of several proteins, including SUV39H1, G9a, and Clr4, have been shown to function as histone methyltransferases (HMTases) (12,13). The significance of chromatin modifications in transcriptional regulation is currently the topic of great interest (14,15), and the presence in Bop of both MYND and SET domains implicated in histone deacetylation and methylation, respectively, suggests a role for Bop in chromatin regulation. Indeed, earlier studies have shown that m-Bop functions as a histone deacetylase-dependent transcriptional repressor (16). Targeted inactivation of the *Bop* gene in mice resulted in embryonic death of *Bop*^{-/-} null mice at approximately day E10.5 accompanied by failure of right ventricular development and accumulation of excessive extracellular matrix (16). *Bop*-null mice failed to express the bHLH-transcriptional regulator Hand2 in the heart, but not in the lateral plate mesoderm, suggesting that m-Bop plays an important specific role in the cardiac transcriptional cascade that leads to a functional adult heart (16).

The current studies demonstrate that m-Bop associates with the skeletal muscle- and heart-specific transcription factor skNAC. skNAC is an isoform of the ubiquitous protein, α NAC (nascent polypeptide-associated complex) that was initially isolated as a heterodimeric complex that binds newly synthesized polypeptides emerging from ribosomes (17). Further studies suggested that skNAC functions as a transcriptional co-activator (18). skNAC has been implicated in controlling myogenic differentiation, and skNAC transcripts are expressed at high levels only in skeletal and cardiac muscle (18,19). skNAC mRNA is also induced in myoblasts of the panniculus carnosus within 12 hours following skin injury, a finding that may reflect an

early role for skNAC in muscle repair (19). We report here that m-Bop and skNAC interact *in vitro* and *in vivo* and display similar kinetics of expression during skeletal myogenesis in culture, suggesting an associated role for m-Bop and skNAC in striated muscle myogenesis. As observed for BS69 and related MYND domain-containing proteins, interaction of m-Bop and skNAC requires an intact m-Bop MYND domain and the presence of a PXLXP motif in skNAC.



Experimental procedures

Yeast two-hybrid screen

Protocols required for yeast growth, transformation, and reporter analysis were performed according to standard procedures (20). Yeast plasmid isolation was performed using a modified boiling lysis method (21). The GAL4 Matchmaker yeast two-hybrid system (Clontech, Palo Alto, CA) was used to identify m-Bop2 interacting proteins. The yeast strains Y190 and Y187 were used for library transformations and interaction confirmations. Library transformations were plated on plates supplemented with 25 mM 3-AT. Positive clones were identified by DNA sequencing (DNA Sequencing Facility, UT Austin) and queried using BLAST at the National Center for Biotechnology Information (NCBI) web site (<http://www.ncbi.nlm.nih.gov/>).

Cell culture

Cell lines were incubated at 37° C and maintained in an atmosphere of 5% CO₂ in air. Cells were cultured in DMEM media (Life Technologies, Rockville, MD) supplemented with 10% fetal bovine serum (Hyclone, UT), 1 mM L-glutamine, 1% non-essential amino acids (Life Technologies), penicillin, streptomycin, and fungizone (Life Technologies). 293T, a derivative of the human embryonic kidney cell line 293 and the mouse myoblast cell line C2C12 (cat # CRL-1772) were obtained from ATCC (Manassas, Virginia). C2C12 myoblasts were induced to differentiate by placing 80-90% confluent cells in DMEM supplemented with 2% horse serum.

Constructs

The sequences of all constructs were confirmed by DNA sequencing. m-Bop2 and t-Bop GAL4 fusion proteins were generated by PCR and restriction digestion with SmaI/SalI followed by sub-cloning into pAS2-1. The skeletal muscle cDNA library was cloned into pGAD10 using the

Two-Hybrid cDNA Library Construction Kit (Clontech). The mammalian expression vector pBK-CMV-(1-4-1)-t-Bop was described previously (Hwang, 1997). pBK-CMV-m-Bop2 was constructed by PCR using the 5' primer (rsBopskm2: GCTCTAGAGCACCATGGAGAACGTGGAGGTCTTC) and 3' primer (138165: TCACAGGAGCAGTCAA) using the mouse cDNA clone m2-5.2 as the template. The PCR product was digested with XbaI/BamHI and sub-cloned into pBK-CMV-1-4-1 (t-Bop) after cutting with NheI/BglII. m-Bop1 was made by digesting pBK-CMV-1-4-1 with AccI/SacI and sub-cloning into pBK-CMV-m-Bop2 cut with AccI/SacI. The pBK-CMV-m-Bop2 MYND mutants were generated by PCR using pBK-CMV-t-Bop MYND mutants as templates originally created by site-directed mutagenesis. The PCR products were cut with PstI/AccI and sub-cloned into pBK-CMV-m-Bop2 digested with PstI/AccI.

Full-length pCI-skNAC and pBK-CMV- α NAC vectors were supplied by Dr. René St-Arnaud (Shriners Hospital, Montréal, Québec; 18). FLAG-tagged α NAC was generated by digestion of pBK-CMV- α NAC with EcoRI/XhoI and sub-cloning the fragment into pCMV-Tag2B (Stratagene, La Jolla, CA) cut with EcoRI/XhoI. The FLAG-MscI truncation was generated by digesting pCI-skNAC with MscI/SmaI and sub-cloning the insert into pFLAG-CMV4 cut with EcoRI and blunt ended. To produce FLAG-E34, pGAD10-E34 (two-hybrid library) was cut with NotI and treated with Klenow fragment. The resulting fragment was inserted by blunt end ligation into pFLAG-CMV4 that had been digested with BglII. FLAG-N3 was generated by PCR and sub-cloned into FLAG-CMV4 cut with EcoRI/BglII. The FLAG-N4 and FLAG-N5 constructs were made by PCR and sub-cloned into pCMV-Tag2A (Stratagene) cut with EcoRI. m-Bop2-6XHis was cloned into pTP17 by PCR and digestion with SpeI/XhoI. A leucine to alanine point mutation in FLAG-E34 was generated to create FLAG-L1952A using

the GeneEditor in vitro Site-Directed Mutagenesis System (Promega, Madison, WI) according to the instructions from the manufacturer.

Transient transfection

LipofectAMINE (Life Technologies) was used to transiently transfect 293T cells according to the instructions of the manufacturer. 80-90% confluent cells were harvested and plated at a density of $2-2.5 \times 10^6$ cells per 100 mm plate 24 hours prior to transfection. 4-8 ug of total DNA was used per 100 mm plate. Cells were harvested 48 hours after transfection.

Antibodies

The hamster anti-Bop monoclonal antibody used for Western blotting, immunoprecipitation, and immunofluorescence has been described previously (Hwang, 1997). Rabbit anti-skNAC polyclonal antibody (UT143) was produced by immunization with a fusion protein consisting of GST fused to amino acids 1688-1995 of skNAC (Cocalico Biologicals, Reamstown, PA). The mouse monoclonal anti-FLAG antibody M5 (cat # F4042) used in Western blotting was purchased from Sigma (Saint Louis, MO).

Immunoprecipitations

Transiently transfected 293T cells were harvested 48 hours after transfection. Cell pellets were lysed in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% DOC, 50 mM Tris pH 8, 0.1% SDS) containing protease inhibitors (Roche Molecular Biochemicals, Indianapolis, IN) for 30 minutes on ice. Supernatants were incubated with primary antibody for 1 hour on ice. Lysates were centrifuged for 10 minutes at 4° C and the supernatants were incubated with 25 μ l protein A-Sepharose (Sigma, Saint Louis, MO) at 4° C with rotation. Immune complexes were washed 3-5

times with 1 ml low IPB (25 mM Tris, pH 7.5; 150 mM NaCl; 2 mM EDTA; 0.5% NP-40) or RIPA buffer. Immunoprecipitated proteins were resuspended in 40 μ l of 2X SDS loading buffer, boiled for 5 minutes, and resolved electrophoretically by 6-10% SDS-PAGE.

Western Blotting

Following SDS-PAGE, proteins were transferred to a nitrocellulose membrane (Protran BA, Schleicher and Schuell, NH) using standard electrophoretic transfer techniques. Membranes were blocked with 5% nonfat milk for 1 hour at room temperature with agitation. Membranes were incubated with primary antibody for 1 hour at room temperature with agitation followed by washing with TBS-T (150 mM NaCl, 20 mM Tris pH 8, 0.1% Tween-20). Membranes were incubated with secondary antibody for 1 hour at room temperature with agitation, and washed with TBS-T as described above. Blots were developed using the ECL Western blotting detection reagent (Amersham Pharmacia Biotech, Piscataway, NJ) according to the instructions from the manufacturer.

Immunofluorescence

1.5×10^4 C2C12 myoblasts per well (24-well) were plated on coverslips coated with laminins (GibcoBRL, cat # 23017-015). Cells were fixed with 3.7% paraformaldehyde (0.37 g paraformaldehyde, 1 ml 10X PBS, 10 μ l 5N NaOH, 8.6 ml dH₂O, 25 μ l 2N HCl) for 10 (myoblasts) or 20 minutes (myotubes). Cells were blocked with 10% normal goat serum for 1 hour at room temperature, washed, and incubated with primary antibody overnight at 4° C (anti-Bop 1:250; UT143 1:2000). Following washing, cells were incubated with 50 μ l secondary antibody 1.5 to 2 hours at 4° C (TRITC anti-rabbit 1:1000; Cy2 anti-hamster 1:100) and washed.

50 μ l DAPI (1 μ l in 1 ml PBS) was added and incubated for 5 minutes. The cells were washed extensively, mounted, and imaged using a Zeiss Axioskop fluorescence microscope.

In vitro interaction assay

Recombinant m-Bop2-6XHis was produced using the Bac-to-Bac Baculovirus Expression System (Life Technologies) and purified using Ni-NTA agarose beads (Qiagen, Valencia, CA). The TnT Coupled Wheat Germ Extract System (Promega, Madison, WI) was used for ³⁵-S labeled *in vitro* transcription/translation. 10 μ g of dialyzed recombinant m-Bop2-6XHis or Mre11-6XHis (kindly provided by Dr. Tanya Paull) was incubated with 25-100 μ l of translated protein for 2 hours at 4 °C with rotation. 15 μ l of Ni-NTA agarose beads were added to the protein mixture and incubated for an additional 1.5 hours. Protein complexes were washed 3 times, eluted, and analyzed by SDS-PAGE and autoradiography.

Results

m-Bop and skNAC interact in the yeast two-hybrid system

In order to identify proteins that interact with m-Bop, the GAL4 yeast two-hybrid system was used to screen a skeletal muscle library. The GAL4-DNA binding domain (DBD) was fused in frame to full-length m-Bop2 and t-Bop. m-Bop2 is an isoform of m-Bop that lacks 13 amino acids found within the S-ET domain of m-Bop1 and t-Bop. When expressed in the yeast strain Y190, the GAL4-Bop fusion proteins migrated at the predicted molecular weight (data not shown). From approximately 25 million independent clones screened with the GAL4-m-Bop2 construct, four clones displayed specific association with GAL4-m-Bop2 and none of these interacted with GAL4-t-Bop or control plasmids (Table 1). The clones isolated encode polypeptides from the carboxy terminus of the muscle-specific transcription factor, skNAC (Fig. 1). Clones E34 and E49 encode amino acids 1857 to 2187 and amino acids 1847 to 2187 of skNAC, respectively, and include the entire carboxy-terminal region common with α NAC (residues 1996 to 2187). Clone E120 (amino acids 1824 to 1965) terminates before the carboxy terminal α NAC residues, and the sequence of clone G38 includes amino acids 1829 to 1972 of skNAC and diverges inexplicably thereafter. The isolation of the E120 and G38 clones that lack α NAC residues suggests that these amino acids are not required for association with m-Bop2. The 108 amino acids that are common to all four positive clones contain an unusually high proportion of prolines (22%). Furthermore, clone G38 was shown to interact with m-Bop2, but not t-Bop, in the LexA yeast two-hybrid system (data not shown).

m-Bop and skNAC interact in mammalian cells

To test whether m-Bop associates with skNAC fragments in mammalian cells, immunoprecipitation assays were performed using transiently transfected 293T cells. Anti-Bop

antibodies coprecipitated FLAG-tagged E34 from cells co-transfected with both constructs (Fig. 2B). FLAG- α NAC did not associate with m-Bop2 in similar experiments, suggesting that α NAC sequences are not sufficient to mediate interaction with m-Bop (Fig. 2B). Interestingly, while E120 interacted in the yeast two-hybrid system, it did not coprecipitate with m-Bop2 in 293T cells (data not shown).

Expression of full-length skNAC was not detected by Western blot following transfection of numerous cell lines tested with pCI-skNAC (R. St-Arnaud) or pCMV-Tag2-skNAC (FLAG-tagged); both constructs were carefully verified by sequencing. Therefore, to determine whether m-Bop interacts with full-length skNAC, *in vitro* assays were performed. *In vitro* translated FLAG-N4, a fragment encompassing amino acids 1928-2187 of skNAC (Fig. 4A), and *in vitro* translated full-length skNAC bound to recombinant m-Bop2-6XHis, but not to a control recombinant protein (Mre11-6XHis) (Fig. 2C). Furthermore, *in vitro* translated NF- κ B1, a protein not expected to interact with m-Bop, did not bind to m-Bop2-6XHis (Fig. 2C).

Residues near the amino terminus of m-Bop are critical for skNAC interaction

To delineate the critical skNAC interaction domain(s) of m-Bop, FLAG-E34 was tested for its ability to associate with various Bop protein isoforms by coimmunoprecipitation. While m-Bop1 and m-Bop2 displayed similar binding to skNAC, t-Bop, which lacks the forty amino acid S region present in m-Bop1 and m-Bop2 (Fig. 3A), did not coprecipitate FLAG-E34 (Fig. 3B). Mutations were generated in the MYND domain of m-Bop2 to test the importance of this domain in skNAC association (Fig. 3C). Cysteine to serine mutations in the first or second predicted zinc fingers of the MYND domain abolished the ability of m-Bop2 to bind skNAC in immunoprecipitation assays. The MYND mutants were expressed in lower quantity compared to wild type m-Bop, but no traces of coprecipitating FLAG-E34 was detected, even at longer

exposures (Fig. 3D). Therefore, an intact MYND domain and amino terminal S sequences of m-Bop proteins are required for interaction with skNAC in mammalian cells.

skNAC interacts with m-Bop through a carboxy-terminal PXLXP motif

Data from the yeast two-hybrid system suggested that a region near the carboxy terminus of skNAC mediates its association with m-Bop2 (Fig. 1). To identify the m-Bop association domain of skNAC, coimmunoprecipitation experiments were performed using 293T cells transiently transfected with a series of skNAC amino-terminal deletion constructs (Fig. 4A). The FLAG-N4 truncation was the smallest skNAC fragment that coprecipitated with m-Bop2 in mammalian cells (Fig. 4B). Examination of the sequences present in FLAG-N4, but absent in FLAG-N5, revealed a PXLXP motif (PPLIP) recently identified as a MYND-binding motif (Fig. 4C) (10). In that study, a leucine to alanine mutation in the PXLXP motif abolished interaction. A point mutation was generated in the PPLIP motif that created a similar leucine to alanine substitution within the skNAC fragment FLAG-E34 (Fig. 4C). Coimmunoprecipitation experiments performed in transiently transfected 293T cells identified the PPLIP motif in skNAC as an important m-Bop interaction motif, as FLAG-L1952A failed to associate with m-Bop2 (Fig. 4D).

m-Bop and skNAC are induced and co-localize during skeletal myogenesis

Previous studies have shown that skNAC transcripts are induced early during skeletal myogenesis (18,19). Having obtained evidence for interaction of m-Bop isoforms and skNAC, we examined the kinetics of expression and localization of m-Bop and skNAC in differentiating C2C12 myoblasts. Whole cell extracts from cultures at various time points during myoblast differentiation were probed with specific anti-Bop and anti-skNAC antibodies. To distinguish it

from α NAC, specific anti-skNAC polyclonal antisera were generated using skNAC specific residues (1688-1995). m-Bop was strongly expressed and skNAC was weakly detectable at 24 hours following induction of differentiation; both reached maximum expression at 48 hours after the onset of myogenic conversion (Fig. 5A). Results observed for skNAC are consistent with previously reported studies of kinetics of skNAC mRNA expression (19). m-Bop2 expression gradually decreased after 48 hours, but remained high at two weeks following induction of differentiation (Fig. 5A). In contrast, skNAC expression remained high from 48 to 144 hours following induction, but was only weakly detectable after two weeks (Fig. 5A).

To determine the sub-cellular localization of m-Bop and skNAC during myogenesis, C2C12 myoblasts undergoing differentiation were stained using double immunofluorescence. 24 hours after the onset of differentiation, m-Bop and skNAC were present in both the nucleus and cytoplasm (Fig. 5B). At 96 hours following the switch to differentiation medium, m-Bop and skNAC displayed high levels of cytoplasmic staining whereas the nuclear staining of both was either absent or very low compared to the staining in the cytoplasm (Fig. 5B).

Discussion

MYND and SET domains similar to those present in m-Bop are found in proteins linked to transcriptional regulation and are involved in various cellular processes including differentiation, proliferation, and chromatin stability. The *Bop* gene is expressed in skeletal muscle, heart and cytotoxic T cells. MYND and SET domains of other proteins have been shown, respectively, to recruit HDACs and to have HMT activity, and we have shown previously that m-Bop itself recruits HDACs and can act as a repressor of reporter gene expression in mammalian cells (16). *Bop* null mice died *in utero* at approximately day E10.5 with abnormal hearts having the following characteristics at day E9.5: absence of a right ventricle, poor trabeculation of the left ventricle, and accumulation of an abnormally large amount of extracellular matrix (cardiac jelly) (16). Additionally, Hand2 expression in the developing heart was shown to be dependent on an intact *Bop* gene (16). Furthermore, the homeobox protein IRX4, shown previously to be Hand2-dependent (22), was down-regulated in *Bop* null mice, suggesting that IRX4 is regulated, in part, by a m-Bop-Hand2 regulatory cascade (16).

In the present studies, the yeast two-hybrid system was employed to identify m-Bop-interacting proteins that are expressed in mouse skeletal muscle. Although m-Bop is expressed equally strongly in heart, skeletal muscle was chosen for study initially because of the availability of mouse cell lines such as C2C12 and 10T1/2 that can be induced to undergo myogenesis in tissue culture. These systems would allow the possibility of over-expression of wild type or mutant m-Bop, its interaction partners, and their fragments to assess effects on myotube formation.

The muscle-specific transcription factor skNAC was isolated from a skeletal muscle library screen using GAL4-m-Bop2 as bait (Table 1). skNAC has been implicated in activating transcription in a sequence-specific manner and controlling skeletal muscle myogenesis (18),

although its function is poorly understood. Previous studies reported that skNAC activates the myoglobin promoter in a sequence specific manner, suggesting that it is a sequence specific transcriptional activator (18). It was further reported that both skNAC and α NAC bind to the same consensus DNA sequence, thus ascribing the DNA-binding properties of skNAC to residues common with α NAC (18). However, a recent report demonstrated that α NAC binds nucleic acids non-specifically (23), and α NAC has also been reported to bind to four-way junction DNA (24), reminiscent of HMG-box proteins and the SWI/SNF complex (25).

The skNAC fragments isolated using the two-hybrid system associated with m-Bop in 293T cells in coimmunoprecipitation assays (Fig. 2). Whereas, both m-Bop1 and m-Bop2 associated with skNAC, t-Bop, which lacks the heart and skeletal muscle-specific amino terminal S region (Fig. 3A), failed to interact with skNAC in the two-hybrid system and in animal cells (Table 1; Fig. 3B). Since we were unable to obtain expression of full-length skNAC in mammalian cells, m-Bop interaction with *in vitro* translated full-length skNAC was examined. Recombinant m-Bop2-6XHis was found to interact with *in vitro* translated full-length skNAC in a specific manner (Fig. 2).

Structural mapping revealed that an intact MYND domain and amino terminal S sequences of m-Bop were required for interaction with skNAC in mammalian cells (Fig. 3). It is likely that these m-Bop domains cooperate with one another to create a structural motif not present in t-Bop, which contains only the MYND domain. The m-Bop interaction domain of skNAC was found to be located in a carboxy terminal region of skNAC (Fig. 4A and 4B). Truncation and site-directed mutagenesis studies demonstrated that a PXLXP sequence (PPLIP) encoded near the end of the large skNAC-specific exon was necessary for m-Bop association (Fig. 4C and 4D). The PXLXP motif was previously shown to be essential for interaction of the closely-related MYND domains of the transcriptional co-repressor protein and potential tumor

suppressor, BS69, and the *C. elegans* proteins, Bra-1 and Bra-2, with viral oncoproteins and the Myc-related cellular protein, MGA(10). The present results further support a commonality in the interactions of MYND-containing proteins with their protein partners. A second PXLXP motif (PPLEP) is present near the amino terminus of the skNAC-specific exon, suggesting a second potential m-Bop-binding motif. This may explain m-Bop2 binding to premature transcription/translation skNAC fragments *in vitro* (Fig. 2C). Additional sequences of skNAC may contribute to m-Bop association since FLAG-N5 weakly bound to m-Bop2-6XHis *in vitro*, although to a substantially lower extent than FLAG-N4 (data not shown).

During skeletal muscle differentiation, distinct transcriptional programs direct the proper formation of mature myotubes. In previous studies, skNAC was reported to adversely affect myogenesis when overexpressed in differentiating myotubes (18). In the present studies, western blots demonstrate that m-Bop and skNAC are induced within the first 24 hours of myogenesis observed in cell culture (Fig. 5). Both proteins continue to be expressed at high levels up to six days after induction of differentiation. While m-Bop protein levels remain high, skNAC expression is reduced dramatically two weeks after the onset of myogenesis (Fig. 5). Immunofluorescence studies indicated that m-Bop and skNAC were localized in the nucleus early in C2C12 differentiation, followed by greatly enhanced expression in the cytoplasm as myogenesis progressed (Fig. 5). Studies in progress with adenovirus vectors expressing skNAC fragments with the wild type (PPLIPP) or mutant (PPAIPP) m-Bop interaction motifs should yield information on the functional significance of m-BOP/skNAC interaction during skeletal muscle myogenesis. Since m-Bop and skNAC are expressed in the heart, and recent studies suggest that they are co-localized in the embryonic heart (S. Pierce, R. Sims, D. Srivastava and P. Gottlieb, unpublished studies), the present studies of skeletal muscle myogenesis should shed light on the role of these proteins in cardiogenesis as well.

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Figure Legends

Figure 1. skNAC clones isolated from a GAL4 yeast two-hybrid skeletal muscle library screen using GAL4-m-Bop2 as bait.

The sequence of E120 terminates at residue 1965; the sequence of G38 diverges at residue 1972. Sequences common to α NAC are denoted by black boxes (23 N-terminal amino acids and 192 C-terminal amino acids). The two-hybrid interaction domain (gray box) corresponds to overlapping skNAC sequences (residues 1857-1965) from the four isolated cDNA clones. The GenBank accession number for skNAC is AAB18732 (*Mus musculus*).

Figure 2. Interaction of m-Bop and skNAC.

(A) Comparisons of skNAC, clone E34, and α NAC. (B) m-Bop2 interacts with FLAG-E34 in mammalian cells. 293T cells were transiently co-transfected with pBK-CMV or m-Bop2 together with FLAG-E34 or FLAG- α NAC. Cell extracts were immunoprecipitated using an anti-Bop antibody, subjected to SDS-PAGE, and detected by Western blot probed with an anti-FLAG antibody. Whole cell extracts (WCE) represent 5% of the total protein used for each immunoprecipitation assay. Proline-rich proteins are known to migrate unpredictably during SDS-PAGE. (C) *In vitro* interaction assay demonstrating full-length skNAC interaction with m-Bop2. Recombinant m-Bop2-6XHis or Mre11-6XHis (control) protein was incubated with 35 S labeled *in vitro* translated NF- κ B1, FLAG-N4, or skNAC. 6XHis tagged proteins were bound with Ni-agarose beads, and analyzed by SDS-PAGE and autoradiography. Input represents 10% of the 35 S translated protein used in the interaction assay.

Figure 3. Amino terminal residues of m-Bop are required for skNAC interaction.

(A) Schematic representation of Bop isoforms; evolutionarily conserved motifs are indicated. (B) skNAC interacts with m-Bop isoforms. 293T cells were transiently cotransfected with Bop isoforms and FLAG-E34. Cell extracts were immunoprecipitated using an anti-Bop monoclonal antibody, subjected to SDS-PAGE, and analyzed by Western blot using an anti-FLAG antibody. Whole cell extracts (WCE) represents 5% of the total protein used for each immunoprecipitation assay. (C) Schematic representation of m-Bop2 zinc finger point mutants. (D) An intact MYND domain is necessary for skNAC association. 293T cells transiently cotransfected with wild type or mutant m-Bop2 together with FLAG-E34. Extracts were immunoprecipitated using a Bop-specific antibody, analyzed to SDS-PAGE, and detected via Western blot utilizing a FLAG-specific antibody. Whole cell extracts (WCE) represent 5% of the total protein used for each immunoprecipitation assay.

Figure 4. Mapping the m-Bop interaction domain of skNAC.

(A) Schematic representation of the skNAC N-terminal truncations. (B) Coimmunoprecipitations of 293T cells co-transfected with m-Bop2 and FLAG-tagged skNAC N-terminal truncations. Cell extracts were immunoprecipitated with an anti-Bop antibody, subjected to SDS-PAGE, and analyzed by Western blot using an anti-FLAG antibody. Whole cell extracts (WCE) represent 5% of the total protein used for each immunoprecipitation assay. (C) Sequence of the PXLXP motif near the carboxy terminus of skNAC. The L1952A point mutation is indicated. The first amino acid of the skNAC FLAG-N5 truncation is shown. (D) Coimmunoprecipitations of 293T cells co-transfected with pBK-CMV or m-Bop2 along with FLAG-E34 or the FLAG-L1952A mutant. Cell extracts were immunoprecipitated with an anti-Bop antibody, subjected to SDS-PAGE, and analyzed by Western blot using an anti-FLAG antibody. Whole cell extracts (WCE) represent 5% of the total protein used for each immunoprecipitation assay.

Figure 5. m-Bop and skNAC are induced and co-localize during skeletal myogenesis.

(A) Western blot of whole cell extracts from distinct time points during C2C12 myoblast differentiation. Blots were probed for Bop using a Bop-specific antibody, stripped, and re-probed with an anti-skNAC antibody. (B) Double immunofluorescence staining of m-Bop and skNAC during C2C12 myogenic conversion. C2C12 cells were induced to differentiate for 24 and 96 hours, fixed and stained using a Bop-specific antibody, specific anti-skNAC antibodies, and DAPI. The phase contrast image is indicated. Images were magnified at 133X.

TABLE I
Interaction of m-Bop2 and skNAC in the GAL4 Yeast Two-Hybrid System

DBD Plasmid		AD Plasmid		β -Galactosidase Filter Assay
p53	(pAS2-1)	SV40	(pACT2)	++++
empty vector	(pAS2-1)	empty vector	(pGAD10)	-
m-Bop2	(pAS2-1)	empty vector	(pGAD10)	-
m-Bop2	(pAS2-1)	G38, E34, E49, E120	(pGAD10)	+++
empty vector	(pAS2-1)	G38, E34, E49, E120	(pGAD10)	-
pLAM5-1	(pAS2-1)	G38, E34, E49, E120	(pGAD10)	-
t-Bop	(pAS2-1)	G38, E34, E49, E120	(pGAD10)	-

Yeast cells (Y187) were transformed with the DBD plasmid and AD plasmid (Y190) shown above. Transformants were then mated and plated onto SD/-Trp/-Leu/-His + 25 mM 3-AT plates. Yeast were scored colorimetrically: (++++) represents blue color in 30 min, (+++) represents blue color in 30 min to 1 hour, (-) represents no blue color after 8 hours.

Figure 1. Sims et al.

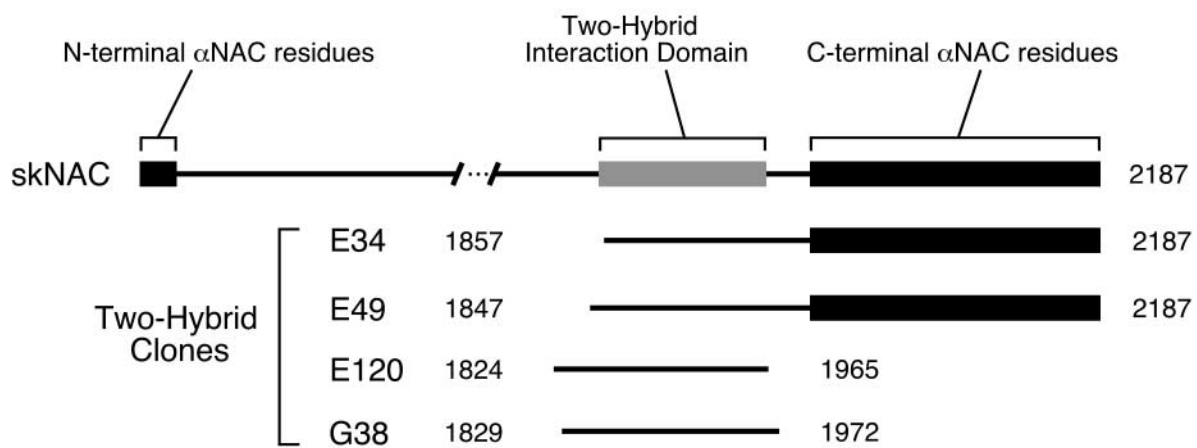


Figure 2. Sims et al.

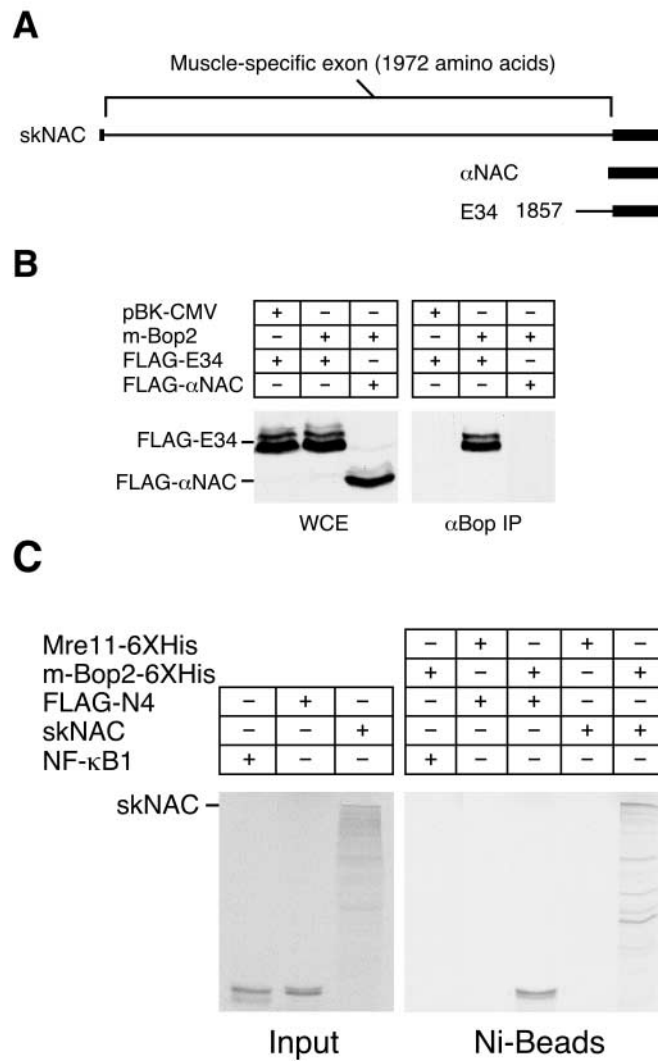
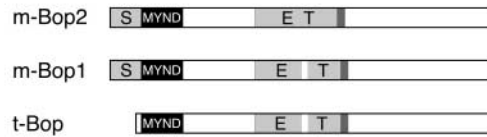
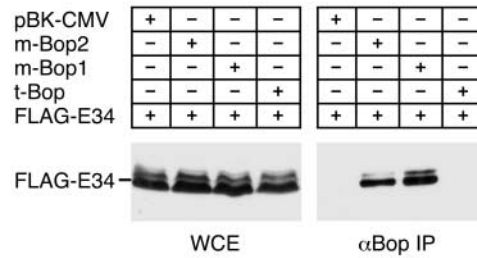


Figure 3. Sims et al.

A



B



C

m-Bop2	C H T C F K R Q E K L H R C G Q C K F A H Y C D R T C Q K D A W L N H K N E C											
MYND-Mut1	C	C			S	S			C	C	H	C
MYND-Mut2	C	C			C	C			S	S	H	C
MYND-Mut1/2	C	C			S	S			S	S	H	C

D

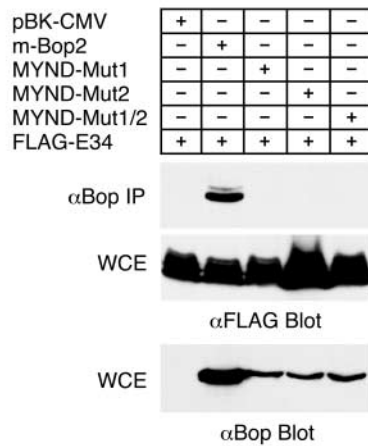


Figure 4. Sims et al.

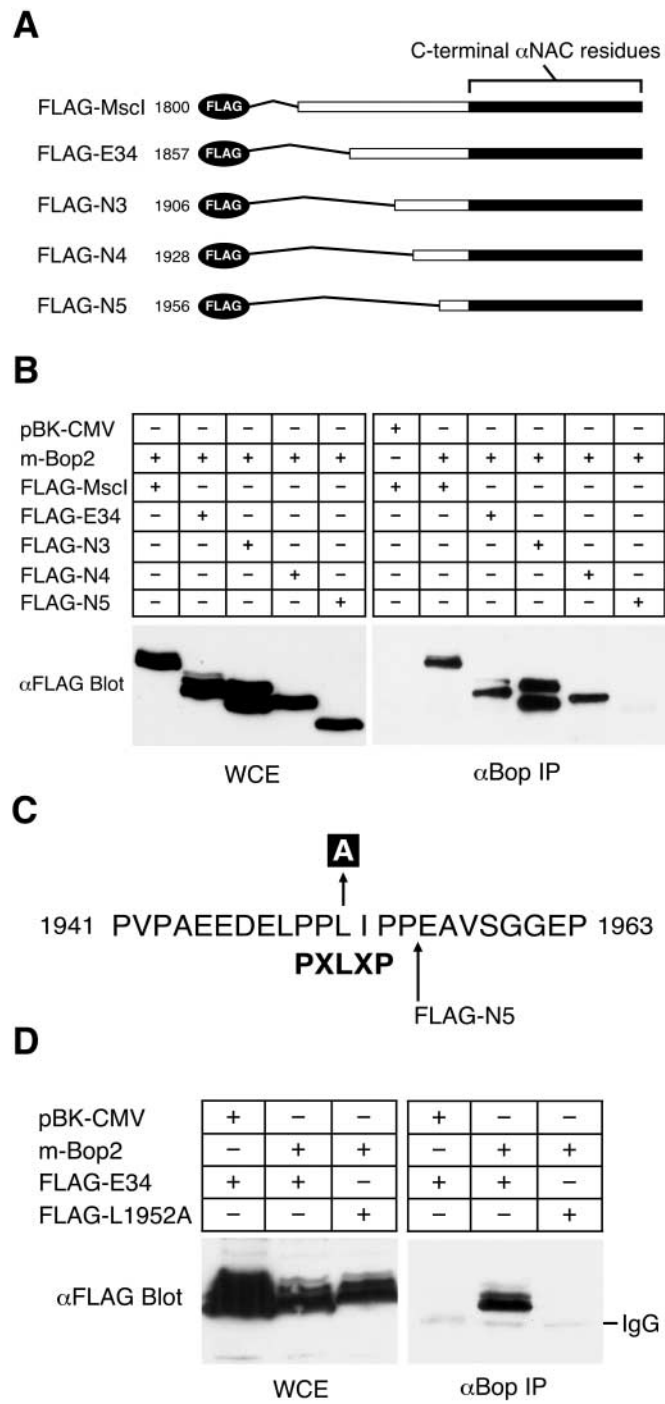
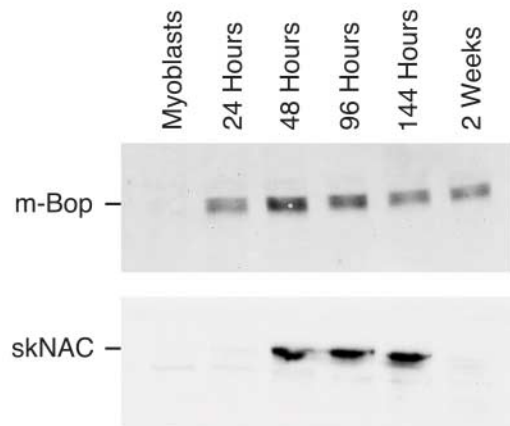


Figure 5. Sims et al.

A



B

