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X-linked intellectual disability gene CUL4B targets Jab1/CSN5 for degradation and regulates bone morphogenetic protein signaling

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ABSTRACT

Cullin 4B (CUL4B) is a scaffold protein involved in the assembly of cullin-RING ubiquitin ligase (E3) complexes. Contemporary reports have identified multiple mutations of *CUL4B* gene as being causally associated with X-linked intellectual disability (XLID). Identifying the specific protein substrates will help to better understand the physiological functions of CUL4B. The current study identified Jun activation domain-binding protein (Jab1/CSN5) in the COP9 signalosome (CSN) complex as a novel proteolytic target for the CUL4B ubiquitin ligase complex. The impaired degradation of Jab1 was observed in cells after RNAi-mediated CUL4B depletion. Integrity of DDB1-CUL4B-ROC1 was further demonstrated to be indispensable for the degradation of Jab1. In addition, the degradation of Jab1 is independent of CUL4A, a cullin family member closely related to CUL4B. *In vitro* and *in vivo* ubiquitination assays revealed that CUL4B promoted the polyubiquitination of Jab1. Interestingly, *CUL4B*-silenced cells were shown to exhibit abnormal upregulation of bone morphogenetic protein (BMP) signaling. Furthermore, *in vivo* studies of embryonic fibroblasts in *Cul4b*-deficient mice demonstrated Jab1 accumulation and increased activation of the BMP signaling pathway. Together, the current findings demonstrate the CUL4B E3 ubiquitin ligase plays a key role in targeting Jab1 for degradation, potentially revealing a previously undocumented mechanism for regulation of the BMP signaling pathway involved with the CUL4B-based E3 complex. This observation may provide novel insights into the molecular mechanisms underlying CUL4B-associated XLID pathogenesis.

1. Introduction

Ubiquitination-dependent protein degradation in the proteasome plays key roles in numerous biological processes, including cell cycle progression, transcription, signal transduction and development [1,2]. E3 ubiquitin ligase forms the final step in the process of polyubiquitin conjugation, mediating the transfer of the ubiquitin molecule from the E2 region onto the substrate protein [3]. In higher eukaryotes, cullins are a family of hydrophobic proteins that have been evolutionarily conserved to provide the scaffolding structure that organizes reactive modules in the largest known E3 ubiquitin ligases, the cullin-RING ubiquitin ligases (CRLs).

The human genome encodes at minimum of seven known cullins (CUL1, CUL2, CUL3, CUL4A, CUL4B, CUL5, and CUL7), each of which is composed of a conserved C-terminal domain that binds the RING finger protein ROC1 (also known as Rbx) to catalyze polyubiquitination [4,5]. In these proteins, divergence at the N-terminal domain allows for association with a large variety of specificity factors [6]. Of the cullin family, CUL4 of mammalian cells is unique in that it consists of the two closely related paralogs CUL4A and CUL4B [7]. CUL4A and CUL4B share approximately 80% protein sequence homology, and both are bound to a DDB1-binding WD40 (DWD) protein module at the N-terminal domains. Thus, these proteins are involved in the placement of a WD40 protein, a member of a large family of substrate-recruiting factors, within the proximity of ROC1 [8–10]. ROC1 then serves to recruit the E2-conjugating enzyme involved in substrate ubiquitination. CUL4A and CUL4B have been shown to important mediators of the ubiquitination of the same target proteins associated with cell cycle regulation, chromatin modification, DNA replication and repair [11]. Thus, CUL4A and CUL4B carry out many overlapping functions in mammalian cells.

Despite the largely redundant functions of the two paralogs, CUL4B has a distinct N-terminus that is 149 amino acids longer than that of CUL4A [12], indicating that CUL4B E3 ligase may selectively

Abbreviations: CUL4B, Cullin 4B; XLID, X-linked intellectual disability; Jab1, Jun activation domain-binding protein; CSN, the constitutive photomorphogenic-9 (COP9) signalosome; BMP, bone morphogenetic protein; CRLs, the cullin-RING ubiquitin ligases; DWD, DDB1-binding WD40; ROS, reactive oxygen species; JAMM, Mpr1-Pad1-N-terminal (MPN) domain metalloenzyme motif; Smurf, Smad ubiquitin regulatory factor; MEF, mouse embryonic fibroblasts; siRNA, small interfering RNA; CHX, cycloheximide

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ubiquitinate specifically recruited substrates. Notably, genetic analysis has linked XLID, a condition characterized by short stature, intellectual disability and other physical abnormalities, to point mutations or deletions of the *CUL4B* gene [13–19]. Conversely, no germ-line mutations have been documented in the human *CUL4A* gene. Recent reports have revealed *Cul4b* performs critical developmental roles in the extra-embryonic tissues and regulates the number of parvalbumin (PV)-positive GABAergic neurons of the hippocampus, and constitutive *Cul4b* null mice resulted in embryonic lethality [20–23]. However, *Cul4a* null mice were shown to display no gross phenotypic abnormalities throughout their lifespan, with the exception of meiotic defects in male mice [20,24–26]. These findings suggest that the CUL4B complex may operate as a distinct E3-ubiquitin ligase. Based on these findings, identification of the specific substrates of CUL4B is of paramount importance in improving understanding of the physiological functions of CUL4B.

Only a few specific substrates for CUL4B E3 ligase have been identified despite its demonstrated association with XLID. The CUL4B/AhR/DDB1 complex promotes ubiquitination and degradation of the estrogen receptor α in response to environmental toxins [27]. CUL4B-deficiency patient-derived cells exhibit impaired camptothecin (CPT)-induced topoisomerase I (Topo I) degradation and ubiquitination and increased Topo I-mediated DNA breakage [28]. Furthermore, the CUL4B-based E3 ligase mediates reactive oxygen species (ROS) during neurogenesis via ubiquitination and degradation of Peroxiredoxin III (PrxIII) [29]. The H3K4 methyltransferase component WDR5 is another specific substrate of CUL4B ligase that is important in regulating neuronal gene expression [30]. Model eukaryotic organisms may contain dozens or even hundreds of WD40 proteins. These variable substrate-recruiting specificity factors may provide CUL4B-based E3 ligase with opportunities to access a wide array of substrates [11,31]. Therefore, identification of additional specific substrates for CUL4B could potentially lead to delineation of the pathogenesis observed in patients with mutations in *CUL4B*.

This study is directed toward the issue and has identified Jab1 as a novel and specific substrate for CUL4B. Jab1, as the fifth component of the CSN complex, is commonly referred to as Jab1/CSN5 [32]. Jab1 contains an Mpr1-Pad1-N-terminal (MPN) domain metalloenzyme motif (JAMM) responsible for the removal of Nedd8 from cullin-RING ubiquitin ligases [32,33]. Notably, Jab1 also plays an essential role in regulation of BMP signaling pathways by promoting degradation of the BMP signaling inhibitor Smad7 through Smad ubiquitin regulatory factor (Smurf), a C2-WW-HECT domain ubiquitin ligase-based degradation pathway [34].

Here, the experimental evidences showed that CUL4B, but not CUL4A, mediates the proteasomal degradation of Jab1. Furthermore, the effect of increased Jab1 levels on Smad7 suppression and aberrant activation of BMP signaling in response to *CUL4B* silencing was explored. Moreover, to prevent embryonic lethality, *Cul4b*-deficient mice with conditional deletion in the epiblast were generated. Using embryonic fibroblasts in the *Cul4b*-deficient mice, the accumulation of Jab1 and enhanced activation of the BMP signaling pathway were observed as well. Collectively, the present studies identify Jab1 as a novel substrate for CUL4B E3 ligase and may potentially offer further insight into the pathogenesis caused by CUL4B deficiency in humans.

2. Materials and methods

2.1. Plasmid construction, cell culture and generation of Cul4b-deficient mice

Plasmid-methods for generating expression constructs of human CUL4B (pcDNA3.1/Myc-HisA-CUL4B) and expression constructs used in rescue experiments (pFLAG-CUL4B, pFLAG-CUL4A and pFLAG-CUL4B 150–895) were conducted as previously described [12]. Construction of pcDNA3.1/myc-His-Jab1 was accomplished by subcloning a PCR-amplified in frame Jab1 fragment into the pcDNA3.1-myc-His A vector (Invitrogen) between BamHI and HindIII sites using HEK293 cDNA as a template. HEK293 cell lines were maintained in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum plus penicillin and streptomycin in a humidified incubator at 37 °C with an atmosphere of 5% CO_2. *Cul4b*-floxed mice were generated [22] and *Sox2-Cre* mice expressing Cre exclusively in the epiblast were purchased from Jackson (The Jackson Laboratory). *Cul4b*$^{-/Y}$ mouse embryonic fibroblasts (MEFs) were taken from *Cul4b*$^{-/Y}$ mice derived from a *Cul4b*$^{f/Y}$ × *Sox2-Cre* cross.

2.2. RNA interference

Small interfering RNA (siRNA) duplexes were used to knockdown CUL4B, CUL4A, DDB1, ROC1, and Jab1. The *CUL4B*, *CUL4A*, *DDB1*, *ROC1*, *Jab1*, and negative control siRNA duplexes were purchased from GenePharma, and oligonucleotide sequences were as follows: CUL4B, 5′-CAAUCUCCUUGUUUCAGAATT-3′; CUL4A, 5′-CCAUGUAAGUAAACG CUUATT-3′; DDB1, 5′-CGUUGACAGUAAUGAACAATT-3′; ROC1, 5′-GAA GCGCUUUGAAGUGAAATT-3′; Jab1, 5′-CCAGACUAUUCCACUUAAUTT-3′; and negative controls, 5′-UUCUCCGAACGUGUCACGUTT-3′.

2.3. Real-time PCR assay

Total RNA from cultured cells was isolated using the TRIzol reagent (Invitrogen), and RNA was then treated with RNase-Free DNase (Promega) to eliminate genomic DNA contamination. Real-time quantitative PCR (RT-PCR) was performed using an ABI Prism 7500 instrument (Applied Biosystems). Human GAPDH was used as an endogenous control. The levels of specific mRNA were measured using the 2 × SYBR Green RT-PCR Master Mix (Applied Biosystems). Primers were designed using Primer 5.0 (Premier) software (primers are available upon request).

2.4. Antibodies and immunological procedures

Antibodies against the following proteins were purchased: anti-CUL4B (C9995, polyclonal antibody produced in rabbit), anti-FLAG (F7425, antibody produced in rabbit) and anti-FLAG (M8823) M2 magnetic beads (Sigma Aldrich); anti-Jab1 (ab124720, monoclonal antibody produced in rabbit), anti-CSN2 (ab77303, monoclonal antibody produced in mouse), anti-ROC1 (ab86862, polyclonal antibody produced in rabbit), and anti-CUL4A (ab34897, polyclonal antibody produced in rabbit) (Abcam); anti-6× His-tag (#2365, polyclonal antibody produced in rabbit), anti-Smad1 (#9743, polyclonal antibody produced in rabbit), and anti-phospho-Smad1/Smad5/Smad8 (#9511, polyclonal antibody produced in rabbit) (Cell Signaling Technology); anti-CSN4 (NB100640, polyclonal antibody produced in rabbit) (Novus), CSN6 (SC-137153, monoclonal antibody produced in mouse), anti-DDB1 (SC-136180, monoclonal antibody produced in mouse), anti-Smad7 (SC-11392, polyclonal antibody produced in rabbit), anti-β-actin (SC-8432, monoclonal antibody produced in mouse), and anti-Ub (SC-166553, monoclonal antibody produced in mouse) (Santa Cruz Biotechnology). Immunoblotting and immunoprecipitation analyses were performed as described previously [35]. Immunofluorescence stainings were performed as described previously [12]. *Cul4b*$^{+/Y}$ and *Cul4b*$^{-/Y}$ MEFs were fixed with 3.7% PFA (in PBS) and washed with phosphate-buffered saline (PBS) plus 0.1% Triton X-100 and then blocked with 1% BSA in PBS. Cells were subsequently stained with FITC-phalloidin (Beyotime) and washed with PBS. Visualization of the actin cytoskeleton and DAPI was conducted using fluorescence microscopy (Olympus).

2.5. Ubiquitin ligation assays

In vitro and *in vivo* ubiquitination assays were performed as described previously [29,35,36]. For *in vitro* ubiquitination assays,