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COMMD1 expression is controlled by critical residues that determine XIAP binding

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Synopsis

COMM domain-containing (or COMMD) proteins participate in several cellular processes, ranging from NF- κ B regulation, copper homeostasis, sodium transport and adaptation to hypoxia. The best studied member of this family is COMMD1, but relatively little is known about its regulation, except that XIAP functions as its ubiquitin ligase. In this study, we identified that the COMM domain of COMMD1 is required for its interaction with XIAP, and other COMM domain containing proteins can similarly interact with IAPs. Two conserved leucine repeats within the COMM domain were found to be critically required for XIAP binding. A COMMD1 mutant unable to bind to XIAP demonstrated complete loss of basal ubiquitination and great stabilization of the protein.

Underscoring the importance of IAP-mediated ubiquitination, we found that long-term expression of wild-type COMMD1 results in nearly physiologic protein levels due to increased ubiquitination, but this regulatory event is circumvented when expressing a mutant form that cannot bind XIAP.

Altogether, our findings indicate that COMMD1 expression is primarily controlled by protein ubiquitination and its interaction with IAP proteins plays an essential role.

Keywords

COMMD1; COMMD proteins; XIAP; IAP; ubiquitin

Introduction

COMMD proteins are a group of evolutionarily conserved factors present in a wide range of organisms [1]. All ten members of this family are found in vertebrates and are also present in *Dictyostelium discoideum*. In addition, several *COMMD* genes are also identifiable in *Drosophila* and *Caenorhabditis* species, as well as in various unicellular protozoa [2].

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The defining characteristic of all the members of the family is the presence of a highly conserved and unique domain in the extreme carboxy-terminus of these proteins known as the *Copper Metabolism MURR1* or *COMM* domain. The tertiary structure of this domain is not known, but it contains conserved tryptophan and proline residues, as well as a series of conserved leucines. The *COMM* domain not only defines the protein family but provides a critical interface for protein-protein interactions, such as *COMMD1*-*COMMD1* dimer formation and binding to *Cul2*, *Elongin C* and *SOCS1* [1,3-5].

At the present time, a number of seemingly disparate functions have been ascribed to these factors. *COMMD1* has been shown to inhibit HIV-1 replication and expression of several pro-inflammatory mediators through its suppression of NF- κ B transcriptional activity [3,6]. *COMMD1* is also known to promote copper excretion, presumably by regulating the stability of the copper transporter *ATP7B* [7-10]. Indeed, a spontaneous mutation in *COMMD1* is responsible for canine copper toxicosis, a disorder that resembles the effects of *ATP7B* mutations [11,12]. *COMMD1* has been implicated in intracellular sodium regulation through its inhibitory effects on the function of the epithelial sodium channel [13]. Recently, *COMMD1* was also found to regulate the adaptation to hypoxia, by serving as an inhibitor of the HIF1 α transcription factor [14]. Finally, *COMMD5* has been reported to affect cell proliferation and gene expression, but the precise targets responsible for these effects are unknown [15-17].

Little is known about the regulation of *COMMD* proteins themselves. We have previously reported that *XIAP*, a member of the Inhibitor of Apoptosis (*IAP*) family, functions as a ubiquitin ligase for *COMMD1* [8]. However, the ultimate importance of this interaction in the regulation of *COMMD1* protein expression and whether this paradigm is true for other *COMMD* proteins is not completely understood. In this study we report that basal levels of *COMMD1* expression are tightly regulated through ubiquitination, and the ability of *COMMD1* to bind to *XIAP* seems to be critical. We mapped the interaction between *COMMD1* and *XIAP* to the *COMM* domain and identified that leucine repeats within the *COMM* domain are required for this interaction. A mutant version of *COMMD1* that cannot bind to *XIAP* lost any appreciable basal ubiquitination, was greatly stabilized and could be stably overexpressed. The data collectively indicate that the *XIAP*-*COMMD1* interaction is critical to the regulation of *COMMD1* protein levels.

Materials and Methods

Plasmids

The plasmids pEBB, pEBG, pEBB-*COMMD1*-Flag, pEBB-*COMMD1*-GST, pEBB-*COMMD1*-GST have been previously described [1,8,18-20]. In addition, we utilized pEBB-*COMMD1* N-term (amino acids 1-118) and pEBB *COMMD1* *COMM* domain (amino acids 119-190) [3]. The plasmids pEBB-*COMMD1* W/P (W124A, P141A), Mut1 (I145A, L147A, L149A), Mut2 (L172A, L175A, V178A) and Mut1/2 were generated by site directed mutagenesis, and were subsequently subcloned into the pGEX-4T1 vector for bacterial expression. The expression vectors pEBB-*XIAP*, pEBB-Flag-*XIAP* pEBG-*XIAP*, pEBB-*XIAP* H467A and pEBG-*XIAP* H467A have been previously described [8]; pEBB Flag c-IAP1 H588A and pEBB Flag c-IAP2 H574A were kindly provided by Dr. Colin Duckett. The plasmid pCW7-His₆-Myc-Ubiquitin was previously described [21].

Cell culture and transfection

Human embryonic kidney 293 and 293T cells were obtained from ATCC. Those cell lines were cultured in DMEM supplemented with 10% FBS and L-glutamine. Treatment with the proteasome inhibitor MG-132 (Boston Biochem) consisted of culturing cells for 3 hour in media containing a 40 μ M concentration of the drug (or the vehicle DMSO as a control). A

standard calcium phosphate transfection protocol [20] was used to transfect plasmids into 293 and 293T cells. Generation of stable lines was performed after lentiviral infection and selection using the transient transfection of the pBABE-Puro plasmid and the corresponding expression vector in a ratio of 1:5. Cells were then placed under Puromycin selection for 6 weeks.

RT-PCR

Total RNA was extracted from 293 cells using the RNeasy procedure (Qiagen) according to the manufacturer's instructions. Yield and purity was determined by measuring OD_{260/280} of RNA diluted in water. Quantitative RT-PCR of *COMMD1* utilizing Taqman probes has been previously described [1]. Quantitative RT-PCR for total and endogenous *COMMD1* mRNA was performed with Sybr-Green based detection using primers located in the transcripts as indicated in Figure 4C: (1) GGACACTTTCCACGGGTAC, (2) ATCCATGTCTGCAGACGCAA, (3) TGTGTGCCATTATAGAGCTGG, and (4) CTTAGAAAAGGTCAGTGGGG.

Immunoblotting and immunoprecipitation

Cell lysates were prepared by adding Triton lysis buffer [8]; immunoblotting and GSH precipitations were performed as previously described [8]. Denaturing immunoprecipitations were performed after boiling cell lysates for 10 minutes in a buffer containing 1% SDS, as previously described [3]. Antibodies against β -Actin (Sigma, A5441), *COMMD1* [1], [12] (Abnova, H00150684-M01), Flag (Sigma, A8592), GST (Santa Cruz, sc-459), *SCHAD* [12], Ubiquitin (Stressgen, SPA-205), and XIAP (BD Pharmingen, 610716) were used as indicated.

Ubiquitination assays

293 cells were transfected with His₆-Ubiquitin along with the indicated plasmids in each experiment. 48 hours later, the cells were lysed with a protein denaturing buffer (8M urea, 50mM Tris, pH 8.0, 300mM NaCl, 50 mM NaPO₄, 0.5% NP-40). The lysate was sonicated for 20 seconds, and centrifuged to obtain a cleared lysate. This was applied to Ni-NTA-Agarose beads (Invitrogen) and rocked at room temperature for 2 hours. The lysate was subsequently aspirated, and the beads washed 4 times in the same buffer at room temperature. The recovered ubiquitinated material was then resuspended in LDS gel loading buffer (Invitrogen) and separated by SDS-PAGE for subsequent immunoblotting. *In vitro* ubiquitination was performed as previously described [8].

In vitro binding assays

Recombinant proteins (GST, *COMMD1*-GST, and *COMMD1* Mut1/2 – GST) were prepared in *E. coli* as previously described [22]. GST fusion proteins bound to GSH sepharose were incubated at room temperature with BSA (1 mg/mL) in 200 μ L of Z-buffer (25 mM HEPES, pH 7.5, 12.5 mM MgCl₂, 150 mM KCl, 0.1% NP-40, 20% glycerol). After 15 min, a cell lysate from cells transfected with an XIAP expression vector was added and incubated for another 45 min at room temperature. The beads were then washed four times with 1 mL NETN buffer (100 mM NaCl, 1 mM EDTA, 0.5% NP-40, 20 mM Tris-HCl, pH 8.0). Bound proteins were resolved by SDS-PAGE and subjected to western blot analysis.

Results

XIAP and c-IAP1 promote *COMMD1* degradation

We have previously reported that *COMMD1* interacts with the BIR3 domain of XIAP, a pro-survival member of the IAP (Inhibitor of Apoptosis) family [23]. Through its RING domain, XIAP functions as an E3 ubiquitin ligase for *COMMD1*. Other IAP family members, such as c-IAP1 and c-IAP2, also contain the BIR3 and RING domains and can function as E3 ubiquitin

ligases [24]. Therefore, we examined first if c-IAP1 or c-IAP2 could similarly promote COMMD1 degradation. To that end, COMMD1 was co-expressed with wild-type or a RING inactive mutant version of XIAP, c-IAP1, or c-IAP2. As was described previously [8], wild-type XIAP expression reduced COMMD1 levels, while the RING inactive mutant XIAP H467A promoted COMMD1 accumulation (Figure 1A). Expression of c-IAP1 resulted in a similar effect, even though the wild-type form of c-IAP1 was expressed at very low levels that were only detectable in very long exposures (data not shown). In contrast, the highly homologous c-IAP2 protein did not promote COMMD1 degradation.

The COMM domain mediates IAP binding

Given the effects of other IAP members on COMMD1 expression, we examined the COMMD1-IAP interaction in more detail. To that end, we first determined the domain in COMMD1 required for this interaction. The amino terminus (amino acids 1-118) or the COMM domain (amino acids 119-190) of COMMD1 were expressed in 293 cells fused with glutathione S-transferase (GST). The expressed proteins were subsequently precipitated from cell lysates with glutathione (GSH) sepharose beads and the presence of co-precipitated XIAP was determined by immunoblotting. As can be seen in Figure 1B, the COMM domain was necessary and sufficient for XIAP binding and proteasomal blockade enhanced the COMMD1-XIAP interaction, as previously described [8].

The identification of the COMM domain as the binding interface in the COMMD1-XIAP interaction suggested that other COMM-domain containing proteins might interact with XIAP. Similarly, given the effects of c-IAP1 on COMMD1 expression, we examined the potential interaction of all COMMD proteins with the XIAP homologs c-IAP1 and c-IAP2. All ten COMMD proteins fused to GST were expressed in 293 cells and subsequently precipitated from cell lysates. The presence of co-precipitated IAP proteins was determined by immunoblotting. The expressed IAPs contained a histidine to alanine point mutation in their RING domains that inactivate their ubiquitin ligase activity [25,26]. This has been previously shown to stabilize the COMMD1-XIAP interaction [8], and allow for detectable expression of the very unstable c-IAP proteins as previously reported [26] (and also shown in Figure 1A). Selected COMMD proteins, namely COMMD1, 2, 4, 5, and 10, demonstrated the ability to bind to IAP proteins, and interestingly, the binding patterns were similar for XIAP, c-IAP1 and c-IAP2 (Figure 1C).

Consistent with their interaction with XIAP and the known role of this protein as a ubiquitin ligase, these COMMD proteins were expressed at lower levels in cells concomitantly transfected with wild-type XIAP (Figure 1D). The mutant XIAP H467A, which is devoid of ubiquitin ligase activity, did not promote COMMD degradation (data not shown), similar to its effect on COMMD1 [8]. Altogether, these findings indicate that like COMMD1, other COMMD proteins also interact with IAPs and undergo similar regulation.

Leucine repeats in the COMM domain are required for COMMD1-XIAP interactions

The identification that COMMD-IAP interactions are mediated by the COMM domain suggested that conserved elements in this domain were likely required for these interactions. The COMM domains of COMMD1, 2, 4, 5, and 10 were aligned as previously described [1]. As shown in Figure 2A, invariably conserved tryptophan and proline residues were identified. In addition, the same alignment demonstrated two conserved leucine repeats. Interestingly, the leucine repeats were also evaluated for their possible role in determining the nucleocytoplasmic distribution of COMMD1 and were found to function as nuclear export signals (Muller and Klomp, unpublished observations).

Point mutations in these areas of conservation were introduced to generate the indicated mutants (Figure 2B), and the ability of these mutants to bind to XIAP was evaluated. As shown in Figure 2C, COMMD1 wild-type demonstrated robust binding to XIAP H467A as expected. However, mutations on the first leucine repeat (Mut1 or Mut1/2) abrogated this interaction. The ability of COMMD1 wild-type or Mut1/2 to bind to XIAP was evaluated again, this time examining their ability to interact with XIAP wild-type as well as XIAP H467A (Figure 2D). In both cases, COMMD1 Mut1/2 failed to bind to XIAP confirming the results shown in Figure 2C. Given that mutations in the leucine repeats also affect the nucleo-cytoplasmic distribution of COMMD1 (Muller and Klomp, unpublished observations), we examined whether the loss of a COMMD1-XIAP interaction could be due to sequestration within the cell. To that end we examined the ability of recombinant COMMD1-GST fusion proteins prepared in *E. coli* to bind to XIAP *in vitro*. As shown in Figure 2E, recombinant wild-type COMMD1 bound to XIAP *in vitro*, while the mutant Mut1/2 failed to do so, consistent with the notion that the observations made in transfected cells represented an intrinsic loss of XIAP binding ability for this mutant. Altogether, these experiments indicated that areas of conservation in the COMM domain, particularly its first leucine repeat, are critical for IAP binding.

Mutations that disrupt IAP binding prevent basal ubiquitination of COMMD1

Since XIAP functions as an E3 ubiquitin ligase for COMMD1, the loss of COMMD1-XIAP interactions would be predicted to decrease the ubiquitination of COMMD1. To test this notion, we evaluated the basal rate of ubiquitination of wild-type COMMD1 or the COMM domain mutant forms. To that end, cells were transfected with His₆-tagged ubiquitin along with COMMD1 wild-type or the indicated mutants. Ubiquitinated proteins were subsequently precipitated from cell lysates using Nickel-agarose beads and the recovered polyubiquitinated fraction was immunoblotted for COMMD1 (Fig 3A, upper panel). As can be appreciated, mutations in either leucine repeat (Mut1, Mut2 or Mut1/2) resulted in dramatic reductions in the amount of ubiquitinated COMMD1 recovered. This was the case even though there was equal recovery of polyubiquitinated proteins in each precipitation (Fig 3A, middle panel) and the expression levels for these mutants were comparable to the wild-type protein (Fig 3A, lower panel). Notably, the W/P mutant, which binds well to XIAP did not demonstrate this phenotype.

Our previous experiments (Figure 2B) demonstrated that Mut2 is capable of binding to XIAP, albeit with lesser efficiency than the wild-type protein when taking into account its input levels. Therefore, the apparent lack of basal ubiquitination for this mutant (Figure 3A) was initially surprising. To examine if some degree of ubiquitination of Mut2 was still possible, the mutants were expressed at increasing levels to detect basal polyubiquitination as before (Figure 3B). This experiment demonstrated that some degree of ubiquitination could be observed for Mut2, and to a lesser extent, Mut 1 could also be ubiquitinated. However, a combined mutation in both leucine repeats (Mut1/2) was resistant to ubiquitination. In addition as shown in Figure 3C, wild-type COMMD1 could be ubiquitinated *in vitro* as previously reported [8]; however, under the same conditions a mutation in both of the leucine repeats prevented this protein from being ubiquitinated.

Consistent with their effects on ubiquitination, the mutations on the leucine repeats of COMMD1 greatly enhanced basal protein expression, an effect that was most pronounced for Mut1/2 (Figure 3D). Similarly, expression levels of wild-type COMMD1 were reduced after XIAP transfection (Figure 3E), an effect mediated by the E3 ubiquitin ligase activity of XIAP [8]. However, Mut1/2 was resistant to XIAP-mediated degradation and the mutations in either leucine repeat (Mut 1 or Mut 2) demonstrated an intermediate phenotype (Figure 3E). Altogether, these results indicated that disruption of the COMMD1-XIAP interaction resulted in decreased ubiquitination of COMMD1 and concomitant protein stabilization.

COMMD1 expression is controlled by post-transcriptional mechanisms

As shown in Figure 3, mutations in the leucine repeats of COMMD1 prevented basal ubiquitination with consequent stabilization of the protein. We examined these effects further by generating stable cell lines expressing either wild-type COMMD1 or the Mut1/2 version. As shown in Figure 4A, stable expression of Mut1/2 resulted in greater protein levels to that of the wild-type protein, although the mRNA expression of both constructs was comparable (Figure 4B). Interestingly, repeated attempts at generating cell lines that stably overexpress wild-type COMMD1 resulted in very modest or no overexpression of the wild-type protein (Figures 4A and 4D). This was the case irrespective of the cell type utilized (293 cells, Hep 3B and Jurkat cells) or whether plasmid transfection or lentiviral infection were used to achieve stable expression (data not shown). In addition, we invariably observed that long-term expression of wild-type COMMD1 resulted in concomitant suppression of endogenous COMMD1 levels, such that the overall cellular levels of COMMD1 were only modestly elevated compared to the control cell lines (Figures 4A and 4D).

The suppressed expression of endogenous *COMMD1* after stable expression of the wild-type protein suggested that cells possess a critical mechanism responsible for the maintenance of nearly physiologic levels of COMMD1 protein. Since the endogenous gene and the exogenous constructs utilize different promoters and UTR elements, we reasoned that mRNA expression was unlikely to account for the reduction of endogenous *COMMD1* expression. To examine this notion, we devised a strategy to measure either total *COMMD1* mRNA (total expression) or expression resulting from the endogenous gene only (endogenous expression, see Figure 4C). As shown in Figure 4D, total levels of *COMMD1* mRNA were elevated by nearly 50-fold in the COMMD1 stable cell line compared to the control, even though the protein levels were only modestly elevated. Importantly, mRNA expression of the endogenous *COMMD1* gene was nearly identical to the vector control line, despite the fact that the endogenous protein was almost undetectable.

COMMD1 ubiquitination controls basal levels of expression

These findings indicated that COMMD1 protein levels are kept within a physiologic range primarily through a post-transcriptional mechanism. Since this is only observed when expressing wild-type COMMD1 and not the ubiquitination resistant-mutant, we reasoned that ubiquitination might be primarily responsible for the physiologic control of COMMD1 levels. To test this notion, COMMD1 was immunoprecipitated from vector control or COMMD1 stable cell lines with a rabbit polyclonal antibody and the recovered material was immunoblotted with a mouse monoclonal antibody against COMMD1. As expected, COMMD1 was recovered after immunoprecipitation with the rabbit antibody, but not the preimmune serum control (Figure 5A, bottom panel). In addition, significant amounts of modified high molecular weight COMMD1 was recovered only from the COMMD1 wild-type stable cell line and not the vector control or the COMMD1 Mut1/2 lines (Figure 5A, top panel). Immunoblotting with a Flag antibody similarly demonstrated the presence of modified high molecular weight in the COMMD1 wild-type cell line and not the COMMD1 Mut1/2 stable line (data not shown). Aided by the fact that Mut1/2 cannot be ubiquitinated under basal conditions as shown before (Figure 3), we concluded that this material is ubiquitinated COMMD1 and that stable expression of COMMD1 results in an increase in the rate of ubiquitination of this protein.

The notion that stable COMMD1 expression increases its rate of ubiquitination was examined further. To that end, we determined the recovery of COMMD1 from Triton-insoluble fractions, a common feature observed for polyubiquitinated proteins [27]. As can be appreciated in Figure 5B, Triton-insoluble COMMD1 was recovered from cells stably overexpressing wild-type protein and not the Mut1/2 version. This was true despite the fact that the latter was expressed

at significantly higher levels. Similarly, the endogenous protein was largely excluded from the insoluble compartment even under conditions in which its expression levels are similar to those achieved in the COMMD1 wild-type transfected cells.

Altogether, these findings are consistent with the notion that increased ubiquitination of the protein might be responsible for maintaining COMMD1 levels near the physiologic range. In addition, these experiments showed that a mutant of COMMD1 that cannot interact with XIAP escapes this mechanism of control. Finally, we examined whether this increase in COMMD1 ubiquitination was mediated by upregulation of IAP levels. Basal expression of XIAP, c-IAP1 or c-IAP2 was unchanged in the COMMD1 stable cell lines, indicating that the mechanism that accelerates the ubiquitination of COMMD1 is not simply the abundance of its ligase in the cell (Figure 5C).

Discussion

COMMD1, the prototype member of the *COMMD* family, was first identified due to its proximity to the imprinted *U2af1-rs1* gene in the mouse [28]. However, the first functional information about this gene was gleaned when it was identified that *COMMD1* participates in copper metabolism [11,12]. Soon after that report, the ability of COMMD1 to inhibit NF- κ B was identified, and presumably through this effect, COMMD1 was found to limit HIV-1 replication in CD4⁺ T cells [6]. Other functions for COMMD1 such as inhibition of HIF1 α -mediated gene expression [14] and inhibition of the epithelial sodium channel [13] have been reported as well. The mechanism that underlies all these various functions is not completely understood, but COMMD1 has been shown to accelerate the rate of degradation of its interacting partners, namely NF- κ B subunits [3] and ATP7B [7]. In the case of NF- κ B, COMMD1 was found to be a subunit of a Cul2-containing ubiquitin ligase for this transcription factor, and presumably a similar mechanism may underlie its effects on ATP7B and other pathways.

Little is known about the regulation of COMMD1 itself. This factor is ubiquitously expressed and its basal levels are controlled by XIAP, a ubiquitin ligase that belongs to the Inhibitor of Apoptosis family. In this study we examined in greater detail the regulation of basal levels of COMMD1. The data presented here demonstrate that the interaction with IAP proteins is critical for this regulation. We determined that the COMM domain is necessary and sufficient for binding to XIAP, and in particular, we identified that the leucine repeats in the COMM domain play a crucial role in XIAP binding. Mutations in the leucine repeats prevented XIAP-mediated degradation, greatly stabilized the protein, and abrogated basal ubiquitination of COMMD1, highlighting the importance of IAP interactions in the regulation of COMMD1 levels.

In addition, our studies indicate that cellular levels of COMMD1 are tightly regulated within a physiologic range and stable overexpression is controlled by increased ubiquitination. It is unclear what upstream signals might stimulate the ubiquitination of COMMD1. While stable overexpression of COMMD1 increased the ubiquitination of this protein, the levels IAP proteins themselves do not account for this effect, indicating that additional mechanisms to accelerate the rate of COMMD1 ubiquitination must exist.

An additional corollary of our studies is the identification that a subset of COMMD proteins can similarly bind to IAP proteins. It is interesting to note that while many of the conserved elements in the COMM domain are also present in COMMDs that did not interact with IAPs, a complete second leucine repeat was absent in COMMD3, 6, 7, and 8. Nevertheless, a clear dichotomy between these two groups of COMMDs cannot be established on the basis of these conserved elements, indicating that the leucine repeats are primarily necessary but not

sufficient to confer IAP binding. Altogether, these findings suggest that ubiquitination by IAP proteins might be a mechanism of regulation for several COMMDs, a notion that will need further investigation.

The interactions between IAP and COMMD proteins can be best demonstrated when expressing the RING inactive IAP mutants, partly because of stabilized IAP expression, but also probably as a result of inactivating IAP-mediated COMMD ubiquitination. Indeed, we initially failed to identify the interaction between COMMD1 and wild-type c-IAP1 [8]; however, the interaction between these two proteins is apparent when utilizing the RING mutant of c-IAP1, and the ability of wild-type c-IAP1 to induce COMMD1 degradation is also consistent with this interaction. On the other hand, an interaction between COMMD1 and c-IAP2 was also shown in our prior report [8]. However, we did not observe c-IAP2 mediated downregulation of COMMD1 expression, or other COMMDs it interacts with (data not shown), suggesting that unlike XIAP or c-IAP1, c-IAP2 does not behave as an E3 ligase in this context, a finding that deserves additional attention in the future.

Finally, COMMD proteins are involved in a growing number of protein-protein interactions and these have been frequently mapped to the COMM domain [1,3,4,6,8,9,13]. Attempts at obtaining structural information regarding the COMM domain have failed thus far, but the mutational analysis performed here indicates that conserved leucine repeats are critical for their ability to interact with XIAP. In addition, the same regions can function as nuclear export signals (Muller and Klomp, unpublished observations), suggesting that perhaps ubiquitination and cellular distribution might be linked events. Once structural information about the COMM domain is available, we anticipate that the leucine repeats will be found to be critical for the interface between COMMD and IAP proteins.

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Abbreviations used

ATP7B	ATPase, Cu ⁺⁺ transporting, beta polypeptide
BIR	baculovirus IAP repeat
c-IAP	cellular inhibitor of apoptosis
COMMD	Copper Metabolism Murr1 Domain containing
Cul2	Cullin2
DMEM	Dulbecco's Modified Eagle's Medium
GSH	glutathione

GST	glutathione s-transferase
HIF1α	hypoxia inducible factor 1 α
IAP	inhibitor of apoptosis
LDS	Lithium dodecyl sulfate
NF-κB	nuclear factor κ B
NTA	Nitrilotriacetic acid
RING	really interesting new gene
RT-PCR	reverse transcription PCR
SCHAD	short chain 3-hydroxyacyl-CoA dehydrogenase
SOCS1	suppressor of cytokine signaling 1
XIAP	X-linked inhibitor of apoptosis

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Figure 1. IAPs regulate COMM domain containing proteins

(A) XIAP and c-IAP1 promote COMMD1 degradation. Cells were transfected with COMMD1-Flag along with the wild-type or RING inactive mutants of the indicated Flag-tagged IAP proteins (XIAP, c-IAP1, or c-IAP2). One day after transfection, the cells were lysed and the levels of the indicated proteins were analyzed by western blot (Flag or β -Actin immunoblotting). **(B) The COMM domain mediates the COMMD1-XIAP interaction.** Wild-type XIAP was co-expressed with full-length COMMD1 (F.L.), the amino-terminal 118 amino acids (N-term), or the COMM domain (amino acids 119-190) fused with GST. Cells were treated with MG-132 or the corresponding vehicle, and cell lysates were subsequently prepared for COMMD1 precipitation using glutathione (GSH) beads. **(C) Other COMM**

domain containing proteins can interact with IAPs. As in (B), COMMD-GST fusion proteins and the indicated IAP proteins were co-expressed in 293 cells. Point mutations in the RING domains of XIAP, c-IAP1 and c-IAP2 were utilized (see text for details). Cell lysates were subsequently prepared and COMMD-GST proteins were precipitated. The presence of co-precipitated IAP proteins was detected by immunoblotting. Expression of COMMD-GST fusion proteins was confirmed by immunoblotting. **(D) XIAP mediates the degradation of interacting COMMDs.** COMMD1, 2, 4, 5, or 10 were expressed in 293 cells along with XIAP. Cell lysates were subsequently subjected to SDS-PAGE and immunoblotting for COMMD levels (Flag antibody), β -Actin (loading control) or XIAP.

Figure 2. Leucine repeats in the COMM domain determine XIAP binding

(A) **Sequence alignment.** The sequences for COMMD1, 2, 4, 5, and 10 were aligned across their COMM domains as previously described [1]. Highly conserved motifs are noted in the alignment. (B) **Mutations introduced in the COMM domain.** The alanine substitutions introduced are depicted. The W/P mutant targets W124 and P141, Mut1 targets the first leucine repeat (I145, L147, and L149A), Mut2 targets the second leucine repeat (L172, L175, and V178), and Mut1/2 targets both leucine repeats. (C) and (D) **Effects of COMM domain mutations on XIAP binding.** COMMD1 wild-type (WT) and the indicated mutations were expressed along with XIAP H467A in untreated cells (C) or along with either XIAP wild-type or XIAP H467A in cells treated with MG-132 for 3 hours (D). COMMD1 was subsequently

precipitated from cell lysates and the presence of XIAP was determined by immunoblotting. **(E) In vitro binding between XIAP and COMMD1.** Recombinant COMMD1-GST fusion proteins were prepared in *E. coli* (wild-type or Mut1/2 versions) and were subsequently mixed with a lysate from cells expressing XIAP. After precipitation of COMMD1 and extensive washing of the GSH-sepharose beads, the material was resolved by SDS-PAGE and immunoblotted for XIAP (top panel) or for GST (bottom panel).

Figure 3. Mutations in the leucine repeats of COMMD1 stabilize the protein by impairing its ubiquitination

(A) and (B) Basal ubiquitination is greatly affected by mutations in the leucine repeats.

COMMD1 was expressed in 293 cells and ubiquitinated proteins were subsequently precipitated from cell lysates with nickel-agarose beads (to precipitate expressed His₆-tagged ubiquitin). The precipitated material was immunoblotted for COMMD1, and for ubiquitin (to ensure adequate precipitation). All mutants generated were tested in (A) and the mutations involving the leucine repeats were tested again in (B) in cells expressing increasing amounts of COMMD1. **(C) Lack of ubiquitination of Mut1/2 *in vitro*.** Radiolabeled wild-type of Mut1/2 COMMD1 were generated by *in vitro* translation and were subsequently offered as

substrate for an *in vitro* ubiquitination reaction. At the end of the reaction, the material was resolved by SDS-PAGE and the proteins were detected by auto-radiography. Unmodified COMMD1 is indicated by arrows, while the ubiquitinated products are noted by markers to the left of the gel. **(D) Mutations of the leucine repeats stabilize COMMD1.** The wild-type protein or its mutants were transfected in a dose titrated manner and the resulting expression of COMMD1 was determined by immunoblotting. β -Actin was utilized as a loading control. **(E) Mutations in the leucine repeats prevent XIAP-mediated degradation.** COMMD1 wild-type or the indicated mutants were expressed along with XIAP and the levels of expression were determined by immunoblotting (upper panel). Expression of XIAP was confirmed (middle panel) and β -Actin was utilized as a loading control (bottom panel).

Figure 4. COMMD1 expression is controlled by post-transcriptional mechanisms
(A) and (B) Stable expression of COMMD1 Mut1/2 results in greater protein levels than COMMD1 wild-type. 293 cells were stably transfected with COMMD1 wild-type or Mut1/2 for over a month. Cell lysates for mRNA and protein extraction were prepared. *COMMD1* mRNA levels were determined by qRT-PCR and normalized to the levels of *GAPDH* (top graph). COMMD1 protein levels were determined by immunoblotting, using β -Actin as a loading control (bottom panels). **(C) RT-PCR detection strategy.** Relative positions of oligonucleotides that recognize both endogenous and exogenous mRNA for *COMMD1* are indicated. **(D) Decreased expression of endogenous COMMD1 is not due to decreased endogenous mRNA expression.** Utilizing the strategy depicted in (C), *COMMD1* mRNA expression was determined by quantitative real-time RT-PCR (left and middle panels, total and endogenous expression). COMMD1 protein levels are also shown (right panel, SCHAD is a loading control).

Figure 5. COMMD1 ubiquitination is critical for controlling basal levels of COMMD1 expression
(A) Expression of wild-type COMMD1 results in increased ubiquitination. Cell lysates from the indicated stable cell lines were subjected to denaturing immunoprecipitation of COMMD1 using a rabbit polyclonal antibody. The recovered material was immunoblotted for COMMD1 using a mouse monoclonal antibody. Non-modified COMMD1 was readily detectable at the bottom of the gel (bottom panel) and modified high molecular weight (HMW) material was noticeable at the top of the gel on the WT lane only (upper panel). **(B) Recovery of COMMD1 from a Triton-insoluble compartment.** Lysates from the indicated stable cell lines were prepared with a Triton containing buffer (soluble fraction, middle panels), and the remaining pellet was lysed with an 8M urea buffer (insoluble fraction, bottom panels). In addition, a whole cell lysate was prepared by lysing cells directly in an 8M urea buffer (WCL, top panels). COMMD1 levels were determined by immunoblotting; β-Actin and histone 4 were utilized as loading controls. **(C) IAP expression levels were unaffected in COMMD1 stable lines.** Cell lysates were subjected to SDS-PAGE and were immunoblotted with an XIAP antibody (top panel) and then re-probed with an antibody that reacts with both c-IAP1 and c-IAP2 (middle panel). β-Actin serves as a loading control.