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KBTBD13 interacts with Cullin 3 to form a functional ubiquitin ligase

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ABSTRACT

Autosomal dominant mutations in BTB and Kelch domain containing 13 protein (KBTBD13) are associated with a new type of Nemaline Myopathy (NEM). NEM is a genetically heterogeneous group of muscle disorders. Mutations causing phenotypically distinct NEM variants have previously been identified in components of muscle thin filament. KBTBD13 is a muscle specific protein composed of an N terminal BTB domain and a C terminal Kelch-repeat domain. The function of this newly identified protein in muscle remained unknown. In this study, we show that KBTBD13 interacts with Cullin 3 (Cul3) and the BTB domain mediates this interaction. Using ubiquitination assays, we determined that KBTBD13 participates in the formation of a Cul3 based RING ubiquitin ligase (Cul3-RL) capable of ubiquitin conjugation. Confocal microscopy of transiently expressed KBTBD13 revealed its co-localization with ubiquitin. Taken together, our results demonstrate that KBTBD13 is a putative substrate adaptor for Cul3-RL that functions as a muscle specific ubiquitin ligase, and thereby implicate the ubiquitin proteasome pathway in the pathogenesis of KBTBD13-associated NEM.

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1. Introduction

We recently identified dominant mutations in an unknown gene, eventually designated as Kelch-repeat and BTB (POZ) domain containing 13 gene (KBTBD13) that are associated with a new form of Nemaline Myopathy type 6 (NEM6) [1,2]. NEM is a heterogeneous group of inherited myopathies that mostly affect infants and children. NEM is characterized by slowly progressive muscle weakness and the presence of thread- or rod-like so called nemaline bodies in affected muscle [2–4]. Mutations causing phenotypically distinct NEM variants have been identified in genes encoding components of skeletal muscle sarcomeric thin filaments or regulators of their assembly and as such NEM is considered a disease of muscle thin filament [3,4].

KBTBD13 encodes the KBTBD13 protein which is highly expressed in striated muscle [1]; however the role and function of this protein are completely unknown. KBTBD13 contains an N-terminal BTB/POZ domain, named after the *Drosophila melanogaster* transcription factors, Bric-a-brac, Tramtrack, and Broad-complex [5,6]. The BTB domain is a conserved protein–protein interacting motif that is involved in a variety of cellular functions. Proteins containing the BTB domain often serve as substrate specific adaptors for Cullin 3 (Cul3) based RING ubiquitin ligases (Cul3-RLs) that target proteins for proteasomal degradation [7–9]. The BTB domain is structurally similar to the Cul1 binding domain of Skp1, and forms analogous interactions with Cul3. Skp1 is an integral component of Skp1/Cul1/F-box (SCF) ubiquitin ligases that bridge Cul1 to an F-box substrate adaptor [10]. In contrast to Skp1, BTB proteins contain additional protein–protein interacting domains such as Kelch or MATH [5,6,11] that can bind directly to substrates without the need for an intervening F-box protein.

KBTBD13 contains a C-terminal Kelch domain consisting of five Kelch repeats. The Kelch domain was originally discovered in galactose oxidase in *D. melanogaster* and is thought to mediate protein–protein interactions [11]. Kelch domain containing proteins are implicated in a broad variety of biological processes, including cytoskeleton modulation, regulation of gene transcription, ubiquitination, cell migration and myofibril assembly [11,12].

The presence of BTB and Kelch domains in KBTBD13 led us to propose that KBTBD13 interacts with Cul3, a core component of Cul3-RL. In this study, we present the first biochemical studies of KBTBD13 protein, its interaction with other proteins and localization in mammalian cells. We show that KBTBD13 directly interacts

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with Cul3 and forms a functional Cul3-RL complex. Our results suggest that KBTBD13 is a putative adaptor for Cul3-RL and raise the possible involvement of the ubiquitin–proteasome machinery in the pathogenesis of NEM.

2. Materials and methods

2.1. Construction of expression plasmids

The expression construct for C-terminal Myc-Flag tagged human KBTBD13 in pCMV6-vector (KBTBD13-Myc-Flag) has previously been described [1]. A plasmid expressing full length human Cul3 was purchased from OriGene. Vector expressing Myc tagged Ringbox 1 protein (Myc-Rbx1) was obtained from Conaway [13]. Construct, pCMV-(HA-Ub)$_8$, expressing hemagglutinin (HA) tagged octameric ubiquitin was a kind gift from Bohmann [14]. The integrity of all plasmids was confirmed by DNA sequencing.

The N-terminal domain (1-383) of Cul3 followed by a hexa-histidine tag was cloned into the NotI and SbfI sites of pMAL-c5X (New England Biolabs) to generate MBP-Cul3(1-383)-(His)$_6$. The BTB domain (6-132) of KBTBD13 followed by a hexa-histidine tag was cloned into the NcoI and NdeI sites of pMAL-c5X to generate MBP-KBTBD13(6-132)-(His)$_6$. The co-expression construct was generated by inserting downstream from the multiple cloning site in pMAL-c5X (MCS-1), a linker, *tac* promoter, and second multiple cloning site (MCS-2). KBTBD13(6-132) was cloned into NcoI and NdeI sites of MCS-1 and Cul3(1-383)-(His)$_6$ was cloned into the NotI and SbfI sites of MCS-2.

2.2. Expression and purification of recombinant proteins

pMAL-c5X plasmids encoding KBTBD13 and Cul3 proteins were transformed into the NEB Express strain (New England Biolabs) or the BL21 strain (Invitrogen) of *Escherichia coli* and grown in LB medium. Protein expression was induced with 0.1 mM isopropyl β-D-1-thiogalactopyranoside. Bacteria were lysed in buffer containing 20 mM Tris pH 8.0, 500 mM NaCl, 0.05% Tween-20, 10 mM 2-mercaptoethanol, 10% glycerol, and supplemented with Complete Protease Inhibitor Cocktail (Roche Applied Sciences). Fusion proteins were purified using nickel agarose (Qiagen) and amylose resin (New England Biolabs). Cleavage of the MBP tag was performed by overnight digestion at 16 °C with factor Xa. After passage through the amylose resin, eluted proteins were concentrated and loaded onto a Superose 6 size exclusion column (GE Healthcare) equilibrated with the lysis buffer. The purified proteins were subjected to SDS–PAGE and Colloidal Blue staining (Invitrogen). Protein samples were used immediately for analysis by sedimentation velocity analytical ultracentrifugation or stored at −80 °C.

The co-expression plasmid encoding MBP-KBTBD13(6-132) and Cul3(1-383)-His$_6$ was used to transform the BL21 strain of *E. coli*. Bacteria were cultured at 37 °C in 2×YT medium until the OD reached 0.4. The temperature was decreased to 23 °C and protein expression induced for 4 h by addition of 0.5 mM isopropyl β-D-1-thiogalactopyranoside. The bacterial pellet was lysed in a buffer containing 20 mM HEPES pH 8.0, 20 mM NaCl, 200 μM tris-carboxyethyl phosphine, and supplemented with DNase, RNase, and Complete Protease Inhibitor Cocktail. Protein complex was purified as described above except that the MBP tag was not cleaved. Protein samples were used immediately for analysis by sedimentation velocity analytical ultracentrifugation or were frozen at −80 °C.

2.3. Sedimentation velocity analytical ultracentrifugation of the BTB–Cul3 complex

Sedimentation velocity experiments were conducted at 20.0 °C on a Beckman Coulter Proteome XL-A analytical ultracentrifuge using the absorbance optical detection system. Samples were loaded into two-channel, 12 mm path length sector shaped cells (400 μL). Scans were acquired at 4 min intervals and rotor speeds of 45,000 rpm; absorbance data were collected as single absorbance measurements at 280 nm using a radial spacing of 0.003 cm. Data were analyzed in SEDFIT 11.9b [15] in terms of a continuous $c(s)$ distribution. Solution density (ρ) and viscosity (η) were calculated using SEDNTERP 1.2 [16], as was the partial specific volume (v) of the protein complex. The $c(s)$ analyses were carried out using an s range of 0–25 with a linear resolution of 200 and maximum entropy regularization confidence levels (F-ratio) of 0.68. Sedimentation coefficients were corrected to standard conditions at 20.0 °C in water, $s_{20,w}$.

2.4. Cell cultures, transfection and immunofluorescence

293T, NIH3T3 and C2C12 cells were grown in Dulbecco's modified eagle medium (DMEM, Invitrogen) containing 10% fetal bovine serum. About 60–70% confluent 293T and NIH3T3 cells were transfected with Lipofectamin 2000 and C2C12 cells were transfected with Lipofectamin LTX according to manufacturer's protocol. At 48 h post-transfection, cells were fixed in 3.8% paraformaldehyde, permeabilized in PBS with 0.2% TritonX-100. Cells were blocked in 5% goat serum (DAKO) and incubated with a rabbit antibody to the Myc (Abcam) or Flag (Sigma) tag for 1 h followed by a mouse ubiquitin antibody (Chemicon) staining. The secondary antibodies, Alexa 488 and Alexa 594 (Invitrogen) were applied for 30–60 min. Cell nuclei were counterstained using Prolong Gold antifade reagent with DAPI (Invitrogen). Cells were visualized and analyzed with a Zeiss LSM 510 confocal microscope.

2.5. Immunoprecipitation and immunoblot analysis

At 48 h post-transfection, the cells were lysed in cold lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 or 1% NP40, 1 mM PMSF, and protease inhibitor mixture) and immunoprecipitated using appropriate antibodies followed by protein-A or -G beads (Invitrogen) per manufacturer protocol. Flag-tagged proteins were immunoprecipitated with anti-Flag M2 magnetic beads (Sigma) according to product guidelines. Immunoprecipitates were analyzed by standard immunoblotting. Briefly, membranes were incubated in blocking buffer followed by antibody and anti-mouse or anti-rabbit IgG conjugated to horseradish peroxidase (HRP) (GE Healthcare). Detection of the HPR-conjugated antibody was done using a Super Signal West Pico kit (Pierce Biotechnology) and exposure to chemiluminescent film (GE Healthcare).

2.6. Ubiquitination assay

Ubiquitination assays were performed according to published protocols [17,18]. For detection of ubiquitinated protein *in vivo*, 293T cells were transfected with expression vectors for KBTBD13-Myc-Flag, Cul3, Rbx1-Myc and pCMV-(HA-Ub)$_8$. The octameric ubiquitin is more efficiently conjugated to the protein than monomeric ubiquitin and can be processed *in vivo* by cellular ubiquitin C-terminal hydrolases [12]. Transfected cells were treated with 50 μM MG132 proteosome inhibitior (Calbiochem) for 3 h prior lysis. The cells were lysed in buffer containing 2% SDS, 50 mM buffer Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM DTT. Lysates were immediately denatured to preserve ubiquitin modification of protein in the cells. The lysates subjected to immunoprecipitation with anti-Flag beads overnight at 4 °C. Ubiquitinated proteins were determined by immunoblotting with anti-HA antibodies (Sigma).

For detection of ubiquitinated protein *in vitro*, 293T cells were transfected with expression vectors for KBTBD13-Myc-Flag, Cul3