Characterization of Cullin-box Sequences that Direct Recruitment of Cul2-Rbx1 and Cul5-Rbx2 Modules to Elongin BC-based Ubiquitin Ligases

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The Elongin BC-box protein family includes the von Hippel-Lindau (VHL) tumor suppressor and Suppressor of Cytokine Signaling (SOCS) proteins, which are substrate recognition subunits of structurally related classes of E3 ubiquitin ligases composed of Elongin C-Elongin B-Cullin 2-Rbx1 (Cul2 ubiquitin ligases) or of Elongin C-Elongin B-Cullin 5-Rbx2 (Cul5 ubiquitin ligases). These ubiquitin ligases, the Elongin BC complex acts as an adaptor that links a substrate recognition subunit to heterodimeric modules composed of either Culin 2 (Cul2) and RING finger protein Rbx1 or Culin 5 (Cul5) and Rbx2. Kamura et al. (Genes Dev. 18, 3055-3065) recently demonstrated that interaction of several BC-box proteins with their cognate Cul/Rbx module is determined by specific regions, called Cul2- or Cul5-boxes, located immediately downstream of their BC-boxes. In this report, we investigate further the mechanisms governing assembly of BC-box proteins with their specific Cul-Rbx modules. Through purification and characterization of a larger collection of BC-box proteins that serve as substrate recognition subunits of CBC ubiquitin ligases and through structure-function studies, we define Cul2- and Cul5-boxes in greater detail. Although it previously appeared that there was little sequence similarity between Cul5- and Cul2-box motifs, analyses of newly identified BC-box proteins that assemble into Cul2 ubiquitin ligases reveal that residues conserved in the Cul2-box represent a subset of those conserved in the Cul5-box. Notably, the sequence motif LPφP, which is conserved in most Cul5-boxes and has been suggested to specify assembly of Cul5 ligases, is compatible with Cul2 interaction. Finally, the spacing between BC- and Culin-boxes is much more flexible than has been appreciated and can vary from as few as three and as many as ~80 amino acids. Taken together, our findings shed new light on the mechanisms by which BC-box proteins direct recruitment of Culin/Rbx modules during reconstitution of ubiquitin ligases.
members of the Cul5 family of E3 ubiquitin ligases are the SH2 domain-containing Suppressors of Cytokine Signaling (SOCS) proteins (7), which negatively regulate Jak/STAT signaling by inhibiting the activities and reducing the levels of Jak or receptor tyrosine kinases (8-10). Sequence comparison of the VHL and SOCS-box proteins revealed that they each include a degenerate, ~12 amino acid sequence motif with consensus sequence [S,T,P]Lxxx[C,S,A]xxxI. This motif, which is referred to as the BC-box, is required for binding to the Elongin BC complex. Solution of a cocystal structure of the VHL-Elongin BC complex revealed that binding of Elongin BC to the BC-boxes of VHL (11), SOCS2 (12), and SOCS4 is governed by interaction of an invariant leucine at the N-terminus of the BC-box with a hydrophobic pocket created by residues in the C-terminal half of Elongin C. The ubiquitin-like domain of Elongin B interacts with a short N-terminal Elongin C region, while the Elongin B C-terminal extension can be seen to make limited contacts with SOCS2 and SOCS4 outside of the BC-box (12). To date, more than 30 different BC-box proteins have been identified using bioinformatic or biochemical approaches, and a number of these have been shown to function as components of Cul2 and Cul5 ubiquitin ligases.

The initial observation that some BC-box proteins assemble into Cul2 ubiquitin ligases whereas others assemble into Cul5 ubiquitin ligases raised questions about how specificity governing recruitment of Cul2 or Cul5 is determined. Kamura and coworkers (13) recently proposed that assembly of Elongin BC-box proteins into Cul5 or Cul2 ubiquitin ligases can be determined by the presence of specific motifs, termed "Cullin-boxes" located downstream of the BC-box. The Cul5-box corresponds to the C-terminal portion of the canonical SOCS-box defined by Hilton and coworkers and has the consensus sequence ψxxLPϕPxxϕxx[Y/F][L/I] (13,14). Structure-function studies of several SOCS-box proteins suggested that the central LPϕP might play a particularly important function as a determinant of Cul5 binding (13). Although a region downstream of the BC-box is clearly needed for interaction with Cul2, it has been difficult to define a specific sequence motif that correlates with Cul2 binding since only three BC-box proteins, VHL, LRR1/PPIL5, and FEM1b, that assemble into Cul2 ubiquitin ligases had been identified when this study was initiated. In addition, assembly of Cul5 ubiquitin ligases containing primate lentiviral Vif proteins has been shown to depend critically on a conserved zinc binding motif located upstream of the BC-box (15-17).

To define in more detail the primary structural determinants governing assembly of Cul2 and Cul5 ubiquitin ligases, we have purified and characterized a larger collection of BC-box proteins and performed structure-function analyses of sequence motifs responsible for their interactions with Cul2 or Cul5. Here we describe the results of these experiments, which have provided new insights into the nature of the Cul5-box and which have identified a consensus Cul2-box present in a large fraction of BC-box proteins that assemble into Cul2 ubiquitin ligases.

**EXPERIMENTAL PROCEDURES**

**Antibodies.** Mouse monoclonal Anti-Flag M2 antibody was obtained from Sigma, HSV-Tag monoclonal antibody from Novagen, c-myc monoclonal antibody from Roche, rabbit polyclonal anti-Cul-5 (H300) from Santa Cruz, rabbit polyclonal anti-Cul-2 from Zymed Laboratories and a mouse monoclonal antibody against Elongin C/SIII p15 from BD Transduction Laboratories.

**Generation and Growth of 293 and M1 Cell Lines Stably Expressing FLAG-tagged Proteins.** Full-length cDNAs encoding Elongin B or Elongin BC-associating proteins were obtained from the American Type Culture Collection, subcloned with N-terminal FLAG epitope tags into pcDNA5/FRT (Invitrogen) or the retroviral vector pMXs-neo (18), and stably introduced either into Flp-In™ 293 cells (Invitrogen) using the Invitrogen Flp-in™ system or into M1 cells by retroviral infection. 293 cell lines were grown on plates or in roller bottles in Dulbecco’s modified Eagle’s medium with 5% glutamax and 10% fetal bovine serum. M1 cells were grown in flasks or in roller bottles with the same media as the 293 cells.

**Transient Transfections.** HEK293T cells grown to ~50% confluence in 10 cm plates were transiently transfected with pcDNA5/FRT constructs encoding full length BC-box proteins or various mutants using Fugene6 transfection reagent (Roche) according to the manufacturer’s
instructions. Cells were collected 48 hours after transfection and processed using the high salt extraction protocol described below. Mutagenesis to generate BC-box, Cul2-box, or Cul5-box mutants was performed using the QuikChange® II XL Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer’s instructions.

**Anti-FLAG Agarose Chromatography.** To prepare protein samples for mass spectrometry, 293 cells were grown to 70-80% confluence in 4-5 15-cm plates or 1-2 roller bottles. M1 cells were grown in 1-2 roller bottles to a density of approximately 10⁶ cells/ml. Cells in plates or roller bottles were washed with phosphate-buffered saline and then lysed by resuspension in lysis buffer (1 ml/plate or 3 ml per roller bottle). Two different protocols were used, a low salt extraction (0.15 M NaCl), low speed centrifugation or a high salt extraction (0.42 M NaCl), high speed centrifugation method. In the low salt extraction, low speed centrifugation method, cells are lysed in 40 mM HEPES-NaOH (pH 7.9), 0.15 M NaCl, 1.5 mM MgCl₂, 10% glycerol, 1 mM dithiothreitol, and 0.2% Triton X-100, and the cell lysate is spun at 14,000 x g for 30 min in a Beckman-Coulter 5417R refrigerated microfuge. In the high salt extraction, high speed centrifugation method, cells are lysed in 20 mM HEPES-NaOH (pH 7.9), 0.42 M NaCl, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, and 0.2% Triton X-100, and the cell lysate was spun for 30 min at 40,000 x g in a Beckman-Coulter Optima XL-100K refrigerated ultracentrifuge in a Ti 70.1 rotor. For both methods, supernatants were mixed with anti-FLAG (M2) agarose beads (Sigma), equilibrated in 10 mM HEPES-NaOH (pH 7.9), 0.2% Triton X-100, 0.3 M NaCl, 10 mM KCl, 1.5 mM MgCl₂, and protease inhibitor cocktail (Sigma cat number P8340) in a ratio of 100 µl packed beads/3 ml of supernatant and gently rocked overnight at 4°C. The beads were washed 4 times with a 50-fold excess of the same buffer. Proteins were eluted by incubation for 30 min at 4°C with one packed bead volume of 10 mM HEPES-NaOH (pH 7.9), 0.05% Triton X-100, 0.1 M NaCl, 1.5 mM MgCl₂, protease inhibitor cocktail, and 0.2 mg/ml FLAG peptide (Sigma). Beads were removed by centrifugation and the elution was repeated an additional 2 times.

**Mass Spectrometry and Data Analysis.** Identification of proteins in purified samples was accomplished by MudPIT (19,20). TCA-precipitated proteins were reduced, alkylated, and digested with endoproteinase LysC and trypsin (Roche Applied Science). Peptide mixtures were applied to a three-phase microcapillary column (21) and eluted into an LCQ Deca-XP ion trap mass spectrometer equipped with a nano-liquid chromatography electrospray ionization source (ThermoFinnigan) as described (22).

SEQUEST (23) was used to match tandem mass spectra to 82242 amino acid sequences consisting of 40877 *H. sapiens* protein sequences downloaded from the National Center for Biotechnology (2006-03-03 release), 177 usual contaminants, 102 custom sequences for epitope tagged proteins, as well as randomized versions of each non-redundant protein entry to estimate False discovery rates (FDR). Spectra/peptide matches were sorted, filtered and compared using DTASelect/CONTRAST (24). The chosen selection criteria led to FDRs of at most 0.6% (Supplementary Tables). Normalized Spectral Abundance Factors (NSAFs) were calculated as described (25).

**RESULTS**

**Identification and Characterization of New Members of the Families of Cul2- and Cul5-containing Ubiquitin Ligases**

As described above, a major impediment to efforts to define sequence elements that support recruitment of Cul2-Rbx1 rather than Cul5-Rbx2 modules to certain ubiquitin ligases was lack of a sufficient number of known Cul2-containing ubiquitin ligases. At the time this work began, only the VHL tumor suppressor protein and the LRR1 and FEM1B proteins were known to contain functional Cul2-boxes (13). Consequently, we sought to identify additional BC-box proteins that contain functional Cul2-boxes.

To identify new members of the BC-box family of proteins, we generated human embryonic kidney Flp-In™ 293 and murine lymphoblastic (myeloid leukemia) M1 cell lines stably expressing Elongin B with an N-terminal FLAG epitope tag. FLAG-Elongin B-associating proteins were purified by anti-FLAG agarose immunoaffinity chromatography from cell lysates prepared from 10 independent 293 cell preparations and 7...
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independent M1 cell preparations. Immunopurified Elongin B-associating proteins were then identified by MudPIT (multidimensional protein identification technology) mass spectrometry. In a MudPIT mass spectrometry experiment, a mixture of proteins is digested into peptides without prior separation by one- or two-dimensional polyacrylamide gel electrophoresis. Peptides are then fractionated by two-dimensional chromatography and identified by tandem mass spectrometry. As a control for the specificity of our immunoaffinity purifications, anti-FLAG agarose eluates from cell lysates of parental 293 and M1 cells not expressing a FLAG-tagged protein were also analyzed by mass spectrometry.

As expected, anti-FLAG agarose eluates from FLAG-Elongin B expressing 293 and M1 cells contained Elongin B and Elongin C, Cul2, Cul5, Rbx1, and Rbx2. In addition, these eluates contained a collection of additional proteins (Supplementary Tables 1-4). Twenty of these had been previously reported to be BC-box proteins based on data from either biochemical or bioinformatic studies, and about half of these had been shown to be able to bind directly to Elongins B and C and may function as substrate recognition subunits of Cul2 or Cul5 ubiquitin ligases (Fig. 1, protein names in bold text). In many of the remaining proteins, we identified short sequences that could be aligned with the BC-boxes of previously described Elongin BC binding proteins (Fig. 1), suggesting that most of them are likely to have BC-boxes that can bind to Elongins B and C and may function as substrate recognition subunits of Cul2 or Cul5 ubiquitin ligases. These new BC-box proteins could be classified into several groups based on the presence of specific structural domains. In addition to new members of the ankyrin repeat-containing and leucine-rich repeat (LRR)-containing families of BC-box proteins, these included proteins containing PCMT (protein-L-isoaspartate carboxylmethyltransferase) domains, KELCH repeats, ZSWIM (SWI2/SNF2 MuDR zinc finger) domains, armadillo repeats, tetratricopeptide repeats (TPRs), and two proteins containing both LXXLL nuclear receptor binding motifs and domains resembling serine/threonine protein kinase catalytic domains (S_TKc), none of which had previously been found in BC-box proteins (Fig. 2).

To determine whether the putative BC-boxes found in Elongin B-associating proteins were functional, selected candidates were tested for their ability to bind to the Elongin BC complex when coexpressed with Elongins B and C in 293 cells. As shown in Fig. 3, FLAG-tagged NRBP1 (nuclear receptor binding protein 1), APPBP2 (amyloid precursor protein binding protein 2), ZYG11BL, and PCMTD2 all coimmunoprecipitated with Elongins B and C, as did the Elongin BC binding proteins VHL and FEM1B. Confirming that the putative BC-boxes were important for binding of each protein to Elongins B and C, mutation of key residues in the BC-box motifs of VHL, PCMTD2, FEM1B, ZYG11BL, APPBP2, and NRBP1 interfered with binding of Elongins B and C to each protein (Fig. 3).

To determine whether the newly identified BC-box proteins assemble into Cul2- or Cul5-containing ubiquitin ligases, we generated 293 cell lines stably expressing Cul2, Cul5, or selected candidate proteins with N-terminal FLAG tags and purified their associated proteins from cell lysates by anti-FLAG agarose chromatography. Copurifying proteins were identified by MudPIT mass spectrometry. VHL and FEM1B were known to interact with Cul2 (2,3,13), while ASB1 and WSB1 interact with Cul5 (7). Accordingly, cell lines expressing FLAG-tagged VHL, FEM1B, ASB1, and WSB1 were used as controls in these experiments. The results are summarized in Fig. 4 and in Supplementary Table S5.

All of the FLAG-tagged proteins analyzed copurified with Elongins B and C. As expected, FLAG-VHL and FLAG-FEM1B copurified with Cul2, while the SOCS-box proteins FLAG-ASB1 and FLAG-WSB1 copurified with Cul5. In addition, ASB3, which like ASB1 is a member of the SOCS-box protein family, copurified with Cul5. Of the additional Elongin BC-box proteins that were expressed in 293 cells as FLAG-tagged proteins, PCMTD2 copurified with Cul5, and APPBP2, KLHDC2, KLHDC3, and ZYG11BL copurified with Cul2. Consistent with our observation that ZYG11BL is associated with Cul2, Vasudevan et al recently reported that ZYG11 family members are components of Cul2-containing ubiquitin ligases (26). Neither FLAG-Muf1, FLAG-Elongin A, nor FLAG-NRBP1 copurified with Cul2 or Cul5. Muf1 was recovered, however, by anti-FLAG agarose chromatography of lysates from 293 cells stably
expressing FLAG-Cul5, as were TULP4 (Tubby-like 4), PCMTD1, and several previously known SOCS-box proteins (Supplementary Table S5). Notably, although no Cullin protein copurified with full-length FLAG-Elongin A, two different Elongin A mutants containing functional BC-box motifs but lacking the highly conserved SII-like N-terminal domain copurified with Elongins B and C and with Cul5 (Fig. 4), consistent with our previous observation that a potential Elongin A-containing ubiquitin ligase can be reconstituted in insect cells with Elongins B and C, Cul5, and Rbx1 (7) and suggesting that the Elongin A N-terminus may negatively regulate the assembly of Elongin A into a ubiquitin ligase.

Characterization of Sequence Motifs Governing Interaction with Cul2 or Cul5

The Cul5-box corresponds to the C-terminal portion of the canonical SOCS-box defined by Hilton and coworkers (14) and has the consensus sequence ΦxxLPΦPxxΦxx[Y/F][L/I] (13). In all previously described SOCS-box proteins, the Cul5-box is located immediately C-terminal to the BC-box. Comparison of newly identified BC-box proteins that assemble with Cul5 revealed that they all included an easily recognizable Cul5-box. Surprisingly, however, the Cul5-box in two of them, PCMTD1 and PCMTD2, is located ~90 amino acids downstream of the BC-box.

Although sequences downstream of the BC-box have been shown to be needed for interaction with Cul2, it has been difficult to define a specific sequence motif that correlates with Cul2 binding (13) since only a limited number of BC-box proteins that assemble into Cul2 ubiquitin ligases had been identified prior to this study. In an effort to identify such a sequence motif, we generated a multiple sequence alignment of VHL, MED8, PPIL5 (LRR1), and FEM1B with Cul2 interacting BC-box proteins ZYG11BL, APPBP2, KLHDC2, and KLHDC3 from several species. Also included in the alignment were additional members of the leucine-rich repeat, FEM1, and ZYG11 families of BC-box proteins. As shown in Fig. 1, we were able to define in each of these proteins a sequence motif, which we designate the Cul2-box. The Cul2-box is located ~8-23 amino acids C-terminal to the BC-box and in each protein is evolutionarily conserved in sequence and position. This motif shares some sequence similarity with the Cul5-box and has the consensus ΦPxxΦxxxΦ, where the first position is most frequently a leucine. Although we have not directly demonstrated that ZSWIM family members assemble into Cul2-containing ubiquitin ligases, it is noteworthy that they include a motif that can be aligned with the Cul2-box of other BC-box proteins that have been shown to interact with Cul2.

Finally, human NRBP1 does not include a well-conserved Cullin-box, consistent with our observation that it does not detectably interact with either Cul2 or Cul5. Although NRBP1 does contain a sequence that is distantly related to the Cul2-box immediately following its BC-box (Fig. 1), this sequence is not conserved throughout evolution, suggesting it may not be important for NRBP1 function.

As part of our effort to understand how Cullin-boxes direct recruitment of Cul2 and Cul5 to their respective ubiquitin ligases, we sought to identify Cul2- and Cul5-box residues critical for these interactions. For these studies, we chose as model BC-box proteins the Cul5-box protein PCMTD2 and the Cul2-box proteins KLHDC2 and KLHDC3. The closely related PCMTD1 and PCMTD2 proteins have potential Cul5-boxes that conform well to the consensus Cul5-box motif (Fig. 5A), but, as noted above, are separated from the BC-box by an ~90 amino acid spacer, rather than the 2-10 amino acid spacer observed for other Cul5-box proteins. The potential Cul2-boxes of the KLHDC2 and KLHDC3 proteins are composed of distinct but related amino acid sequences located just downstream of their BC-boxes (Fig. 5A). To confirm the functional significance of these putative Cullin-boxes and to identify Cul2- and Cul5-box residues critical for their ability to direct Cullin recruitment, we constructed a systematic series of FLAG epitope-tagged PCMTD2, KLHDC2, and KLHDC3 mutants with either alanine substitutions or direct swaps of predicted key Cul2- and Cul5-box residues. Wild type and mutant PCMTD2, KLHDC2, and KLHDC3 were introduced by transient transfection into 293T cells and tested using FLAG-immunoprecipitation assays for their abilities to assemble with endogenous Cul2 or Cul5.

The results of these structure-function experiments are shown in Fig. 5 and can be
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summarized as follows. First, several mutations in the predicted PCMTD2 Cul5-box abolished the ability of the protein to assemble with Cul5 without significantly affecting its ability to interact stably with the Elongin BC complex (Fig. 5B), supporting the identification of this sequence as important for Cul5 binding and suggesting that, at least in this case, the Cul5-box can function as a modular unit that supports assembly of a Cul5 ubiquitin ligase even when located as far as 90 amino acids away from the BC-box. Previous structure-function studies of the SOCS-box proteins SS1, RAR3, and SOCS1 supported a role for the conserved LPφP sequence in Cul5 interaction and suggested that the second LP in this motif is particularly important as a determinant of Cul5 binding (13). Consistent with these earlier results, mutation of the central LPLP in the PCMTD2 Cul5-box to AAAP abolished Cul5 binding. We observe, however, that an LPLP to AALP mutation or an LPLP to LPAA had little effect on the interaction of PCMTD2 with Cul5, indicating that a single LP in either position can support Cul5 interaction (Fig. 5B, middle panel). Mutations of the LP motif in the predicted Cul2-boxes of KLHDC2 and KLHDC3 strongly reduced Cul2 binding, indicating that, like the Cul5-box, these Cul2-boxes require a single, similarly positioned LP (Figs. 5C and 5D). The KLHDC3 mutations SCLP to SCLA, SCAA, or AAAA markedly reduced Cul2 binding. In addition, mutation of the KLHDC3 SCLP to LPLP, which rendered the Cul2-box more similar to a Cul5-box, neither reduced Cul2 binding nor enhanced Cul5 binding by KLHDC3. Similarly, mutation of the PCMTD2 LPLP to SCLP neither reduced Cul5 binding nor enhanced Cul5 binding by PCMTD2. Taken together, these observations argue that sequences outside the central LP-containing region are responsible for discriminating between Cul2 and Cul5.

Comparison of the Cul2 and Cul5 binding properties of PCMTD2 mutants M5, M6, M7, and M8, all of which bound Elongin C, suggested that the conserved hydrophobic residue, V, at the N-terminus of the PCMTD2 Cul5-box is important for Cul5 binding, whereas the conserved [Y/F][L/I] residues at the C-terminus are much less important. PCMTD2 mutant M7, in which the N-terminal V remained unchanged but the four C-terminal residues of the Cul5-box were replaced by the four C-terminal residues of the KLHDC2 Cul2-box, bound Cul5 similarly to wild type PCMTD2. In contrast, PCMTD2 mutants M5, M6, and M8, in which various combinations of these residues plus the N-terminal V were mutated to residues found in the Cul2-box of KLHDC2, failed to bind either Cul5 or Cul2 (Fig. 5B, lower panel).

Unlike the Cul5-box, which appears to require a hydrophobic residue at its N-terminus, the Cul2-box can accommodate either a hydrophobic L in the case of KLHDC2 or a non-hydrophobic N in the case of KLHDC3. Nevertheless, we observe that binding of Cul2 to KLHDC2 was severely defective when residues on either the N-terminal or the C-terminal side of the LP were mutated to residues found in the corresponding positions of the PCMTD2 Cul5-box. Thus, although there is no obvious sequence conservation in residues N-terminal to the Cul2-box LP, mutations in this region affect Cul2-box function. Finally, we observe that mutations that made the KLHDC2 and KLHDC3 Cul2-boxes more similar to a Cul5-box did not enhance Cul5 binding, even when, as in the case of KLHDC2 M2, the Culin-box sequence of the mutant protein includes all of the conserved residues of the Cul5-box motif.

**DISCUSSION**

In summary, in this report we have investigated recruitment of Cullin-Rbx modules to Elongin BC-based E3 ubiquitin ligases. In particular, we have investigated how a given BC-box substrate recognition subunit of these ubiquitin ligases is able to direct recruitment of either a Cul2-Rbx1 or Cul5-Rbx2 module. In a recent study, Kamura and coworkers identified in several BC-box proteins a 10-20 amino acid region referred to as a Cul2- or Cul5-box, which is located immediately downstream of the BC-box and which determines whether a given BC-box protein assembles into a Cul2- or Cul5-containing ubiquitin ligase (13).

Here, through purification and analysis of a larger collection of Cul2- and Cul5-containing ubiquitin ligases and through structure-function studies, we have defined the Cul2- and Cul5-box motifs in greater detail. Based on an earlier comparison of the Cul2-box regions of VHL, PPL5, and FEM1B with the Cul5-boxes of a variety of SOCS-box proteins, there appeared to be
little similarity between residues conserved in Cul2- and Cul5-boxes (13). With the identification of additional BC-box proteins that assemble into Cul2 ubiquitin ligases, however, it has become clear that residues conserved in the Cul2-boxes of many BC-box proteins are a subset of those conserved in the Cul5-box. Interestingly, many Cul2-boxes include a single LP, IP, or VP in place of the conserved Cul5-box LP\(\text{I}P\) motif, which has been proposed to play an important role in Cul5 recruitment. We observe, however, that a single LP in the Cul5-box of PCMTD2 will support Cul5 recruitment, and an LP\(\text{I}P\) in the Cul2-box of KLHDC3 will support Cul2 recruitment.

Finally, a by-product of our studies has been the identification of new Elongin BC-box proteins, most of which are members of the families of Cul2- and Cul5-containing ubiquitin ligases. Notably, many of the new BC-box proteins belong to protein families not previously found to function together with Elongins B and C. Among these proteins are members of the ZSWIM (27), TPR, and kelch repeat families. In addition, two of the proteins contain canonical isoaspartate methyltransferase (PCMT) domains and would be predicted to catalyze methylation of target proteins, perhaps linking the ubiquitination and methylation pathways. Other new BC-box proteins are previously uncharacterized members of the leucine-rich repeat and ankyrin-repeat protein families. Although the biological functions of most BC-box proteins are unknown, several of the new BC-box proteins have been implicated in specific biochemical pathways. APPBP2 was originally isolated as a protein that binds to the C-terminal intracellular domain of amyloid precursor protein (28), which is believed to be the major neurodegenerative agent in Alzheimer’s disease (29). The NRBP1 and NRBP2 proteins contain two LXXLL nuclear receptor binding motifs and a serine/threonine kinase-like domain, although it is not clear whether the latter domain is catalytically active (30). NRBP1, also called MADM (MLF1-adaptor molecule) interacts in cells with activated Rac3 and with myeloid leukemia factor 1 (MLF1), and it appears to promote phosphorylation of the MLF1 14-3-3 binding site (31). In addition, NRBP1 has recently been shown to play a role in eye development in *Xenopus laevis* (32).

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**FOOTNOTES**

1. The abbreviations used are: APPBP2, amyloid precursor protein binding protein 2; Cul2, Cullin 2; Cul5, Cullin 5; MudPIT, multi-dimensional protein identification technology; NRBP, nuclear receptor binding protein; Rbx1, RING box protein 1; Rbx2, RING box protein 2; RING, really interesting new gene; SOCS, suppressor of cytokine signaling; TSC22, TGF-β1 stimulated clone 22; VHL, Von Hippel-Lindau

2. Research Collaboratory for Structural Bioinformatics Protein Databank = PDB #2izv

**FIGURE LEGENDS**
Cul2 and Cul5 Cullin-Boxes

Fig. 1. **Multiple Sequence Alignment of Selected Elongin BC-associating Proteins.** Human sequences were obtained from the GenBank accession numbers indicated in Table 1 and sequences for the closest orthologs in other species were obtained using Protein-protein BLAST (blastp) from NCBI. Sequences were first aligned using the blosum62mt2 alignment matrix with the AlignX program of the Vector NTI software package. Identical amino acids are highlighted in yellow, very similar ones in blue and similar amino acids are shown in green based on the AlignX default similarity table. The names of BC-box proteins known to assemble into Cul2 or Cul5 ubiquitin ligases prior to the initiation of this study are shown in bold type; names of BC-box proteins demonstrated in this study to assemble into Cul2 or Cul5 ubiquitin ligases are shown in red. BC-box proteins detected in MudPIT analyses are indicated with an asterisk. The canonical BC-box and Cul5-box sequences and conserved Cul2-box residues are indicated below each alignment. hs: Homo sapiens, mm: Mus musculus, xl: Xenopus laevis, dm: Drosophila melanogaster, dp: Drosophila pseudoobscura, oa: Ornithorhynchus anatinus, ce: Caenorhabditis elegans, xt: Xenopus tropicalis.

Fig. 2. **Domain Organization of Some Cul2-box and Cul5-box proteins.** The BC-box, Cul2-box and Cul5-box are represented as black, gray and hatched boxes, respectively. LRR, Leucine Rich Repeats; ANK, Ankyrin Repeats; ARM, Armadillo; TPR, tetratricopeptide repeats; K, KELCH domain; PCMT, protein-L-isoaspartate carboxymethyltransferase; RAB, Rab-like GTPase domain; SII, transcription factor SII-like domain; SH, SH2 phosphotyrosine binding domain; SW, SWIM (SWI2/SNF2 MuDR) zinc fingers; TUB, Tubby-like domain; LXXLL, LXXLL Nuclear Receptor Binding motifs; SPRY, SP1a and ryanodine receptor domain; S_TKc, serine/threonine protein kinase catalytic domain.

Fig. 3. **BC-Box Mutations Interfere with Binding of Newly Identified Elongin BC-Associating Proteins to Elongins B and C**. HEK293T cells were co-transfected with pcDNA5 constructs encoding c-myc-tagged Elongin B, HSV-tagged Elongin C, and FLAG-tagged wild type or mutant versions of the indicated proteins. Cells were lysed using the low salt extraction, low-speed centrifugation protocol described in Experimental Procedures. Proteins were immunoprecipitated using anti-FLAG agarose, eluted from anti-FLAG beads with FLAG peptide, and subjected to Western blotting with anti-FLAG, anti-c-myc, or anti-HSV antibodies. IP, immunoprecipitation; WB, western blotting.

Fig. 4. **BC-box Proteins and Their Associated Cullins.** MudPIT analyses of immunopurified material from Flp-In™ 293 cell lines stably expressing FLAG-tagged Elongin BC-associating proteins. The numbers shown correspond to the number of spectra detected in MudPIT runs. Detailed supporting data is provided in Supplementary Table S5.

Fig. 5. **Characterization of sequences essential for Cul2 or Cul5 binding.** (A) Alignment of amino acid sequences of the consensus Cul-2 and Cul-5 boxes with the Cullin-boxes of PCMTD2, KLHDC2 and 3. Conserved amino acids are indicated in bold. (B,C) Lysates of HEK293T cells transiently expressing wild type Flag-tagged PCMTD2, KLHDC2, KLHDC3 or various mutant versions of these proteins as indicated in the top panel were subjected to immunoprecipitation with anti-Flag agarose. Proteins were eluted from anti-FLAG beads with FLAG peptide, and subjected to Western blotting with anti-FLAG, Anti-Cul5, Cul2 or Elongin C antibodies. Mutated amino acids are shown in red lowercase type. Anti-Cul5 blots were developed with SuperSignal West Dura Extended Duration Substrate (Pierce Biotechnology) and exposed to film for approximately 5 min (B) or SuperSignal West Femto Maximum Sensitivity Substrate and exposed to film for approximately 30 min (C and D). Anti-Cul2 blots were developed with SuperSignal West Femto Maximum Sensitivity Substrate and exposed to film for approximately 30 min (B) or SuperSignal West Dura Extended Duration Substrate (Pierce Biotechnology) and exposed to film for approximately 5 min (C and D).
### Fig. 1

#### Cul2

| Gene       | Accession   | Start   | End     | BC-box | Cullin-box |
|------------|-------------|---------|---------|--------|-----------|
| VHHLhs*    | NP_000542   | 155     | 189     |        | 189       |
| FPIL5hs*   | NP_689542   | 320     | 352     |        | 352       |
| PPIL5dp     | XP_01508344 | 234     | 276     |        | 276       |
| LRRC14hs   | NP_055480   | 83      | 119     |        | 119       |
| PPIL5hs     | NP_689542   | 320     | 352     |        | 352       |
| PPIL5dp     | EAL33104    | 330     | 360     |        | 360       |
| LRRC14xl   | NP_001087894| 330     | 360     |        | 360       |
| LRRC14hl   | NP_055480   | 83      | 119     |        | 119       |
| LRRC28hs   | NP_055480   | 83      | 119     |        | 119       |
| LRRC28xl   | NP_001087894| 330     | 360     |        | 360       |
| LRRC28hl   | NP_055480   | 83      | 119     |        | 119       |
| LRRC42hs   | NP_055480   | 83      | 119     |        | 119       |
| LRRC42xl   | NP_001087894| 330     | 360     |        | 360       |
| LRRC42hl   | NP_055480   | 83      | 119     |        | 119       |
| LRRC58hs   | NP_055480   | 83      | 119     |        | 119       |
| LRRC58xl   | NP_001087894| 330     | 360     |        | 360       |
| LRRC58hl   | NP_055480   | 83      | 119     |        | 119       |
| FEM1Bhs*   | NP_056137   | 594     | 625     |        | 625       |
| FEM1Ahs*   | NP_061178   | 636     | 667     |        | 667       |
| FEM1Ace    | CAI26025    | 638     | 669     |        | 669       |
| FEM1Chs*   | NP_064562   | 583     | 614     |        | 614       |
| FEM1Cxl    | NP_001090163| 583     | 614     |        | 614       |
| FEM1Bhs*   | NP_078922   | 583     | 614     |        | 614       |
| FEM1Bxl    | AAI26060    | 636     | 667     |        | 667       |
| ZYG11BLhs* | NP_006327   | 15      | 51      |        | 51        |
| ZYG11BLmm* | NP_001028806| 15      | 51      |        | 51        |
| ZYG11BLdm  | AAF47500    | 15      | 51      |        | 51        |
| ZYG11ce    | NP_495677   | 15      | 51      |        | 51        |
| ZYG11xl    | AAH57742    | 15      | 51      |        | 51        |
| ZYG11Bhs*  | NP_078922   | 15      | 51      |        | 51        |
| ZYG11Bxl   | AAI26060    | 15      | 51      |        | 51        |
| APPBP2hs*  | NP_006371   | 10      | 44      |        | 44        |
| APPBP2dm   | NP_572328   | 10      | 44      |        | 44        |
| KLHDC2hs*  | NP_055130   | 361     | 395     |        | 395       |
| KLHDC2mm*  | NP_081393   | 361     | 395     |        | 395       |
| KLHDC2xl   | AAH73038    | 362     | 396     |        | 396       |
| KLHDC3hs*  | NP_476502   | 335     | 365     |        | 365       |
| KLHDC3dm   | AM50253     | 357     | 387     |        | 387       |
| KIAA0913hs*| NP_055852   | 73      | 109     |        | 109       |
| KIAA0913dm*| NP_001090163| 73      | 109     |        | 109       |
| ZSWIM5hs*  | NP_065934   | 303     | 339     |        | 339       |
| ZSWIM5xl   | NP_001090163| 303     | 339     |        | 339       |
| ZSWIM5hm   | NP_001090163| 303     | 339     |        | 339       |
| ZSWIM6hs*  | NP_001090163| 303     | 339     |        | 339       |
| ZSWIM6xl   | NP_001090163| 303     | 339     |        | 339       |
| MED8hs     | NP_000101654| 140     | 183     |        | 183       |

#### Cul5

| Gene       | Accession   | Start   | End     | BC-box | Cullin-box |
|------------|-------------|---------|---------|--------|-----------|
| SOCS2hs*   | NP_003868   | 160     | 192     |        | 192       |
| SOCS6hs*   | NP_004223   | 497     | 531     |        | 531       |
| WSB1hs*    | NP_055413   | 515     | 549     |        | 549       |
| ASB8hs*    | NP_055413   | 515     | 549     |        | 549       |
| ASB9hs*    | NP_057198   | 296     | 333     |        | 333       |
| ASB8hs*    | NP_057198   | 296     | 333     |        | 333       |
| ASB13hs*   | NP_078977   | 239     | 285     |        | 285       |
| ASB8hs*    | NP_078977   | 239     | 285     |        | 285       |
| SSB3hs*    | NP_543137   | 239     | 285     |        | 285       |
| TULP4hs*   | NP_064630   | 189     | 223     |        | 223       |
| MUF1hs*    | NP_006360   | 43      | 79      |        | 79        |
| PCMTD1hs*  | NP_443169   | 240     | 352     |        | 352       |
| PCMTD1xl   | AAH81122    | 240     | 352     |        | 352       |
| PCMTD1rm*  | NP_89844    | 240     | 352     |        | 352       |
| PCMTD2hs*  | NP_060727   | 240     | 352     |        | 352       |
| PCMTD2rm*  | NP_060727   | 240     | 352     |        | 352       |
| ANKRD9hs*  | NP_689539   | 275     | 313     |        | 313       |
| ANKRD9xt   | AA135836    | 275     | 313     |        | 313       |
| EloAh*     | NP_003189   | 548     | 581     |        | 581       |
| EloA2hs*   | NP_057511   | 526     | 559     |        | 559       |
| EloA3hs*   | NP_061628   | 526     | 559     |        | 559       |

#### No Cullin Detected

| Gene       | Accession   | Start   | End     | BC-box | Cullin-box |
|------------|-------------|---------|---------|--------|-----------|
| NRFB1hs*   | NP_037524   | 330     | 357     |        | 357       |
| NRFB1xl    | NP_001005659| 330     | 357     |        | 357       |
| NRFB1dm    | NP_649581   | 296     | 401     |        | 401       |
**Fig. 2**

### Cul2

- PPIL5
- LRRC14
- LRRC28
- LRRC42
- LRRC58
- FEM1A
- FEM1B
- FEM1C
- APPBP2
- ZYG11B
- ZYG11BL
- KHLDC2
- KHLDC3
- KIAA0913
- ZSWIM5
- ZSWIM6

### Cul5

- SOCS6
- SOCS7
- ASB3
- ASB6
- ASB8
- ASB13
- SSB3
- TULP4
- RAB40C
- PCMTD1
- PCMTD2
- ANKR69
- ELOA
- ELOA2
- ELOA3

### No Cullin detected

- NRBPI
Fig. 3

**IP: anti-Flag**

| Sample       | Mock | f-vHl l-wf | f-vHl l-C162F | f-PCMTD2 l-wf | f-PCMTD2 L243P, A247F |
|--------------|------|------------|---------------|----------------|------------------------|

**WB: anti-Flag**

- EloB

**WB: anti c-myc**

- EloC

**WB: anti HSV**

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### Fig. 4

| Protein Name | None | VHL | FEM1B | APPBP2 | ZYG11BL | KLHDC2 | KLHDC3 | ASB1 | ASB3 | WSB1 | RAB40B | PCMTD2 | MUF1 | Elongin A | Elongin A 388-772 | Elongin A 470-772 | NRB1 |
|--------------|------|-----|-------|--------|---------|--------|--------|------|------|------|--------|--------|------|-----------|-------------------|-------------------|------|
| Bait         | 33   | 710 | 156   | 1360   | 91      | 43     | 269    | 32   | 229  | 68   | 248    | 242    | 683  | 183       | 105               | 231               |      |
| Cul2         | 117  | 598 | 2     | 25     | 13      | 29     |        |      |      |      |        |        |      |           |                   |                   |      |
| Cul5         | 4    |     | 4     | 303    | 208     |        | 33     | 119  | 126  | 19   | 22     |        |      |           |                   |                   |      |
| Elongin B    | 28   | 36  | 31    | 216    | 13      | 6      | 28     | 22   | 9    | 11   | 121    | 47     | 9    | 111       | 33                | 48                |      |
| Elongin C    | 30   | 59  | 60    | 123    | 21      | 6      | 40     | 77   | 95   | 28   | 68     | 20     | 135  | 75        | 72                | 39                |      |
**Supplementary Table S1. Potential Elongin BC-associating proteins identified by MudPIT analysis of Elongin B preparations from FLAG-Elongin B-expressing human Flp-In™ 293 or murine M1 cell lines.**

FLAG-Elongin B-associating proteins were purified by anti-FLAG agarose immunoaffinity chromatography from cell lysates from 10 independent preparations of 293 cells (5 grown without and 5 grown with 50 μM of the proteasome inhibitor MG132 for 4 hr prior to cell lysis) and 7 independent preparations of M1 cells grown without MG132. As a control for the specificity of immunoaffinity purifications, combined data from 293 cells or M1 cells. "Times detected" represents the number of MudPIT runs in which a particular protein was detected out of a total of 10 or 7 independent FLAG-Elongin B purifications from 293 or M1 cells, respectively. Human GenBank accession numbers are listed. The proteins detected in samples from M1 cells are the murine orthologs of the human proteins listed; murine accession numbers are provided in Table S2. Detailed supporting data is provided in the supplementary tables. The numbers in parentheses are supporting references. 

| Protein   | Other names | Gen Bank acc. no. (H. sapiens) | Conserved Domains | 293-FRT Total Spectra | 293-FRT Times Detected | M1 Total Spectra | M1 Times Detected | Cullin |
|-----------|-------------|-------------------------------|-------------------|------------------------|------------------------|------------------|------------------|--------|
| NCBI | NCBI | NCBI | NCBI | NCBI | NCBI | NCBI | NCBI | NCBI |

**New Elongin BC-associating proteins**

| Protein   | Other names | Gen Bank acc. no. (H. sapiens) | Conserved Domains | 293-FRT Total Spectra | 293-FRT Times Detected | M1 Total Spectra | M1 Times Detected | Cullin |
|-----------|-------------|-------------------------------|-------------------|------------------------|------------------------|------------------|------------------|--------|
| NCB1 | NCB1 | NCB1 | NCB1 | NCB1 | NCB1 | NCB1 | NCB1 | NCB1 |

**Shared subunits of Cul2/5 ubiquitin ligases**

| Protein   | Other names | Gen Bank acc. no. (H. sapiens) | Conserved Domains | 293-FRT Total Spectra | 293-FRT Times Detected | M1 Total Spectra | M1 Times Detected | Cullin |
|-----------|-------------|-------------------------------|-------------------|------------------------|------------------------|------------------|------------------|--------|
| NCBI | NCBI | NCBI | NCBI | NCBI | NCBI | NCBI | NCBI | NCBI |

**BC-box proteins identified prior to initiation of this study**

| Protein   | Other names | Gen Bank acc. no. (H. sapiens) | Conserved Domains | 293-FRT Total Spectra | 293-FRT Times Detected | M1 Total Spectra | M1 Times Detected | Cullin |
|-----------|-------------|-------------------------------|-------------------|------------------------|------------------------|------------------|------------------|--------|
| NCBI | NCBI | NCBI | NCBI | NCBI | NCBI | NCBI | NCBI | NCBI |

**293-FRT and Table S5; cThe indicated Elongin BC-binding proteins copurified with FLAG-Cul2, supporting data in Table S5; bSupporting data in Tables S3 and S4.** Table 293-FRT shows the results of the MudPIT analysis of Elongin B preparations from FLAG-Elongin B-expressing human Flp-In™ 293 or murine M1 cell lines. **The last column lists the Cullin protein shown to be associated with the particular protein detected.**
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Characterization of cullin-box sequences that direct recruitment of Cul2-Rbx1 and Cul5-Rbx2 modules to elongin BC-based ubiquitin ligases

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