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COMMD1: a modulator of immunity and NF-κB activity

Ezra Burstein

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Preface

Brief Introduction

Immunity to pathogens involves innate responses driven by pattern recognition receptors able to respond to microbial determinants without the need of prior exposure. Thereafter, adaptive responses based on the stimulation of T and B cells able to specifically recognize unique molecular characteristics of the offending agent give rise to individualized responses and immune memory.

NF-κB is a conserved transcription factor that regulates expression of genes critical to both adaptive and innate immunity. As such, NF-κB plays a central role in the response to microbial infection, while its inappropriate activation is important in the pathogenesis of multiple diseases, including immune disorders and cancer. Thus, the pathophysiologic consequences of NF-κB activation in human disease are vast, and this remains an area of intense study. NF-κB activity is primarily controlled by IκB proteins, which prevent nuclear accumulation of NF-κB dimers. IkB degradation leads to activation of this pathway, and occurs through a phosphorylation-dependent ubiquitination event. The kinase responsible, known as the IκB kinase (IKK), is a trimeric complex containing IKKα, IKKβ, and the scaffold protein NEMO. Upon IκB degradation, NF-κB dimers accumulate in the nucleus where they bind to promoter sites, activating transcription of gene targets.

Comparatively, less is known about the mechanisms that subsequently terminate NF-kB initiated transcription. It is known that NF-κB binding to chromatin occurs within a physiologic time window. The NF-κB mediated resynthesis of IκB is thought to mediate export of nuclear NF-κB dimers into the cytosol, and by inference, the release of chromatin bound NF-κB. In addition, NF-κB also promotes the expression of the IKK inhibitory factors CYLD and A20, which serve to attenuate IKK activity and IκB degradation. Altogether, these mechanisms promote the resynthesis of IκB and the cytosolic export of NF-κB, and are regarded as the main homeostatic mechanisms.

In addition to IκB dependent termination mechanism, it has been demonstrated that the ubiquitin dependent degradation of DNA-bound RelA is also critical to the termination of NF-κB activity. Moreover, a role for NF-κB phosphorylation mediated by IKKα in these termination events has also been demonstrated. Nevertheless, the identity of the ubiquitin ligase responsible for NF-κB termination, its regulation, and the biologic significance of this pathway in immunity were not previously known.

Scope of this Thesis

My work has focused on understanding the termination of NF-κB activity through the ubiquitination of RelA. We have demonstrated that RelA ubiquitination, degradation and transcriptional termination are mediated by a ligase complex containing the co-factor COMMD1. This ubiquitously expressed protein is the prototype member of the COMMD protein family, a group of highly conserved and still poorly understood factors that we also identified. ***This thesis will present the cumulative evidence that demonstrates that COMMD1 plays a critical role in the termination of NF-κB activity and the control of inflammation, acting through the ubiquitination pathway***. In Chapter 1, the main events that mediate the inhibitory activity of COMMD1 in the NF-κB pathway are discussed. The discovery of COMMD1 as an inhibitor of NF-κB was made from an initial observation in a Yeast-2-hybrid screen with the anti-apoptotic protein XIAP. In Chapter 2 these initial studies are presented, which led to the appreciation of the broad role of COMMD1 (known at the time as MURR1) as an NF-κB inhibitor. Moreover, these studies demonstrated that COMMD1 affects HIV-1 replication, a viral pathogen that requires NF-κB to complete its life cycle. Subsequent to the discovery of COMMD1 as an NF-κB inhibitor, I identified that this factor is the prototype member of a conserved family of homologous factors termed COMMD proteins. This discovery is presented in Chapter 3. Although initially thought to act at the level of IκB degradation, subsequent studies revealed that COMMD1 does not substantially affect nuclear translocation of NF-κB, indicating that a nuclear mechanism must be at play. Studies in my laboratory demonstrated that COMMD1 promotes the ubiquitination and degradation of NF-κB subunits through a Cullin-containing ligase, and these are displayed in Chapter 4. Chapter 5 describes recent studies that delineate the molecular mechanism by which COMMD1 activates ubiquitin ligases of this class, primarily through the displacement of a ligase inhibitor. Chapter 6 describes the identification that phosphorylation of the NF-κB subunit RelA is required for its degradation and that this is mediated by the same COMMD1 complex acting in concert with the transcriptional regulator, GCN5. More recently, a collaborative study with Dr. Van de Sluis' group (UMCG, Groningen, The Netherlands) identified that Commd1 plays a critical role in inflammatory responses *in vivo* and that this gene is frequently repressed in the setting of human inflammatory bowel disease. This is presented in Chapter 7. Finally, in Chapter 8 all these results and the role of COMMD1 in immunity will be discussed.

Chapter 1

COMMD proteins and the control of the NF-κB pathway

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Perspective

COMMD Proteins and the Control of the NFκB Pathway

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ABSTRACT

The COMM domain containing (COMMD) family of proteins represents a recently discovered set of evolutionarily conserved factors characterized by the presence of a defining carboxy-terminal motif. In vertebrates, there are ten members of the family, and among their emerging functions the control of the transcription factor NFκB has been most extensively studied. NFκB plays a critical role in a number of homeostatic processes in multicellular organisms, including the regulation of immunity and cell survival. COMMD proteins inhibit NFκB mediated gene expression, and recent mechanistic studies have revealed that COMMD1 controls the ubiquitination of NFκB subunits, an event linked to transcriptional termination. COMMD1 binds to a multimeric ubiquitin ligase containing Elongins B/C, Cul2 and SOCS1 ($\mathrm{ECS}^{\mathrm{SOCS1}}$). In this complex, COMMD1 facilitates the binding of NFκB subunits to the ligase, thereby promoting their ubiquitination and degradation. Additional insights gained from these studies indicate that COMMD proteins likely play a broader role in cellular homeostasis through their participation in the ubiquitination pathway.

KEY WORDS

COMMD1, MURR1, ubiquitination, Cullin, NFκB

NFκB: CURRENT PARADIGM

NFκB is a dimeric transcription factor formed by members of a highly conserved family of proteins that share a ~300 amino acid sequence termed the Rel Homology Domain (RHD). In mammals, there are five genes that encode members of this family: *RELA, RELB, REL, NFKB1* and *NFKB2*. Through transcriptional regulation of many gene products, NFκB participates in a number of biologic processes including innate and adaptive immune responses, programmed cell death, transcriptional regulation of viral replication, cell cycle progression, and oncogenesis.[1-5]

NFκB mediated gene expression is regulated to a large extent by cytoplasmic sequestration of the NFκB complex. In the canonical NFκB pathway, this is the result of interactions between the inhibitory IκB proteins and NFκB complexes (Fig. 1).[6,7] Activation of a multimeric kinase known as the IκB Kinase (IKK) complex, results in phosphorylation of IκB. Once phosphorylated, IκB is ubiquitinated by a multimeric ubiquitin ligase containing Cul1 (known as $\mathrm{SCF}^{\beta\text{-TrCP}}$), targeting it for proteasomal degradation.[8,9] IκB degradation enables the translocation of NFκB complexes to the nucleus where they bind to cognate DNA sequences present in an array of promoters, ultimately resulting in induction of gene expression. This is mediated through a series of complex events at the chromatin level that involve the removal of repressive complexes containing histone deacetylases (HDACs) and their replacement with NFκB in association with various transcriptional co-activators.[10-13] Once transcription has occurred, termination of NFκB activity is also an important regulatory step. This is largely mediated by re-synthesis of IκB proteins, which facilitate nuclear export of NFκB.[14] In addition, ubiquitination of chromatin-bound NFκB is also required for termination of NFκB dependent gene expression, and the mechanism responsible for this ubiquitination event has begun to be elucidated.[15,16]

COMMD1 AS AN INHIBITOR OF NFκB

In a search for regulators of the anti-apoptotic factor XIAP, we identified an interaction between XIAP and the *MURR1* gene product (now referred to as COMMD1).[17] At the time of the identification of this interaction, the *MURR1* locus had no known function, but soon thereafter, *MURR1* was shown to participate in copper metabolism, as mutations in canine *MURR1* result in a copper overload disorder in Bedlington terriers.[18,19]

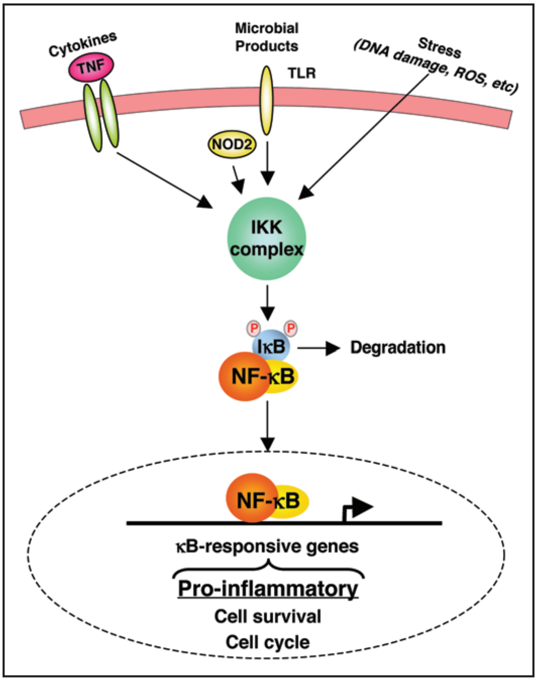

Figure 1. Schematic representation of the canonical NFκB pathway. In resting cells, NFκB exists as dimers associated with IκB proteins. Cell stimulation by a broad range of factors promotes the activation of the IKK complex. IκB proteins become phosphorylated by IKK, resulting in their ubiquitination and proteasomal degradation. This enables NFκB to translocate to the nucleus and induce expression of pro-inflammatory genes.

An analysis of the functional consequence of the XIAP-COMMD1 interaction demonstrated that it had no impact on the anti-apoptotic properties of XIAP, but rather COMMD1 blocked XIAP-mediated NFκB activation.[20] As an extension of those observations, it was appreciated that COMMD1 functions as a more global inhibitor of NFκB activation mediated by a variety of stimuli including TNF, IL1β, phorbol esters, and ectopic expression of IKK subunits.[21] Interestingly, COMMD1 binds to the NFκB complex, a fact that is likely linked to its global effect on NFκB mediated transcription.[20]

More recently, we have demonstrated that COMMD1 controls the expression of a number of endogenous NFκB inducible gene products.[22] TNF stimulation of cells deficient in COMMD1 resulted in greater accumulation of various endogenous κB-inducible transcripts, including *ICAM1*. Furthermore, conditioned media from COMMD1 deficient cells promoted enhanced chemotaxis of freshly isolated peripheral blood mononuclear cells across a membrane barrier. This increase in chemotaxis rate correlated with heightened secretion of NFκB inducible chemokines such as CCL2.

In addition to these effects of COMMD1 on NFκB mediated events, a role for this factor in controlling the HIV-1 life cycle has been previously demonstrated.[21] Expression levels of COMMD1 in freshly isolated naïve CD4$^+$ lymphocytes modulated the rate of HIV-1 replication. Cells overexpressing COMMD1 demonstrated blunted HIV-1 replication, while decreased expression of COMMD1 following RNA interference (RNAi) resulted in greater rates of replication. This finding is consistent with the known role of NFκB in the life cycle of HIV-1, as well as with gene expression data in animal models of SIV disease progression.[23] Specifically, macaques that experienced slow disease progression after SIV infection had the highest levels of COMMD1 expression before infection and during the seven weeks of disease progression, compared to animals with typical or accelerated rates of disease progression (GEO database, record GDS172). Altogether, these findings establish a role for COMMD1 in the control of NFκB mediated transcription, which in turn regulates events such as pro-inflammatory gene expression and the rate of HIV-1 replication, and possibly others that remain to be elucidated.

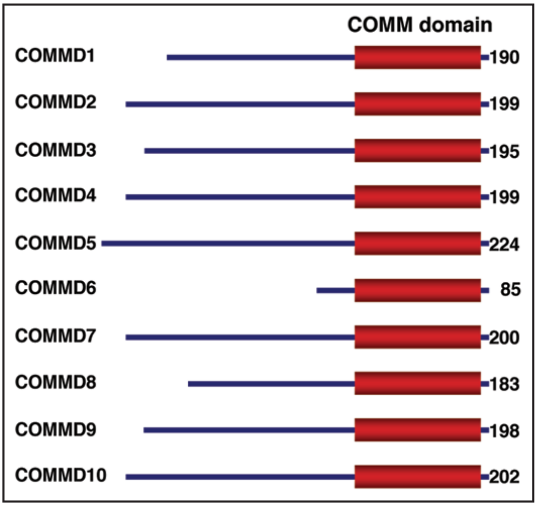

Figure 2. Schematic representation of the COMMD family of proteins. The conserved COMM domain is shown in red, along with the respective amino acid length of each protein in humans.

DISCOVERY OF THE COMMD PROTEIN FAMILY

A biochemical screen for COMMD1 associated factors revealed the presence of three COMMD1-associated proteins, which notably had sequence homology to COMMD1.[20] Additional efforts to search for COMMD1 homologous proteins in public databases identified a total of ten factors in vertebrates, which were subsequently designated as COMMD proteins (COpper Metabolism MURR1 Domain containing). The defining characteristic of all COMMDs is the presence of a ~70 amino acid region of high homology in their extreme carboxy-termini, designated as the COMM domain (Fig. 2). This motif serves as an interface for COMMD proteins to interact with each other, and is important for interactions with other proteins, including NFκB. With the exception of COMMD6, which consists primarily of a COMM domain, all other COMMD proteins possess additional amino-terminal structures which are not homologous amongst family members, but remain highly conserved across multiple species. The predicted structure of the amino-termini of all COMMDs suggests the presence of tandem α-helices. This is consistent with the recently reported solution structure of the amino-terminus of COMMD1.[24] However, the structure of the COMM domain itself remains to be elucidated.

In addition to their shared COMM domain, and their ability to interact with each other through this domain, COMMD proteins other than COMMD1 can also bind to NFκB subunits and inhibit NFκB activation.[20] Cells transfected to express COMMD proteins demonstrated suppression of κB-dependent expression of a reporter gene in response to TNF, and decreased expression of COMMD1, 4 and 6 after RNAi resulted in enhanced TNF-induced expression of the NFκB responsive gene *BIRC3/c-IAP2*. Interestingly, the COMM domain seems to play a critical role in suppresion of NFκB, as mutations in the conserved tryptophan and proline residues within the COMM domain of COMMD6 abrogated the inhibitory activity of the molecule.[25]

COMMD PROTEINS SUPPRESS NFκB IN A MANNER DISTINCT FROM IκB PROTEINS

Stimulation-dependent degradation of the IκB proteins allows translocation of NFκB dimers to the nuclear compartment of cells, a critical step for the initiation of κB-mediated gene expression. Turnover of IκB proteins is mediated by a Cul1-containing multimeric ubiquitin ligase called SCF$^{\beta\text{-TrCP}}$, which promotes poly-ubiquitination of the phosphorylated form of IκB, targeting it for proteasomal degradation.[6,26] While COMMD1 can stabilize phosphorylated IκBα presumably through an interaction with Cul1,[21] this event does not seem to be physiologically dominant since nuclear translocation of RelA is unaffected by COMMD1 expression.[20] Interestingly, an additional indication that COMMD proteins operate in a different manner than IκB is the fact that COMMD1 interacts with an amino-terminal motif present in all NFκB subunits that is distinct from the motif that the IκB proteins associate with.[20]

In addition to cytosolic sequestration of NFκB, other mechanisms of transcriptional suppression involve control of the activity of chromatin bound NFκB subunits through various pathways.[13,15,27,28] Evidence indicates that COMMD1 regulates NFκB after its nuclear entry.[20] The interaction of RelA (the most abundant NFκB subunit) with chromatin is regulated by COMMD1. Increased expression of COMMD1 decreased the binding of RelA to chromatin and conversely, decreased endogenous expression of COMMD1 after RNAi intensified the association of RelA to the promoter site. In addition to these findings, COMMD1 was found to be recruited to the promoter itself in a stimulus-dependent manner. However, the mechanism responsible for the effects of COMMD1 on RelA-chromatin interactions remained elusive until recently.

COMMD1 INHIBITS NFκB THROUGH A UBIQUITINATION PATHWAY

Ubiquitination and proteasomal degradation of DNA-bound RelA has been shown to control RelA-chromatin interactions independent of the IκB pathway, similar to the described effects of COMMD1 on RelA-chromatin binding.[16] Therefore, the possibility that ubiquitination of RelA might be a mechanism for the inhibitory effect of COMMD1 on κB-mediated transcription was recently examined.[22] Indeed, multiple data demonstrated that COMMD1 promotes NFκB ubiquitination, as cells transfected to express COMMD1 accumulated greater amounts of ubiquitinated RelA and conversely, depletion of endogenous COMMD1 by RNAi decreased the amount of ubiquinated RelA recovered.

Although COMMD1 can accelerate the ubiquitination of RelA, it remained to be determined whether this was a direct effect on the ubiquitination reaction itself or an indirect effect that ultimately facilitates this step. In support of the first possibility, endogenous COMMD1 immunoprecipitates can catalyze the formation of polyubiquitin chains in an in vitro ubiquitination reaction when provided with the necessary co-factors (E1 and E2 enzymes, ubiquitin, and ATP), indicating that COMMD1 associates with a complex that has E3 ubiquitin ligase activity. The identity of this complex(es) would likely explain the ability of COMMD1 to promote RelA ubiquitination.

COMMD1 PROMOTES NFκB UBIQUITINATION THROUGH A DISTINCT MULTIMERIC UBIQUITIN LIGASE

It has been demonstrated that a protein called SOCS1 promotes the ubiquitination and proteasomal degradation of RelA, although the context of this activity was unclear.[15] SOCS1 is part of a larger family of proteins containing the conserved carboxy-terminal SOCS box domain.[29] Through their SOCS-box, these factors associate with Cullin-containing multimeric ubiquitin ligases. These complexes are referred to as ECS and contain Elongins B and C, Cullin 2 or 5, and a SOCS box containing protein.[30]

Given that COMMD1 promotes RelA ubiquitination and its immunoprecipitates contain ubiquitin ligase activity, the notion that COMMD1 may associate with the ECS$^{\text{SOCS1}}$ complex was examined. Indeed, endogenous COMMD1 co-precipitated with endogenous SOCS1 and Cul2, with the latter interaction being an inducible event critical to the assembly of the mature ECS$^{\text{SOCS1}}$ complex. In addition, COMMD1 was shown to interact with core components of the ECS$^{\text{SOCS1}}$ complex in cotransfection experiments, including Elongin C, Cul2, SOCS1, and Rbx1. Furthermore, the data indicate that COMMD1 requires the ECS$^{\text{SOCS1}}$ complex to promote the ubiquitination of RelA. Suppression of Cul2 or SOCS1 expression after RNA interference abrogated the COMMD1-mediated accumulation of ubiquitinated RelA in cells. In addition, COMMD1 immunoprecipitates from cells expressing ECS$^{\text{SOCS1}}$ and not control transfected cells, efficiently promoted polyubiquitination of RelA in an in vitro ubiquitination reaction. These data firmly establish that the association of COMMD1 with ECS$^{\text{SOCS1}}$ facilitates ubiquitination of NFκB subunits. Additional studies also demonstrated that binding of COMMD1 to the ECS$^{\text{SOCS1}}$ complex facilitates RelA binding to the ligase.

CONCLUSION

The majority of the work on the regulation of NFκB has focused on how this transcription factor becomes activated to promote induction of gene expression.[5,31] These include pathways that regulate the translocation of NFκB from the cytosol to the nucleus and the assembly of complexes on chromatin that promote transcription. Once NFκB activation has taken place the termination of transcription is an equally important regulatory step, which is partly mediated by re-synthesis of IκB proteins and export of nuclear NFκB complexes. However, other mechanisms that are critical for the termination of the NFκB-response are beginning to be uncovered.

It has been demonstrated that ubiquitination and proteasomal degradation of chromatin-bound RelA is required for normal transcriptional termination independent of IκB mediated nuclear export. Furthermore, in the study by Saccani et al, this ubiquitination event

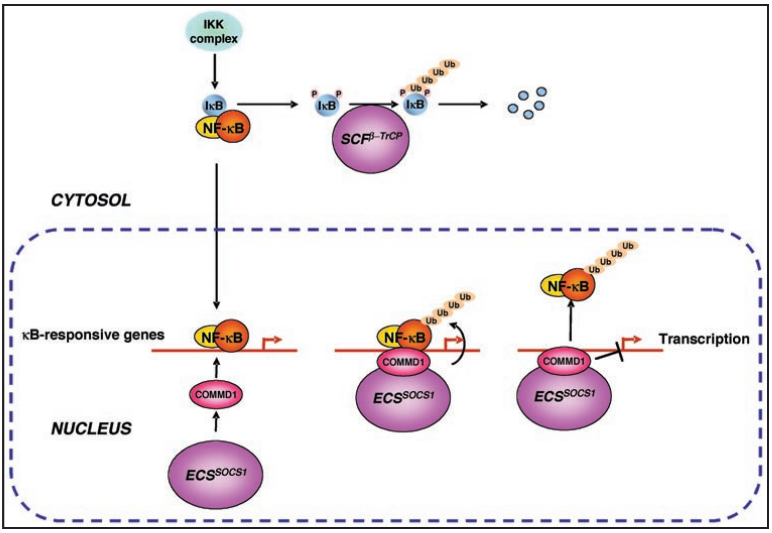

Figure 3. Model of COMMD1-mediated negative regulation of NFκB. Activation of the NFκB pathway promotes ubiquitination and degradation of IκB proteins by the SCF$^{β\text{-TrCP}}$ ubiquitin ligase, enabling translocation of NFκB dimers to the nucleus where they bind cognate promoter elements to induce gene expression. COMMD1 is recruited to chromatin, which later becomes associated with the ECS^{SOCS1} ubiquitin ligase. We speculate that this results in the recruitment of the entire ubiquitin ligase complex to these promoter sites, facilitating poly-ubiquitination of NFκB subunits. This event ultimately targets NFκB for proteasomal degradation and termination of transcription.

required RelA binding to DNA and seemed to be orchestrated locally at the site of the gene promoter, as proteasome subunits were shown to be recruited to the promoter itself.[16] However, the identity of the ubiquitin ligase responsible for this event was not elucidated at that time. Ryo and collaborators demonstrated that the SOCS1 protein can mediate the ubiquitination of RelA, although the precise context of this event was not further delineated.[15] Interestingly, we identified that COMMD1 acts as an accessory subunit to a SOCS1 containing complex (known as ECS^{SOCS1}) that targets RelA for ubiquitination.[22] We observed that COMMD1 deficiency led to more persistent nuclear accumulation of RelA following TNF stimulation, but did not affect the early entry of NFκB into the nucleus. Furthermore, our data indicate that at least the COMMD1 subunit of this complex is recruited to chromatin in a stimulus dependent fashion, and that levels of COMMD1 control the duration of RelA-chromatin association.

A model that would bridge the findings of Saccani, Ryo and our own work suggests that in response to NFκB recruitment to promoter sites, a ubiquitin ligase containing COMMD1 is recruited to ubiquitinate DNA-bound RelA (Fig. 3). Importantly, this ligase also undergoes assembly in response to NFκB activation, as evidenced by increased interaction between COMMD1 and Cul2, the main scaffold protein in the complex. This inducible interaction peaks at about two hours after NFκB activation, suggesting that COMMD1-directed ubiquitination of NFκB may be critical for late transcriptional termination, after IκB-mediated export has been completed. Indeed, our data indicates that for a number of genes, NFκB mediated transcription continues to take place even after the total levels of nuclear RelA have returned to the pre-stimulation state. This suggests that active NFκB complexes are probably retained in the nucleus and that ubiquitination is an important mechanism for their removal.

The COMMD1-ECS^{SOCS1} complex represents the second Cullin-containing ubiquitin ligase that regulates the NFκB pathway. The Cul1-containing SCF$^{β\text{-TrCP}}$ complex plays a critical role in the ubiquitination of IκB proteins and the NFκB precursor subunits, p100 and p105.[6,7,32] Interestingly, COMMD1 binds to additional Cullins, including Cul1, Cul2, Cul3 and Cul5, a fact that is not entirely surprising since the Cul2-COMMD1 interaction was mapped to the conserved Cullin-homology domain. Given the multitude of targets that these ubiquitin ligases have, it can be speculated that COMMD1 likely plays a role outside of regulating NFκB, and this may underlie other reported activities of this protein such as its involvement in copper metabolism or the regulation of ENaC.[17,33] Similarly, the COMMD family contains 9 additional members, suggesting the possibility of broader biologic roles for this protein family through their involvement in the ubiquitination cascade.

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Chapter 2

The gene product Murr1 restricts HIV-1 replication in resting CD4+ lymphocytes

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The gene product Murr1 restricts HIV-1 replication in resting CD4$^+$ lymphocytes

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Although human immunodeficiency virus-1 (HIV-1) infects quiescent and proliferating CD4$^+$ lymphocytes, the virus replicates poorly in resting T cells[1–6]. Factors that block viral replication in these cells might help to prolong the asymptomatic phase of HIV infection[7]; however, the molecular mechanisms that control this process are not fully understood. Here we show that Murr1, a gene product known previously for its involvement in copper regulation[8,9], inhibits HIV-1 growth in unstimulated CD4$^+$ T cells. This inhibition was mediated in part through its ability to inhibit basal and cytokine-stimulated nuclear factor (NF)-κB activity. Knockdown of Murr1 increased NF-κB activity and decreased IκB-α concentrations by facilitating phospho-IκB-α degradation by the proteasome. Murr1 was detected in CD4$^+$ T cells, and RNA-mediated interference of Murr1 in primary resting CD4$^+$ lymphocytes increased HIV-1 replication. Through its effects on the proteasome, Murr1 acts as a genetic restriction factor that inhibits HIV-1 replication in lymphocytes, which could contribute to the regulation of asymptomatic HIV infection and the progression of AIDS.

Murr1 is a highly conserved 190-amino-acid protein that does not have any identifiable motifs, and a homozygous deletion in the gene encoding canine Murr1 leads to copper toxicosis in Bedlington terriers[8]. In this study, Murr1 was initially identified in a two-hybrid screen by binding the X-linked inhibitor of apoptosis, a known activator of NF-κB (refs 10, 11, and E.B., unpublished observations). To study its effect on NF-κB, HIV-1 reporter plasmids with wild-type or mutant (ΔκB) sites[2] were co-transfected with control or Murr1 expression plasmids in the different cell lines. Murr1 inhibited both basal and tumour necrosis factor (TNF)-α-dependent HIV-1 transcription from the wild-type but not the κB-mutant reporter in Jurkat T-leukaemia and 293T renal epithelial cell lines (Fig. 1a, left and middle panels). In contrast, Murr1 did not substantially inhibit tumour growth factor-β-dependent transcription in HepG2 cells, confirming its specificity (Fig. 1a, right panel). The κB effect was dose-dependent and observed with other inducers of NF-κB, including interleukin-1 (IL-1) and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Fig. 1b). Murr1 modulated the expression of endogenous κB-regulated genes: transfection into 293T cells decreased the endogenous cell-surface expression of major histocompatibility complex (MHC) class I, in contrast to CD9, which is independent of NF-κB (Fig. 1c).

Its site of action in the NF-κB signalling pathway was further defined by co-transfection of different regulators with an NF-κB reporter in Jurkat T cells. Whereas Murr1 inhibited both IKK-1- and IKK-2-induced NF-κB activity (Fig. 1d, middle and right panels), it failed to block RelA-mediated transcription (Fig. 1d, left panel), indicating that Murr1 might interact downstream of the IκB kinase signalosome. As determined by immunoprecipitation, co-transfected haemagglutinin (HA)-tagged Murr1 and Myc-tagged IKK-2 did not associate *in vivo* (Fig. 2a, lane 2, left panel). Although IKK-1 also did not associate with Murr1 (data not shown), an interaction between transfected HA-tagged Murr1 and endogenous IκB-α was readily detected (Fig. 2a, lane 6). The ankyrin domain of IκB-α was required for association with Murr1, as were amino acids 1–160 of Murr1 (Supplementary Fig. 1a).

A polyclonal antibody against Murr1 demonstrated the association between endogenous Murr1 and IκB-α *in vivo*. RelA antibody immunoprecipitated IκB-α, IκB-β and Murr1 (Fig. 2b, lane 10). IκB-α antibody also pulled down RelA and Murr1 (Fig. 2b, lane 12), but the IκB-β antibody did not precipitate Murr1 (Fig. 2b, lane 14), suggesting that Murr1 interacted preferentially with the NF-κB–IκB-α complex. This association was confirmed *in vivo* by confocal microscopy with fluorescent fusion proteins (Supplementary Fig. 1b), similarly to the pattern of RelA association with IκB-α[12–14].

The physiological consequences of these interactions were determined by knockdown of endogenous Murr1 in 293T cells using control and Murr1-specific short interfering RNA (siRNA) duplexes. The specificity of two such siRNAs, Murr1-1 and Murr1-2, directed to different Murr1 sequences, was first confirmed by transfecting 293T cells with wild-type or mutant siRNAs along with wild-type or mutant Murr1 complementary DNAs modified at the siRNA target site (Supplementary Fig. 2). Transient transfection of Murr1-specific siRNA duplexes downregulated endogenous Murr1 and IκB-α, had little effect on IκB-β, p65 or IKK-2 (Fig. 3a, left panel), and increased κB-dependent reporter activity (Fig. 3a, right panel).

To investigate the mechanism of Murr1 action, 293T cells were transfected with a control or Murr1 siRNA. Four days after transfection, cells were treated with the proteasome inhibitor MG132 for 2 h or with vehicle alone and stimulated with TNF-α. Cells depleted of Murr1 showed a decrease in basal IκB-α (Fig. 3a) and an increase and persistence of phospho-IκB-α in response to stimulation with TNF-α (Fig. 3b, left panel). This effect was observed in the absence of a proteasome inhibitor, MG132, but not in its presence (Fig. 3b, right panel), indicating that Murr1

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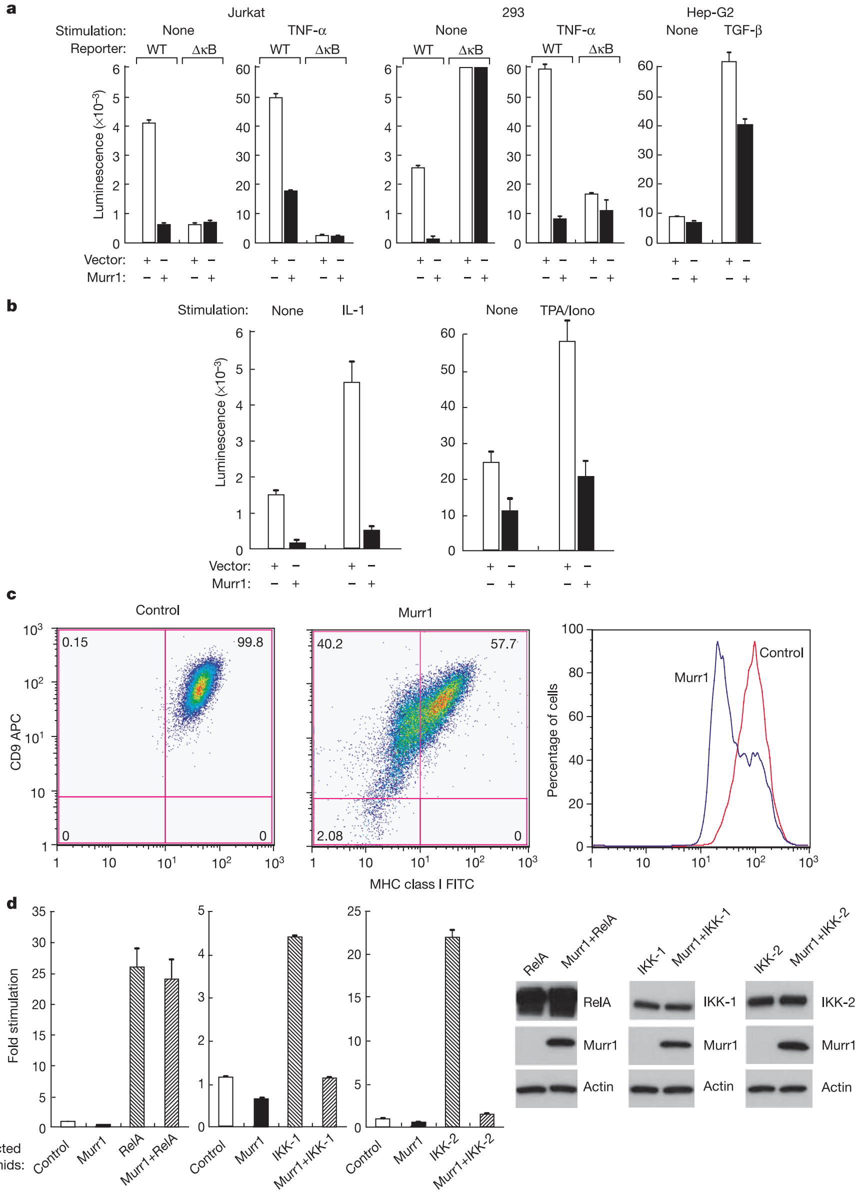

Figure 1 Murr1 suppresses NF-κB-dependent activity from independent stimuli by acting downstream of IκB kinase. **a**, Murr1 inhibits κB-dependent gene expression in Jurkat T-leukaemia cells (left) or 293T human embryonic kidney cells (middle) transfected with HIV (WT) or ΔκB-luciferase reporter (ΔκB) and Murr1 (solid bars) or vector plasmid (open bars). HepG-2 cells (right) were transfected with p3TP-Lux reporter. Cells were treated with the indicated cytokines after 24 h, and luciferase activity was measured at 36 h. **b**, Murr1 inhibits NF-κB induced by IL-1 and TPA in 293 cells. **c**, Murr1 decreases the expression of NF-κB-dependent endogenous class I MHC in 293T cells transfected with HA–LacZ (left) or HA–Murr1 (middle) by flow cytometry for class I and CD9 in HA-positive cells. The decrease in MHC class I expression is summarized (right). **d**, Murr1 blocks the activation by IKK-1 and IKK-2 but does not inhibit RelA transactivation. Fold stimulation (left) and expression (right) are shown. An average of three independent experiments in triplicate, standardized for transfection efficiency, are shown (**a**, **b**, **d**).

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might act similarly to a proteasome inhibitor to enhance and sustain phospho-IκB. Phospho-IκB-α is degraded by ubiquitin–protein conjugates that require three enzymes, which participate in a cascade of ubiquitin transfer reactions: a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2) and a ubiquitin ligase (E3). The specificity of protein ubiquitination and 26S proteasome degradation is determined by the E3 enzymes[15]. Skp1/Cul1/β-TrCP1 (F-Box) complexes constitute a class of E3 enzymes that are required for the degradation of phospho-IκB-α[16]. The possible association of endogenous Murr1 with Skp1/Cul1/β-TrCP1 (F-box) complexes was examined. As determined by immunoprecipitation followed by western blotting, Murr1 interacted biochemically with the Cul1 and not the Skp1 component of the E3 ligase complex, in contrast to a denatured negative control (Fig. 3c). Although Murr1 binds to common constituents of E3 ligase, it nevertheless showed specificity because knockdown did not alter steady-state concentrations of β-catenin, the target of proteasomal degradation of the Wnt signalling pathway (Fig. 3d). Taken together, these results suggest that Murr1 blocked NF-κB activation through its ability to interact with E3 ligase and inhibit the proteasomal degradation of IκB.

To determine whether Murr1 was detectable in T cells, lymphocytes from normal healthy individuals were examined. Both CD4$^+$ and CD8$^+$ cells expressed Murr1, with higher concentrations in CD4$^+$ cells (Fig. 4a), raising the possibility that Murr1 might affect HIV-1 replication in these cells. This possibility was examined first by transfection of Murr1 in primary T cells and compared with another inhibitor of NF-κB activation, the IκB super-repressor (IκB-SR). Expression of recombinant Murr1 or IκB-SR inhibited HIV-1 replication in activated T cells similarly relative to controls (Fig. 4b; $P = 0.02$; paired *t*-test), indicating that Murr1 might inhibit HIV-1 replication comparably to NF-κB inhibitors in primary activated T cells. To determine its effect on HIV-1 replication in resting primary T cells, CD4$^+$ cells from HIV-1-negative donors were transfected with an siRNA for Murr1 (Murr1-1) or a negative control. To identify transfected cells, siRNAs were mixed with an unrelated fluorescent Cy3-labelled luciferase siRNA at a ratio of 2:1, and more than 30% of cells displayed fluorescence. In Murr1 siRNA-transfected cells, a maximum decrease in Murr1 protein concentration was achieved between 48 and 72 h after electroporation, and these cells did not display T-cell activation markers—CD69, CD25 and HLA-DR—at the time of HIV-1 infection (data not shown). Murr1 siRNA increased dose-dependent HIV-1 replication in primary resting CD4$^+$ T cells from three separate cell donors (Fig. 4c; $P = 0.0007$, 0.0024 and 0.0014, respectively; paired *t*-test), implicating Murr1 in the regulation of HIV infection in these cells.

Whereas HIV-1 entry into activated CD4$^+$ lymphocytes leads to a productive infection[1], the virus remains latent in resting CD4$^+$ lymphocytes[17], existing as a preintegration complex awaiting cell stimulation, which facilitates the transition to productive replication[3,4,6,7,18]. The molecular mechanisms responsible for HIV-1

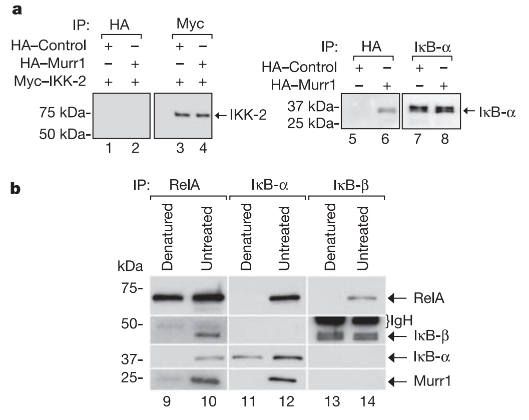

Figure 2 Murr1 associates with the NF-κB–IκB-α complex. **a**, Murr1 does not associate with IKK-2 but interacts with endogenous IκB-α. Lysates from transfected 293T cells, treated with lactacystin (10 μM) 16 h before harvesting, were immunoprecipitated (IP) as indicated and immunoblotted with antibody against IKK-2 (left) or IκB-α (right). **b**, Immunoprecipitation of Murr1–NF-κB–IκB-α in 293T cells with antibodies against indicated proteins. IgH, immunoglobulin heavy chain.

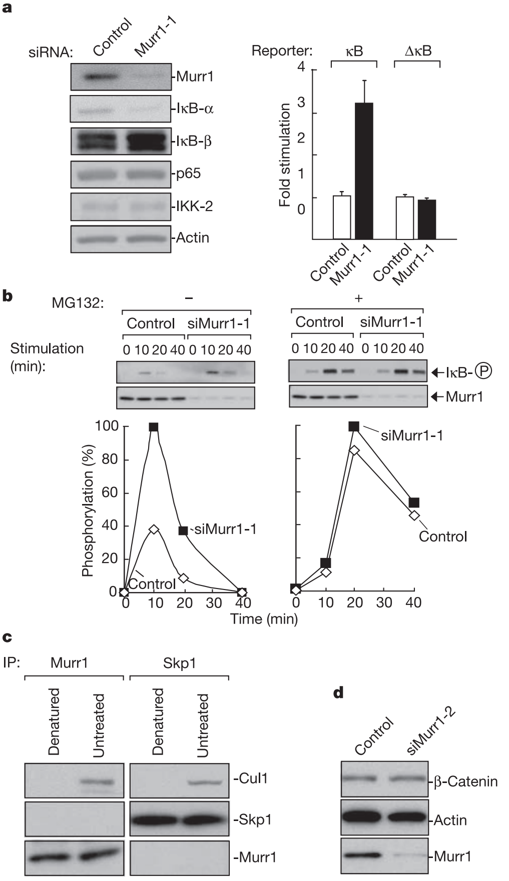

Figure 3 Murr1 regulates IκB-α turnover through effects on proteasomal degradation. **a**, Knockdown of Murr1 decreases cellular IκB-α and increases basal NF-κB activity in 293T cells transfected with control or Murr1 siRNAs, analysed by immunoblotting to the indicated proteins at 48 h (left). Fold stimulation of the indicated co-transfected plasmids is shown (right). **b**, Murr1 alters IκB-α phosphorylation in 293T cells transfected with control or Murr1 siRNAs. At 90 h after transfection, cells were treated with vehicle (left) or MG132 (right) for 2 h, followed by stimulation with TNF-α and immunoblotting for Murr1 and phospho-IκB-α (top) and quantification (bottom). **c**, Immunoprecipitation (IP) of Skp1–Cul1–β-TrCP1 complexes in 293T cell extracts with antibodies against Murr1 or Skp1, immunoblotted for Cul1, Skp1 or Murr1. **d**, Knockdown of Murr1 by Murr1-2 siRNA compared with a negative control (GFP) does not alter β-catenin concentrations.

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latency are not well understood. Among the factors that can activate HIV-1 replication are host transcription factors such as NF-κB (ref. 2), epigenetic modifications at the site of integration[19], premature termination of transcription due to the absence of sufficient concentrations of Tat and NF-κB (refs 20, 21), and inefficient export of RNAs for structural proteins[5]. The roles of these factors in resting CD4$^+$ T cells are inferred from cell culture experiments and affect viral transcription, translation or RNA transport. Here we show that Murr1 regulates IκB-α turnover, which inhibits the productive HIV-1 infection of resting T lymphocytes. Expression of Murr1 in activated HIV-infected T cells also inhibited virus replication, indicating that it might also have a function in limiting the extent of viral replication, or burst size. NF-κB is sequestered in the cytoplasm by a 450-kDa IκB complex[12,22–25] and, after activation of T cells, phosphorylation of IκB-α (ref. 26) in this complex by two inducible kinases, IKK-1 and IKK-2, leads to the subsequent ubiquitination and degradation of IκB-α by the 26S proteasome[27]. Degradation of IκB-α by the proteasome releases NF-κB, which then translocates to the nucleus to stimulate the synthesis of genes involved in immune activation. NF-κB enhances transcription and subsequently recruits several transcriptional activators, including positive transcription elongation factor b, which is ubiquitinated and degraded by the proteasome[28]. Inhibition of proteasomal activity by Murr1, after it rebounds from its initial degradation and after this processive transcription complex has formed, could subsequently stabilize it and enhance transcription. The proteasome, through the ubiquitin E3 ligase complex, is also involved in viral processing and the maturation of Gag[29]. Because Murr1 inhibits proteasomal degradation, it could further block HIV-1 infection by decreasing the proteasome-dependent degradation and maturation of Gag-associated factors required for viral replication, and although Murr1 acts selectively on the NF-κB and not the Wnt signalling pathway (Fig. 3), it remains possible that it might yet affect other such proteins degraded by the proteasome. Such an example is APOBEC3G, shown recently to associate with a Cul5 E3 ligase[31]. Blocks to viral replication in resting lymphocytes, as well as in cells containing latent provirus, might affect disease progression and viral rebound after the discontinuation of anti-retroviral therapy (reviewed in ref. 7). A more precise understanding of the molecular regulation of these events might identify novel genetic restriction factors, like Murr1, through which anti-viral drugs might delay the progression of HIV-1 infection to AIDS. ☐

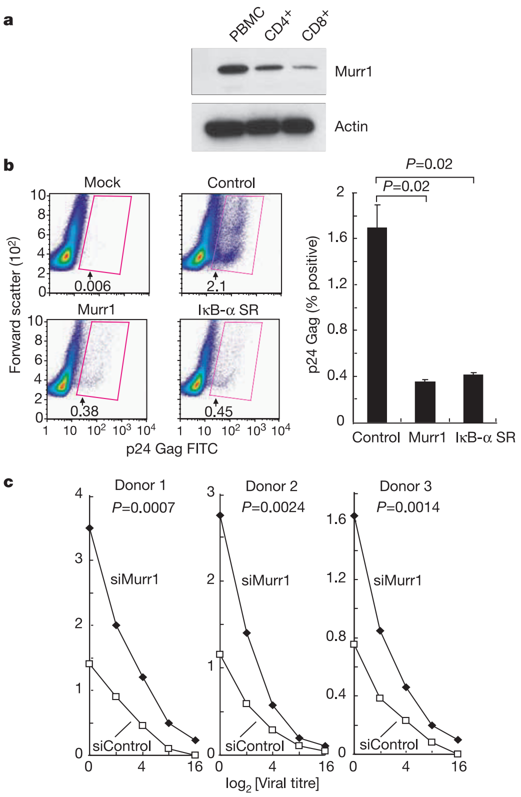

Figure 4 Murr1 inhibits productive HIV-1 infection in CD4$^+$ lymphocytes. **a**, Murr1 concentrations in CD4$^+$ and CD8$^+$ T cells shown by immunoblotting with Murr1 antisera and reprobing with β-actin antibody. **b**, Murr1 inhibits HIV-1 replication in phytohaemagglutinin/IL-2-stimulated human CD4$^+$ cells transfected with plasmids encoding HA–LacZ (control), HA–Murr1 or HA–IκB-SR, infected with HIV-1$_{BaL}$ (p24 $= 75$ ng ml^{-1}) 36 h later. HIV-1 replication was analysed 48 h after infection by staining for intracellular p24. A representative of three independent HIV-1-negative donors is shown ($P = 0.02$, control versus Murr1). **c**, Murr1 siRNA-transfected resting CD4$^+$ T lymphocytes from three healthy donors show increased susceptibility to HIV-1 infection after transfection with siRNA for Murr1 (Murr1-1) compared with GFP (control). Cy3 luciferase siRNA was mixed with siRNAs (2:1) to label transfected cells. Single-round HIV-1 replication was measured in labelled cells at 48 h. The multiplicity of infection of undiluted virus was ~1.0.

Methods

Plasmids, siRNA and cell transfections

The gene encoding chloramphenicol acetyltransferase (CAT) in HIV-LTR–CAT and HIV-LTR-ΔκB–CAT[2] was replaced with the luciferase gene from pGL3 basic (Promega) to construct HIV-LTR–Luc and HIV-LTR-ΔκB–Luc. The HA-IκB-α SR, p3TP-Lux reporter, green fluorescent protein (GFP)–RelA, pRK-Myc-IKK-1, pRK-Myc-IKK-2, p65 and IκB-α cDNAs are described in Supplementary Methods, as are siRNA sequences and the cell transfection techniques.

T-cell purification and transfection

To purify resting T cells, CD4$^+$ T lymphocytes were isolated from Ficoll-purified peripheral blood mononuclear cells (PBMCs) by negative selection with a CD4$^+$ T-cell isolation kit containing CD8, CD11b, CD16, CD19, CD36 and CD56 antibodies (Miltenyi Biotech). CD25$^+$ and HLA-DR$^+$ cells were then removed from this population by using CD69, CD25 and HLA-DR antibodies (Miltenyi Biotech) by negative selection. The purity of the isolated resting T cells was assessed by fluorescence-activated cell sorting (FACS) analysis for CD4, CD69, CD25 and HLA-DR (BD-Pharmingen); 95% of the cells were CD4$^+$ and there were less than 0.1% of activated T cells as defined by the presence of CD69, CD25 or HLA-DR. Resting CD4$^+$ T cells (10^7) were transfected with control or Murr1 siRNA (Dharmacon). For each transfection, 1.2 μM siRNA (800 nM unlabelled siRNA and 400 nM Cy3-labelled siRNA) was used. Transfections were performed with a 2:1 mixture of unlabelled siRNA and Cy3 luciferase. To transfect the resting CD4$^+$ T cells with siRNA, 10^7 cells were resuspended in 100 μl T-cell Nucleofector reagent and immediately electroporated with the recommended protocol U-14 on the Nucleofector instrument (Amaxa Biosystems). The electroporated cells were washed 4 h after transfection and incubated in 1.5 ml RPMI and infected with HIV-1 48 h after electroporation. FACS analysis was performed with CD69, CD25 and HLA-DR antibodies (BD Pharmingen) to check the activation status of the cells at the time of infection. For plasmid transfections, CD4$^+$ T lymphocytes isolated from Ficoll-purified PBMCs by negative selection were stimulated with phytohaemagglutinin (10 μg ml^{-1}) (Calbiochem) and 40 U ml^{-1} IL-2 (Peprotech) for 24 h and maintained in 40 U ml^{-1} IL-2 for 4 days. Cells were then washed to remove IL-2, and 10^7 cells were resuspended in 100 μl T-cell Nucleofector reagent containing 0.5 μg of the respective plasmids and immediately electroporated with recommended protocol T-20 on the Nucleofector instrument. The electroporated cells were washed 12 h after transfection and incubated in 1.5 ml of RPMI supplemented with 40 U ml^{-1} IL-2 and infected with HIV-1 24 h after electroporation.

HIV-1 infection and flow cytometric analysis for expression of p24-Gag

At 48 h after siRNA transfection or 24 h after plasmid transfection, HIV-1 infection of CD4$^+$ T lymphocytes was performed in 96-well round-bottomed culture plates by combining 40 μl virus stock with 20 μl CD4$^+$ T lymphocytes (1.5×10^5 cells). The multiplicity of infection of undiluted virus was ~1.0. After incubation for 2 h at 37 °C, cells were washed twice and continued in culture with indinavir at 1 μM. CD4$^+$ T lymphocytes were harvested for intracellular p24-Gag staining 48 h after exposure to virus. In brief, cells were stained with anti-CD4-PE and ethidium bromide monoazide (EMA) for 10 min. EMA was cross-linked to the cells by exposure to a bright light source for 15 min. Cells were washed once, fixed and permeabilized with Cytoperm/Cytofix (BD Pharmingen) for

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20 min. Cells were then stained for p24 Gag (KC-57 fluorescein isothiocyanate (FITC); Coulter) for 20 min and washed once in 1 × Perm/wash buffer (BD Pharmingen). Cells in each group were analysed by flow cytometry for intracellular HIV-1 p24 and CD4 after the exclusion of dead cells by their affinity for EMA. For a detailed description of HIV-1 production, infection and flow cytometric analysis see ref. 30.

Antibodies, western blots and immunoprecipitations

Cell lysis for western blots and immunoprecipitations were performed in cell lysis buffer (Cell Signal). Antibodies against β-actin (Sigma), His (Invitrogen), HA, HA-PE, p65, IκB-α, IκB-β, Cul1, Skp1 (Santa Cruz), IKK1, IKK2, Ser-32-phospho IκB-α, β-catenin (Cell Signal) and HLA Class1 FITC (Biosource) were used in flow cytometry, immunoblotting and immunoprecipitations in accordance with the manufacturer's instructions. Polyclonal Murr1 antibody has been described previously[9].

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Inheritance of a pre-inactivated paternal X chromosome in early mouse embryos

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In mammals, dosage compensation ensures equal X-chromosome expression between males (XY) and females (XX) by transcriptionally silencing one X chromosome in XX embryos[1]. In the prevailing view, the XX zygote inherits two active X chromosomes, one each from the mother and father, and X inactivation does not occur until after implantation[2–6]. Here, we report evidence to the contrary in mice. We find that one X chromosome is already silent at zygotic gene activation (2-cell stage). This X chromosome is paternal in origin and exhibits a gradient of silencing. Genes close to the X-inactivation centre show the greatest degree of inactivation, whereas more distal genes show variable inactivation and can partially escape silencing. After implantation, imprinted silencing in extraembryonic tissues becomes globalized and more complete on a gene-by-gene basis. These results argue that the XX embryo is in fact dosage compensated at conception along much of the X chromosome. We propose that imprinted X inactivation results from inheritance of a pre-inactivated X chromosome from the paternal germ line.

In mice, X-chromosome inactivation (XCI) takes on two lineage-specific forms. Random XCI[1], in which both X chromosomes have an equal chance of being inactivated, occurs in the epiblast (embryo proper). In contrast, imprinted XCI[7] leads to paternal X-chromosome (X^P) silencing in the extraembryonic tissues (placenta). Although the imprint is set in gametes, classical studies support a view in which both X^P and X^M (maternal X chromosome) are transmitted to the zygote in an active form: the absence of a late-replicating X chromosome in pre-implantation embryos[2] suggests equal X-transcriptional status, and bimodal distributions of enzymatic activities for two X-linked genes (*Hprt*[3,4], *Gla*[5]) suggest twice as much expression in XX as compared with XY embryos. Thus, the prevailing view postulates that XX and XY embryos have a twofold imbalance of X dosage until implantation, when XCI takes place for the first time in the extraembryonic and embryonic lineages[6,8–10]. However, some recent observations have not been explained easily[11]. Studies of the X-linked *Pgk1* (refs 12, 13) revealed that, whereas the maternal allele is expressed during pre-implantation development, the paternal allele is silent. Furthermore, one *Xist* allele is expressed at high levels in pre-implantation embryos[9], leading to the idea that the X chromosomes may be transcriptionally distinct[6,11]. Indeed, the maternal and paternal haplogenomes can behave differently in early

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Chapter 3

COMMD proteins, a novel family of structural and functional homologs of MURR1

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COMMD Proteins, a Novel Family of Structural and Functional Homologs of MURR1* S

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MURR1 is a multifunctional protein that inhibits nuclear factor κB (NF-κB), a transcription factor with pleiotropic functions affecting innate and adaptive immunity, apoptosis, cell cycle regulation, and oncogenesis. Here we report the discovery of a new family of proteins with homology to MURR1. These proteins form multimeric complexes and were identified in a biochemical screen for MURR1-associated factors. The family is defined by the presence of a conserved and unique motif termed the COMM (copper metabolism gene MURR1) domain, which functions as an interface for protein-protein interactions. Like MURR1, several of these factors also associate with and inhibit NF-κB. The proteins designated as COMMD or COMM domain containing 1–10 are extensively conserved in multicellular eukaryotic organisms and define a novel family of structural and functional homologs of MURR1. The prototype of this family, MURR1/COMMD1, suppresses NF-κB not by affecting nuclear translocation or binding of NF-κB to cognate motifs; rather, it functions in the nucleus by affecting the association of NF-κB with chromatin.

NF-κB is a dimeric complex formed by members of a highly conserved family of proteins that share a defining motif designated the Rel homology domain (RHD).[1] Through transcriptional regulation of many gene products, NF-κB participates in a number of biological processes including innate and adaptive immune responses, programmed cell death, cell cycle progression, and oncogenesis (1–6). Additionally, by its ability to regulate transcription of various viral genomes including human immunodeficiency virus-1 (HIV-1) (7–10), NF-κB also participates in viral cycle progression.

Studies into the regulation of NF-κB activation have largely focused on the role of cytoplasmic sequestration of the NF-κB complex as a mainstay level of control. In most cells NF-κB is localized in the cytoplasm through the interaction of the complex with members of the IκB family (11). These proteins contain ankyrin repeats that allow their interaction with NF-κB and mask the nuclear localization signal present in the RHD. Phosphorylation of IκB by a multimeric kinase known as the IκB kinase complex targets these proteins for ubiquitination and proteasomal degradation (3, 12). This allows the translocation of NF-κB to the nucleus where it binds to cognate DNA sequences present in an array of gene promoters.

MURR1 is a recently identified factor that has been shown to participate in two apparently distinct activities, regulation of the transcription factor NF-κB and control of copper metabolism (13). Mutations in *MURR1* are responsible for copper toxicosis in an inbred canine strain (Bedlington terriers) (14), and an interaction between MURR1 and the copper transporter ATP7B (15) has been recently reported.

In addition to its role in copper metabolism in mammals, more recent studies implicate MURR1 in the regulation of the transcription factor NF-κB (13, 16). MURR1 was found to be a broad inhibitor of NF-κB, affecting κB-responsive transcription from endogenous and viral promoters including the HIV-1 enhancer (16). Through this effect, MURR1 can function as a factor that limits HIV-1 replication in resting CD4$^+$ lymphocytes.

Here we report the discovery of a family of proteins structurally and functionally related to MURR1. These factors contain a unique and defining domain termed the COMM (copper metabolism gene MURR1) domain, and thus, these proteins have been named COMM domain-containing or COMMD proteins. Similar to MURR1/COMMD1, several of these factors associate with NF-κB and inhibit its transcriptional activity. In addition, we find that COMMD proteins form heteromeric complexes that are mediated by the COMM domain. The prototype of the family, MURR1/COMMD1, exerts its ability to inhibit κB-mediated transcription without affecting nuclear translocation but through nuclear regulation of NF-κB. We show here that MURR1/COMMD1 is recruited to chromatin of a κB-responsive promoter upon NF-κB activation and negatively regulates the association of RelA to chromatin. Therefore, this work identifies a novel family of factors that regulate NF-κB-mediated transcription by controlling the occupancy of NF-κB on chromatin.

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[1] The abbreviations used are: RHD, Rel homology domain; EGFP, enhanced green fluorescence protein (GFP); TAP, tandem affinity purification; GST, glutathione *S*-transferase; TNF, tumor necrosis factor; MS, mass spectroscopy; RT, reverse transcription; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation; RNAi, RNA interference; siRNA, small interfering RNA; HIV-1, human immunodeficiency virus-1.

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EXPERIMENTAL PROCEDURES

Plasmids—The plasmids pEBB, pEBG, pEBB-MURR1-Flag and pEBB-MURR1-GST, pEBB-T7-IκB-αS.D., 2κB-luc, and EGFP-p65 (kindly provided by Dr. Rainer de Martin) have been described previously (17–22). pEBB-COMMD1-GST vectors expressing exon 1, exon 2-3, and exon 1-3 were generated by PCR amplification using pEBB-MURR1-Flag as template with the boundaries outlined in Fig. 3*C*. pEBB-MURR1-TAP was constructed by subcloning MURR1 into pEBB-TAP, which was generated by PCR amplification of the coding sequence for the tandem affinity purification (TAP) tag using pBS1539 as template (23). Expression vectors for COMMD proteins in fusion with Flag and glutathione *S*-transferase (GST) (pEBB-COMMD-Flag or pEBB-COMMD-GST) were generated by PCR amplification of the coding sequences for each of these proteins. To that effect the following full-length IMAGE clones were used as templates to amplify COMMD2 through COMMD10, respectively: 4443942, 3531636, 5743903, 6644608, 1692591, 5275167, 4051246, 4333615, and 3683093. pEBG-RelA-(1–305), pEBG-RelA-(306–551), and pEBG-RelA-(1–180), pEBG-c-Rel-(1–180), pEBG-RelB-(97–267), and pEBG-p50-(1–233) and pEBG-p52-(1–212) were generated by PCR using the vectors RSV-RelA, RSV-c-Rel, RSV-RelB, RSV-p50, and RSV-p52 as templates, respectively (kindly provided by Dr. Neil Perkins) (24).

Cell Culture, Transfection, and Luciferase Assays—Human embryonic kidney 293 cells and prostate cancer and DU145 cells were obtained from ATCC. 293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and L-glutamine, and DU145 cells were cultured in minimum Eagle's medium supplemented with 10% fetal bovine serum, L-glutamine, sodium bicarbonate, and pyruvate. A standard calcium phosphate transfection protocol (20) was used to transfect plasmids and siRNA oligonucleotides into 293 cells. Delivery of siRNA oligonucleotides into DU145 cells was performed using Oligofectamine (Invitrogen) as specified by the manufacturer. For luciferase reporter experiments, cells were seeded in 6-well plates and transfected with 4 μg of pEBB plasmid, and 25 ng of the reporter plasmid 2κB-luciferase. Luciferase assays were performed as described previously (25). TNF (Roche Applied Science) treatments consisted of 1000 units/ml for 12 h. For immunoprecipitation experiments cells were seeded in 10-cm plates and transfected with a total of 12 μg of plasmid. Finally, suppression of endogenous COMMD expression was achieved by transfection of 293 cells seeded in 6-well plates with 2 μg of the corresponding siRNA oligonucleotides (Qiagen).

TAP Screening—293 cells seeded in 15-cm plates were transiently transfected with pEBB-MURR1-TAP (15 μg of plasmid/plate) and 2 days later were lysed in Triton lysis buffer (25 mM HEPES, 100 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Triton X-100, protease inhibitors). The lysate was supplemented with NaCl and Nonidet P-40 and applied to a chromatography column containing IgG-Sepharose beads (Amersham Biosciences). After 2 h of incubation at 4 °C the column was drained and washed with IPP150 buffer (10 mM Tris-HCl, pH$^+$ 8.0, 150 mM NaCl, 0.1% Nonidet P-40, protease inhibitors) and TEV cleavage buffer (10 mM Tris-HCl, pH$^+$ 8.0, 150 mM NaCl, 0.1% Nonidet P-40, 0.5 mM EDTA, 1 mM dithiothreitol, protease inhibitors). After incubation for 2 h at 16 °C in TEV cleavage buffer supplemented with TEV enzyme (Invitrogen), the eluate was collected and supplemented with $CaCl_2$ and IPP150 calmodulin binding buffer (10 mM Tris-HCl, pH$^+$ 8.0, 150 mM NaCl, 0.1% Nonidet P-40, 1 mM magnesium acetate, 1 mM imidazole, 2 mM $CaCl_2$, 10 mM β-mercaptoethanol). This was then applied to a chromatography column containing calmodulin 4B beads (Amersham Biosciences) and incubated at 4 °C for 1 h. The column was then drained and washed with IPP150 calmodulin binding buffer. After incubation at 4 °C with IPP150 calmodulin elution buffer (10 mM Tris-HCl, pH$^+$ 8.0, 150 mM NaCl, 0.1% Nonidet P-40, 1 mM magnesium acetate, 1 mM imidazole, 2 mM EGTA, 10 mM β-mercaptoethanol), a final eluate was collected. Proteins were precipitated by adding cold 10% trichloroacetic acid in acetone; after overnight incubation at −20 °C, the precipitate was collected by centrifugation at 4 °C (10,000 × *g* for 30 min), rinsed in 100% acetone, and allowed to air dry. These samples were then submitted to the Proteomics Centre at the University of Victoria for further processing, including tryptic digestion, high performance liquid chromatography separation, and tandem mass spectrometry (MS/MS) to determine peptide sequences.

RT-PCR and Expression Data—Total RNA was extracted from 293 cells using RNeasy (Qiagen) according to the manufacturer's instructions. Yield and purity was determined by measuring $A_{260/280}$ of RNA diluted in water. Oligonucleotides and internal probes for RT-PCR and quantitative RT-PCR of *COMMD* transcripts were designed with the use of the automated primer design tool, AutoPrime (www.autoprime.de). Detailed information about sequences and cycling parameters are available upon request. For non-quantitative RT-PCR, Titan One-Tube RT-PCR (Roche Applied Science) was used according to the manufacturer's instructions. For quantitative RT-PCR reactions, an RT reaction with 500 ng of total RNA in 25 μl was performed using random hexamers and Taqman reverse transcription reagents (Applied Biosystems). This was followed by quantitative PCR performed in the 7500 real time PCR system (Applied Biosystems). In all reactions, Taqman PCR Master Mix with the appropriate primers and probes was used. Primers and probe sets for *c-IAP2* (*BIRC3*) and *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) as an internal control were obtained from Applied Biosystems. Expression data in normal tissues were obtained from the Genomics Institute of the Novartis Research Foundation (26), downloaded, and further analyzed.

Immunoblotting and Immunoprecipitation—Cell lysates were prepared by adding Triton lysis buffer; immunoblotting and GSH precipitations were performed as previously described (19). A polyclonal COMMD1 antiserum was raised by immunizing New Zealand White rabbits. Recombinant protein, which was used as immunogen, was produced in *Escherichia coli* by expressing GST-COMMD1 using the pGEX-4T1 bacterial expression vector (Amersham Biosciences). GST-COMMD1 was purified over a glutathione-Sepharose chromatography column (Amersham Biosciences), and COMMD1 was generated by thrombin cleavage of the GST affinity tag according to the manufacturer's instructions. Antibodies against Flag (Sigma, A8592), RelA (BD Transduction Laboratories, 610868), c-Rel (Santa Cruz, sc-6955), RelB (Santa Cruz, sc-226), p50 (Upstate Biotechnology, 06–886), p52 (Upstate Biotechnology, 05–361), IκB-α (Upstate Biotechnology, 06–494), GST (Santa Cruz, sc-459), α-tubulin (Molecular Probes, A11126), and GCN5 (Santa Cruz, sc-20698) were used as indicated.

Confocal and Fluorescence Microscopy—293 cells were plated in chambered cover glass plates or 6-well plates and transfected with EGFP-p65 (25 or 50 ng/well, respectively). Morphological assays for nuclear translocation of EGFP-p65 were performed by observing cells with a Zeiss Axiovert 100 M confocal microscope before and after treatment with TNF. Representative images were obtained, and 250–400 cells were observed and scored accordingly.

Electrophoretic Mobility Shift Assay (EMSA)—293 cells were seeded in 10-cm dishes and transfected as indicated. TNF stimulation, when performed, consisted of treating cells with 1000 units/ml for 30 min before lysis. The preparation of nuclear extracts and EMSA have been described previously (24). For our studies, a double-stranded oligonucleotide encompassing a canonical κB sequence was used as probe (forward sequence, AGCTTACAAGGGACTTTCCGCTGGGGACTTTCCAGGG).

Cellular Fractionation—293 cells were plated in 10-cm plates 48 h before the procedure. Medium was aspirated, and the cells were rinsed in phosphate-buffered saline, scraped, and collected in a microcentrifuge tube. The cells were resuspended in 200 μl of buffer 1 (25 mM HEPES, 5 mM KCl, 0.5 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and protease inhibitors). After that, 200 μl of buffer 2 were added (Buffer 1 with 1% Nonidet P-40), and the cells were incubated with constant rotation at 4 °C for 15 min. The samples were centrifuged for 1 min at 600 × *g*, and the supernatant, corresponding to the cytoplasmic fraction, was collected. The precipitated material was gently rinsed in 100 μl of buffer 3 (1:1 mixture of buffers 1 and 2). After centrifuging the samples again as before, the supernatant was collected as part of the cytoplasmic fraction. The remaining precipitated material was then treated by the addition of 500 μl of buffer 5 (25 mM HEPES, 10% sucrose, 350 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 0.01% Nonidet P-40, protease inhibitors) and incubated with constant rotation at 4 °C for 60 min. After this, the samples were centrifuged for 10 min at 16,100 × *g*. The supernatant, corresponding to the nuclear fraction, was collected separately.

Chromatin Immunoprecipitation—Subconfluent DU145 cells were treated with TNF (1000 units/ml) before cross-linking for chromatin immunoprecipitation (ChIP) analysis. For attachment assays, 293 cells were re-plated in serum-free media on laminin-coated plates (Discovery Labware) as previously described (27). ChIP protocol and primers sequences have been previously described (28). Antibodies used in the ChIP studies include COMMD1 (described above), M2 Flag (Sigma, F3165), RNA polymerase II (Santa Cruz, SC-9001), and RelA (Upstate, 06-418).

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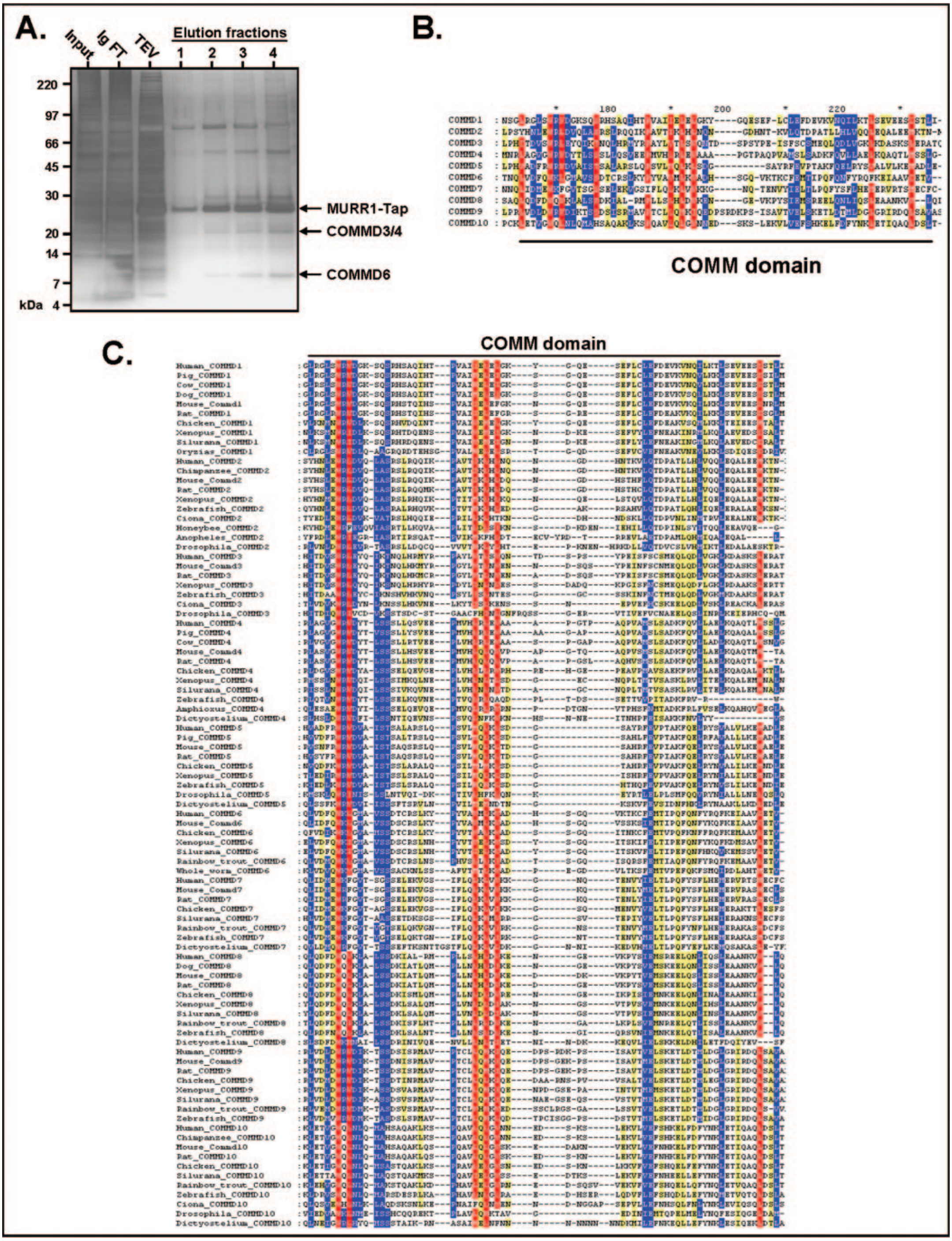
A.
Input
Ig FT
TEV
Elution fractions
1
2
3
4
220
97
66
45
30
20
14
7
kDa 4
MURR1-Tap
COMMD3/4
COMMD6
B.
180
200
220
COMMD1
COMMD2
COMMD3
COMMD4
COMMD5
COMMD6
COMMD7
COMMD8
COMMD9
COMMD10
COMM domain
C.
COMM domain

FIG. 1. *A*, identification of the COMMD protein family. Fractions obtained during TAP purification were separated by SDS-PAGE, and the gel was silver-stained. The lysate was subjected first to an immunoglobulin column, and the flow-through (*Ig FT*) and eluate (*TEV*) were collected. This eluate was further purified over a calmodulin column, and the eluates were collected in four fractions (*Elution fractions*). COMMD3, -4, and -6 were identified in the final eluate by liquid chromatography-MS/MS. *B*, human COMMD proteins. Analysis of the human protein databases using

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RESULTS

Biochemical Screen for MURR1-associated Factors—To further understand the cellular activities of MURR1, a biochemical screen for associated proteins was performed based on the TAP scheme that has been previously described (23). Briefly, MURR1 in fusion with the TAP affinity tag was transiently expressed in 293 cells, and MURR1-TAP was subsequently purified from cells lysates using two sequential chromatography columns containing IgG and calmodulin beads, respectively (Fig. 1*A*). The material obtained was subjected to tryptic digestion, and the peptides generated were then identified by tandem mass spectrometry (MS/MS) after initial separation using liquid chromatography. A number of associated factors were identified, including three proteins that upon close inspection demonstrated the presence of a region with close homology to MURR1 in their carboxyl termini (Fig. 1, *A* and *B*). These factors were later designated as COMMD3, -4, and -6 (see below).

Identification of the COMMD Protein Family—After the identification of three MURR1 homologous factors in our biochemical screen, we performed an extensive search of the sequence databases for additional homologs. Through this approach we were able to identify 10 proteins in humans, including MURR1, that contain highly conserved carboxyl-terminal sequences (Fig. 1*B*). The majority of these genes were only known as open reading frames and had not been previously characterized.

Further analysis of orthologs present across multiple species demonstrated that the area of close homology in the carboxyl termini of these proteins represents a previously unrecognized, unique, and highly conserved motif (Fig. 1*C*). This leucine-rich, 70–85 amino acid long sequence is predicted to form a β-sheet. We have termed this region the copper metabolism gene MURR1 (COMM) domain.

The designation of homologs of MURR1 identified here required the generation of a new nomenclature. *Murr1* derived its name from its proximity to the *U2af1-rs1* locus in mice (mouse U2af1-rs1 region 1); however, this genomic organization is not observed in other organisms including humans. In addition, an unrelated gene that also lies in close proximity to *U2af1-rs1* has been designated *Murr2*, precluding the use of this name for MURR1 homologs (29). In consultation with the HUGO gene nomenclature committee, the term COMMD (COMM Domain containing) is proposed to designate these factors based on the shared structural domain that defines this family of proteins and has been adopted in NCBI public databases. The name COMMD1 is suggested for MURR1 as a means to standardize the nomenclature to designate this protein family and will be used hereafter.

With the exception of COMMD1, no other COMMDs have been previously described in any detail. *COMMD5* was identified as an open reading frame that is overexpressed in naturally hypertensive rats and suppressed in a number of primary tumors and cancer cell lines (30). The expressed protein localizes to the nucleus, although a direct role in transcription had not been previously demonstrated. *COMMD6* is orthologous to a mouse gene located in a region that is necessary for normal embryonic development, although it is unclear whether *COMMD6* itself is required for normal embryogenesis (31). Similarly, *COMMD3* was previously identified as a locus with close proximity to the Polycomb-group gene *Bmi-1* (32). Finally, an expressed sequence tag corresponding to *COMMD7* was found to be consistently repressed in an experimental system designed to screen for factors involved in leukemogenesis (33).

COMMD Genes Are Highly Conserved—We found that all 10 genes have been conserved throughout vertebrate evolution, as can be gleaned from orthologs found in *Silurana tropicalis, Xenopus laevis, Danio rerio*, *Oncorhynchus mykiss,* and *Oryzias latipes* (see the supplemental table). In general, mammalian sequences are about 90% conserved when compared with their human orthologs. Furthermore, lower metazoans, including insects, worms, and molds, also possess *COMMD* genes; however, none of these genes were identified in unicellular eukaryotic organisms or bacteria. Five of these genes were found in *Drosophila melanogaster* (*COMMD2*, *-3*, *-4*, *-5*, and *-10*) and *Dictyostelium discoideum* (*COMMD4*, *-5*, *-7*, *-8*, and *-10*). Overall, eight of the *COMMD* genes can be found in lower metazoans, with *COMMD1* and *COMMD9* orthologs being restricted to vertebrate species (see the supplemental figure).

Despite the presence of a conserved and defining motif in all these proteins, a significant proportion of the sequence of each COMMD protein is composed of unique regions that are divergent across members of the family. For example, human and zebrafish COMMD1 are 72% conserved, whereas human COMMD1 and COMMD10 are only 34% conserved when regions outside the COMM domain are included in the comparison (data not shown).

COMMD Genes Are Widely Expressed—Given that COMMD proteins were initially identified as COMMD1-associated factors in 293 cells, we first investigated the pattern of expression of human *COMMD* genes in this cell line. To this end, we designed primers for RT-PCR of each one of these genes including in each case one primer that was selected at an exon-exon junction. This strategy minimizes the possibility of spurious amplification from contaminating genomic DNA because such junctions are generated only after splicing. In addition, the potential for mispriming against the intronic boundary was taken into account in the design. With this algorithm we identified primers for all 10 human *COMMD* genes, with the amplicon size and position of exon-exon primers indicated in Fig. 2*A*. Using these primers and total RNA extracted from 293 cells we were able to amplify the appropriate size products for each of the *COMMD* genes (Fig. 2*B*). Template-lacking negative controls did not amplify these products (data not shown). This indicated that 293 cells express all *COMMD* genes, a fact that was also confirmed by publicly available expression data (not shown here).

Next, the level of *COMMD* expression in multiple tissues was analyzed using data available from the Genomics Institute of the Novartis Research Foundation. Utilizing oligonucleotide arrays, expression levels for more than 44,000 mRNA transcripts across 79 human tissues were determined (26). This raw data were downloaded, and the corresponding probes for most *COMMD* genes (with the exception of *COMMD6*) were identified. Expression levels in 13 selected tissues were further analyzed and are presented in Fig. 2*C*. As shown, *COMMDs* are

BLAST allowed for the identification of six additional proteins with sequence homology to MURR1. A partial alignment of all 10 human COMMD proteins is shown, demonstrating a region of higher conservation (COMM domain). The accession numbers for the 10 human mRNA sequences are NM_152516, AY542158, AY542159, AY542160, NM_014066, AY542161, AY542162, AY542163, AY542164, and AY542165, corresponding to COMMD1–10, respectively. The degree of conservation of each amino acid residue among these sequences is indicated based on Dayhoff PAM 250 scoring matrices (*red*, 90% conserved; *blue*, 70% conserved; *yellow*, 50% conserved). *C*, alignment of 91 COMMD proteins from multiple species across the COMM domain. All protein sequences spanning through the COMM domain were aligned using the ClustalV algorithm, and this alignment was then refined using the SAM program. The resulting alignment was then annotated using the GeneDoc program. The degree of conservation of each amino acid residue among these sequences is indicated based on Dayhoff PAM 250 scoring matrices as before.

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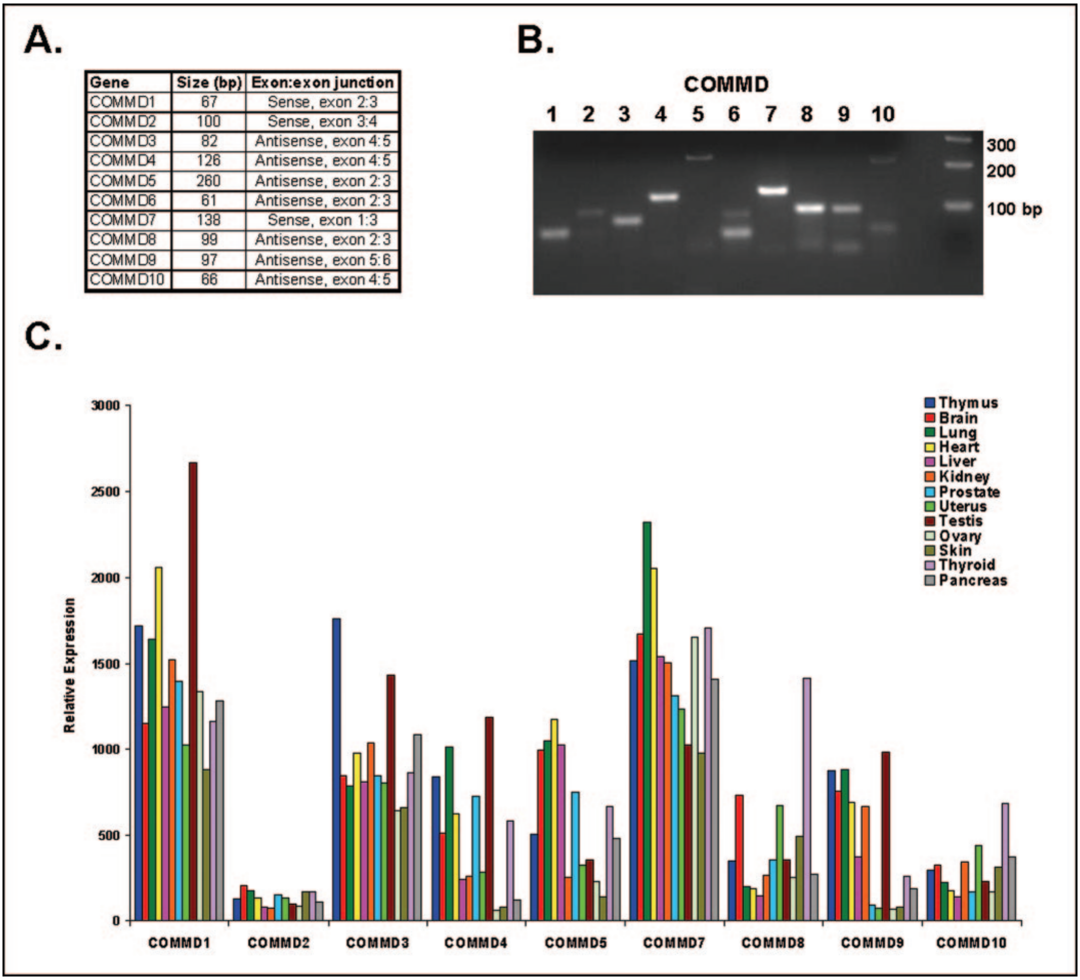

Gene	Size (bp)	Exon:exon junction
COMMD1	67	Sense, exon 2:3
COMMD2	100	Sense, exon 3:4
COMMD3	82	Antisense, exon 4:5
COMMD4	126	Antisense, exon 4:5
COMMD5	260	Antisense, exon 2:3
COMMD6	61	Antisense, exon 2:3
COMMD7	138	Sense, exon 1:3
COMMD8	99	Antisense, exon 2:3
COMMD9	97	Antisense, exon 5:6
COMMD10	66	Antisense, exon 4:5

FIG. 2. *A*, amplicon size and position of mRNA-specific primers utilized for RT-PCR. *B*, *COMMD* genes are expressed in 293 cells. Using the primer sets described, RT-PCR was performed with total RNA extracted from 293 cells serving as template. The presence of amplified products of the proper sizes was determined by agarose gel electrophoresis as shown here. *C*, expression of *COMMD* genes in 13 normal tissues. Levels of mRNA expression were determined using oligonucleotide microarrays.

widely expressed in human tissues, but the relative abundance of any given *COMMD* mRNA is different across the samples. For example, whereas *COMMD1* expression is highest in the testis, *COMMD3* is the highest expressed in the thymus, *COMMD7* in the lung, and *COMMD8* in the thyroid. Conversely, any given tissue has a complement of *COMMD* genes that demonstrate highest expression, and these subsets are not identical in each case (data not shown).

COMMD1 Can Associate with Other COMMD Proteins—COMMD3, -4, and -6 were initially identified biochemically by their ability to interact with COMMD1. Therefore, the ability of all the members of the family to interact with COMMD1 was evaluated. Each of the 10 COMMD proteins was fused to GST and expressed in 293 cells. COMMD-GST fusion proteins were then precipitated from cell lysates with glutathione-Sepharose beads, and the recovered material was immunoblotted for endogenous COMMD1 (Fig. 3*A*). COMMD1–8 and COMMD10 could readily precipitate endogenous COMMD1; COMMD9 also co-associates with COMMD1 but to a lesser extent (not shown here). These experiments demonstrated that COMMD1 can interact with itself and with all other COMMD proteins, consistent with the interactions detected in the initial TAP screen.

COMMD-COMMD Protein Interactions Are Mediated by the COMM Domain—To define the domain(s) required for COMMD multimer formation, a variety of deletion constructs of COMMD1 were tested for their ability to bind COMMD1 and COMMD3. The coding regions corresponding to each exon of COMMD1 were used as boundaries in constructs expressing fusion proteins with GST (Fig. 3*B*). The hereditary canine copper toxicosis mutation described previously consists of a genomic deletion encompassing exon 2 of *COMMD1* such that the expressed open reading frame lacks 94 amino acid residues (14). This protein product was also expressed in fusion with GST and similarly tested for its ability to bind COMMD1 and COMMD3.

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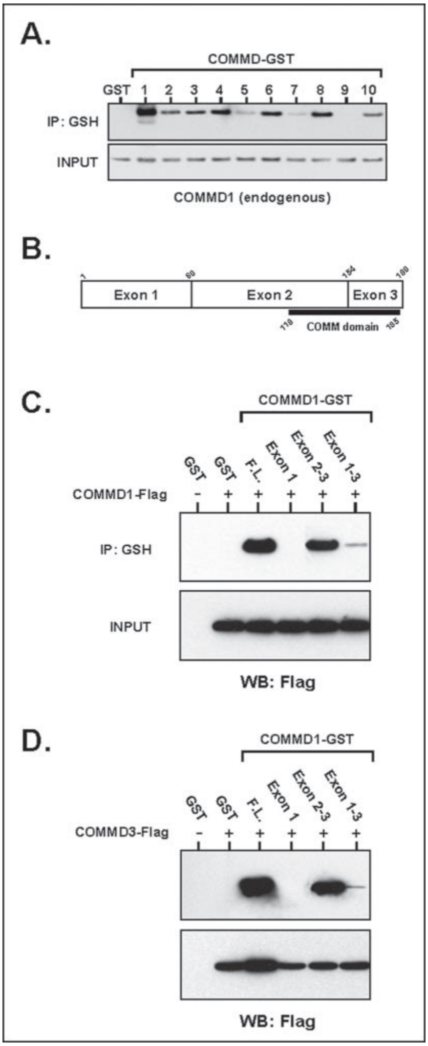

FIG. 3. *A*, COMMD-COMMD interactions. Each of the 10 COMMD proteins was expressed in 293 cells in fusion with GST and precipitated (*IP*) from cell lysates by glutathione-Sepharose beads. The presence of endogenous COMMD1 in the precipitates was determined by immunoblotting. *B*, schematic representation of COMMD1 and the amino acid residues that are the boundaries for interaction mapping experiments. *C* and *D*, the COMM domain is required for the association of COMMD proteins. COMMD1-Flag (*C*) or COMMD3-FLAG (*D*) were expressed in 293 cells along with various regions of COMMD1 in fusion with GST as indicated. COMMD1-GST was precipitated from cell lysates by glutathione-Sepharose beads and the presence of COMMD1 or -3 in the precipitates was determined by immunoblotting (*WB*) with a Flag antibody.

These fusion proteins were expressed in 293 cells and precipitated with glutathione-Sepharose beads. The ability of these fragments to support an interaction was determined by the presence of COMMD1 or COMMD3 in the precipitate (Fig. 3, *C* and *D*). The carboxyl terminus of COMMD1 (exon 2-3), which contains the COMM domain, was found to be sufficient for interactions with COMMD1 and COMMD3. The lack of the COMM domain in the exon 1-GST fusion protein abrogated binding in both cases. Furthermore, the exon 1-3 product, replicating the protein product in dogs with copper toxicosis and lacking part of the COMM domain, had a significant impairment in binding. These data demonstrated that the COMM domain that defines this protein family serves as an interface for COMMD-COMMD interactions.

Several COMMD Proteins Suppress κB-mediated Transcription—MURR1/COMMD1, the prototype member of the family, was recently reported to inhibit κB-mediated transcription from endogenous and viral promoters (16). Therefore, the ability of other COMMD proteins to inhibit NF-κB was investigated. Cells were transfected with a κB-responsive reporter plasmid (2κB-luc) along with each of the COMMD proteins, and the response to TNF stimulation was subsequently evaluated. As shown in Fig. 4*A*, COMMD1, -2, -4, -7, -9, and -10 were capable of strongly inhibiting TNF-mediated NF-κB activation, whereas COMMD3 and -8 inhibited NF-κB weakly in this assay. Similarly, most COMMD proteins also inhibited basal levels of κB-mediated transcription.

Next, the ability of these proteins to control transcription of an endogenous κB-responsive gene was evaluated. Expression levels of endogenous *COMMD1*, *-4,* and *-6* were decreased by the use of RNA interference (RNAi), and the induction of *c-IAP2* mRNA levels in response to TNF was evaluated (Fig. 4, *B* and *C*). The effectiveness of RNAi against *COMMD1*, *-4*, and *-6* was confirmed by quantitative RT-PCR (Fig. 4*C*). When compared with mRNA levels present in control samples, transfection with siRNA oligonucleotides against *COMMD1*, *-4,* and *-6* resulted in a 75–95% suppression of mRNA expression, consistent with efficient RNAi of these transcripts. Next, the effect of reduced COMMD expression on *cIAP-2* transcription was evaluated (Fig. 4*B*). In cells transfected with oligonucleotides against an irrelevant target (GFP), treatment with TNF resulted in a 9-fold increase in *c-IAP2* mRNA levels, as measured by quantitative RT-PCR, a fold increase that is identical to that observed in untransfected cells (data not shown). Decreased *COMMD* levels resulted in heightened expression of *c-IAP2* after TNF treatment, with this effect being most notable after *COMMD6* RNAi. Therefore, these factors not only share a common domain but have similar functional properties in the regulation of NF-κB transcriptional activity.

COMMD Proteins Associate with the NF-κB Complex—The ability of COMMD1 to inhibit κB-mediated transcription was previously found to depend on its association with the NF-κB complex (16). Indeed, precipitation of endogenous COMMD1 with the use of rabbit polyclonal sera against COMMD1 resulted in the co-precipitation of endogenous RelA and c-Rel (Fig. 5*A*).

The possibility that other COMMD proteins can interact with the NF-κB complex was evaluated using fusion proteins with GST expressed in 293 cells. Precipitations with glutathione-Sepharose beads were performed followed by immunoblotting for detection of endogenous NF-κB subunits RelA, c-Rel, RelB, NF-κB1/p105, and NF-κB2/p100. As was the case for COMMD1, most COMMD proteins were also capable of precipitating NF-κB complexes (Fig. 5*B*). Although the intensity of recovery of NF-κB subunits correlated with the level of expression of the COMMD-GST proteins themselves (data not shown), the pattern of association with NF-κB subunits was different between the various COMMDs. Some COMMD proteins favor complexes containing RelB and NF-κB1/p105 (as in the case of COMMD3 and 9), whereas others seem to interact preferentially with RelA-containing complexes (as in the case of COMMD6 and -7). COMMD1, -2, -4, -5, -8, and -10 could associate more broadly with NF-κB subunits, although pattern differences were still evident. COMMD1 could asso-

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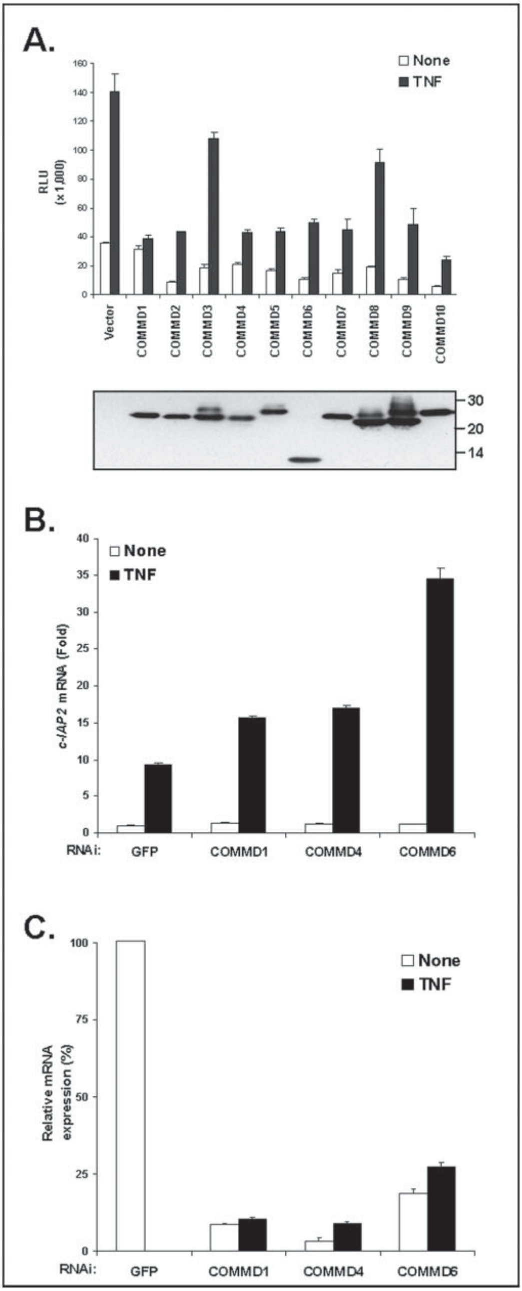

FIG. 4. *A*, COMMD proteins suppress κB-mediated transcription. Cells were transfected with the 2κB-luciferase reporter and COMMD proteins and were treated with TNF (1000 units/ml) for 12 h. Transcriptional activation of NF-κB was determined by luciferase assay (*top panel*), and expression of COMMD proteins in these lysates was determined by Flag immunoblotting (*bottom panel*). *RLU*, relative luminescence units. *B*, effect of COMMDs on *c-IAP2* expression. Endogenous levels of COMMD1, -4, and -6 were decreased with the use of siRNA oligonucleotides. The effects of TNF treatment on the expression levels of *c-IAP2*, a known NF-κB responsive gene, were evaluated by quantitative RT-PCR. *C*, efficiency of *COMMD* RNAi. Levels of expression of *COMMD1*, *-4,* and *-6* were determined by quantitative RT-PCR and compared with the corresponding control samples transfected with the GFP siRNA oligonucleotide. The data are presented as a percentage of the control samples, which was standardized to 100%.

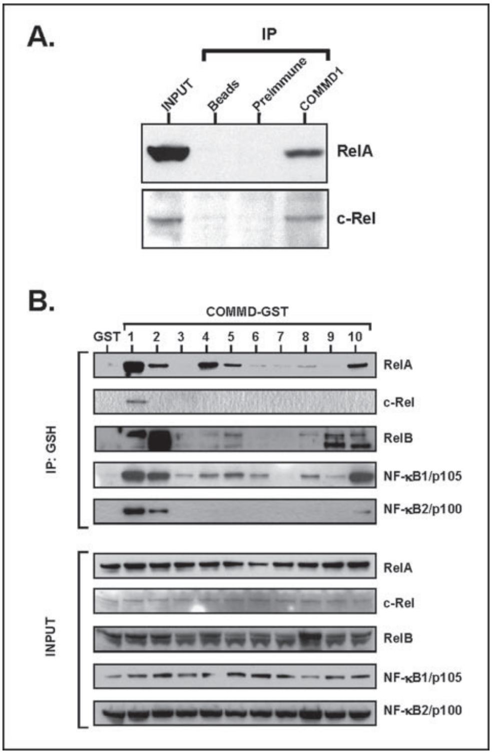

FIG. 5. *A*, COMMD1 associates with endogenous NF-κB subunits. Endogenous COMMD1 was immunoprecipitated (*IP*) from cell lysates prepared from 293 cells. This material was immunoblotted for endogenous RelA (*top panel*) and c-Rel (*bottom panel*). *B*, other COMMDs also associate with NF-κB. GST fusions with all COMMD proteins were expressed in 293 cells and precipitated from cell lysates by glutathione-Sepharose beads, and the presence in the precipitates of endogenous RelA, c-Rel, RelB, NF-κB1/p105, and NF-κB/p100 was determined by immunoblotting.

ciate with all five subunits and was the only COMMD able to precipitate c-Rel, whereasCOMMD2 also associated broadly with NF-κB subunits but interacted more strongly with RelB-containing complexes.

COMMD1-NF-κB Interactions Can Be Mapped to Distinct Domains—The ability of various regions of COMMD1 to sustain an interaction with endogenous RelA was evaluated next. In these experiments GST fusions of various domains of COMMD1, as depicted in Fig. 3*B*, were used for co-precipitation experiments. Unlike COMMD1-COMMD interactions that seem to require only the COMM domain (Fig. 3, *C* and *D*), COMMD1-RelA interactions are only detectable with full-length COMMD1 (Fig. 6*A*). Therefore, the interaction between COMMD1 and RelA relies on the presence of elements other than the COMM domain.

Next, the interaction between COMMD1 and RelA was evaluated further by determining the domains in RelA that are required for this interaction (Fig. 6*B*). Different domains of RelA in fusion with GST were expressed in 293 cells and

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precipitated with glutathione-Sepharose beads; the presence of COMMD1, p50 or its precursor p105, and IκB-α in the precipitates was determined by immunoblotting. As shown in Fig. 6*C*, the RHD of RelA was sufficient for binding to COMMD1, p50 or p105, and IκB-α. The carboxyl-terminal 246 amino acids of RelA (306–551), which correspond to the transactivation domain of the molecule (TAD), did not bind any of these molecules. Within the RHD, the first 180 amino acids comprise the DNA binding domain, whereas the remainder of the RHD (181–305) contains residues involved in IκB binding and dimerization with other subunits and the nuclear localization signal. Interestingly, the first 180 amino acids of RelA were capable of binding COMMD1 but not p50/p105 or IκB-α, probably because of the absence of the dimerization sequences. Reciprocal experiments, in which precipitation of COMMD1-GST was performed to determine its ability to bind to different domains of RelA, also supported these results (data not shown).

The RHD is highly conserved in all five NF-κB subunits (34). Therefore, the possibility that COMMD1 could also bind to similar sequences present in the RHD of other NF-κB subunits was similarly evaluated. Sequences contained in c-Rel-(1–180), RelB-(97–267), p50-(1–233), and p52-(1–212) were found to be homologous to RelA-(1–180) by aligning all five subunits using the ClustalV algorithm (data not shown). Each one of these regions in fusion with GST was expressed in 293 cells and precipitated from cell lysates with glutathione-Sepharose beads, and the presence of COMMD1 was determined by immunoblotting. As shown in Fig. 6*D*, these homologous regions, present in the RHD of all five NF-κB subunits, were capable of binding to COMMD1.

COMMD1 Does Not Affect Nuclear Translocation of NF-κB—In most cells NF-κB is ordinarily localized in the cytoplasm through interactions with members of the IκB family, which results in masking of the nuclear localization signal and cytoplasmic sequestration of the complex (11). Because the translocation of NF-κB from the cytosol to the nucleus is a well characterized regulatory step in the activation of κB-dependent transcription, the ability of COMMD1 to affect this process was investigated.

First, cellular localization of a fusion protein between RelA and EGFP was assessed by fluorescence microscopy before and after TNF treatment. TNF stimulation resulted in nuclear translocation of EGFP-RelA, a process that was inhibited in cells cotransfected with a superdominant form of IκB-α (Fig. 7, *A* and *B*). In contrast, the distribution of EGFP-RelA was unchanged by COMMD1 transfection, suggesting that unlike IκB-α, COMMD1 does not suppress the nuclear translocation of the NF-κB complex.

The translocation of NF-κB complexes into the nucleus and their DNA binding capacity was also evaluated by EMSA. Cells transfected with a control vector and subsequently treated with TNF demonstrated increased κB binding activity in nuclear extracts (Fig. 7*C*). As a control, this induction was blocked by

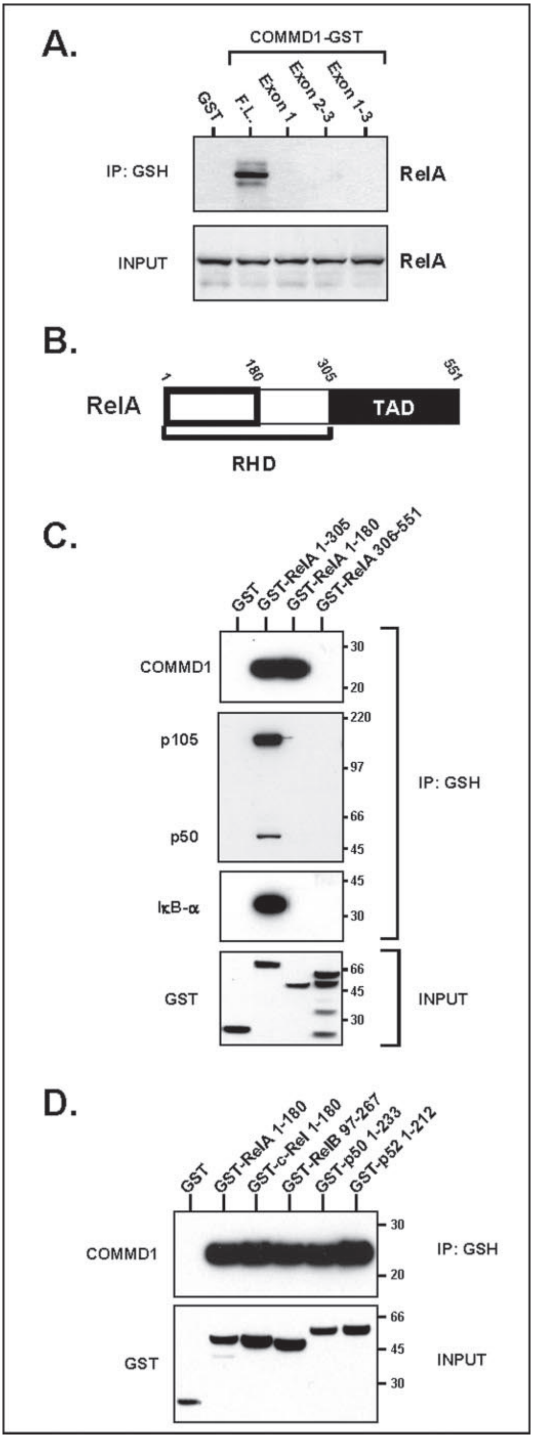

FIG. 6. *A*, domains involved in the interaction between COMMD1 and RelA. Deletion constructs of COMMD1 in fusion with GST corresponding to the boundaries described in Fig. 3*B* were utilized for co-precipitation experiments. The presence of endogenous RelA in the precipitate was determined by immunoblotting. *B*, shown is a schematic depicting the boundaries of the RHD and transactivation domain (*TAD*) of RelA. *C*, the ability of COMMD1 to bind to different regions of RelA was evaluated. Different domains of RelA were expressed in fusion with GST in 293 cells. These fusion proteins were precipitated from cell lysates by glutathione-Sepharose beads, and the presence of COMMD1, p50/p105, and IκB-α was determined by immunoblotting. Input levels for COMMD1, p50, p105, and IκB-α were identical in all samples (data not shown). *D*, regions derived from other NF-κB subunits that are homologous to RelA-(1–180) were expressed in fusion with GST and precipitated from cell lysates with glutathione-Sepharose beads. The presence of COMMD1 in the precipitate was determined by immunoblotting. Input levels for COMMD1 were identical in all samples (data not shown). *IP*, immunopreipitate.

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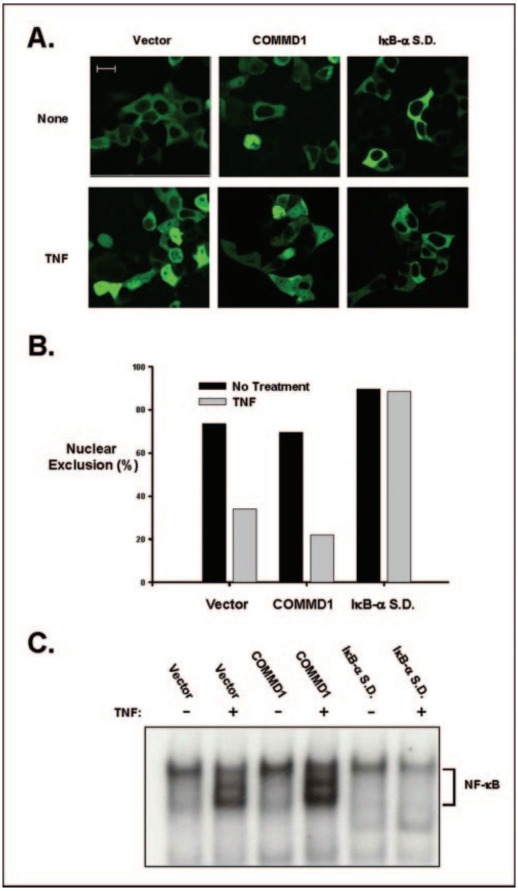

FIG. 7. *A*, COMMD1 does not prevent nuclear translocation of NF-κB. 293 cells were plated in cover glass-chambered slides and transfected with a vector encoding for RelA in fusion with EGFP and with COMMD1, IκB-αS.D. or a vector control (pEBB). Twenty-four hours after transfection cells were treated with TNF for 1 h. Representative images are shown (magnification bar corresponds to 18 μm). *B*, quantification of nuclear translocation. 293 cells were plated 6-well dishes, transfected as described in *A* and treated with TNF for 1 h. Before and after treatment, 250–400 cells in each group were counted under the microscope and rated for the presence or absence of appreciable nuclear signal from RelA, the absence of which was recorded as nuclear exclusion. The data are presented as a percentage of the total number of cells counted in each group. *C*, nuclear translocation was also evaluated by EMSA. 293 cells plated in 10-cm plates were transfected with the constructs indicated in the figure. TNF stimulation was performed 30 min before extraction of nuclear proteins, which were used for EMSA.

transfection of superdominant IκB-α. However, COMMD1 transfection had no appreciable effects on the induction of κB binding activity in nuclear extracts after TNF treatment. In addition, whereas RNAi of COMMD1 led to transcriptional activation of NF-κB, this did not lead to induction of κB binding by EMSA (data not shown).

COMMD1 Binds to κB-responsive Promoter Regions and Affects NF-κB Binding to Chromatin—Although COMMD proteins can interact with the NF-κB complex, the ability of COMMD1 to inhibit transcriptional activation is independent of the nuclear translocation of NF-κB. This suggested that the activity of NF-κB complexes in the nucleus was compromised by COMMD1. To investigate this possibility, the presence of nuclear COMMD1 was first established. Subcellular fractions were prepared from 293 cells during timed stimulation with TNF (including an early and late time point). As shown in Fig. 8*A*, COMMD1 was present in both nuclear and cytosolic fractions, and its quantity seemed slightly increased in the late time point (180 min). As expected, TNF stimulation also resulted in nuclear translocation of RelA and early degradation of IκB-α with late accumulation of this protein in the nuclear fraction. The purity of the fractions was determined by immunoblotting for tubulin (cytosolic marker) and GCN5 (nuclear marker).

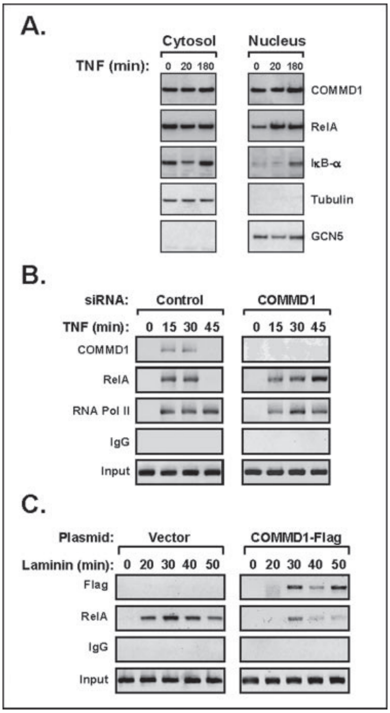

FIG. 8. *A*, presence of a nuclear pool of COMMD1. Cell lysates were fractionated to obtain nuclear and cytosolic fractions during timed stimulation with TNF. These fractions were used for immunoblotting to determine the cellular localization of endogenous COMMD1. As a control for the of TNF stimulation, the effects on RelA and IκB-α levels were also determined by immunoblotting. Tubulin and GCN5 served as cytosolic and nuclear markers, respectively. *B* and *C*, COMMD1 inhibits chromatin occupancy by NF-κB. ChIP analysis on the *cIAP-2* promoter after TNF stimulation was performed on DU145 cells transfected with siRNA oligonucleotides to COMMD1 or GFP (control). (*B*). ChIP analysis was performed on the *cIAP-2* gene using 293 cells expressing either Flag-tagged COMMD1 or empty vector control. Cell adhesion to a laminin-coated plate was used as the NF-κB activating stimulus. *Pol*, polymerase.

Based on the above findings, the effects of COMMD1 on NF-κB nuclear function were investigated by examining the recruitment of RelA to chromatin. Utilizing ChIP, the effects of COMMD1 levels on the recruitment of RelA to the κB-responsive *c-IAP2* promoter were determined. The effects of stimulation with TNF were compared after transfection of control and COMMD1 siRNA oligonucleotides (Fig. 8*B*). Decreases in endogenous COMMD1 levels resulted in prolongation of the occupancy time of RelA on the *c-IAP2* promoter. In addition, COMMD1 itself was found to be recruited to this κB-responsive site after TNF stimulation. A complementary set of experiments was also performed to determine if increases in

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COMMD1 protein levels would have the converse effect on RelA recruitment to chromatin. In this case, cell adhesion to laminin was used as the stimulus for NF-κB activation, as this results in robust and sustained recruitment of RelA to the *c-IAP2* promoter site. As shown in Fig. 8*C*, overexpression of COMMD1 resulted in a marked decrease in the duration of RelA association with chromatin after NF-κB activation; again, COMMD1 recruitment to chromatin in response to stimulation was also detected.

Taken together, these data indicate that COMMD1 affects the half-life of the RelA-chromatin complex that is recruited in response to NF-κB-activating stimuli. Because COMMD1 itself is recruited to chromatin and remains associated even after NF-κB has been displaced, this suggests that COMMD1 either alone or through the recruitment of other factors can affect the affinity of NF-κB for chromatin.

DISCUSSION

In this report we describe the identification of a novel and conserved family of homologs of MURR1. These factors are defined by the presence of a unique carboxyl-terminal motif termed the COMM domain. The existence of this protein family and any insights into the cellular functions of these factors were largely unknown up to this point.

We show here that all COMMD proteins can interact with MURR1/COMMD1. Interestingly, whereas all 10 factors are expressed in 293 cells, only COMMD3, -4, and -6 were identified as COMMD1-associated factors in our TAP screen, suggesting that certain associations might be preferentially present in cells. In addition, other COMMD-COMMD complexes that do not include COMMD1 are likely to occur, and indeed, our search of the *Drosophila* protein interaction map recently published (35) predicts an interaction between *Drosophila* COMMD2 and COMMD3. Therefore, once antibodies to all 10 proteins are available, the composition of physiologic COMMD-COMMD complexes would be able to be identified. We also show that interactions between COMMD proteins are mediated by the COMM domain.

Several COMMD proteins are functionally similar to the prototype member of the family, MURR1/COMMD1, and bind to and suppress NF-κB. We studied in further detail the binding mechanism by which COMMD1 interacts with RelA and demonstrate that this is distinct from dimerization to other NF-κB subunits or binding to IκB-α (Fig. 6*C*), indicating that these two events are not required for the interaction of COMMD1 with NF-κB. This is similarly supported by the observation that COMMDs can also associate with RelB (Fig. 5*B*), an NF-κB subunit that does not associate with IκB-α (36). These findings suggest that the prior identification of an association between IκB-α and COMMD1 that requires the ankyrin repeats of IκB-α could be potentially explained as a tertiary complex, since the ankyrin repeats are required for IκB-α binding to the NF-κB subunits (16).

Unlike the association between COMMD1 and itself or COMMD3, COMMD1-RelA interactions are not supported by the COMM domain alone (Figs. 3, *C* and *D*, and 6*A*), indicating that the association of COMMDs to NF-κB complexes is likely mediated by a mechanism distinct from COMMD-COMMD interaction. Similarly, the pattern of COMMD association to NF-κB could not be simply explained by COMMD multimer formation. Despite the demonstrated interaction between COMMD1 and COMMD3, only COMMD1 could precipitate RelA. In addition, COMMD9 could readily precipitate RelB, whereas COMMD6 did not despite the stronger COMMD6-COMMD1 interaction (Figs. 3*A* and 5*B*).

NF-κB-mediated transcription is largely controlled by the cytoplasmic sequestration of NF-κB complexes through the binding to ankyrin repeat-containing inhibitory molecules such as the IκBs (11, 37). Activation of the IκB kinase complex in response to a variety of extracellular signals initiates a cascade of events leading to IκB degradation and nuclear translocation on NF-κB. Whereas a role for MURR1/COMMD1 in the regulation of IκB-α degradation has been demonstrated (16), our findings here suggest that this effect is not sufficient to explain its ability to inhibit NF-κB-mediated transcription, since nuclear translocation of NF-κB subunits is unaffected by COMMD1. Rather, we find that COMMD1 can regulate the nuclear function of NF-κB through its recruitment to κB-responsive promoters where it affects the duration of RelA binding to chromatin.

Once activated, NF-κB can mediate expression of multiple gene targets. However, the observed responses are often specific to the cell type and stimulus in question. For example, NF-κB has been reported to be able to activate transcription of both pro- and anti-apoptotic factors in various settings (5, 6). The regulation of NF-κB solely by IκB-mediated sequestration of the complex is unlikely to account for these differences in gene expression. In this regard, post-translational modifications of NF-κB (38) and preferential promoter binding by different subunits have been shown to participate in the regulation of certain promoters in response to specific stimuli (36, 39). However, additional layers of regulation that could account for the tissue- and promoter-specific nature of the response are likely at play. The identification here of the COMMD family reveals an additional regulatory level that might be important in this regard. The conservation of all 10 *COMMD* genes through vertebrate evolution and the differential pattern of association to NF-κB complexes suggest that despite their similarities, COMMD proteins probably serve unique and nonredundant functions. A thorough understanding of the mode of action of these factors in different cell types may help to account for the precision and selectivity by which expression of the multitude of NF-κB-responsive genes can be orchestrated in response to a diverse range of stimuli.

Unlike other nuclear regulators of NF-κB such as histone acetylases and deacetylases, the COMMDs themselves possess no apparent intrinsic enzymatic activity. The transcriptional inhibitory functions of the COMMDs must in turn be regulated in response to certain stimuli, possibly by post-translational modifications. XIAP, a stress responsive pro-survival factor (40), can ubiquitinate COMMD1, resulting in its degradation (19), suggesting that the regulation of COMMD proteins might be integrated into cellular stress responses that are known to activate NF-κB.

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Supplemental Data

Supplemental Table: All ten human *COMMD* loci are presented, along with orthologs to these genes identified in other species. Altogether, 94 *COMMD* genes were identified, including 84 genes in non-human species. The proteins encoded by these orthologous genes were compared to the human proteins using the "BLAST 2 sequences" program available at NCBI. The degree of identity and homology is indicated.

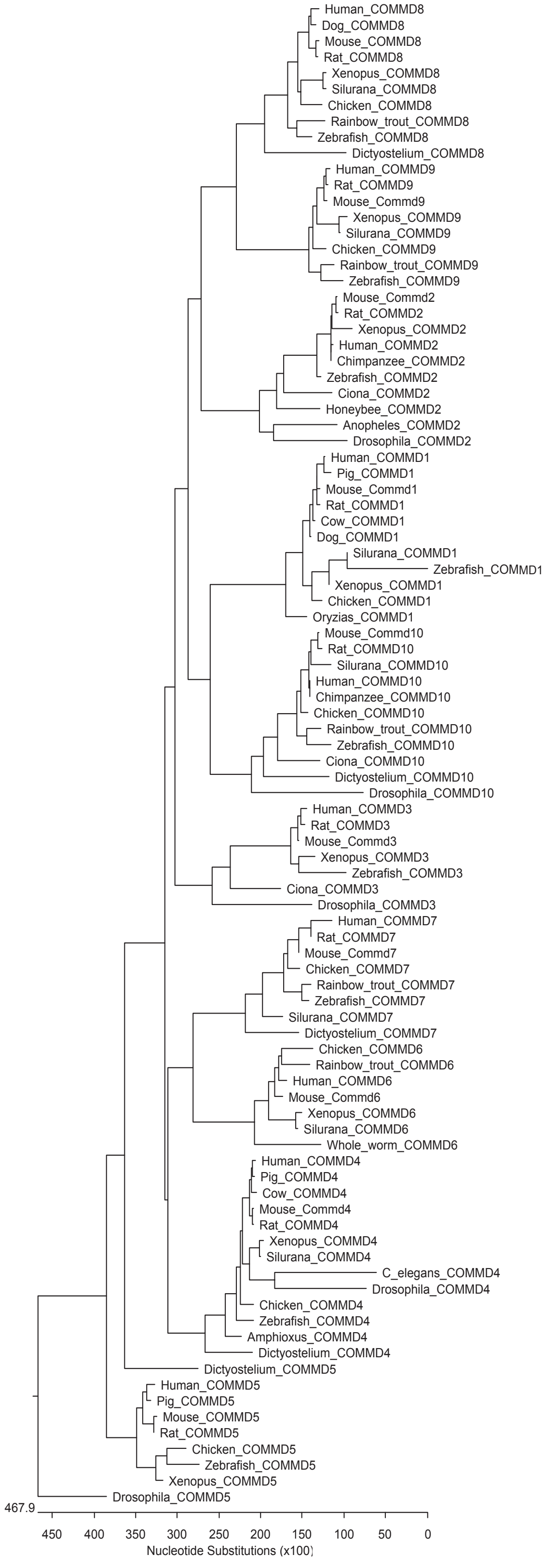
Supplemental Figure: A phylogenetic tree indicating the relationships between all 94 COMMD proteins was generated. First, these sequences were aligned using the ClustalV algorithm, and this alignment was then refined using the SAM program. The resulting refined alignment was used to generate the phylogenetic tree utilizing the MegAlign program (DNAStar).

Supplemental Table

Proposed name	**COMMD1**	**COMMD2**	**COMMD3**	**COMMD4**	**COMMD5**	**COMMD6**	**COMMD7**	**COMMD8**	**COMMD9**	**COMMD10**
Published name	**MURR1**		**Bup**		**HcaRG**	**AK000009**				
Locus in human genome	2p15	3q25.1	10pter-q22.1	15q23	8q24-qter	13q22	20q11.22	4p12	11p13	5q23.1

Species					**Orthologs (Identity/Homology %)**					
Pan troglodytes		98/99								99/99
Sus scrofa	91/95			94/97	86/92					
Bos taurus	88/93			89/94						
Canis familiaris	88/93							85/92		
Mus musculus	86/92	92/97	89/93	90/95	79/88	87/93	88/94	76/86	89/93	89/94
Rattus norvegicus	85/90	92/97	90/93	89/94	80/89		90/95	79/87	93/95	89/93
Gallus gallus	68/81			73/83	54/76	64/75	74/86	64/79	72/82	77/91
Xenopus laevis	61/83	75/85	63/78	65/83	52/75	76/89		57/73	64/82	
Silurana tropicalis	60/81			67/83		65/89	52/73	57/73	66/80	75/87
Oryzias latipes	55/76									
Oncorhynchus mykiss						75/88	62/81	54/70	61/77	68/81
Danio rerio	46/72	76/88	52/71	66/79	46/70		62/82	48/70	60/77	60/76
Branchiostoma belcheri				61/78						
Ciona intestinalis		45/67	33/51							47/65
Apis mellifera		42/64								
Anopheles gambiae		30/47								
Drosophila melanogaster		30/46	22/40	37/59	24/51					28/53
Caenorhabditis elegans				30/52						
Lumbricus rubellus						51/67				
Dictiostelium discodeum				39/58	31/56		39/61	31/63		32/55

Supplemental Figure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Dog_COMMD8
Mouse_COMMD8
Rat_COMMD8
Xenopus_COMMD8
Silurana_COMMD8
Chicken_COMMD8
Rainbow_trout_COMMD8
Zebrafish_COMMD8
Dictyostelium_COMMD8
Human_COMMD9
Rat_COMMD9
Mouse_Commd9
Xenopus_COMMD9
Silurana_COMMD9
Chicken_COMMD9
Rainbow_trout_COMMD9
Zebrafish_COMMD9
Mouse_Commd2
Rat_COMMD2
Xenopus_COMMD2
Human_COMMD2
Chimpanzee_COMMD2
Zebrafish_COMMD2
Ciona_COMMD2
Honeybee_COMMD2
Anopheles_COMMD2
Drosophila_COMMD2
Human_COMMD1
Pig_COMMD1
Mouse_Commd1
Rat_COMMD1
Cow_COMMD1
Dog_COMMD1
Silurana_COMMD1
Zebrafish_COMMD1
Xenopus_COMMD1
Chicken_COMMD1
Oryzias_COMMD1
Mouse_Commd10
Rat_COMMD10
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Rainbow_trout_COMMD10
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Human_COMMD3
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Rainbow_trout_COMMD7
Zebrafish_COMMD7
Silurana_COMMD7
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Chicken_COMMD6
Rainbow_trout_COMMD6
Human_COMMD6
Mouse_Commd6
Xenopus_COMMD6
Silurana_COMMD6
Whole_worm_COMMD6
Human_COMMD4
Pig_COMMD4
Cow_COMMD4
Mouse_Commd4
Rat_COMMD4
Xenopus_COMMD4
Silurana_COMMD4
C_elegans_COMMD4
Drosophila_COMMD4
Chicken_COMMD4
Zebrafish_COMMD4
Amphioxus_COMMD4
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Human_COMMD5
Pig_COMMD5
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Rat_COMMD5
Chicken_COMMD5
Zebrafish_COMMD5
Xenopus_COMMD5
Drosophila_COMMD5
467.9
450
400
350
300
250
200
150
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Nucleotide Substitutions (x100)

Chapter 4

COMMD1 promotes the ubiquitination of NF-κB subunits through a cullin-containing ubiquitin ligase

Maine GN, Mao X, Komarck CM, Burstein E.
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COMMD1 promotes the ubiquitination of NF-κB subunits through a cullin-containing ubiquitin ligase

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NF-κB is a pleiotropic transcription factor involved in multiple processes, including inflammation and oncogenesis. We have previously reported that COMMD1 represses κB-dependent transcription by negatively regulating NF-κB–chromatin interactions. Recently, ubiquitination of NF-κB subunits has been similarly implicated in the control of NF-κB recruitment to chromatin. We report here that COMMD1 accelerates the ubiquitination and degradation of NF-κB subunits through its interaction with a multimeric ubiquitin ligase containing Elongins B and C, Cul2 and SOCS1 ($\mathrm{ECS}^{\mathrm{SOCS1}}$). COMMD1-deficient cells demonstrate stabilization of RelA, greater nuclear accumulation of RelA after TNF stimulation, de-repression of several κB-responsive genes, and enhanced NF-κB-mediated cellular responses. COMMD1 binds to Cul2 in a stimulus-dependent manner and serves to facilitate substrate binding to the ligase by stabilizing the interaction between SOCS1 and RelA. Our data uncover that ubiquitination and degradation of NF-κB subunits by this COMMD1-containing ubiquitin ligase is a novel and critical mechanism of regulation of NF-κB-mediated transcription.

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Subject Categories: chromatin & transcription; proteins
Keywords: COMMD1; cullin; NF-κB; transcription; ubiquitination

Introduction

NF-κB is a dimeric transcription factor that controls expression of genes involved in multiple processes including immunity, apoptosis, and cell cycle progression (Karin and Lin, 2002). In mammals, NF-κB subunits are encoded by five genes (*RELA, RELB, REL, NFKB1*, and *NFKB2*). All subunits share an $\sim$300 amino-acid region of homology termed the Rel homology domain (RHD) that mediates DNA binding and dimerization. The *NFKB1* and *NFKB2* genes encode large precursor polypeptides known as p105 and p100, which are cleaved into the mature p50 and p52 subunits, respectively.

NF-κB is ordinarily sequestered in the cytoplasm in a transcriptionally inactive form as a result of interactions with IκB proteins or, in the case of RelB, through its dimerization with p105 or p100, which contain IκB-like domains in their carboxy-termini. In response to a variety of stimuli, transient nuclear translocation of NF-κB dimers takes place, a prerequisite for κB-mediated transcription to occur. The translocation event is triggered by phosphorylation of IκB proteins by the multimeric IκB kinase, which leads to their ubiquitination by the $\mathrm{SCF}^{\beta\text{-TrCP}}$ ubiquitin ligase (Henkel *et al*, 1993; Chen *et al*, 1995; Yaron *et al*, 1998). Ubiquitinated IκB is then quickly degraded allowing nuclear translocation of NF-κB to occur. Once in the nucleus, NF-κB complexes bind to cognate DNA sequences present in an array of promoters resulting in gene expression.

In addition to nuclear translocation, transcriptional activation by NF-κB requires recruitment of co-activators and the displacement of co-repressor complexes. This is mediated through an intricate set of nuclear events that involve post-translational modifications of NF-κB subunits, including phosphorylation and acetylation (Sakurai *et al*, 1999; Chen *et al*, 2001; Zhong *et al*, 2002).

In order to achieve controlled expression of its gene targets, the termination of NF-κB-mediated transcriptional responses is tightly regulated. Nuclear export of NF-κB complexes is a major step in the regulation of this pathway. Through a negative feedback loop, NF-κB induces the resynthesis of IκB proteins, ultimately driving nuclear export of NF-κB and termination of transcription (Hoffmann *et al*, 2002). Despite the important role that IκB proteins play in regulating the nuclear pool of NF-κB, other factors are likely involved. For example, NF-κB remains largely excluded from the nucleus in cells that are profoundly deficient in IκB proteins, in contrast to the mostly nuclear localization that results from stimulus-dependent degradation of IκB (Tergaonkar *et al*, 2005), suggesting that other pathways are also involved in the control of nuclear NF-κB levels.

We have previously reported that a factor named MURR1, now referred to as COMMD1, is a ubiquitously expressed inhibitor of NF-κB (Ganesh *et al*, 2003; Burstein *et al*, 2005). COMMD1 interferes with HIV-1 replication, a process that is dependent on κB-mediated transcription (Ganesh *et al*, 2003). More recently, we identified that COMMD1 is the prototype of a family of 10 factors known as COMMD proteins that also function to repress NF-κB (Burstein *et al*, 2005). The defining characteristic of all family members is the presence of an $\sim$70 amino-acid region of high homology in their extreme carboxy-termini termed the COMM domain, which serves as an interface for COMMD–COMMD protein interactions (Burstein *et al*, 2005). COMMD proteins interact with NF-κB subunits but, unlike IκB-α, do not control the translocation of NF-κB from the cytosol to the nucleus (Burstein *et al*, 2005).

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Rather, COMMD1 is recruited to κB-responsive promoters and decreases the duration of RelA–chromatin interactions (Burstein *et al*, 2005). However, the precise mechanism for this effect remained elusive.

Recent work has demonstrated that ubiquitination and proteasomal degradation of nuclear RelA also participate in the termination of NF-κB–chromatin interactions (Saccani *et al*, 2004), prompting us to investigate a potential role for COMMD1 in RelA ubiquitination. Indeed we demonstrate here that COMMD1 promotes the ubiquitination and degradation of NF-κB subunits. COMMD1 deficiency results in stabilization of NF-κB and increased nuclear accumulation of RelA following TNF stimulation. Furthermore, NF-κB activation in COMMD1-deficient cells results in de-repressed κB-dependent transcription and enhanced release of chemokines. We show that endogenous COMMD1 immune complexes contain E3 ubiquitin ligase activity, as well as components of a Cullin-containing ubiquitin ligase comprised of Elongins B and C, Cul2 and SOCS1 (also known as $\mathrm{ECS}^{\mathrm{SOCS1}}$), which has been previously implicated in RelA ubiquitination. Immunoprecipitation of COMMD1 from cells expressing Cul2 or the $\mathrm{ECS}^{\mathrm{SOCS1}}$ complex recovered ubiquitin ligase against RelA *in vitro*. Finally, our data indicate that the interaction of COMMD1 with $\mathrm{ECS}^{\mathrm{SOCS1}}$ serves to stabilize SOCS1–RelA interactions, thereby promoting binding of the substrate to the enzyme. Overall, these studies define a novel pathway by which κB-mediated transcription is regulated through COMMD1-mediated ubiquitination of NF-κB subunits.

Results

COMMD1 promotes the ubiquitination of NF-κB subunits

We have previously reported that COMMD1 inhibits κB-mediated transcription by decreasing the duration of RelA–chromatin interactions (Burstein *et al*, 2005). Recently, ubiquitination and proteasomal degradation of DNA-bound RelA was implicated in the control of RelA–chromatin interactions (Saccani *et al*, 2004). Therefore, we explored the possibility that ubiquitination of RelA might be a mechanism for the inhibitory effects of COMMD1 on κB-mediated transcription.

To investigate this, we examined the effect of COMMD1 expression on the levels of ubiquitinated NF-κB. First, ubiquitination of ectopically expressed, GST-tagged RelA was evaluated by precipitating RelA and immunoblotting the recovered material for polyubiquitin (Figure 1A). Heavily ubiquitinated RelA was recovered after proteasomal blockade only in cells coexpressing COMMD1. Utilizing a similar approach, we next examined the effect of COMMD1 expression on the ubiquitination of endogenous RelA (Figure 1B). In this case, endogenous RelA was immunoprecipitated and the recovered material immunoblotted for polyubiquitin. Again, expression of COMMD1 resulted in increased recovery of ubiquitinated RelA. To corroborate that the ubiquitinated material recovered in Figure 1A and B was RelA itself, a complementary approach was utilized. Here ubiquitinated proteins were recovered by precipitating ubiquitin (ectopically expressed as a fusion protein containing the His_6 affinity tag, His_6-Ubi) and detecting the presence of RelA in this fraction by immunoblotting. As shown in Figure 1C, precipitation of cell lysates with Ni^{+}-NTA agarose beads resulted in the recovery of polyubiquitinated proteins only when the cells were transfected to express His_6-Ubi (and not from vector-transfected cells). Slight and nonspecific binding of RelA to the beads was detected, but modified RelA consisting of material of increasing molecular weight was only recovered from cells expressing His_6-Ubi, indicating that this material is ubiquitinated RelA (Figure 1C, upper panel). As in Figure 1A and B, increased expression of COMMD1 accelerated RelA ubiquitination (Figure 1C, left panel), whereas decreased expression of endogenous COMMD1 after RNA interference (RNAi) diminished the amount of ubiquitinated RelA (Figure 1C, right panel). Importantly, these changes in the recovery of ubiquitinated RelA were not the result of uneven expression of RelA or uneven precipitation of polyubiquitinated proteins across the samples.

COMMD1 is a member of a larger protein family, which also shares the ability to bind to and inhibit NF-κB activity; therefore, we investigated the possibility of a similar effect by other COMMD family members. Decreased expression of COMMD6, 9, and 10 also resulted in decreased recovery of ubiquitinated RelA (Supplementary Figure S1). Finally, using a similar approach, we observed that COMMD1 expression accelerated the ubiquitination of two other NF-κB subunits, RelB and p52 (Figure 1D). Altogether, these results indicate that COMMD1 promotes the ubiquitination of NF-κB.

COMMD1 affects the stability of RelA

If COMMD1-mediated ubiquitination of NF-κB subunits results in their degradation, we reasoned that COMMD1 should alter the half-life and possibly the steady-state levels of NF-κB subunits in cells. We examined this possibility in U2OS cells with stable RNAi of COMMD1, resulting in nearly undetectable COMMD1 protein expression (Figure 2A) and 94% suppression of the *COMMD1* transcript (Figure 2B). In these cells, basal levels of RelA protein were increased (Figure 2A) and were not accompanied by changes in *RELA* mRNA expression, suggesting a post-transcriptional effect of COMMD1 on RelA (Figure 2B). In addition, similar increases in the steady-state protein levels of RelB, p105, and p100 were observed (Figure 2A) in the absence of transcriptional upregulation of their respective genes (data not shown).

This suggested an effect of COMMD1 on the protein stability of RelA, and therefore the half-life of the protein was examined. After metabolic labeling with ^{35}S-labeled methionine and cysteine, cell lysates were prepared at different time points and endogenous RelA was immunoprecipitated, resolved by SDS–PAGE, and then detected by autoradiography. Consistent with the previous results, the half-life of RelA was prolonged in COMMD1-deficient cells (Figure 2C). Altogether, this indicated that COMMD1 destabilizes RelA by promoting its ubiquitination and targeting the protein for proteasomal degradation.

COMMD1 controls endogenous κB-mediated transcription and cellular responses

The predicted functional effect of the stabilization of RelA that we observed in COMMD1-deficient cells would be an increase in κB-dependent transcription. Therefore, we examined the inducible mRNA expression of NF-κB-responsive genes following TNF stimulation. Treatment with TNF resulted in time-dependent increases in mRNA expression of known NF-κB target genes such as *ICAM1*, *BIRC3* (encoding

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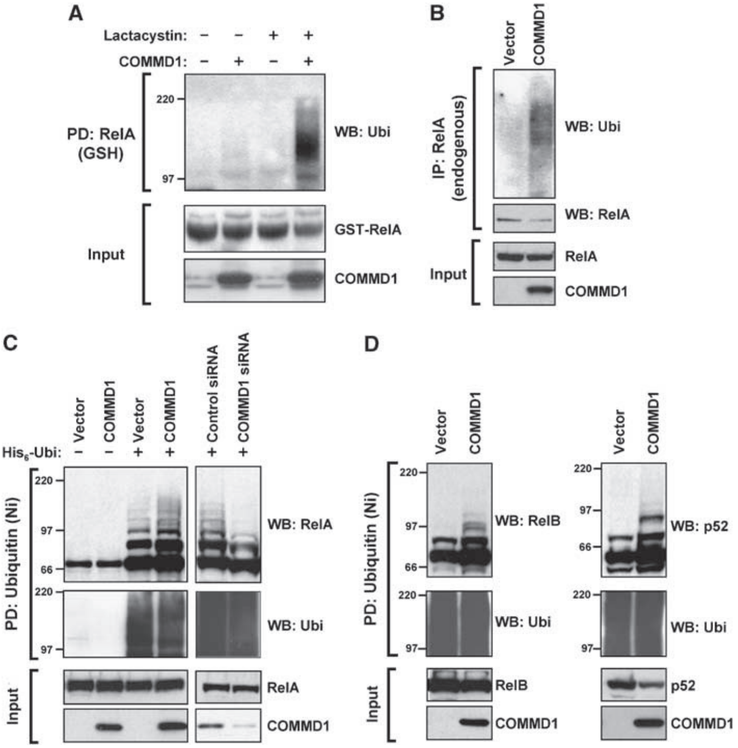

Figure 1 COMMD1 promotes the ubiquitination of NF-κB subunits. (**A**) Ubiquitination of RelA. HEK 293 cells were transfected with COMMD1 or a vector control, along with GST-RelA. Cells were treated with Lactacystin (10 μM) for 3 h before lysis with Triton X-100 buffer. RelA was precipitated (PD) using glutathione (GSH) beads and the recovered material was immunoblotted for ubiquitin (Ubi). (**B**) Ubiquitination of endogenous RelA. Cells were transfected with COMMD1 (or vector control) as in (A), and endogenous RelA was immunoprecipitated from cell lysates prepared with Triton X-100 buffer. The recovered material was immunoblotted for ubiquitin. (**C**) Effects of overexpression and underexpression of COMMD1 on RelA ubiquitination. HEK 293 cells were transfected with His_6-ubiquitin (His_6-Ubi) or a vector control, along with HA-RelA. These cells were co-transfected with a vector control or COMMD1 (left panel), or with control or COMMD1-specific siRNA (right panel). Ubiquitinated material was precipitated from cell lysates prepared with Triton X-100 buffer, using Ni-NTA agarose beads. The recovered material was immunoblotted for RelA (HA) and ubiquitin. (**D**) Effects on other NF-κB subunits. HEK 293 cells were transfected as in (C), except that HA-RelB (left panel) or HA-p52 (right panel) was used.

c-IAP2), *CXCL1*, and *CCL2* (Figure 2D). In each case, COMMD1-deficient cells demonstrated increased transcription of these genes, consistent with the notion that endogenous levels of COMMD1 serve to restrict κB-mediated transcription. Interestingly, the transcriptional effects observed varied in each case (Figure 2D), and for other NF-κB-regulated genes such as *IL8* or *TNF*, the effects were minimal (Supplementary Figure S2). These data indicate that endogenous levels of COMMD1 serve to repress transcription of a specific subset of NF-κB-inducible genes.

NF-κB-mediated transcription plays a critical role in a number of cellular events, particularly the expression of mediators of the inflammatory response *in vivo*, including the production of several chemokines. Given the increased rates of NF-κB-mediated transcription observed in COMMD1-deficient cells (Figure 2D), we anticipated that these cells might produce larger amounts of chemokines. To test this possibility, we examined the ability of conditioned media to induce chemotaxis of freshly isolated peripheral mononuclear cells across a membrane barrier. As shown in Figure 2E, conditioned media from COMMD1-deficient cells induced a higher rate of chemotaxis than the control cell line, particularly after TNF stimulation. As predicted, this increased chemotaxis rate correlated with increased secretion of NF-κB-inducible chemokines into the media such as CCL2 (Figure 2F), a chemokine whose mRNA expression was also upregulated in these cells (Figure 2D, fourth panel).

COMMD1 deficiency alters the kinetics of nuclear accumulation of RelA

The data thus far indicated that COMMD1 promotes the ubiquitination of RelA and affects κB-mediated transcription and cellular responses, such as the production of chemokines. Given our prior observations that COMMD1 negatively regulates RelA–chromatin interactions (Burstein *et al*, 2005), we reasoned that one potential outcome of COMMD1 deficiency would be decreased RelA ubiquitination leading to enhanced accumulation of nuclear RelA. To study this latter possibility, nuclear extracts of control and COMMD1-deficient cells were prepared at various time points following pulsed TNF stimulation. As shown in Figure 3A, COMMD1

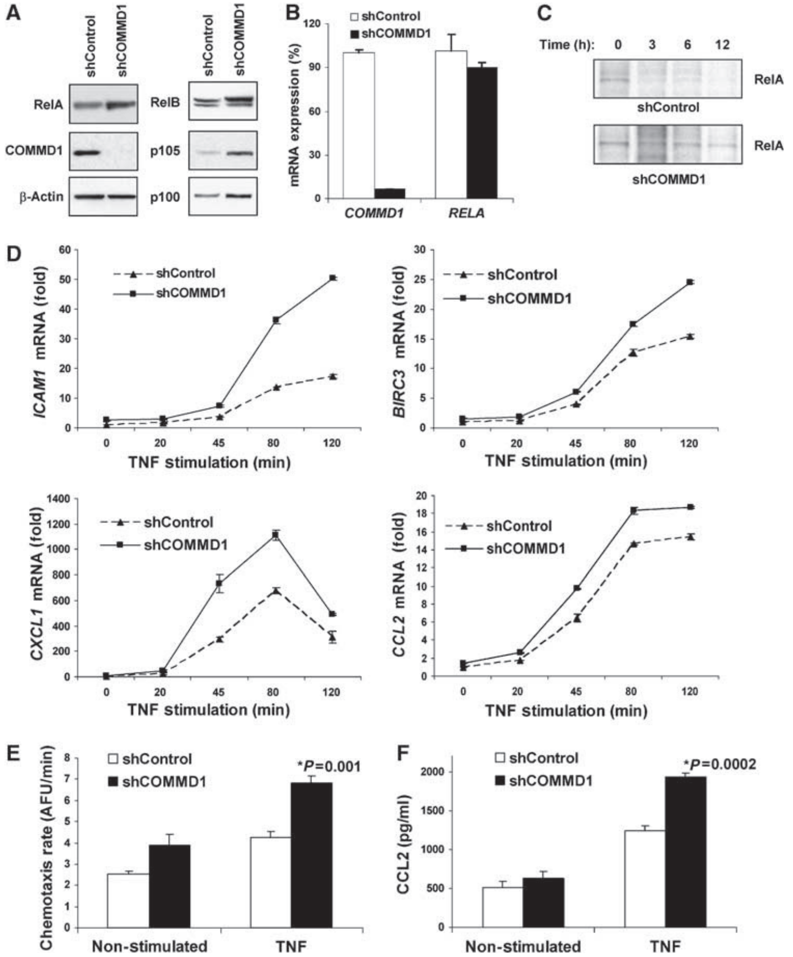

Figure 2 COMMD1 deficiency stabilizes RelA and de-represses endogenous κB-dependent transcription and cellular events. (**A**) COMMD1 deficiency results in higher basal levels of RelA. U2OS cells were stably infected with lentiviruses targeting a control gene (shControl) or COMMD1 (shCOMMD1). Once stable polyclonal cell lines were established, cell lysates were prepared with Triton X-100 buffer for immunoblotting as indicated. (**B**) COMMD1 deficiency does not affect mRNA levels of *RELA*. In the same cells, mRNA was extracted and the levels of *COMMD1* and *RELA* expression were determined by qRT–PCR and expressed as % compared to the control line. (**C**) The half-life of RelA is prolonged in COMMD1-deficient cells. Cells were metabolically labeled with ^{35}S-labeled methionine and cysteine and cell lysates were prepared at increasing time intervals as indicated. After immunoprecipitation of RelA, the recovered material was resolved by SDS–PAGE and subjected to autoradiography. (**D**) COMMD1 deficiency results in enhanced transcription of several κB-responsive genes. COMMD1-deficient and control cell lines were stimulated with a 10 min pulse of TNF, rinsed in PBS, and placed in normal media. Extraction of mRNA was performed for qRT–PCR of the indicated gene transcripts. (**E**) COMMD1-deficient cells produce greater amounts of chemotactic factors. Conditioned media from untreated or TNF-treated cells (12 h) were utilized. The ability of the media to induce chemotaxis was determined by monitoring the migration of fluorescently labeled peripheral mononuclear cells across a membrane barrier (and expressed as a rate of fluorescence change over time). (**F**) COMMD1-deficient cells secrete greater amounts of CCL2. Levels of CCL2 in the conditioned media (prepared similarly as in (E)) were determined by ELISA.

deficiency resulted in greater nuclear accumulation of RelA, and the differences observed were not accounted for by the levels of IκB-α as COMMD1-deficient cells had equal or higher levels of IκB-α at every time point (Figure 3B). Interestingly, despite the prolongation in the half-life of RelA (Figure 2C), the increased accumulation of nuclear RelA in these cells was not a sustained effect and was only noticeable during the peak times of 20 and 45 min post-stimulation. This observation correlated with a faster and higher rate of IκB-α protein resynthesis in COMMD1-deficient cells (Figure 3B), explained in part by modest increases in mRNA expression (Supplementary Figure S3). This is consistent with the known negative feedback loop by which NF-κB activates transcription of *NFKBIA* (the gene encoding IκB-α), an event known to promote nuclear export of NF-κB (Hoffmann *et al*, 2002).

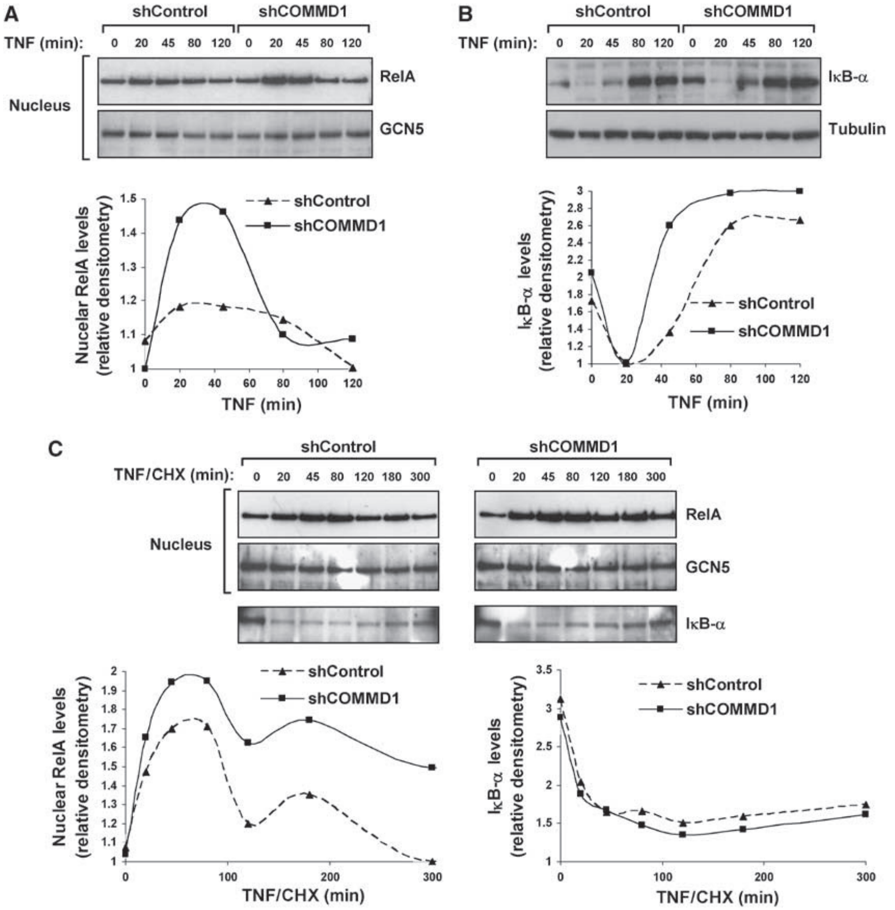

Figure 3 COMMD1 deficiency alters the nuclear accumulation of RelA. (**A**) COMMD1 deficiency results in greater accumulation of nuclear RelA after TNF treatment. U2OS COMMD1-deficient and control cell lines were stimulated with a 10 min pulse of TNF. At the indicated time point, cells were harvested and nuclear extracts were prepared for Western blot analysis of RelA in the nuclear fraction (and GCN5 as a loading control). Densitometry analysis is also presented. (**B**) Greater nuclear accumulation of RelA is not due to alterations in IκB-α degradation or resynthesis. U2OS cells were treated in an identical manner as in the experiment performed in (A) and IκB-α degradation was evaluated by immunoblotting and densitometry. (**C**) COMMD1 deficiency promotes greater nuclear accumulation of RelA independent of IκB-α levels. Similar to (A), U2OS cells were stimulated with a 10 min pulse of TNF, and medium containing CHX (30 μM) was then added. Nuclear extracts were prepared at the indicated time intervals and immunoblotted for RelA. In addition, IκB-α levels were evaluated utilizing the cytosolic fractions (bottom panel). Representative data and densitometry analysis are presented.

In order to examine the effect of COMMD1 on nuclear RelA levels in the absence of IκB-mediated nuclear export, we examined the kinetics of TNF-induced nuclear accumulation of RelA after blocking new protein synthesis with cycloheximide (CHX). The presence of this inhibitor largely abrogated IκB-α resynthesis (Figure 3C compared to B). Under these conditions, nuclear accumulation of RelA following TNF treatment was more sustained and, importantly, remained significantly elevated in COMMD1-deficient cells compared to the control cells (Figure 3C). Altogether, these data indicate that COMMD1-mediated ubiquitination of RelA affects its stability in the nuclear compartment, consistent with the observation that COMMD1 decreases RelA–chromatin interactions.

COMMD1 associates with an endogenous ubiquitin ligase

Although COMMD1 can accelerate the ubiquitination of RelA, it remained to be determined whether this was a direct effect on ubiquitination, or an indirect effect that subsequently facilitates the ubiquitination of RelA. Given that COMMD proteins do not possess any known catalytic activity, we examined first whether COMMD1 could interact with an endogenous ubiquitin ligase. To that end, COMMD1 immunoprecipitates were offered as a potential source for E3 ubiquitin ligase activity to an *in vitro* ubiquitination reaction containing recombinant ubiquitin and the E1 and E2 enzymes. At the end of the reaction, the presence of E3 ubiquitin ligase activity was determined by the formation of

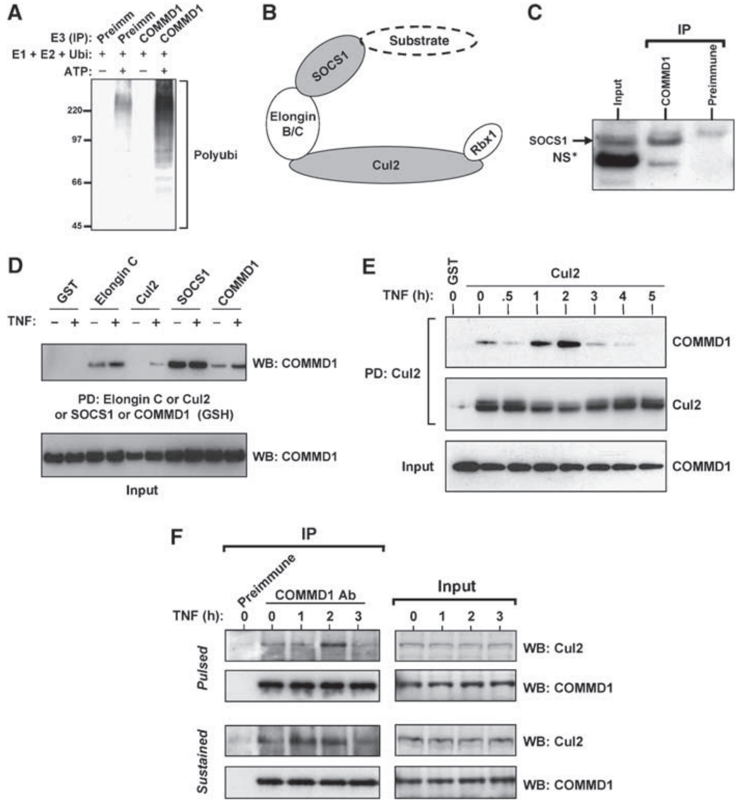

Figure 4 COMMD1 interacts with the Cullin-containing $\text{ECS}^{\text{SOCS1}}$ complex. (**A**) COMMD1 immunoprecipitates possess endogenous E3 ubiquitin ligase activity. An *in vitro* ubiquitination reaction containing recombinant E1, E2, and ubiquitin was supplemented with immunoprecipitates prepared with COMMD1 antibody or preimmune serum control. The presence of E3 activity was determined by the formation of polyubiquitin chains, as detected by immunoblotting. (**B**) Schematic representation of the $\text{ECS}^{\text{SOCS1}}$ complex. SOCS1 is the substrate binding subunit of the complex. An Elongin B and C complex serves as an adaptor linking SOCS1 to Cul2, which in turn interacts with the RING finger protein Rbx1. (**C**) Interaction between endogenous COMMD1 and SOCS1. NIH-SR cells were lysed with Triton X-100 buffer and COMMD1 antibodies or a control preimmune serum was used for immunoprecipitation. The recovered material was immunoblotted for SOCS1. (**D**) COMMD1 interacts with several components of $\text{ECS}^{\text{SOCS1}}$. HEK 293 cells were transfected with plasmids expressing Elongin C, Cul2, SOCS1, or COMMD1 in fusion with GST, along with COMMD1-Flag in each case. TNF stimulation consisted of 30 min. After lysis with RIPA buffer, ECS components (or COMMD1-GST) were precipitated with GSH beads and the recovered material was immunoblotted for COMMD1 (Flag). (**E**) COMMD1–Cul2 interactions are inducible upon TNF stimulation. Similar to the experiment shown in (D), cells were transfected to express GST-Cul2 and COMMD1-Flag. Cells were stimulated with TNF for 10 min and then placed in fresh media. The cells were lysed in RIPA buffer at the indicated time points and Cul2 was precipitated using GSH beads. The recovered material was immunoblotted for COMMD1 (Flag). (**F**) Endogenous COMMD1 and Cul2 interactions. After TNF stimulation, either as a 10 min pulse or a sustained exposure, endogenous COMMD1 was immunoprecipitated at the indicated time points or the recovered material was immunoblotted for endogenous Cul2.

polyubiquitin chains. As can be appreciated in Figure 4A, COMMD1 immunoprecipitates catalyzed the formation of polyubiquitinated material in the presence of ATP. This effect was far stronger than the contaminating activity that co-precipitated with the preimmune serum and indicated that endogenous COMMD1 interacts with a protein complex possessing E3 ubiquitin ligase activity.

COMMD1 interacts with the $\text{ECS}^{\text{SOCS1}}$ complex, a Cullin-containing ubiquitin ligase

Previous work identified that SOCS1 can induce the ubiquitination and degradation of RelA (Ryo *et al*, 2003). SOCS1 is a member of a large family of proteins containing the conserved carboxy-terminal SOCS box domain. These factors can form, through their SOCS box, part of multimeric ubiquitin ligases that contain a Cullin protein. These complexes are referred to as ECS and contain Elongins B and C, Cullin 2 or 5, and a SOCS box containing protein (Figure 4B) and are similar in structure to the better known Cul1-containing SCF complexes (Willems *et al*, 2004). An ECS complex containing SOCS1 (designated as $\text{ECS}^{\text{SOCS1}}$) is involved in the ubiquitination of various targets, including JAK2, IRS1, and IRS2 (Rui *et al*, 2002; Ungureanu *et al*, 2002).

We have previously reported that COMMD1 interacts with Cul1, a member of the Cullin family that is highly homologous to Cul2 (Ganesh *et al*, 2003). Given the finding that COMMD1 immune complexes contain E3 ubiquitin ligase activity and that $\text{ECS}^{\text{SOCS1}}$ has been implicated in the ubiquitination of RelA, we investigated the possibility that COMMD1 could interact with this complex. Immunoprecipitation of endogenous COMMD1 resulted in the co-precipitation of endogenous SOCS1, consistent with an interaction between these two proteins (Figure 4C). Next, various components of the ECS^{SOCS1} complex were expressed in Human embryonic kidney (HEK) 293 cells, precipitated from cell lysates, and the recovered material was immunoblotted for COMMD1. As shown in Figure 4D, Elongin C, Cul2, and SOCS1 (but not the control GST protein) co-precipitated with COMMD1. In addition, the interactions of COMMD1 with Elongin C and Cul2 (but not SOCS1) were enhanced by TNF stimulation. Interestingly, the ability of COMMD1 to interact with itself, an event mediated by the conserved COMM domain (Burstein *et al*, 2005), was also enhanced by TNF stimulation (Figure 4D). In reciprocal experiments, precipitation of COMMD1 resulted in the recovery of components of the ECS complex (including Elongin C, Cul2, SOCS1, and Rbx1) and these interactions were mediated by the COMM domain of COMMD1 (Supplementary Figure S4).

Given the TNF-inducible nature of COMMD1–Cul2 binding, we investigated next the temporal profile of this interaction. Cells expressing Cul2 were stimulated with a pulse of TNF and lysates were prepared at different time points following stimulation. Cul2 was immunoprecipitated and the recovered material was immunoblotted for COMMD1. As before, TNF treatment induced greater recovery of COMMD1 over time, with the peak of the effect taking place 2 h after stimulation (Figure 4E). Similar experiments were performed by immunoprecipitating endogenous COMMD1 and detecting the co-precipitation of endogenous Cul2 after TNF stimulation (Figure 4F). As before, TNF augmented the interaction between COMMD1 and Cul2, and this effect peaked at 2 h, irrespective of whether the stimulation was provided as a pulse or sustained exposure to TNF. Altogether, these data demonstrated a physical interaction between COMMD1 and the ECS^{SOCS1} complex, which is inducible upon activation of the NF-κB pathway, and suggested the possibility that this might be responsible for COMMD1-directed RelA ubiquitination.

COMMD1-directed ubiquitination is mediated through ECS^{SOCS1}

Given the interaction of COMMD1 with the ECS^{SOCS1} complex, previously implicated in RelA ubiquitination, we investigated if COMMD1 could be operating through this ligase. To that end, we examined the ability of COMMD1 to induce RelA ubiquitination after RNAi-mediated suppression of either Cul2 or SOCS1. COMMD1 expression induced the accumulation of ubiquitinated RelA in control cells, whereas this event was prevented in cells deficient in either Cul2 or SOCS1 (Figure 5A). This experiment was consistent with the notion that expression of the endogenous ECS^{SOCS1} complex is required for the effects of COMMD1. Next, we examined the effects of ECS^{SOCS1} expression on the rates of RelA ubiquitination. Expression of Cul2 and SOCS1 increased the recovery of ubiquitinated RelA and this effect was enhanced by concurrent expression of COMMD1, particularly in Cul2-expressing cells (Figure 5B), and to a lesser extent after proteasomal blockade in SOCS1-expressing cells (data not shown).

The effect that the expression levels of ECS^{SOCS1} had on the recovery of ubiquitinated RelA from cells supported the notion that COMMD1-directed ubiquitination is mediated through the ECS^{SOCS1} complex. Therefore, we set out to test this possibility in an *in vitro* ubiquitination system. COMMD1 immunoprecipitates were provided as an E3 ubiquitin ligase to a reaction containing recombinant ubiquitin, the E1 and E2 enzymes, and purified GST-RelA as ubiquitination substrate for the reaction. We prepared COMMD1 immunoprecipitates from cells transfected with Cul1, Cul2, Cul5, or the corresponding expression vector as a control. Despite the ability of COMMD1 to interact with all three Cullins (data not shown), only COMMD1 immune complexes recovered from cells expressing Cul2 were capable of catalyzing detectable polyubiquitination of GST-RelA *in vitro* (Figure 5C). In a similar approach, COMMD1-containing complexes were recovered from cells coexpressing several ECS^{SOCS1} subunits (Elongin C, Cul2, SOCS1, and Rbx1) or transfected with the corresponding expression vector as a control. Again, the immunoprecipitation of COMMD1 from ECS^{SOCS1}-expressing cells was able to recover an activity that ubiquitinates RelA *in vitro* (Figure 5D).

COMMD1 and ECS^{SOCS1} cooperate to inhibit RelA by promoting its degradation

Our data thus far indicated that the interaction of COMMD1 with the ECS^{SOCS1} complex is required for COMMD1-mediated ubiquitination of RelA in cells and *in vitro*. Therefore, we reasoned that these factors should have a synergistic negative impact on NF-κB-mediated transcription. We examined this possibility by determining the effects of COMMD1 and ECS^{SOCS1} components on RelA-mediated *ICAM1* expression (Figure 5E). Cells were transfected with RelA and, as expected, this induced strong expression of the endogenous *ICAM1* gene (Figure 5E, lane 3). Under the conditions of this experiment, expression of COMMD1, Elongin C, or Cul2 by themselves did not have a substantial inhibitory effect on RelA-mediated transcription of *ICAM1* (Figure 5E, lanes 4, 5, and 7). However, when combined with COMMD1, both Elongin C and Cul2 had significant inhibitory activity (Figure 5E, lanes 6 and 8). Similarly, SOCS1-mediated inhibition of *ICAM1* expression was further enhanced by COMMD1 (Figure 5E, lanes 9 and 10).

The inhibition of *ICAM1* expression correlated directly with the ability of COMMD1 and ECS^{SOCS1} components to induce reductions in RelA protein levels (Figure 5E, second and third panels). The decreases in protein levels observed were not due to changes in mRNA levels of RelA, and did not occur with a deletion mutant of SOCS1 that cannot interact with the catalytic components of the ubiquitin ligase (data not shown), consistent with a post-transcriptional effect that requires the activity of the ubiquitin ligase. Altogether, the synergistic effects of COMMD1 and ECS^{SOCS1} on transcription and RelA degradation indicate that these factors cooperatively repress NF-κB.

COMMD1 serves as an adaptor between SOCS1 and Cul2

Next, we sought to understand the role of COMMD1 in the activity of the ECS^{SOCS1} ubiquitin ligase. We began by examining to which component(s) of the complex COMMD1 binds to directly. To this end, we tested the ability of COMMD1 to

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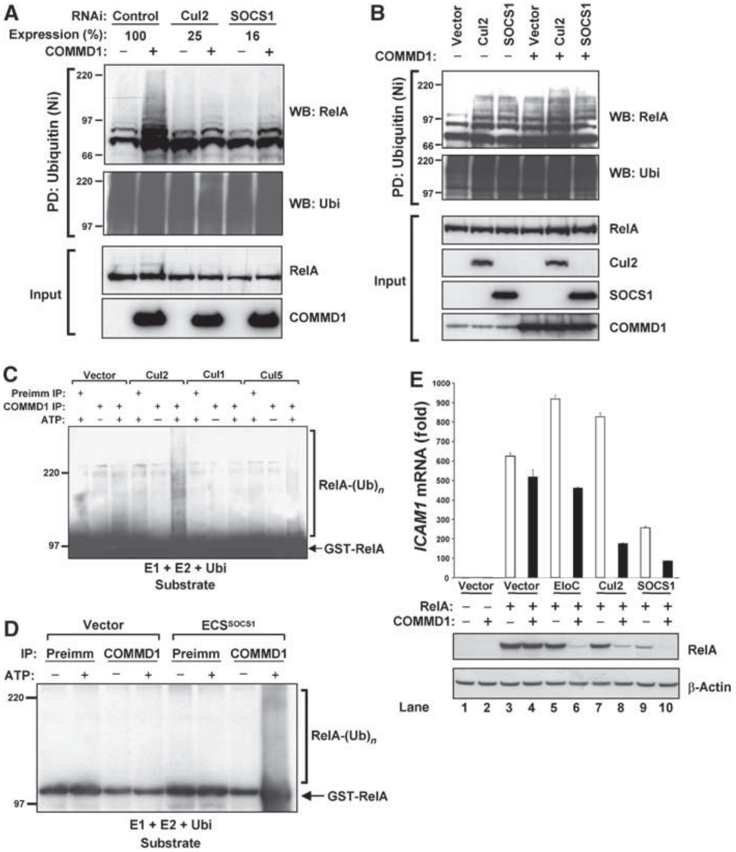

Figure 5 The ECS^{SOCS1} complex is required for COMMD1-mediated ubiquitination of RelA. (**A**) COMMD1-mediated ubiquitination of RelA depends on the endogenous ECS^{SOCS1} complex. HEK 293 cells were transfected with His$_6$-Ubi and RelA as before (Figure 1C), as well as siRNA against Cul2, SOCS1, or a control target. Two days later, mRNA expression levels of Cul2 and SOCS1 were determined by qRT–PCR. In addition, cell lysates were prepared with an 8 M urea buffer and ubiquitinated proteins were precipitated with Ni-NTA beads. The recovered material was immunoblotted for RelA (HA) and ubiquitin. (**B**) COMMD1 and ECS components have additive effects on RelA ubiquitination. As before, cells were transfected with His$_6$-Ubi and RelA, along with SOCS1, Cul2, and COMMD1, as indicated. Lysates prepared with an 8 M urea buffer were utilized for ubiquitin precipitation; the recovered material was immunoblotted for RelA and ubiquitin. (**C**, **D**) *In vitro* ubiquitination of RelA by COMMD1 immunoprecipitates. An *in vitro* ubiquitination reaction containing recombinant E1, E2, ubiquitin, and GST-RelA as substrates was provided with immunoprecipitates prepared with COMMD1 antibody or preimmune serum as a source for E3 activity. In (C), the immunoprecipitates were prepared from cells expressing Cul1, Cul2, Cul5, or a vector control. In (D), the immunoprecipitates were prepared from cells expressing ECS^{SOCS1} subunits (Elongin C, Cul2, SOCS1, and Rbx1) or a vector control. (**E**) COMMD1 and ECS^{SOCS1} cooperatively inhibit RelA-mediated *ICAM1* expression and promote RelA degradation. HEK 293 cells were transfected with RelA, along with Elongin C (EloC), Cul2, SOCS1, or COMMD1 as indicated. Two days later, *ICAM1* mRNA abundance was determined by qRT–PCR and expressed as fold over the vector control sample. In addition, RelA (HA) and β-actin protein levels were concurrently determined by Western blot.

bind to deletion mutants of Cul2 and SOCS1 that cannot interact with the rest of the ECS^{SOCS1} complex (schematic representation of domains involved is shown in Figure 6E). As expected, an amino-terminal deletion of Cul2 (Cul2 ΔN) could not bind to Elongin C; however, COMMD1 binding to this mutant was unaffected (Figure 6A, lanes 2 and 3). This suggested that the carboxy-terminal end of Cul2 is involved in COMMD1 binding and indeed, a carboxy-terminal deletion of Cul2 (Cul2 ΔC) abrogated binding to COMMD1 (Figure 6A, lane 4). Similar studies performed with SOCS1 indicated that a deletion of its carboxy-terminal SOCS box (SOCS1 ΔSB) did not affect COMMD1 binding although as expected it prevented binding to Elongin C (Figure 6A, lanes 5 and 6). In fact, the SH2 domain of SOCS1 was necessary and sufficient for COMMD1 binding (Figure 6A, lanes 7 and 8), even though this region alone is incapable of binding to the ECS complex. Altogether, these studies indicated that COMMD1 can bind independently with two components of the ECS^{SOCS1}, namely Cul2 and SOCS1, resembling the binding characteristics of the adaptor Elongin B/C complex (Figures 4B and 6E).

COMMD1 facilitates the binding of SOCS1 to RelA

SOCS1 is the component of the ECS^{SOCS1} complex that binds to its substrates, including RelA (Ryo *et al*, 2003). Interestingly, COMMD1 associates with both SOCS1 and RelA, and accelerates the ubiquitination of RelA, prompting

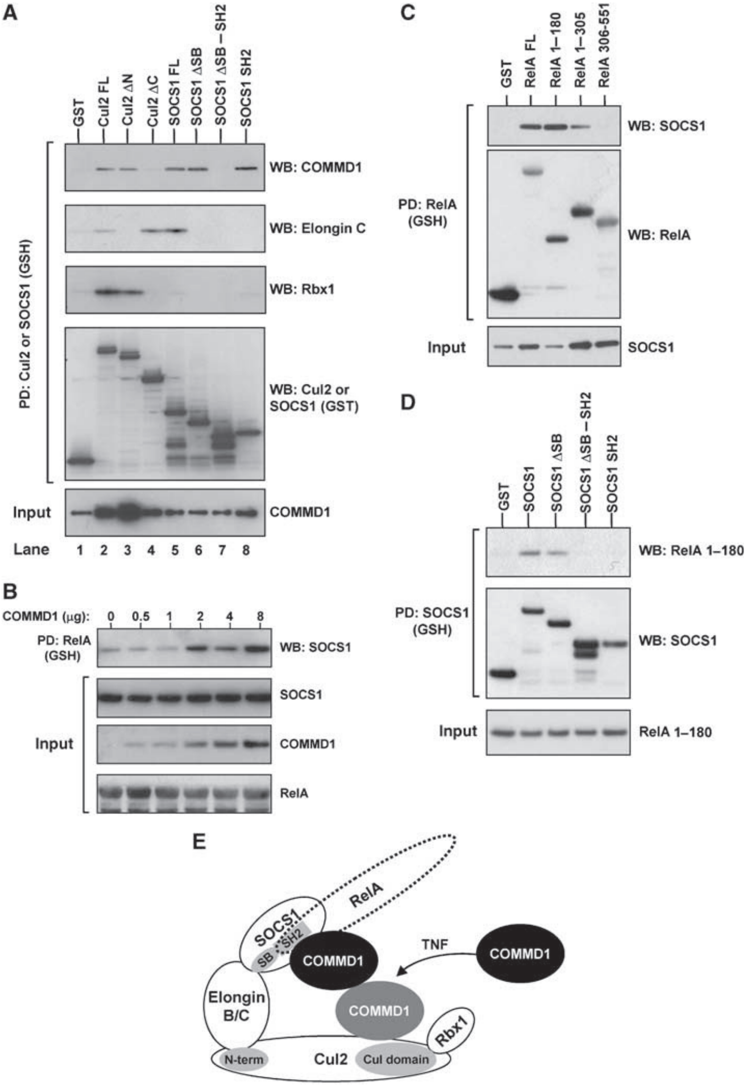

Figure 6 COMMD1 interacts with both Cul2 and SOCS1 and facilitates SOCS1–RelA binding. (**A**) COMMD1 interacts independently with both SOCS1 and Cul2. Full-length and specific deletion mutants of Cul2 and SOCS1 were expressed in fusion with GST, along with COMMD1-Flag. Cul2 and SOCS1 were precipitated from cell lysates (RIPA buffer) and the recovered material was immunoblotted as indicated. (**B**) COMMD1 facilitates SOCS1–RelA interactions. HEK 293 cells were transfected with GST-RelA, SOCS1, and increasing amounts of COMMD1 as indicated. Two days after transfection, the cells were lysed in RIPA buffer. RelA was precipitated from the lysate and the recovered material was immunoblotted for SOCS1 (Flag). (**C**) SOCS1 binds to the amino-terminus of the RHD of RelA. Cells were transfected as in (B), this time utilizing GST-RelA full-length and deletion constructs encompassing the amino acids indicated in the figure. Two days after transfection, cell lysates were prepared with RIPA buffer and RelA was precipitated. The recovered material was immunoblotted for SOCS1 (HA) or RelA (GST). (**D**) RelA binds to the entire amino-terminus of SOCS1. HEK 293 cells were transfected to express GST-SOCS1 or the deletion mutants indicated, as well as RelA 1–180. Two days after transfection, the cells were lysed in RIPA buffer. SOCS1 was precipitated from the lysate and the recovered material was immunoblotted for RelA 1–180 (HA) or SOCS1 (GST). (**E**) Schematic representation of COMMD1 interactions with the ECS^{SOCS1} complex. COMMD1 interacts independently with both SOCS1, at the level of its SH2 domain, and Cul2, at the level of its conserved carboxy-terminus. The interaction between COMMD1 and Cul2 is inducible upon TNF stimulation, and COMMD1 in turn facilitates the binding of RelA to SOCS1.

us to investigate if COMMD1 facilitates SOCS1–RelA binding. To this end, we examined the ability of SOCS1 to co-precipitate with RelA under increasing expression of COMMD1. As shown in Figure 6B, precipitation of RelA resulted in co-precipitation of SOCS1, which was enhanced by increased expression of COMMD1 in a dose-dependent manner.

The ability of COMMD1 to interact with both SOCS1 and RelA and the cooperative effect of COMMD1 on the

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SOCS1–RelA interaction suggested the possibility of a stabilizing protein–protein interaction. Interestingly, SOCS1 co-precipitated with the first 180 amino acids of RelA (Figure 6C), the same amino-terminal motif of the RHD that is also involved in COMMD1 binding (Burstein *et al*, 2005). Similar to the ability of COMMD1 to enhance SOCS1 binding to full-length RelA, expression of COMMD1 also augmented the binding of SOCS1 to this region of RelA (Supplementary Figure S5).

This raised the possibility that SOCS1 binding to RelA might be indirect and mediated through COMMD1. However, unlike SOCS1 binding to COMMD1 that only required the SH2 domain (Figure 6A), the entire amino-terminal end of SOCS1 was required for SOCS1–RelA interactions (Figure 6D). These data indicate that COMMD1 does not mediate but rather enhances binding of SOCS1 with RelA, altogether suggesting that COMMD1 stabilizes the SOCS1–RelA interaction by cooperatively binding to the same amino-terminal motif in RelA (Figure 6E).

Discussion

In this study, we examined the potential mechanism responsible for COMMD1-mediated repression of NF-κB and its ability to accelerate the dissociation of RelA from chromatin (Burstein *et al*, 2005). Interestingly, others had reported that ubiquitination of RelA also accelerates its release from chromatin (Saccani *et al*, 2004), suggesting that ubiquitination of RelA might be responsible for the effects of COMMD1. Indeed, we present evidence that COMMD1 promotes the ubiquitination of NF-κB subunits and our data also indicate that this effect is mediated by the interaction of COMMD1 with ECS^{SOCS1}, a Cullin-containing ubiquitin ligase complex previously implicated in RelA ubiquitination (Ryo *et al*, 2003). These events are physiologically important because COMMD1 deficiency results in de-repression of several endogenous NF-κB-responsive genes and enhanced κB-mediated cellular responses such as the production of chemokines. Finally, our studies indicated that the role of COMMD1 in this process is to induce greater binding of the substrate, RelA, to the SOCS1 subunit, ultimately promoting its ubiquitination.

SOCS1 has been previously implicated in the control of RelA levels and the data presented here indicate that SOCS1 functions in concert with the ECS complex. More importantly, we present data indicating that the ability of COMMD1 to ubiquitinate RelA is mediated through ECS^{SOCS1}. COMMD1 interacts with components of the ECS complex, as shown at the level of endogenous SOCS1 and Cul2 (Figure 4C and F) and overexpressed ECS components (Figures 4D, E, and 6A and Supplementary Figure S4). Similarly, COMMD1-mediated ubiquitination of RelA in cells is hampered by ECS deficiency (Figure 5A) and accentuated by overexpression of ECS components (Figure 5B). Finally, COMMD1 immune complexes contain ubiquitin ligase activity and can mediate the ubiquitination of RelA *in vitro* when recovered from cells expressing Cul2 and not other Cullins, or from cells expressing ECS components (Figure 5C and D).

SOCS1 expression is under the control of the JAK/STAT pathway, which stimulates transcription of the *SOCS1* gene (Naka *et al*, 1997) and promotes the stability of the SOCS1 protein through Pim-mediated phosphorylation (Chen *et al*, 2002). These effects predict that STAT activation should result in negative crosstalk with the NF-κB pathway. In this regard, NF-κB activation results in the expression of various inducers of the STAT pathway (such as IL-6) that can upregulate SOCS1 expression (Zhang *et al*, 1994), possibly providing an autocrine negative feedback loop. This mechanism could be important during inflammatory responses *in vivo*, when multiple cytokines are coordinately activated. In addition, our studies demonstrate that the SOCS1–COMMD1 interaction is mediated by the SH2 domain of SOCS1 (Figure 5A), a region that preferentially interacts with tyrosine phosphorylated targets (Ungureanu *et al*, 2002). This suggests the possibility that activation of tyrosine kinase(s) that might target COMMD1 could enhance this interaction and provide additional crosstalk regulation of the NF-κB pathway.

Our previously reported data suggest that COMMD1-mediated inhibition of NF-κB occurs in the nucleus, a compartment where the protein can be readily identified (Burstein *et al*, 2005). Although it is largely accepted that the cytoplasmic pool of SOCS1 is responsible for its ability to regulate JAK2 kinase activity (Endo *et al*, 1997), the presence of a nuclear pool of SOCS1 is also well documented (Vuong *et al*, 2004). Indeed, our analysis indicates that the main components of the ECS^{SOCS1} complex, including SOCS1, Elongin C, Cul2, and Rbx1, can be found in nuclear extracts (Supplementary Figure S6).

Another important observation in our studies is that there is dissociation between the duration of transcriptional responses and nuclear RelA accumulation. The mRNA levels for all κB-responsive genes evaluated here continued to accumulate at time points when nuclear levels of RelA were back to baseline (after 45 min; see Figures 2D and 3A). This suggests that after RelA has been effectively exported into the cytosol, other mechanisms are likely involved in the late termination of transcription. In fact, the inducible COMMD1–Cul2 interaction peaks at 2 h (Figure 4E and F), a time point when nuclear export of RelA has been completed, suggesting that the physiologic role of NF-κB ubiquitination is in late transcriptional termination.

We have previously reported that COMMD1 binds to Cul1 (Ganesh *et al*, 2003), and more detailed examination indicates that COMMD1 can bind to additional Cullins (GN Maine and E Burstein, unpublished observations). This suggests the possibility that COMMD1 might be a component of various ubiquitin ligases targeting proteins other than NF-κB subunits and might ultimately explain functions of COMMD1, such as its role in copper metabolism, that appear unrelated to its effects on NF-κB (van de Sluis *et al*, 2002; Burstein *et al*, 2004).

Altogether, this study demonstrates that COMMD1, acting via a Cullin-containing ubiquitin ligase, represses κB-mediated transcription through ubiquitination of NF-κB subunits. COMMD1-mediated regulation of NF-κB through ECS^{SOCS1} is part of a larger paradigm of transcriptional regulation. Indeed, various Cullin-containing complexes have been shown to play critical roles in the regulation of other transcription factors such as c-Myc (von der Lehr *et al*, 2003) and HIF1α (Pause *et al*, 1997). Our data uncover that in addition to the well-known events that control IκB protein stability, regulation of the stability of the NF-κB subunits by this COMMD1-containing ubiquitin ligase is a critical event in the termination of κB-mediated transcription.

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Materials and methods

Plasmids and small interfering RNA

Expression vectors for RelB and p52 were generated by PCR amplification of the corresponding coding sequences from plasmid templates kindly provided by Dr Ranjeny Thomas and Dr Colin Duckett, respectively. Expression vectors for Elongin C, Cul2, SOCS1, and Rbx1 were generated by PCR amplification using as templates the following IMAGE cDNA clones: 3923736, 4104375, 5179306/5093311, and 3138751, respectively. Deletion constructs for Cul2 and SOCS1 were similarly generated with the amino-acid boundaries of the encoded mutant proteins being Cul2 $\Delta N = 109$–745, Cul2 $\Delta C = 1$–415, SOCS1 $\Delta SB = 1$–157, SOCS1 ΔSB-$SH2 = 1$–78, SOCS1 $SH2 = 78$–174. Information about the sequences of small interfering RNA (siRNA) oligonucleotides utilized (chloramphenicol acetyl transferase, COMMD1, SOCS1, and Cul2, obtained from Invitrogen) are available upon request. For stable RNAi utilizing lentiviral delivery we introduced a cassette containing the histone 1 promoter and a short hairpin RNA into the FG12 plasmid (kindly provided by Dr David Baltimore). All other plasmids used have been described previously (Burstein *et al*, 2004, 2005).

Cell culture, transfection, and lentiviral production

HEK 293 cells, HEK 293T cells, U2OS cells, and NIH-SR cells were obtained from ATCC. HEK 293, HEK 293T, and U2OS cells were cultured in DMEM supplemented with 10% FBS and L-glutamine (2 mM). NIH-SR cells were cultured in RPMI supplemented with 10% FBS, L-glutamine (2 mM), sodium pyruvate (1 mM), and glucose (4.5 g/l). A standard calcium phosphate transfection protocol was used to transfect plasmids and siRNA into HEK 293 cells (Burstein *et al*, 2004). TNF treatments (1000 U/ml; Roche) were for variable amounts of time, as indicated in each experiment. Production of lentiviruses for stable delivery of short hairpin RNA constructs was performed as previously described utilizing plasmids kindly provided by Dr David Baltimore (Lois *et al*, 2002).

Quantitative RT–PCR

RNA extraction and RT–PCR methods were performed as previously described (Burstein *et al*, 2005). Oligonucleotides and probes for *RELA*, *ICAM1*, *BIRC3*, *CXCL1*, *CCL2*, *TNF*, and *IL8* transcripts were obtained from Applied Biosystems.

Immunoblotting and immunoprecipitation

The compositions of Triton lysis buffer and RIPA buffer have been previously described (Burstein *et al*, 2005). In some experiments, an 8 M urea buffer (8 M urea, 50 mM Tris, pH 8.0, 300 mM NaCl, 50 mM $NaPO_4$, 0.5% NP-40) was used. All buffers were supplemented with 1 mM sodium orthovanadate and protease inhibitors (Roche). Preparation of cytosolic and nuclear extracts, immunoprecipitations, GSH precipitations, Ni-NTA precipitations, and immunoblotting were performed as described previously (Burstein *et al*, 2004, 2005). The following antibodies were used: COMMD1 (Burstein *et al*, 2005; Abnova), Flag (Sigma), HA (Sigma), ubiquitin (Stressgen), RelA (Santa Cruz Biotechnology), RelB (Santa Cruz Biotechnology), p52 (Upstate Biotechnology), Cul2 (Zymed, Upstate Biotechnology, and Abnova), Elongin C (BioLegend), SOCS1 (Zymed, Abcam, Santa Cruz Biotechnology), Rbx1 (LabVision), IκB-α (Upstate Biotechnology), GST (Santa Cruz), β-actin (Sigma), α-tubulin (Molecular Probes), and GCN5 (Santa Cruz).

Metabolic labeling

One day prior, 8×10^5 cells were seeded in a 10cm dish. They were placed in a cysteine- and methionine-deficient medium (with 10% dialyzed FBS) for 30 min, and 500 μCi of ^{35}S-labeled methionine and cysteine (GE Healthcare) was added per plate for 1 h. The media were then replaced with regular growth media supplemented with excess non-radiolabeled methionine (2 mM) and cysteine (2 mM). Denatured lysates were prepared by applying 50 mM Tris, 1% SDS, 5 mM DTT, and boiling the samples for 10 min at 95°C. The buffer was then supplemented with Triton X-100 lysis buffer to decrease the SDS concentration to 0.2%. Samples were pre-cleared with protein G Sepharose beads for 1 h at 4°C and the supernatant obtained after centrifugation was subjected to RelA immunoprecipitation (Santa Cruz Biotechnology, sc-372). The recovered material at the end of immunoprecipitation was resolved by SDS–PAGE and RelA was detected by autoradiography.

In vitro chemotaxis assay and chemokine measurement

Conditioned media before and after 12-h TNF stimulation were used. Peripheral mononuclear cells were freshly isolated after dextran sedimentation and Ficoll gradient centrifugation, as previously described (Diamond *et al*, 1991). The cells were then fluorescently labeled with Calcein-AM (Sigma) and chemotactic migration across a membrane and toward conditioned media was determined utilizing a 96-well FluoroBlock plate pre-coated with fibronectin, according to the manufacturer's instructions (BD Biosciences). The plate was placed at 37°C on a fluorometer (ABI Cytofluor 4000) and cell migration was monitored every 15 min for a total of 2 h. CCL2 levels in conditioned media were determined by ELISA according to the manufacturer's instructions (R&D systems).

In vitro ubiquitination reaction

Each reaction mixture consisted of recombinant ubiquitin (2.5 μg/reaction), E1 (Uba1, 50 ng/reaction), E2 (UbcH5a, 100 ng/reaction), and ATP regenerating buffer (Boston Biochem), resuspended in reaction buffer (40 mM HEPES pH 7.9, 60 mM potassium acetate, 2 mM DTT, 5 mM $MgCl_2$, 10% glycerol). The material obtained from immunoprecipitation of COMMD1 (or control) was added as a source for E3 ubiquitin ligase activity. Recombinant GST-RelA was used as the substrate in these reactions and was prepared by expression in 293 cells and affinity purification using standard protocols.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

Acknowledgements

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Supplementary Figures

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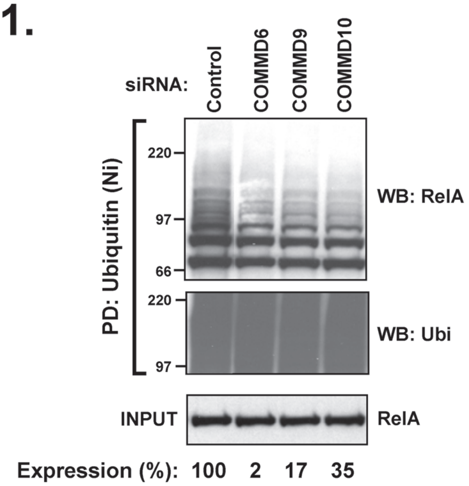
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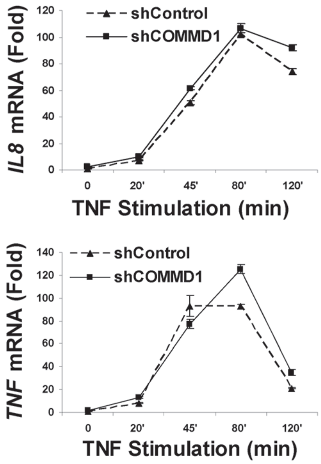

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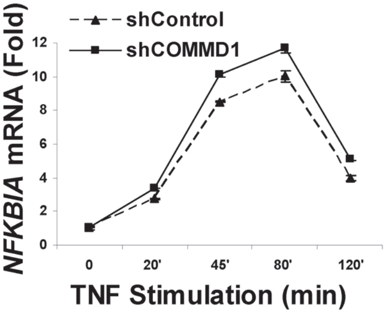

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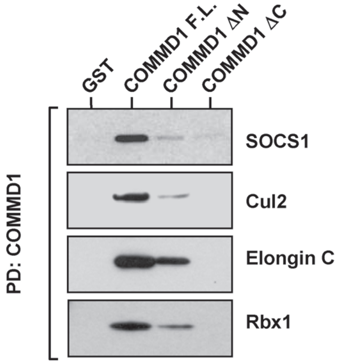
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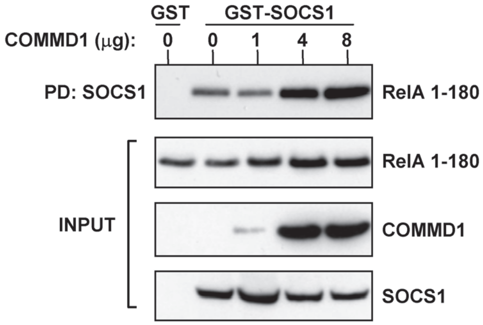
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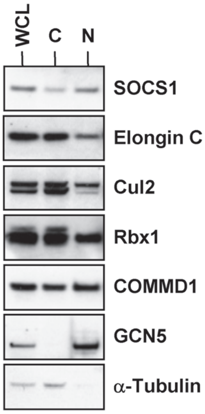
SOCS1

6.


WCL
C
N
SOCS1
Elongin C
Cul2
Rbx1
COMMD1
GCN5
α-Tubulin

SUPPLEMETARY INFORMATION

Figure S1: Underexpression of other COMMDs decreases the ubiquitination of RelA. HEK 293 cells were transfected with HA-RelA and His_6-Ubi, along with siRNA directed against COMMD6, 9, 10 or a control. Ubiquitinated material was precipitated from cell lysates prepared with 8M urea buffer using Ni-NTA agarose beads. The recovered material was immunoblotted for RelA (HA) and ubiquitin. The level of COMMD expression was determined by qRT-PCR and is indicated as % relative to the control (bottom).

Figure S2: COMMD1 deficiency does not potentiate transcription of all κB-responsive genes. COMMD1-deficient and control cell lines were stimulated with TNF for 10 minutes, rinsed in PBS and placed in normal growth media. Following the beginning of the TNF stimulation pulse, mRNA was harvested at the indicated time points and used for quantitative RT-PCR of the *IL8* and *TNF* genes.

Figure S3: COMMD1 deficiency results in a modest up-regulation of *NFKBIA* gene expression. This experiment was performed as described in Figure S2, but in this case mRNA was harvested for qRT-PCR of the *NFKBIA* gene.

Figure S4: COMMD1 association with ECS^{SOCS1} is mediated by the conserved carboxy-terminal COMM domain. HEK 293 cells were co-transfected with vectors encoding an ECS^{SOCS1} subunit (SOCS1, Cul2, Elongin C or Rbx1), and COMMD1 in fusion with GST (either full-length or a truncated form as indicated,). Two days after transfection, the cells were lysed in

RIPA buffer and COMMD1 precipitated using GSH beads. The recovered material was immunoblotted for SOCS1, Cul2, Elongin C, or Rbx1 (Flag).

Figure S5: **COMMD1 stabilizes the SOCS1-RelA interaction.** HEK 293 cells were transfected with GST-SOCS1, RelA 1-180 (the binding domain within RelA), and increasing amounts of COMMD1 as indicated. Two days after transfection, the cells were lysed in RIPA buffer and SOCS1 precipitated using GSH beads. The recovered material was immunoblotted for RelA 1-180 (HA) and the input material was immunoblotted for RelA 1-180, COMMD1 and SOCS1.

Figure S6: **ECS^{SOCS1} can be found in the nucleus.** NIH-SR cells were utilized to prepare whole cell lysates (WCL) in RIPA buffer, or cytosolic (C) and nuclear (N) extracts. Equal amount of protein was loaded into each lane and western blot analysis was performed for the presence of SOCS1, Elongin C, Cul2, Rbx1 or COMMD1. GCN5 and α-Tubulin were utilized as nuclear and cytosolic markers, respectively.

Chapter 5

COMMD1 (Copper Metabolism MURR1 Domain-Containing Protein 1) regulates Cullin RING ligases by preventing CAND1 (Cullin-associated Nedd8-dissociated Protein 1) binding

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Burstein E.
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COMMD1 (Copper Metabolism MURR1 Domain-containing Protein 1) Regulates Cullin RING Ligases by Preventing CAND1 (Cullin-associated Nedd8-dissociated Protein 1) Binding[*][S]

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Cullin RING ligases (CRLs), the most prolific class of ubiquitin ligase enzymes, are multimeric complexes that regulate a wide range of cellular processes. CRL activity is regulated by CAND1 (Cullin-associated Nedd8-dissociated protein 1), an inhibitor that promotes the dissociation of substrate receptor components from the CRL. We demonstrate here that COMMD1 (copper metabolism MURR1 domain-containing 1), a factor previously found to promote ubiquitination of various substrates, regulates CRL activation by antagonizing CAND1 binding. We show that COMMD1 interacts with multiple Cullins, that the COMMD1-Cul2 complex cannot bind CAND1, and that, conversely, COMMD1 can actively displace CAND1 from CRLs. These findings highlight a novel mechanism of CRL activation and suggest that CRL regulation may underlie the pleiotropic activities of COMMD1.

Ubiquitin, a highly conserved 76-amino acid polypeptide, can be conjugated to substrate proteins through an enzymatic machinery present in all eukaryotic cells (1). Ubiquitination can lead to a variety of outcomes, but a particularly notable event is the degradation of the target protein by the proteasome. Ubiquitination of the target protein involves a multistep enzymatic process that requires a ubiquitin ligase, also referred to as an E3 enzyme (2). It is estimated that there are ~500–1,000 ubiquitin ligases (3–5), and among them, a particularly prolific group are the Cullin RING ligases (CRLs)[3] (6). The core CRL complex contains a Cullin protein (such as Cul1, 2, 3, 4A, 4B, or 5 in mammals), and a RING box protein (Rbx1 or Rbx2). In addition, a variety of substrate binding subunits specific to each Cullin contributes to a large repertoire of complexes, estimated at ~300 distinct ligases (7–9). These complexes are designated by the Cullin and the specific substrate subunit that they contain. For example, the well known ligase complex that targets IκB-α, which contains Cul1 and the substrate receptor protein βTrCP, is designated as CRL1-βTrCP.

Structurally, the carboxyl-terminal globular domain of the Cullin protein binds to Rbx1 or Rbx2, whereas the amino-terminal region is a rigid stalk formed by three unique five–helix bundle structures called Cullin repeats 1, 2, and 3 (10). The amino-terminal Cullin repeat 1 is responsible for recruiting the substrate receptor complex (SRC). Displacement of this complex is a major mechanism of inhibition of these ligases and is mediated by CAND1 (Cullin-associated Nedd8-dissociated), a large protein that interacts with all three Cullin repeats and the carboxyl-terminal domain (11–13). Formation of the CRL-CAND1 complex is in turn regulated by the ubiquitin-like protein Nedd8, which upon conjugation to the carboxyl-terminal domain prevents CAND1 binding to the Cullin protein (11, 12). On the other hand, the assembled Cul1-CAND1 complex cannot be effectively neddylated because of steric occlusion of the lysine acceptor site by CAND1. Therefore, the mechanism of dissociation of the Cul1-CAND1 complex remains poorly understood, and the existence of cellular factors that control CRL-CAND1 interactions has been proposed (13).

COMMD (copper metabolism MURR1 domain-containing) proteins are pleiotropic factors present in a wide range of eukaryotic organisms (14) and are defined by the presence of the carboxyl-terminal COMM domain (15, 16). The best studied of these factors is COMMD1, which participates in copper metabolism (17), NF-κB-mediated transcription (18), adaptation to hypoxia (19), and electrolyte transport (20, 21). A large deletion in the canine *COMMD1* gene, which abolishes protein expression, leads to pathologic copper accumulation, cirrhosis, and liver failure in Bedlington terriers (17). Although humans with pathologic copper accumulation caused by *COMMD1* mutations have not been identified (22, 23), a role for this gene in modulating the phenotype of Wilson's disease has been proposed (24). Moreover, COMMD1 has been found to have copper binding activity *in vitro* (25) and can modulate the matura-

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S3.

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[3] The abbreviations used are: CRL, Cullin RING ligase; SRC, substrate receptor complex; RIPA, radioimmune precipitation assay.

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tion of the copper-containing enzyme SOD1 (26). In addition, COMMD1 has been found to play a role in tumor invasion acting as a regulator of both hypoxia-inducible factor (HIF) and NF-κB (27). Decreased expression of COMMD1 in cancer was found to promote tumor invasion in a variety of settings and was associated with a negative impact on patient survival.

Although the mechanisms underlying these pleiotropic activities of COMMD1 remain unclear, this protein has been shown to be an activating co-factor for a Cul2-containing ligase that ubiquitinates NF-κB/RelA (15, 16). However, a broader paradigm that explains the pleiotropic activities of COMMD proteins has not been defined. Here, we report that these factors bind to multiple CRLs and that the prototype member, COMMD1, can activate ligase function through displacement of the CRL inhibitor CAND1.

EXPERIMENTAL PROCEDURES

Plasmids—Expression vectors for Cullin proteins, CAND1, and Skp1 (all of human origin) were generated by PCR amplification of the corresponding coding sequence, which was then introduced into the pEBB-FLAG and pEBG vectors. For Cul1, Pallino-FLAG-Cul1 was used as template (kindly provided by Dr. Michele Pagano). The following IMAGE cDNA clones were obtained from Open Biosystems and used as templates for Cul3, Cul4A, Cul4B, Cul5, Cul7, CAND1, and Skp1: 5784147, 3537176, 5269392, 30331132, 5580027, 5265409, and 6672613, respectively. COMMD-EYFP constructs were generated by subcloning EYFP into pEBB-FLAG expression vectors previously described (15). The plasmid pEBB-COMMD1 (M110A/H134A)-GST was generated by site-directed mutagenesis. Deletion constructs for Cul2 were generated by PCR using pEBB-FLAG-Cul2 as template (16), with the amino acid boundaries of the encoded mutant proteins being: Cul2 1–415, 109–415, 109–745, 415–745, 1–150 (R1), 151–270 (R2), 271–386 (R3), and 151–386 (R2-R3). His_6-tagged versions of COMMD1 and Skp1 were subcloned from the corresponding pEBB vectors into pET30a using the BamHI and NotI sites. All other plasmids have been previously described (15, 16, 28, 29).

Cell Culture and Transfection—HEK 293 cells and HeLa cells were obtained from ATCC. U2OS cells with stable shRNA-mediated repression of COMMD1 have been previously reported (16). HEK 293 cells stably expressing GST or GST-Cul2 were generated by lentiviral infection and selection as previously described (16). All of the cell lines were cultured in DMEM supplemented with 10% FBS and L-glutamine (2 mM). Biotin (Sigma; 4 μM) was added to the medium when fusion proteins with the biotinylation target peptide were expressed. A standard calcium phosphate transfection protocol was used to transfect HEK 293 cells (29). HeLa cells were transfected using FuGENE (Roche Applied Science) according to the manufacturer's instructions.

Confocal and Fluorescence Microscopy—HeLa cells were plated in chambered coverglass plates and transfected with the indicated COMMD-EYFP plasmids (0.5 μg/well). The cells were stained with Hoechst 33342 (8 μM) for 30 min, and images were obtained with a Zeiss LSM 510 META confocal microscope equipped with a Chameleon XR NIR laser.

Immunoblotting and Protein Precipitation—Cell lysate preparation, immunoprecipitations, GSH precipitations, and immunoblotting were performed as previously described (15, 28). For the COMMD1-Cul1 interaction, the cells were sonicated in detergent-free buffer E (25 mM HEPES, 100 mM NaCl, 1 mM EDTA, 5% glycerol). Interactions with Cul2 and Cul3 were studied using buffer F (PBS, 0.8% Nonidet P-40). Other lysis buffer conditions are indicated in the figure legends where appropriate. The precipitation of RelA after denatured lysis of nuclei, which was used to detect ubiquitinated RelA (see Fig. 6), has been previously described (30). The following antibodies were utilized in our studies: CAND1 (Novus, H00055832-MO1; Santa Cruz, sc-10672), COMMD1 (15), Cul1 (Santa Cruz, sc-12761), Cul2 (Zymed Laboratories Inc., 51-1800), Cul3 (kindly provided by Dr. Matthias Peter) (31), Elongin C (BioLegend, 613101), FLAG (Sigma, A8592 and F1804), GST (Santa Cruz, sc-459), HA (Sigma, H6533; Covance, MMS101R), RelA (Santa Cruz, sc-372), and Rbx1 (LabVision, 127-075-160).

Recombinant Protein Preparation—HA-tagged Skp1 and COMMD1 proteins were expressed in HEK 293 cells and purified from cell lysates. After incubation with an anti-HA affinity matrix (Roche Applied Science), the purified material was extensively washed four times with RIPA lysis buffer and then eluted in dissociation buffer (40 mM HEPES, pH 7.9, 60 mM potassium acetate, 10% glycerol) containing HA peptide as a competitor (1 mg/ml). Elution was performed three times at 37 °C with agitation. The combined eluate was used for further experiments. His_6-tagged COMMD1 or Skp1 were expressed and purified from *Escherichia coli* (BL21 strain, Stratagene) using the nickel-nitrilotriacetic acid purification system (Invitrogen). The final imidazole eluate was loaded onto an Amicon centrifuge filter (Millipore) to eliminate imidazole, and the purified protein was redissolved in PBS buffer with 10% glycerol through sequential filtration and buffer exchange.

Bimolecular Affinity Purification of the Cul2-COMMD1 Complex—HEK 293 cells were transfected with GST or GST-tagged Cul2 and COMMD1 fused to TB (a biotinylation target peptide), grown in biotin-supplemented media, and lysed in Triton buffer 2 days later. The Cul2-COMMD1 complex was purified through sequential affinity purification using glutathione and streptavidin columns as previously described (29).

In Vitro Ubiquitination Assays—Each reaction mixture consisted of recombinant HA-human ubiquitin (2.5 μg), human His_6-E1 (Uba1, 50 ng), various human E2 enzymes as indicated (UbcH5a, 5b, 5c, 7, and 10, 100 ng; UbcH3, 150 ng), and an ATP regenerating buffer (all obtained from Boston Biochem). These were mixed in reaction buffer (40 mM HEPES, pH 7.9, 60 mM potassium acetate, 2 mM DTT, 5 mM $MgCl_2$, 10% glycerol) and agitated at 30 °C for 60 min (16). Polyubiquitin chain formation was detected by Western blotting with an anti-HA antibody as described above.

In Vitro Displacement of CAND1—The CAND1 dissociation reaction was based on the prior report by Zheng *et al.* (12). For displacement of endogenous CAND1 (see Fig. 4*B*), HEK 293 cells were transfected with GST-Cul1 or GST-Cul2 and lysed in a hypotonic buffer (20 mM HEPES, pH 7.2, 5 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT) using a Dounce apparatus. The lysate was cleared by centrifugation at 27,000 × *g* (4 °C for 30 min). In the

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case of displacement of overexpressed FLAG-CAND1 (see Fig. 4, *C* and *D*), the cells were similarly transfected but lysed in a Triton X-100-containing buffer (25 mM HEPES, pH 7.9, 100 mM NaCl, 10% glycerol, 1% Triton X-100). All of the buffers were supplemented with protease inhibitors (Roche Applied Science) and DTT (10 mM). Cul1 or Cul2 complexes were purified by GSH-Sepharose (GE Healthcare). The GSH beads were aliquoted and incubated at 30 °C for 30 min with ATP in dissociation buffer (40 mM HEPES, pH 7.9, 60 mM potassium acetate, 10% glycerol), with the indicated recombinant proteins (Skp1 or COMMD1). Thereafter, the beads were washed twice with Triton X-100 lysis buffer (25 mM HEPES, 100 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Triton X-100). The leftover material was utilized for Western blot analysis.

RESULTS

COMMD1 Associates with CRL Complexes—Given the diverse activities of COMMD1 (32), we initially explored the possibility that this factor might play a broader role in the regulation of ubiquitination of various targets by CRLs. To this end, we examined in more detail the interaction between COMMD1 and other Cullin proteins. We found that endogenous COMMD1 could be co-precipitated with endogenous Cul1, Cul2, or Cul3 (Fig. 1*A*). The Cul1-COMMD1 interaction was best visualized in a detergent-free buffer, whereas the Cul2 and Cul3 interactions were best seen with a glycerol-free buffer containing low amounts of Nonidet P-40 (see “Experimental Procedures”). These interactions were also evident using a buffer containing 1% Triton X-100 (supplemental Fig. S1). Utilizing expression vectors for all five canonical Cullins (Cul1, 2, 3, 4A, 4B, and 5), we could easily recapitulate the interaction between COMMD1 and all of the Cullin family members tested (Fig. 1*B*).

Other COMMD Proteins Also Interact with CRLs—Other COMMD proteins have largely undefined functions at this point. Using a similar approach, it was found that other COMMDs may also interact with CRLs (Fig. 1*C*). Interestingly, their patterns of Cullin preference were not identical.

Several COMMDs are known to display differential tissue expression (15), yet several of them are ubiquitous and form heterodimers. To try to understand the potential redundancy in this system, we examined the cellular distribution of COMMD proteins expressed with a fused EYFP tag (Fig. 1*D*). Although some similarities were found for several family members, certain COMMD proteins displayed specific and unique cellular distribution. For example, in the case of COMMD2, the protein was consistently excluded from the nucleus, whereas COMMD7 demonstrated a unique punctuate pattern.

COMMD1 Associates with Active CRLs in a Copper-independent Manner—Consistent with the interaction between COMMD1 and CRLs, we found that COMMD1 could precipitate ubiquitin ligase activity when extracted from mammalian cells. COMMD1 was expressed and affinity-purified from HEK 293 cells or *E. coli*, and the recovered proteins were offered to an *in vitro* reaction containing the E1 and E2 (UbcH5a) enzymes, recombinant ubiquitin, and an ATP regenerating buffer. Polyubiquitin chain formation in the presence of ATP was readily detectable when COMMD1 precipitated from mammalian cells was provided as a source of ubiquitin ligase activity (Fig. 2*A*, *left panels*). However, if COMMD1 was precipitated and washed in RIPA buffer, this activity was lost, consistent with the fact that COMMD1-CRL interactions are abolished in this buffer (data not shown). On the other hand, recombinant COMMD1 prepared in *E. coli* was devoid of ligase activity, but when the protein was incubated with a mammalian lysate and then washed, ligase activity was reconstituted (Fig. 2*A*, *right panels*). These results suggested that COMMD1 interacts with cellular factors that provide ligase activity.

Next, we observed that the COMM domain of COMMD1, which is responsible for binding to Cul2 (16), was also necessary and sufficient for recovering ligase activity (Fig. 2*B*, *COMM Domain* or *CD lane*). Given the conservation of the COMM domain across all COMMD proteins and our finding of COMMD-CRL interactions, we speculated that other family members may be similarly linked to active ubiquitin ligases. Indeed, when precipitated from mammalian cells, several COMMD proteins provided a source of ubiquitin ligase activity *in vitro* (Fig. 2*C*). The ligase activity varied as a function of the E2 offered to each COMMD protein (supplemental Fig. S2), consistent with their differential binding to Cullin family members.

Next, we examined whether CRL interactions were responsible for COMMD1-associated ligase activity. To test this possibility, COMMD1 and Rbx1 (the main RING finger subunit in CRLs) were co-expressed in HEK 293 cells, and Rbx1 was immunoprecipitated prior to assessing the E3 activity associated to COMMD1. Depletion of Rbx1 led to a significant drop in COMMD1-associated ubiquitin ligase activity (Fig. 2*D*), consistent with the notion that ligase activity associated with COMMD1 is due to its interaction with CRLs.

Finally, a copper binding activity for COMMD1 has been found *in vitro*, and residues that coordinate copper have been similarly mapped (25). We examined whether copper could modulate COMMD1-CRL interactions. A double mutation in COMMD1 (M110A/H134A) targeting copper-binding residues did not affect Cul2 binding or COMMD1-associated E3 activity (Fig. 2*E*). Similarly, the addition of copper to the growth medium did not affect these interactions (data not shown), indicating that CRL binding is copper-independent.

The Cul2-COMMD1 Complex Excludes the CRL Inhibitor CAND1—Altogether, the data indicated that COMMD1 associates with CRLs, a property that seems to be shared by other COMMD proteins. Nevertheless, the specific function for such interactions was not immediately apparent. To address the latter question, we utilized a bi-molecular tandem affinity purification scheme (29) to isolate a purified Cul2-COMMD1 complex from mammalian cells (Fig. 3*A*). Cul2 was purified using a glutathione-Sepharose column (fraction A); after elution, this material was offered to a streptavidin-agarose column for purification of the COMMD1 subfraction (fraction B). As expected, COMMD1 and Cul2 were readily demonstrated in fraction B (Fig. 3*B*), and based on the amounts recovered, this complex was estimated to represent 2–5% of all cellular Cul2. In addition, fractions A and B provided ubiquitin ligase activity *in vitro* (not shown here), and known components of the active ligase, such as Elongin C, were also present in both fractions (Fig. 3*C*).

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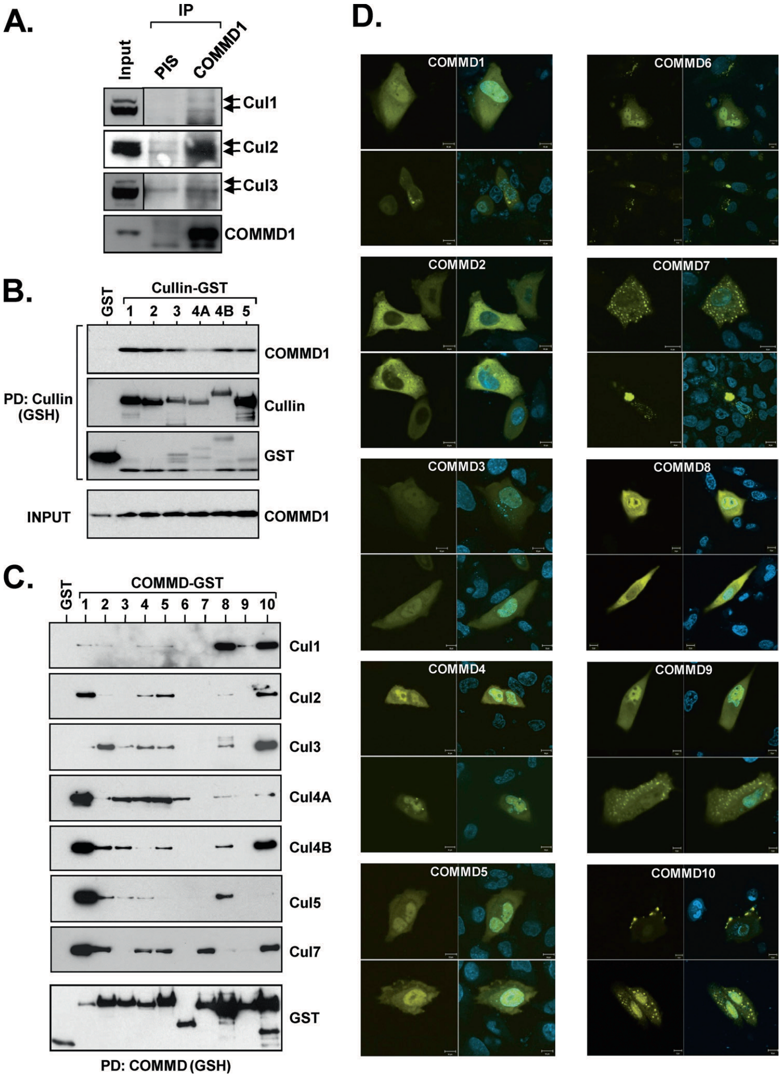
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Input
PIS
COMMD1
Cul1
Cul2
Cul3
COMMD1
B.
GST
Cullin-GST
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COMMD1
PD: Cullin (GSH)
Cullin
GST
INPUT
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1 2 3 4 5 6 7 8 9 10
Cul1
Cul2
Cul3
Cul4A
Cul4B
Cul5
Cul7
GST
PD: COMMD (GSH)
D.
COMMD1
COMMD6
COMMD2
COMMD7
COMMD3
COMMD8
COMMD4
COMMD9
COMMD5
COMMD10

FIGURE 1. **COMMD1 and other COMMD proteins associate with CRL complexes.** *A*, endogenous co-immunoprecipitation (*IP*) of COMMD1 and CRLs. COMMD1 was immunoprecipitated from HEK 293 cell lysates. The precipitated material was probed for the indicated Cullins. *PIS*, preimmune serum. *B*, COMMD1 binds to other Cullin family members. COMMD1 was expressed in HEK 293 cells together with Cullin proteins fused to GST, which were precipitated as above and immunoblotted for COMMD1. *PD*, pulldown. *C*, COMMD proteins interact with Cullins. In each experiment, one FLAG-tagged Cullin was expressed together with COMMD proteins fused to GST, which were subsequently precipitated. The presence of the Cullin in the precipitates was determined by immunoblotting with the FLAG antibody. *D*, cellular distribution of COMMD proteins. The indicated COMMD proteins fused to EYFP were expressed in HeLa cells. The cells were counterstained with Hoechst and imaged in a confocal microscope. The *scale bar* corresponds to 10 μm.

Interestingly, the CRL inhibitor CAND1 was readily identified in fraction A but was absent in Cul2-COMMD1 complexes (Fig. 3*C*).

Next, we sought to ascertain whether the lack of CAND1 in the Cul2-COMMD1 complex was truly due to the purification of a distinct cellular fraction of Cul2 rather than an artifact of the purification scheme itself. To address this question, Cul2 was first purified, eluted, and subsequently offered to a second column for precipitation of either COMMD1 or CAND1. Although COMMD1 and CAND1 were readily co-precipitated with Cul2 in the initial fraction, the second purification step of either COMMD1 or CAND1 resulted in the depletion of the other protein (Fig. 3*D*), consistent with the existence of mutually exclusive pools of COMMD1 and CAND1 when bound with Cul2 in cells.

COMMD1 Promotes CRL E3 Activity—Given the exclusion of CAND1 from Cul2-COMMD1 complexes, we reasoned that COMMD1 might bind to or promote a more active CRL state.

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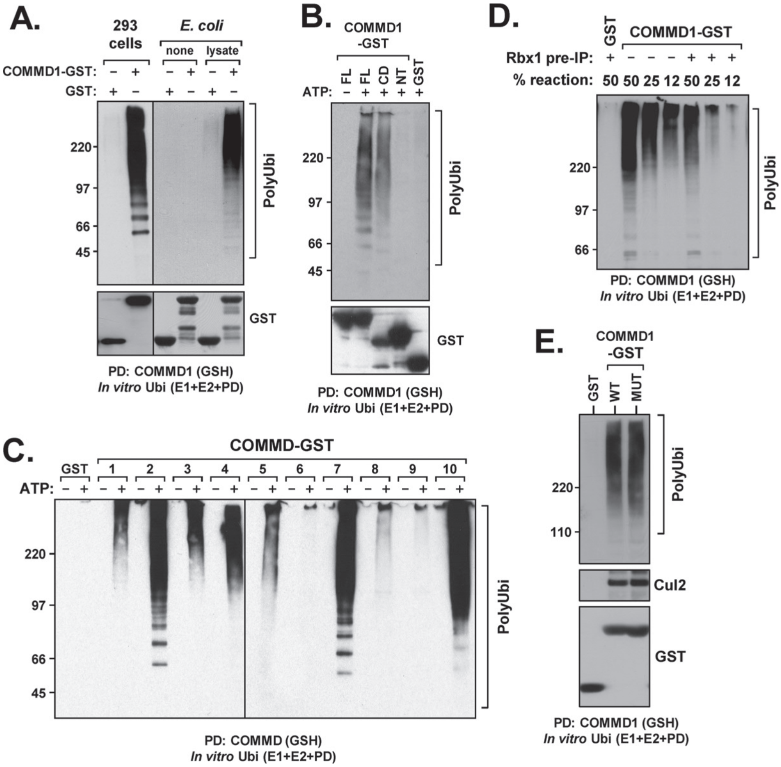

FIGURE 2. **COMMD1 associates with active CRL complexes in a copper-independent manner.** *A*, COMMD1 precipitates cellular factors with ubiquitin ligase activity. *In vitro* ubiquitination reactions were supplemented with precipitated proteins as indicated. Polyubiquitin chain formation was determined by Western blotting as an indication of ubiquitin ligase activity. COMMD1-GST or GST were expressed in HEK 293 cells (*left panels*) or prepared recombinantly in *E. coli* (*right panels*). Recombinant proteins either were offered directly (*none*) or were first mixed with a mammalian lysate and washed prior to the reaction (*lysate*). *PD*, pulldown. *B*, COMMD1 precipitates ubiquitin ligase activity through the COMM domain. COMMD1 full-length (*FL*), its amino terminus (*NT*), or the COMM domain (*CD*) fused to GST were precipitated from transfected HEK 293 cells and added to an *in vitro* ubiquitination reaction as in *A*. *C*, COMMD proteins precipitate ubiquitin ligase activity. COMMD proteins fused to GST were precipitated and added to an *in vitro* ubiquitination reaction as in *A*. *D*, depletion of Rbx1 reduces COMMD1-associated ligase activity. COMMD1-GST was utilized as a source of ubiquitin ligase activity as before. The protein was expressed in HEK 293 cells and either precipitated directly (−) or following immunodepletion of FLAG-Rbx1 (+). *IP*, immunoprecipitation. *E*, copper binding by COMMD1 is not involved in E3 and Cul2 binding. WT COMMD1 or an M110A/H134A mutant (*MUT*) unable to bind copper were expressed in HEK 293 cells. After precipitation, the protein complexes were examined for associated E3 activity as before (*upper panel*) and for the presence of co-precipitated endogenous Cul2 (*middle panel*). *Ubi*, ubiquitination.

To test this notion, Cul2 was co-expressed with COMMD1 and subsequently precipitated from cell lysates directly or through COMMD1. The CRL inhibitor CAND1 was included as a negative control. At the end of the precipitation and after adjusting for equivalent Cul2 recovery, we utilized the isolated complexes as a source for E3 ligase activity in an *in vitro* ligase reaction. These experiments demonstrated that Cul2 associated with COMMD1 was the most active isolate, whereas the CAND1 fraction was relatively devoid of enzymatic activity (Fig. 3*E*).

The preferential binding of COMMD1 to a CAND1-free pool of Cul2 could be responsible for these findings; alternatively, COMMD1 could promote an active conformation for the complex. Therefore, we explored further whether COMMD1 could potentiate CRL E3 activity *in vitro*. GST-Cul1 that was immunoprecipitated from mammalian cells and its E3 activity was examined *in vitro* as before. In these assays, its substrate receptor adaptor Skp1 or COMMD1 were added to the *in vitro* reaction. As can be seen in Fig. 3*F*, the addition of COMMD1 or Skp1 led to enhanced Cul1 E3 activity. This was not due to additive E3 activity precipitated by COMMD1 or Skp1, because there was no associated E3 activity bound to COMMD1 or Skp1 when these preparations were made with RIPA buffer (Fig. 3*F*, *right half* of the gel). Altogether, this indicated that COMMD1 binds to active CRL complexes and can lead to their activation *in vitro*. However, cellular deficiency of COMMD1 did not substantially affect endogenous CRL2 activity (supplemental Fig. S3), potentially because of redundant effects by other COMMDs expressed in these cells.

COMMD1 Promotes the Dissociation of CAND1 from CRL Complexes—Akin to the distinct fractionation between CAND1 and substrate receptor complexes (11–13), our data indicated that CAND1 and COMMD1 do not coexist in the same CRL complexes. To test this notion further, we examined whether the purified Cul2-COMMD1 complex was capable of binding CAND1 *in vitro*. To that end, after purification of fraction B, unbound complexes were offered again to a glutathione-

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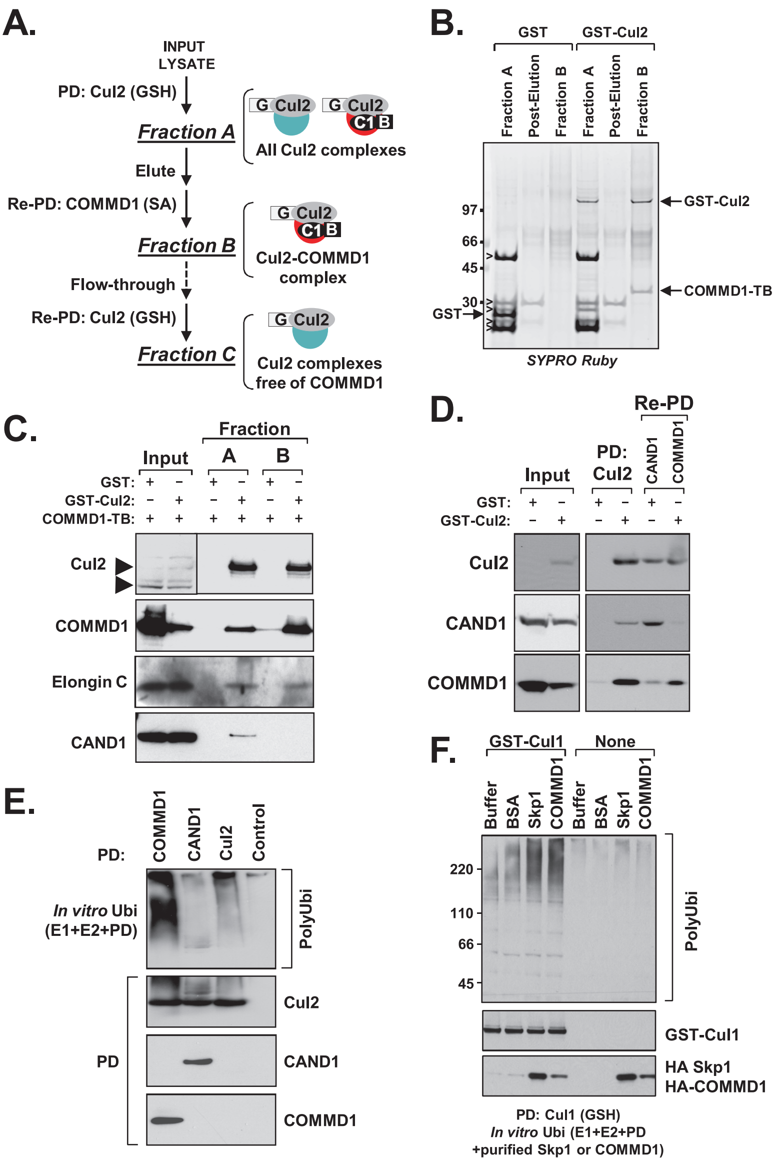

FIGURE 3. **The Cul2-COMMD1 complex excludes the CRL inhibitor CAND1.** *A*, purification strategy used to isolate the Cul2-COMMD1 complex. Cul2 fused to GST (*G*) and COMMD1 fused to a biotinylation tag (*B*) were expressed in HEK 293 cells. Cul2 was purified through a GSH-Sepharose column (fraction A) and subsequently eluted. COMMD1 was precipitated from the eluted material using streptavidin (*SA*)-agarose beads (fraction B). Cul2 remaining in the flow-through after the streptavidin-agarose column was reprecipitated with a GSH column (*Re-PD: Cul2 (GSH)*) resulting in fraction C. *PD*, pulldown. *B*, Cul2-COMMD1 complex purification. Proteins from fractions A and B were separated by SDS-PAGE and stained with SYPRO Ruby; the identity of major bands was determined by paired Western blot analysis (nonspecific bands indicated by *open arrowheads*). *C*, the Cul2-COMMD1 complex excludes CAND1. Fractions A and B were immunoblotted for Cul2, COMMD1, Elongin C, and CAND1. Endogenous Cul2 and GST-Cul2 are noted by *arrowheads* to the *left* of the *top panel*. *D*, COMMD1 and CAND1 exist in separate Cul2 cellular pools. GST-Cul2, COMMD1 fused to a biotinylation tag, and FLAG-CAND1 were co-expressed in HEK 293 cells. Cul2 was purified through a GSH affinity column (*PD: Cul2*) and subsequently eluted. COMMD1 or CAND1 were then precipitated from the eluate using SA agarose beads or FLAG antibody, respectively. The resulting fractions were immunoblotted for Cul2, CAND1, or COMMD1. *E*, Cul2 complexes containing COMMD1 are active. GST-Cul2 was precipitated from transfected HEK 293 cells utilizing GSH beads (Cul2) or using protein G beads as a control (Control). FLAG-COMMD1 or FLAG-CAND1 co-expressed with GST-Cul2 were precipitated utilizing the FLAG antibody. After adjusting for equal Cul2 recovery, the resulting material was added to an *in vitro* ubiquitination reaction as in Fig. 2*A* and immunoblotted for ubiquitin. *F*, purified COMMD1 potentiates Cul1 E3 activity *in vitro*. GST-tagged Cul1 was expressed in HEK 293 cells and purified using GSH affinity matrix. Purified HA-Skp1 or HA-COMMD1 prepared from mammalian lysates was then added to the recovered Cul1 complex, and this preparation was used as a source for E3 activity as before.

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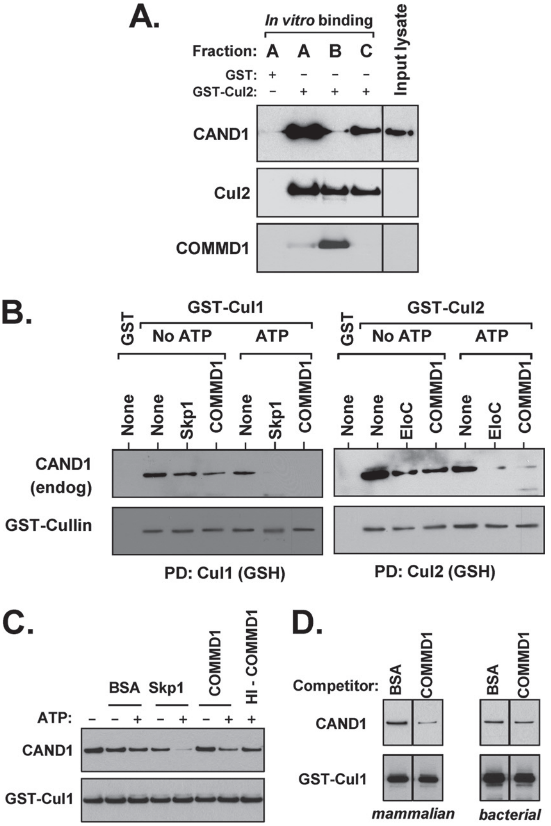

FIGURE 4. **COMMD1 promotes the dissociation of CAND1 from CRL complexes.** *A*, COMMD1 prevents CAND1 binding to Cul2. Fractions A, B, and C were prepared as depicted in Fig. 3*A*, mixed for 2 h with a fresh HEK 293 cell lysate expressing FLAG-CAND1, washed, and immunoblotted for CAND1, Cul2, or COMMD1. *B*, COMMD1 can displace CAND1 from Cul1 or Cul2 complexes. GST-Cul1 or GST-Cul2 were expressed in HEK 293 cells and purified through a GSH affinity matrix. This material was then mixed with HA-Skp1 or HA-COMMD1 purified from mammalian cells. After incubation at 30 °C with ATP (15 mM), the GSH beads were washed, and the presence of endogenous CAND1 still bound to Cul1 or Cul2 was determined by immunoblotting. *PD*, pulldown. *C*, the activity of COMMD1 is heat-labile. GST-Cul1 complexes were purified as before from cells also co-expressing FLAG-CAND1. After a displacement reaction (performed as previously but using 7.5 mM ATP), the presence of remaining FLAG-CAND1 bound to Cul1 was determined by immunoblotting. *HI-COMMD1*, heat inactivated COMMD1 (95 °C for 10 min). *D*, recombinant COMMD1 made in *E. coli* is devoid of activity. Shown is the same reaction as in *C*, but utilizing mammalian HA-COMMD1 or bacterially made His_6-COMMD1. Only the ATP containing reactions are shown; each subpanel was run in the same gel.

Sepharose column to isolate Cul2 devoid of COMMD1 (fraction C; Fig. 3*C*). All of these preparations were mixed with a fresh lysate containing expressed CAND1, and binding *in vitro* was assessed by co-precipitation. This experiment indicated that total Cul2 complexes or Cul2 complexes devoid of COMMD1 (fractions A and C) bound CAND1 well, but the Cul2-COMMD1 complex (fraction B) could not bind CAND1 efficiently (Fig. 4*A*). This was the case despite similar loading of Cul2 among these fractions and the additional purification steps required to isolate fraction C, strongly suggesting that these results were independent of the purification scheme itself.

Next we examined whether COMMD1 could facilitate the dissociation of CAND1 from CRLs. To test this notion, GST-tagged Cul1 or Cul2 was precipitated from transfected HEK 293 cells resulting in the co-precipitation of endogenous CAND1. Next, the precipitated Cul1 or Cul2 complexes were mixed with purified COMMD1 or their respective substrate receptor adaptors, Skp1 or Elongin C, respectively. After incubation at 30 °C and thorough washing, the presence of CAND1 still associated to either Cul1 or Cul2 was assessed by immunoblotting. As has been reported before, substrate receptor adaptors were able to promote the dissociation of CAND1 (12, 33), particularly when the reaction was supplemented with ATP (Fig. 4*B*). Importantly, the same was true of COMMD1, which promoted CAND1 dissociation from either Cul1 or Cul2 (Fig. 4*B*). Interestingly, this displacement reaction was abrogated when COMMD1 had been heat-inactivated (Fig. 4*C*). In addition, recombinant COMMD1 made in *E. coli* was devoid of activity compared with a mammalian preparation (Fig. 4*D*). This is in contrast with Skp1, which, as previously described (12), was active irrespective of it being prepared from *E. coli* lysates (data not shown).

COMMD1 Binds to CRLs in a Manner Distinct from Substrate Receptors—Structural information indicates that Skp1 and CAND1 bind to an overlapping region of Cullin repeat 1 (R1) in Cul1 (13). CAND1 also binds to the carboxyl-terminal domain of Cul1 over its neddylation site and makes extensive contacts with R2 and R3 (13, 34) (Fig. 5*F*). This information explains why CAND1 does not bind to neddylated Cullins and why CAND1 and SRC binding are mutually exclusive.

Next, we tried to ascertain whether competitive binding for a shared domain on Cul2 may be involved in the ability of COMMD1 to displace CAND1. First, it was noted that neither the amino terminus nor the carboxyl terminus of Cul2 were required for COMMD1 binding (Fig. 5*A*). Instead, amino acids 109–415 were found to be sufficient to mediate the COMMD1 interaction. Utilizing an alignment between Cul2 and Cul1 and the published crystal structure of Cul1 as a framework (10), we predicted the boundaries of Cul2 R1, R2, and R3 (Fig. 5*F*). COMMD1 was found to bind preferentially to R2 and not to R1 (Fig. 5*B*).

Based on this finding, we predicted that COMMD1-CRL binding occurs independently of the SRC. To test this notion, we introduced a previously reported point mutation in R1 of Cul1 (10), which disrupted Skp1 binding (Fig. 5*D*). Interestingly, the T47A mutant lost the ability to interact with Skp1 but bound to CAND1 (Fig. 5*C*), consistent with the incomplete overlap between CAND1 and Skp1 binding surfaces (10, 13). Importantly, this Cul1 mutant retained its ability to interact with COMMD1 (Fig. 5*D*), indicating that this interaction is independent of the SRC.

Consistent with the competitive nature of the COMMD1-CAND1 interactions with CRLs, we observed that siRNA-mediated reduction of CAND1 expression increased the Cul2-COMMD1 endogenous binding (Fig. 5*E*). On the other hand, depletion of COMMD1 by siRNA had little effect on the CAND1-Cul1 interaction (data not shown), which may be explained by the potential redundancy of the nine other COMMD family members.

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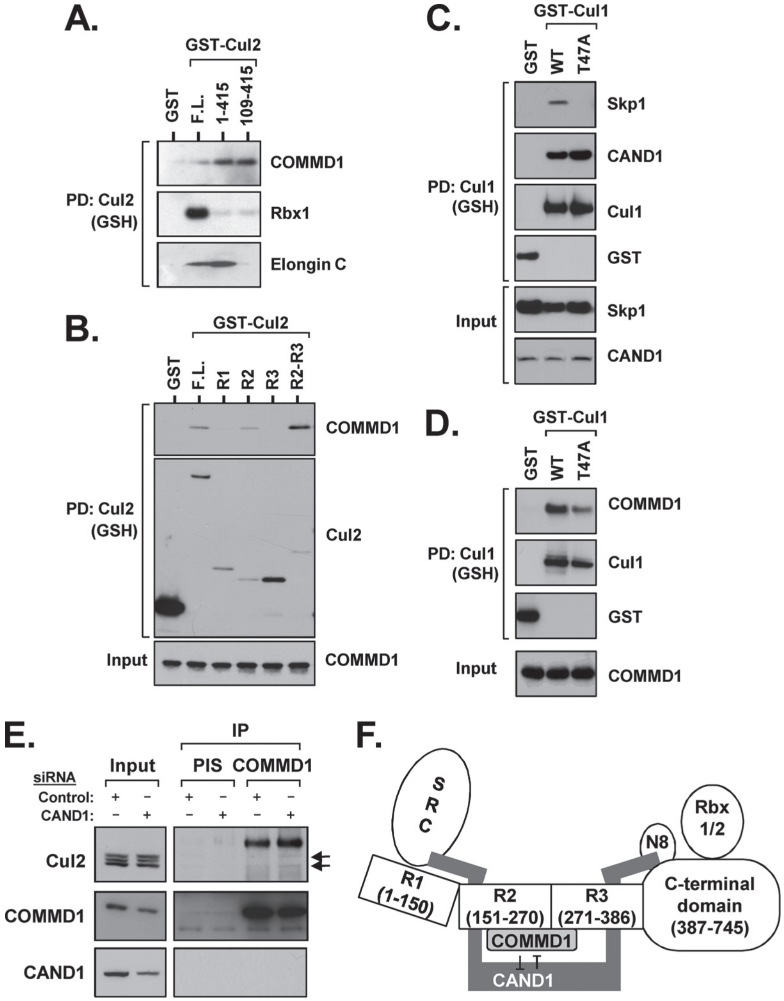

FIGURE 5. **COMMD1 binds to CRLs in a manner distinct from substrate receptors.** *A*, full-length and specific deletion mutants of Cul2 fused to GST were expressed in HEK 293 cells and precipitated from cell lysates with glutathione-Sepharose (*GSH*) beads. The recovered material was immunoblotted for endogenous COMMD1, Rbx1, and Elongin C. *PD*, pulldown. *B*, COMMD1 binds to Cullin repeat 2 in Cul2. COMMD1 was expressed in HEK 293 cells together with Cul2 deletion mutants fused to GST (R1, R2, or R3, Cullin repeats 1, 2, or 3, respectively), which were precipitated as in *A* and immunoblotted for COMMD1. *C* and *D*, Cul1 can bind to COMMD1 independently of Skp1. Cul1 WT or a T47A point mutant fused to GST were expressed in HEK 293 cells. Their interactions with HA-Skp1 or FLAG-CAND1 were tested by co-precipitation (*C*). The same Cul1 proteins were expressed together with HA-COMMD1 and Cul1-COMMD1 interactions were examined by co-precipitation (*D*). *E*, CAND1 deficiency promotes COMMD1-Cul2 binding. HEK 293 cells were transfected with siRNA duplexes targeting CAND1. Endogenous COMMD1 was immunoprecipitated (*IP*), and its interaction with endogenous Cul2 was evaluated as in Fig. 1*A*. *F*, model depicting the various domains in canonical Cullin proteins, their known interacting partners, and the competitive binding by COMMD1 and CAND1 for R2. The amino acid boundaries for R1, R2, and R3 in Cul2 are also depicted. *N8*, NEDD8.

COMMD1 Is Required for the Ubiquitination of Specific Targets in Vivo—We next set out to address the role of COMMD1 on the ubiquitination of endogenous proteins *in vivo*. We examined this question in the context of the NF-κB signaling pathway. As reported previously, we found that COMMD1 is required for NF-κB/RelA ubiquitination (16, 35, 36). Cells with decreased levels of COMMD1 (Fig. 6*A*) demonstrated a profound decrease in the ubiquitination of endogenous RelA (Fig. 6*B*). In addition, the pro-inflammatory cytokine TNF stimulated the binding of COMMD1 to Cul2 (Fig. 6*C*), consistent with its role in promoting NF-κB/RelA ubiquitination and degradation as a mechanism to terminate signaling (16, 35, 36). Interestingly, TNF stimulation had only modest effects on total CRL2 activity (Fig. 6*D*), suggesting that the TNF induction of COMMD1/Cul2 binding more likely activates specific CRL2 complexes, such as those targeting RelA and possibly other targets.

DISCUSSION

Altogether, our studies indicate that COMMD1 can modulate CRL activity through its ability to displace CAND1. This

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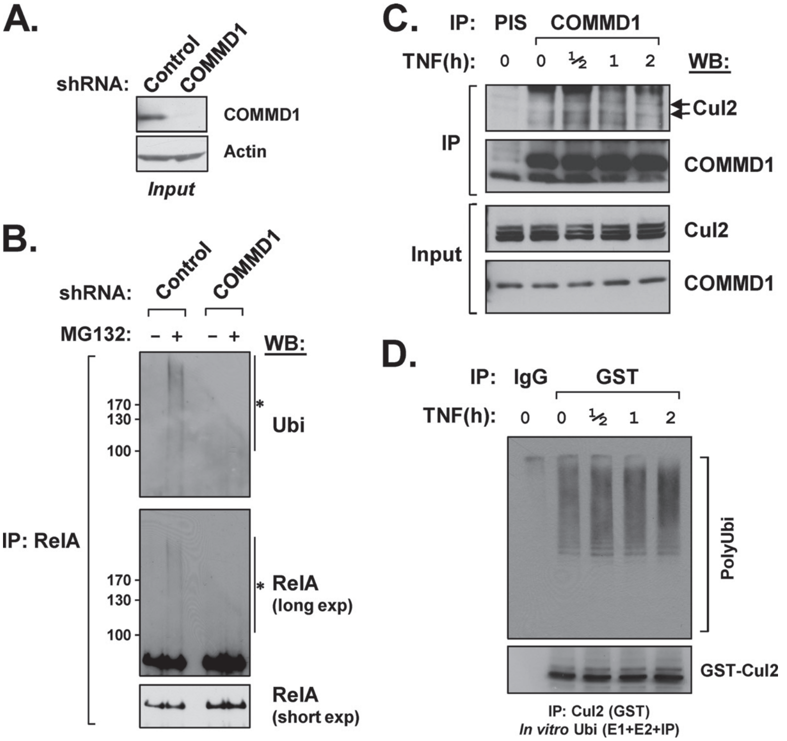

FIGURE 6. **COMMD1 is required for the ubiquitination of specific targets *in vivo*.** *A*, Western blot demonstrating stable shRNA-mediated repression of COMMD1 in U2OS cells. *B*, RelA ubiquitination is greatly impaired in COMMD1-deficient cells. U2OS cells were treated with the proteasome inhibitor MG-132 as indicated, and cell nuclei were isolated and then lysed in a denaturing buffer. RelA was immunoprecipitated from nuclear extracts and immunoblotted for ubiquitin (*top panel*) or for RelA itself (*middle* and *bottom panels*). The *asterisk* indicates the high molecular weight smear consistent with ubiquitinated RelA. *C*, TNF stimulates COMMD1-Cul2 binding. HEK 293 cells were treated with TNF (1,000 units/ml) for the indicated time intervals, and endogenous COMMD1 was subsequently precipitated from cell lysates as in Fig. 1*A*. The recovery of endogenous Cul2 was determined by immunoblotting. *D*, cells stably expressing GST-Cul2 (see supplemental Fig. S3 for details) were treated with TNF (1,000 units/ml) for the indicated time points. Thereafter, GST-Cul2 was immunoprecipitated with a GST antibody. The recovered material was utilized as a source for E3 activity as previously. *WB*, Western blot; *IP*, immunoprecipitation; *PIS*, preimmune serum; *Ubi*, ubiquitination.

finding provides a mechanism for the ability of COMMD1 to promote protein ubiquitination and degradation (16, 36, 37). The data also suggest that COMMD1 activates specific CRL complexes involved in the turnover of specific products, such as RelA. This activation seems to be regulated by the inducible interaction between COMMD1 and certain CRL complexes under specific conditions, as in the case of induced COMMD1-Cul2 interactions after TNF treatment.

In the present model of CRL regulation, CAND1 exerts its inhibitory function on all Cullins, potentially regulating ~300 different ligase complexes by preventing the full assembly of the active ligase (9). This broad activity would have a myriad of effects on cellular physiology, and therefore factors that control CAND1-CRL regulation in a more restricted fashion would make physiologic sense. Indeed, others have proposed the existence of such regulation based on the unexplained variable affinity of CAND1 for different Cullins in different tissues (8). Based on the data presented, our view is that the COMMD protein family, with its variable pattern of tissue and cellular expression and the unique Cullin binding preferences of each COMMD family member, could provide fine tuning of CRL activation by countering CAND1 binding.

Given the myriad of activities regulated by the ~300 CRL complexes existent in mammals, it is anticipated that COMMD proteins would likely affect many physiological processes. Indeed, consistent with this notion, COMMD1 has been implicated in a variety of seemingly unrelated processes such as NF-κB regulation, sodium and electrolyte transport, hypoxia responses, and copper excretion. In most instances, the mechanism has been linked to protein ubiquitination, in agreement with the notion that COMMD1 and other COMMD proteins regulate CRL ubiquitin ligase complexes. Our view is that the biochemical events dissected in this study provide a mechanistic explanation for the various biological activities identified for COMMD1.

Similar to what has been reported for substrate receptor complexes (11, 12, 33), COMMD1 binding to CRLs displaces CAND1, leading to an enzymatically active complex. Based on the structure of Cul1, we speculate that this is mediated by competitive binding for the highly conserved R2 region (Fig.

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5*F*), which interacts extensively with CAND1 (13). The competitive nature of COMMD1/CAND1 binding to CRLs is extensively demonstrated using various *in vitro* systems, and the relevance of these findings is validated by the increased binding of COMMD1 to Cul2 in CAND1-deficient cells.

It is important to note that the biochemical events that lead to CRL-CAND1 dissociation remain quite enigmatic. In the case of both COMMD1 and the SRC components Skp1 or Elongin C, the reaction requires ATP supplementation, yet the basis for this ATP requirement is not evident, because none of these factors have ATPase or kinase activity. In addition, a CRL1-CAND1 complex formed by *E. coli*-expressed recombinant proteins cannot be dissociated by Skp1 or neddylation co-factors (13), and this reaction only takes place when the CRL-CAND1 complex is isolated from mammalian cells (12, 33). For all of these reasons, we speculate that additional factors present in the mammalian preparations are required for CAND1 dissociation, and these factors may explain the requirement for ATP in the reaction. Similarly, we found that COMMD1 was active in this reaction only when extracted from mammalian cells, suggesting either that post-translational modifications of COMMD1 are necessary or that co-factor(s) associated with COMMD1 in mammalian cells are required for this process.

Recent data indicate that the recycling of CRLs between the CAND1-bound and SRC-bound states facilitates the loading of rare SRCs that would otherwise be outcompeted by more abundant co-factors (38). Although the SRC can dissociate the CRL1-CAND1 complex *in vitro*, various *in vivo* experiments indicate that other factors regulate this interaction. Disrupting Cul1-SRC complex formation *in vivo* has limited effects on CRL1-CAND1 interactions (39). Similarly, inhibiting CRL neddylation in cells did not promote CRL1-CAND1 interactions, nor did it destabilize CRL1-SRC complexes (40). These observations support the notion that other factors regulate CRL-CAND1 interactions *in vivo*, which is in keeping with the findings of this study.

At the present time, it remains unclear whether COMMD1 acts to synergize the SRC recycling or whether it constitutes a parallel activation pathway. Until the precise mechanisms of CAND1 displacement are further elucidated, addressing these questions will remain problematic with our current systems. Nevertheless, the fact that COMMD1 binds to Cullins in a manner that is distinct from that used by SRCs suggests that these two mechanisms of CAND1 displacement may be related but are still independent from each other.

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Supplementary Figure 1
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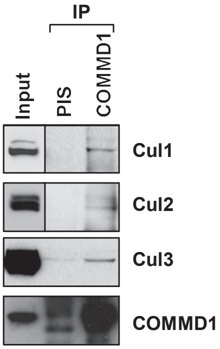

Fig. S1: COMMD1 interacts with endogenous Cullin proteins. Endogenous COMMD1 was immunoprecipitated and the presence of endogenous Cul1, Cul2 and Cul3 was determined by immunoblotting. In this experiment, a buffer containing 1% Triton X-100 was utilized (HEPES 25mM, NaCl 100 mM, EDTA 1 mM, glycerol 20%, Triton X-100 1%).

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Supplementary Figure 2

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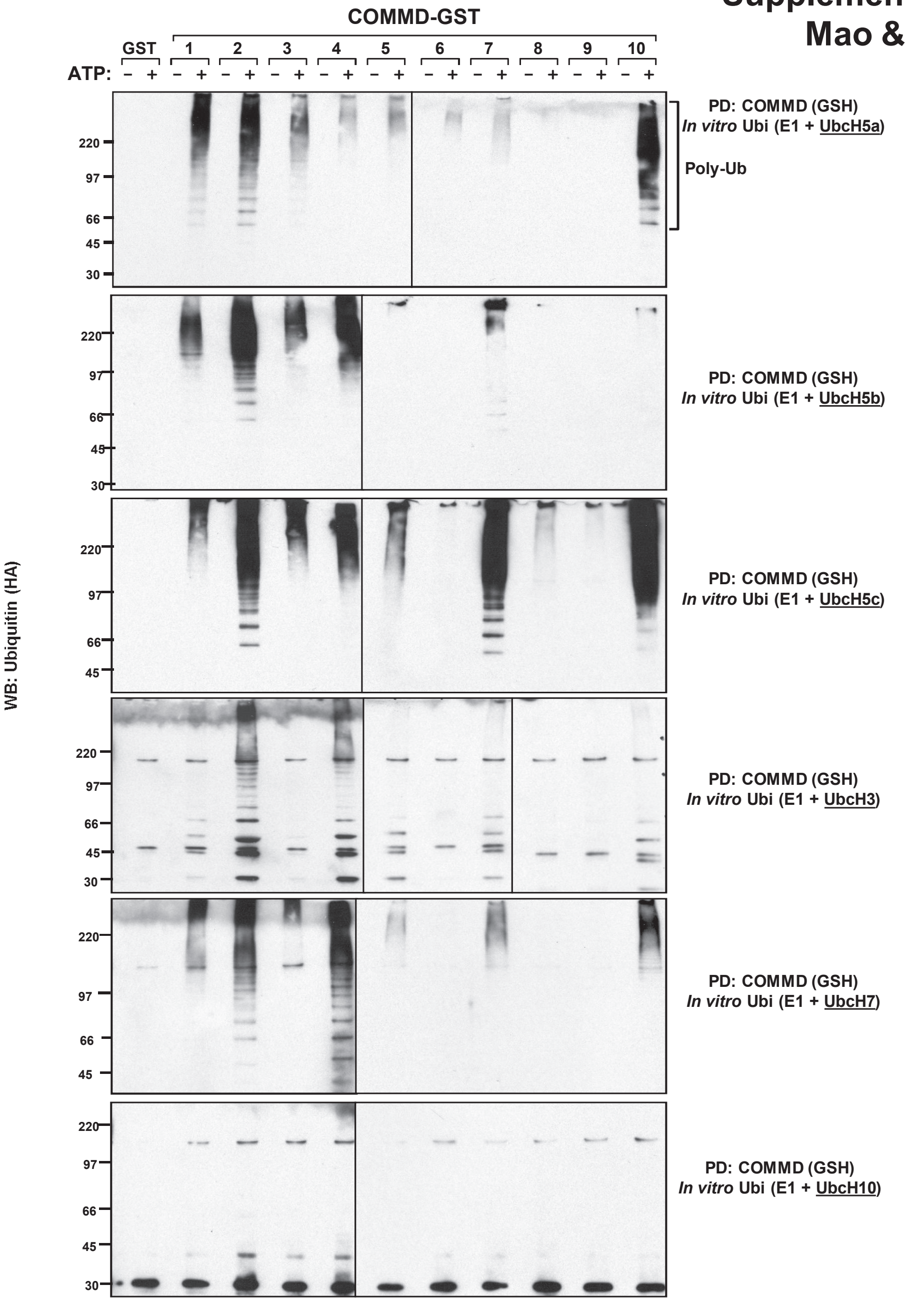
COMMD-GST
GST
1
2
3
4
5
6
7
8
9
10
ATP: – + – + – + – + – + – + – + – + – + – + – +
WB: Ubiquitin (HA)
220
97
66
45
30
PD: COMMD (GSH)
In vitro Ubi (E1 + UbcH5a)
Poly-Ub
PD: COMMD (GSH)
In vitro Ubi (E1 + UbcH5b)
PD: COMMD (GSH)
In vitro Ubi (E1 + UbcH5c)
PD: COMMD (GSH)
In vitro Ubi (E1 + UbcH3)
PD: COMMD (GSH)
In vitro Ubi (E1 + UbcH7)
PD: COMMD (GSH)
In vitro Ubi (E1 + UbcH10)

Fig. S2: COMMD proteins precipitate ubiquitin ligase activity. COMMD proteins fused to GST were precipitated and added to an *in vitro* ubiquitination reaction as in Fig 2C. In these experiments, different recombinant E2 enzymes were utilized as noted.

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Supplementary Figure 3
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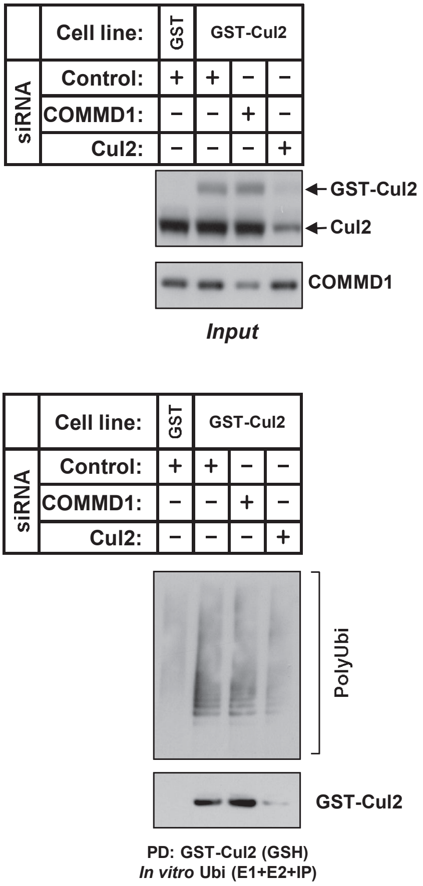
siRNA
Cell line:
GST
GST-Cul2
Control: + + − −
COMMD1: − − + −
Cul2: − − − +
GST-Cul2
Cul2
COMMD1
Input
siRNA
Cell line:
GST
GST-Cul2
Control: + + − −
COMMD1: − − + −
Cul2: − − − +
PolyUbi
GST-Cul2
PD: GST-Cul2 (GSH)
In vitro Ubi (E1+E2+IP)

Fig. S3: Effect of COMMD1 deficiency on CRL2 activity. (A) HEK293 cells stably expressing GST or GST-Cul2 were generated by lentiviral infection and selection. These cells were subsequently transfected with siRNA against COMMD1, an irrelevant sequence (Control), or Cul2 (as an positive control). A western blot against Cul2 was utilized to determine the expression of Cul2 and GST-Cul2 (upper panel). Similarly, a COMMD1 western blot demonstrates the effect of siRNA. (B) Using these cells, GST-Cul2 was precipitated by GSH-agarose beads. The recovered material was provided to an *in vitro* ubiquitination reaction as a source for E3 activity. Polyubiquitin chain formation was determined by immunoblotting.

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Chapter 6

GCN5 is a required cofactor for a ubiquitin ligase that targets NF-κB/RelA

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GCN5 is a required cofactor for a ubiquitin ligase that targets NF-κB/RelA

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The transcription factor NF-κB is a critical regulator of inflammatory and cell survival signals. Proteasomal degradation of NF-κB subunits plays an important role in the termination of NF-κB activity, and at least one of the identified ubiquitin ligases is a multimeric complex containing Copper Metabolism Murr1 Domain 1 (COMMD1) and Cul2. We report here that GCN5, a histone acetyltransferase, associates with COMMD1 and other components of the ligase, promotes RelA ubiquitination, and represses κB-dependent transcription. In this role, the acetyltransferase activity of GCN5 is not required. Interestingly, GCN5 binds more avidly to RelA after phosphorylation on Ser 468, an event that is dependent on IKK activity. Consistent with this, we find that both GCN5 and the IκB Kinase (IKK) complex promote RelA degradation. Collectively, the data indicate that GCN5 participates in the ubiquitination process as an accessory factor for a ubiquitin ligase, where it provides a novel link between phosphorylation and ubiquitination.

[*Keywords*: NF-κB; RelA; GCN5; COMMD1; ubiquitin; Cul2]

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NF-κB is a dimeric transcription factor formed by members of a highly conserved family of proteins that share an ~300-amino-acid region termed the Rel Homology Domain (RHD) (Karin and Lin 2002). The activity of NF-κB is primarily regulated by cytoplasmic sequestration of the NF-κB dimer through its interaction with inhibitory IκB proteins (Baeuerle and Baltimore 1988). Activation of the multimeric IκB Kinase (IKK) results in the phosphorylation of IκB, followed by its ubiquitination and degradation (Henkel et al. 1993; Chen et al. 1995), allowing the translocation of NF-kB into the nucleus. Nuclear NF-kB dimers bind to an array of promoters, ultimately resulting in the induction of genes involved in processes such as immunity, apoptosis, cell cycle progression, and oncogenesis (Silverman and Maniatis 2001). Termination of NF-κB activity is mediated by resynthesis of IκB proteins, which facilitate nuclear export of NF-κB (Arenzana-Seisdedos et al. 1997). More recently, additional mechanisms for transcriptional regulation have been recognized, including post-translational modifications of NF-κB subunits such as phosphorylation (Zhong et al. 1998; Sakurai et al. 1999), acetylation (Chen et al. 2001; Kiernan et al. 2003), prolyl-isomerization (Ryo et al. 2003), and ubiquitination (Saccani et al. 2004; Maine et al. 2007; Tanaka et al. 2007).

Copper Metabolism Murr1 Domain-containing (COMMD) proteins are a group of evolutionarily conserved factors present in a wide range of organisms (Burstein et al. 2005). COMMD1, the prototype member of the family, binds to a conserved domain present in the RHD of all NF-κB subunits (Ganesh et al. 2003; Burstein et al. 2005) and inhibits NF-κB-mediated transcription, leading to decreased proinflammatory gene expression (Maine et al. 2007) and impaired HIV-1 viral replication (Ganesh et al. 2003). We recently reported that COMMD1 inhibits NF-κB by promoting the ubiquitination and proteasomal degradation of NF-κB subunits (Maine et al. 2007). COMMD1 interacts with a multimeric ubiquitin ligase that also contains Elongins B and C, Cul2, and SOCS1 that is capable of ubiquitinating NF-κB subunits (Ryo et al. 2003; Maine et al. 2007). Interestingly, another report implicates IKKα-mediated phosphorylation of RelA to the subsequent degradation of this NF-κB subunit, although the precise

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pathway of degradation has not been elucidated (Lawrence et al. 2005). In particular, any potential links between COMMD1-mediated ubiquitination and the phosphorylation of RelA are not known.

Here we report that GCN5, a histone acetyltransferase (HAT), is a COMMD1-associated factor. Unlike its well-known role as a transcriptional activator (Thomas and Chiang 2006), we find that GCN5 functions as a repressor of NF-κB by promoting the ubiquitination and degradation of RelA, an activity consistent with its interaction with the COMMD1-containing ligase. Interestingly, GCN5 binds primarily to the C terminus of RelA, and this interaction is enhanced by IKK-dependent phosphorylation on Ser 468. The ability of GCN5 to bind to phosphorylated RelA and to the COMMD1 complex provides a link between IKK-mediated phosphorylation, ubiquitination, and the termination of κB-mediated transcription.

Results

Identification of GCN5 as a COMMD1-associated factor

Using tandem affinity purification (TAP), we previously identified a number of putative COMMD1-associated factors (Burstein et al. 2005). The HAT GCN5 was tentatively identified among them through a C-terminal peptide detected by LC/MS-MS (Fig. 1A). This interaction was first confirmed through independent coimmunoprecipitation experiments. Precipitation of GST-tagged COMMD1 from cell lysates resulted in the coprecipitation of GCN5, and, furthermore, binding to GCN5 was mapped to the COMM domain, the homology domain that is present in all COMMD proteins (Fig. 1B). Indeed, another COMM domain-containing protein, COMMD10, was also able to interact with GCN5 (Fig. 1C). Furthermore, immunoprecipitation of endogenous COMMD1 resulted in the coprecipitation of endogenous GCN5, and the converse precipitation also demonstrated the presence of an endogenous COMMD1/GCN5 complex in cells (Fig. 1D).

GCN5 inhibits NF-κB-mediated transcription

Given the role of COMMD1 as an NF-κB inhibitor, we examined whether GCN5 plays a role in the regulation of this pathway. Transfection of 293 cells with siRNA against GCN5 led to exaggerated induction of *TNF* mRNA in response to TNF stimulation (Fig. 1E); *CX3CL1* (fractalkine) and *TNFAIP3* (A20) were similarly up-regulated (Supplemental Fig. S1A,B). Conversely, expression of GCN5 by plasmid transfection inhibited the induction of the *TNF* gene (Fig. 1F). Moreover, concurrent expression of an siRNA-resistant mutant of GCN5 abrogated the effect of the siRNA oligonucleotide on the expression of *TNFAIP3* (Supplemental Fig. S1E). Altogether, these data supported the conclusion that these are specific effects of the siRNA oligonucleotide targeting GCN5. Similar experiments were performed in U2OS cells after lentiviral delivery of a shRNA targeting GCN5 and, once again, GCN5-deficient cells demonstrated exaggerated expression of NF-κB-regulated genes after TNF stimulation, such as *ICAM1* (Fig. 1G) as well as *IL8* and *TNF* (Supplemental Fig. S1C,D). These data indicated that endogenous GCN5 inhibits several TNF-inducible genes in more than one cell line. To address whether the repressive effect of GCN5 is mediated through NF-κB itself, *TNF* gene expression was examined after depletion of RelA, a key NF-κB subunit. The efficiency of the RNAi was demonstrated by Western blotting (Supplemental Fig. S2). As predicted, stimulated *TNF* expression was dampened by RelA deficiency (Fig. 1H, open bars). Interestingly, the exaggerated expression of *TNF* in GCN5-deficient cells was lost upon concurrent deficiency in RelA (Fig. 1H, black bars), supporting the notion that GCN5 acts on RelA to suppress the *TNF* gene.

GCN5 interacts with RelA and promotes its ubiquitination

Given the known interaction between COMMD1 and RelA, we investigated whether GCN5 similarly interacts with RelA. Immunoprecipitation of endogenous GCN5 resulted in the recovery of RelA from whole-cell lysate (WCL) or nuclear extracts, and, in both instances, TNF stimulation resulted in greater coprecipitation (Fig. 2A). Next, we resorted to a sequential affinity purification scheme to examine whether purified GCN5/COMMD1 complexes could interact with endogenous RelA. As expected, GCN5 bound both COMMD1 and RelA (Fig. 2B, PD: GCN5), and importantly, the purified GCN5/COMMD1 complex also contained endogenous RelA (Fig. 2B, Re-PD: COMMD1).

The interaction between GCN5 and COMMD1 and their shared ability to bind to and inhibit NF-κB suggested that these two proteins might work through a common mechanism. Hence, we examined if, similar to COMMD1, GCN5 could promote RelA ubiquitination in the nucleus. Nuclear extracts were prepared and endogenous RelA was precipitated after denaturing the lysate by adding SDS and boiling, and the recovered material was immunoblotted for polyubiquitin. Expression of GCN5 in 293 cells resulted in greater amounts of ubiquitinated RelA (Fig. 2C, right), while the level of ubiquitinated RelA was decreased in U2OS cells deficient in GCN5 (Fig. 2C, left). This finding was confirmed by another approach, where cells were transfected with His$_6$-tagged Ubiquitin and ubiquitinated RelA was detected after precipitating ubiquitinated proteins with nickel agarose beads. As shown in Supplemental Figure S3A, decreased GCN5 expression also led to decreased recovery of ubiquitinated RelA by this approach. Moreover, GCN5 deficiency impaired the ability of COMMD1 to promote RelA ubiquitination (Supplemental Fig. S3B), suggesting that GCN5 is a required cofactor for COMMD1-mediated ubiquitination, an effect that is congruent with its interaction with COMMD1.

GCN5 controls nuclear RelA stability and its interaction with promoter sites

Previous studies indicate that RelA ubiquitination is necessary for the proper termination of RelA–promoter

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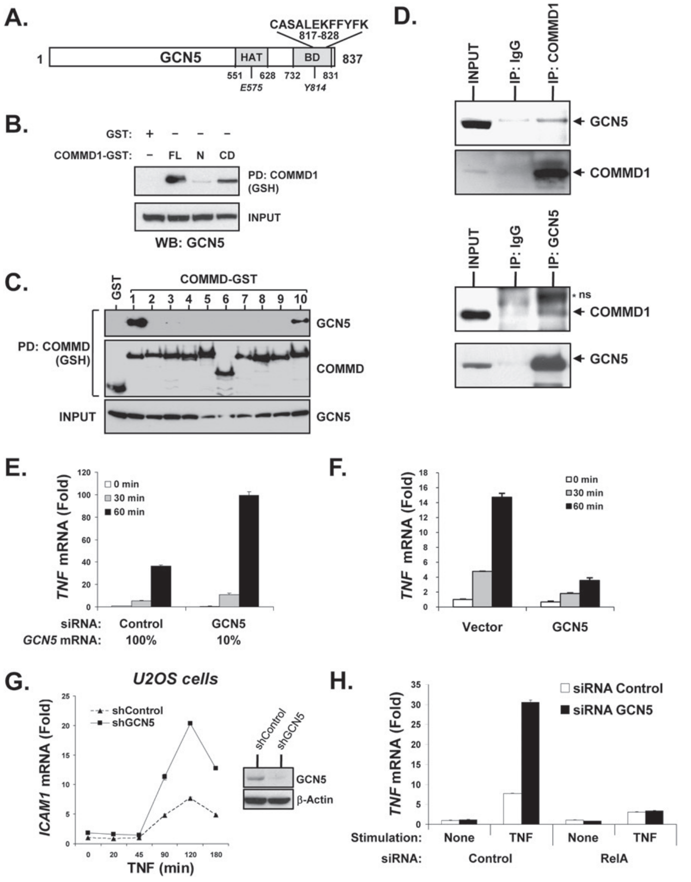

Figure 1. GCN5 binds to COMMD1 and inhibits NF-κB-mediated transcription. (*A*) Identification of GCN5 as a COMMD1-associated factor. Schematic representation of GCN5 with the boundaries of its HAT domain and Bromo domain (BD). The C-terminal peptide of GCN5 (amino acids 817–828) identified by LC/MS-MS is shown. The HAT activity and Bromo domain function are dependent on residues E575 and Y814, respectively. (*B*) COMMD1 binds GCN5 through its COMM domain. GCN5 was coexpressed with full-length COMMD1 (FL), its N terminus (N), or its COMM domain (CD) fused with GST. Subsequently, COMMD1 was precipitated and the recovered material was immunoblotted for GCN5. (*C*) GCN5 interacts with other COMMD proteins. HEK293 cells were cotransfected with GCN5 and the indicated COMMD proteins fused to GST, which were subsequently precipitated from cell lysates using GSH Sepharose beads. The presence of coprecipitated GCN5 was determined by immunoblotting. (*D*) Coprecipitation of endogenous GCN5 and COMMD1. Cell extracts were subjected to immnoprecipitation for COMMD1 (*top* panel) or GCN5 (*bottom* panel). Immunoblotting for GCN5 and COMMD1 was performed as indicated (ns, nonspecific band; input is 0.5% of the IP material). (*E–G*) GCN5 represses NF-κB-dependent gene expression. HEK293 cells were transiently transfected with siRNA against GCN5 (*E*) or a GCN5 expression vector. U2OS cells were stably transduced with a lentivirus expressing shRNA against GCN5 (*F*). After TNF stimulation, the indicated transcript levels were measured by qRT–PCR. (*H*) The effect of GCN5 is dependent on RelA. HEK293 cells were transiently transfected with siRNA against GCN5, and additionally, cells received control siRNA transfection (open bars) or siRNA transfection against RelA (black bars). The effect on *TNF* transcript levels was assessed by qRT–PCR.

interactions (Saccani et al. 2004). Consistent with this, COMMD1 deficiency is associated with prolonged RelA binding to promoter sites (Burstein et al. 2005) and leads to blunted disappearance of RelA from the nucleus (Maine et al. 2007). Therefore, we examined whether GCN5 deficiency would similarly result in both of these events. First, nuclear levels of RelA in GCN5-deficient U2OS cells were compared with the corresponding control. IκB resynthesis and nuclear export, a negative feedback loop in the NF-κB pathway, was inhibited by cycloheximide (CHX). As shown in Figure 2D, GCN5 deficiency led to greater RelA nuclear levels, particularly at late time points after TNF stimulation. Next, we examined RelA recruitment to the *ICAM1* and *IL8* promoters by chromatin immunoprecipitation (ChIP) and found that in both instances, GCN5 deficiency led to prolonged promoter occupancy by RelA (Fig. 2E). In addition, these experiments demonstrated that GCN5 was inducibly recruited to these promoter sites. Altogether, the data indicated that akin to COMMD1, GCN5 participates in controlling the levels of nuclear and chromatin-associated RelA.

The HAT activity of GCN5 is dispensable for its role in RelA degradation

RelA can be acetylated by p300 (Chen et al. 2001, 2002), and the GCN5 homolog PCAF has also been reported to acetylate RelA (Kiernan et al. 2003). Therefore, we evaluated whether GCN5-promoted RelA ubiquitination could be linked to RelA acetylation. While p300 promoted significant accumulation of acetylated RelA, neither GCN5 nor PCAF had an appreciable effect (Supplemental Fig. S4). Nevertheless, the HAT activity of GCN5 could

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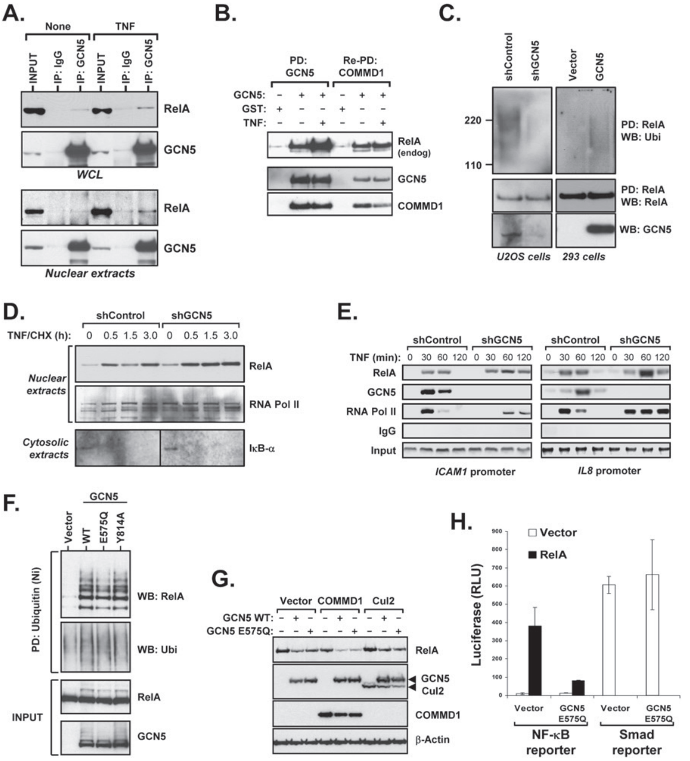

Figure 2. GCN5 interacts with RelA and promotes its ubiquitination. (*A*) Coprecipitation of endogenous RelA and GCN5. WCLs or nuclear extracts were prepared from unstimulated and TNF-stimulated HEK293 cells. GCN5 was immunoprecipitated, and the recovered material was immunoblotted for RelA (input is 0.8% of the IP material). (*B*) The GCN5/COMMD1 complex interacts with endogenous RelA. GCN5 fused with GST and COMMD1 fused to a biotinylation tag were expressed in cells. GCN5 was purified through a GSH affinity column (PD: GCN5); GCN5 was eluted from the column and COMMD1 was precipitated from this fraction using streptavidin-agarose beads (Re-PD: COMMD1). Western blots for endogenous RelA, GCN5, and COMMD1 are presented. (*C*) GCN5 promotes the ubiquitination of endogenous RelA. Nuclear extracts from U2OS GCN5-deficient cells (*left* panels) or HEK293 cells transfected with GCN5 (*right* panels) were prepared. A denatured immunoprecipitation for RelA was subsequently performed and the presence of ubiquitinated RelA was determined by immunoblotting for ubiquitin. The deficiency and overexpression of GCN5 were determined by Western blotting for GCN5. (*D*) GCN5 deficiency increases nuclear RelA level. The GCN5-deficient U2OS cell line or the corresponding control line were treated with TNF for 10 min, followed by cycloheximide (CHX). At the indicated time points, cells were harvested and nuclear extracts were prepared and immunobloted for RelA and RNA Pol II (as a loading control). (*Bottom* panel) IκB-α levels in cytosolic extracts were determined by Western blotting to monitor the effect of TNF and CHX. (*E*) GCN5 deficiency prolongs RelA binding to NF-κB-responsive promoters. The same GCN5-deficient and control U2OS cell lines were treated with TNF as before. At the indicated time points, cells were lysed and used for ChIP. Occupancy of RelA, GCN5, and RNA Pol II on the *ICAM1* and *IL8* gene promoters was analyzed. (*F*) GCN5 promotes RelA ubiquitination despite inactivating mutations in its HAT or Bromo domains. GCN5 wild-type, E575Q (HAT-deficient), or Y814A (Bromo domain-deficient) were cotransfected with HA-RelA and His$_6$-tagged ubiquitin, and the levels of ubiquitinated RelA were determined as before. (*G*) GCN5 promotes the degradation of RelA independently of its HAT activity. HEK293 cells were transfected with RelA along with GCN5 wild-type or E575Q mutant, in combination with COMMD1 or Cul2 as indicated. Cells were lysed and the expression of RelA was determined by Western blot. (*H*) The HAT activity of GCN5 is dispensable for repression of κB-dependent transcription. The effects of GCN5 E575Q on an NF-κB-responsive reporter (3κB-luc) or a Smad-responsive reporter (SBE-JONK) were examined by luciferase assay.

be more broadly involved in RelA ubiquitination, perhaps acting on a different substrate. To test this notion, we introduced an E575Q mutation in GCN5 to inactivate its HAT domain (Tanner et al. 1999) or a Y814A point mutation that disrupts acetylated-lysine recognition by its Bromo domain (Hudson et al. 2000). Interestingly, neither point mutation significantly impaired GCN5-mediated RelA ubiquitination (Fig. 2F). Indeed, the HAT-deficient point mutant E575Q promoted the degradation of RelA to a similar extent as wild-type GCN5 (Fig. 2G) and it inhibited RelA-mediated activation of an NF-κB-responsive reporter plasmid, whereas it did not affect the activity of a Smad-responsive promoter (Fig. 2H). Altogether these results indicated that GCN5-promoted RelA ubiquitination is separable from its known HAT activity or the function of its Bromo domain, establishing this effect as a separable novel property of GCN5.

GCN5 interacts with the COMMD1-containing ligase

COMMD1 promotes RelA ubiquitination through its interaction with a Cul2-containing ubiquitin ligase (Maine et al. 2007), suggesting that GCN5 could also interact with this complex. To investigate this possibility, GCN5 expressed in mammalian cells was precipitated from cell lysates and offered to an in vitro reaction

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containing the E1 and E2 (UbcH5a) enzymes, recombinant ubiquitin, and an ATP regenerating buffer. Ubiquitin ligase activity, evident by polyubiquitin chain formation in vitro, was readily recovered. In contrast, recombinant GCN5 prepared in *Escherichia coli* was devoid of activity (Fig. 3A). However, when recombinant GCN5 was mixed with cellular lysates and subsequently extensively washed, ubiquitin ligase activity was reconstituted, suggesting that that the activity detected was secondary to the association of GCN5 with other cellular factors. Interestingly, its interaction with the substrate, RelA, was not responsible for precipitating this activity, as this was evident when using lysates from *rela*-deficient fibroblasts (Supplemental Fig. S5).

We were particularly interested in knowing whether GCN5 could interact with Cul2 complexes, given the role of Cul2 as the main scaffold subunit of the COMMD1-containing ligase that targets RelA. To address this question, Cul2 complexes were purified from cells, and expected partner proteins such as COMMD1 and Elongin C were identified in this material (Fig. 3B). Importantly, GCN5 was also copurified, while TAF6, a component of GCN5-containing coactivator complexes such as TFTC and STAGA, was absent. Additionally, the converse precipitation of GST–GCN5 from cell lysates also coprecipitated endogenous Cul2 (Fig. 3C) and this was not affected by TNF stimulation (Supplemental Fig. S6). Interestingly, the recovered Cul2 was predominantly the active neddylated form, which is enriched in the cell nucleus, where GCN5 resides (Maine et al. 2007).

Collectively, these data supported the notion that GCN5 does not have intrinsic ligase activity, but interacts with an active Cul2/COMMD1 ubiquitin ligase. However, it was recently reported that PCAF, a close homolog of GCN5, possesses intrinsic ubiquitin ligase activity in its N terminus, a region that is conserved between the two proteins (Linares et al. 2007). Therefore, we examined which region in GCN5 is responsible for its interaction with ubiquitin ligase components. The C terminus of GCN5 (HAT/Bromo, amino acids 491–837) was found to mediate its interactions with COMMD1 and Cul2 (Fig. 3D), and, in contrast to PCAF, this domain, and not the N terminus, was able to provide ligase activity in vitro (Fig. 3E).

RelA phosphorylation enhances its binding to GCN5

While we had determined that GCN5 promotes RelA ubiquitination, a unique role for GCN5 in this process was not yet apparent. To address this question, we examined in more detail the GCN5–RelA interaction, and the mechanism behind its TNF-inducible nature (Fig. 2A,B). First, cells transfected with GCN5 were treated with various IKK-activating stimuli, namely TNF, IL1-β, or Flagellin, and the formation of the GCN5–RelA complex was assessed by coprecipitation of endogenous RelA. All of these forms of cell stimulation resulted in increased recovery of RelA (Fig. 4A). This suggested that the inducible formation of the GCN5–RelA complex could be a downstream consequence of IKK activation, such as the translocation of RelA into the nucleus, where GCN5 resides. Therefore, we first examined whether the effect of TNF could be recapitulated under in vitro conditions, where the cellular redistribution of RelA would not be a factor. Cell lysates were prepared after TNF stimulation and offered to bacterially made recombinant GCN5–GST for in vitro binding. After precipitation of GCN5, endogenous RelA could be easily recovered, and, importantly, TNF stimulation prior to lysis enhanced the ability of GCN5 to bind to RelA (Fig. 4B). Since the interaction in this experiment occurred post-lysis, the effect of TNF could not be explained by cellular redistribution of RelA.

Another consequence of IKK activation is IκB degradation, and, therefore, we speculated that perhaps IκB might regulate GCN5–RelA complex formation. To examine this notion, cells were transfected with IκB-α superdominant (SD), a stable mutant that lacks the IKK phosphorylation sites. As shown in Figure 4C, the interaction between GCN5 and RelA was not impaired by IκB-α SD expression and, in fact, this protein was incorporated into the GCN5–RelA complex, indicating that IκB proteins do not impair the GCN5–RelA interaction.

Finally, we examined whether phosphorylation of RelA itself might be responsible for its increased binding to GCN5. To this end we repeated the in vitro binding experiment after treating cells with Calyculin A, a phosphatase inhibitor that causes dramatic accumulation of phosphorylated RelA (Supplemental Fig. S7). Exposure to this agent prior to cell lysis resulted in very dramatic enhancement of RelA–GCN5 binding in vitro (Fig. 4D). Importantly, treatment of cellular lysates with recombinant λ-protein phoshatase (λ-PPase) prior to incubation with GCN5 reversed the binding of RelA to GCN5 to the unstimulated level (Fig. 4E).

RelA phosphorylation is linked to its ubiquitination and degradation

Since RelA phosphorylation enhances its binding to GCN5, phosphorylation would be expected to trigger RelA ubiquitination. Indeed, prior reports indicate that LPS and other TLR ligands that promote IKK activation and RelA phosphorylation also accelerate its ubiquitination (Tanaka et al. 2007). We examined this notion by determining the effect of Calyculin A on the ubiquitination of RelA in vivo, and found that this agent promoted significant accumulation of ubiquitinated RelA (Fig. 4F). In addition, phosphorylated RelA accumulated more dramatically when a proteasomal inhibitor was also used (Fig. 4G), consistent with the notion that phosphorylated RelA is unstable and undergoes ubiquitination and proteasomal degradation. Finally, we examined the effect of Calyculin A on the ubiquitination of RelA in vitro. To this end, GCN5 was expressed and subsequently precipitated from cell lysates and offered as a source for ligase activity in vitro. Coprecipitation of endogenous RelA was observed when this material was immunoblotted for RelA. Interestingly, high molecular weight material accumulated after the ubiquitination reaction in the

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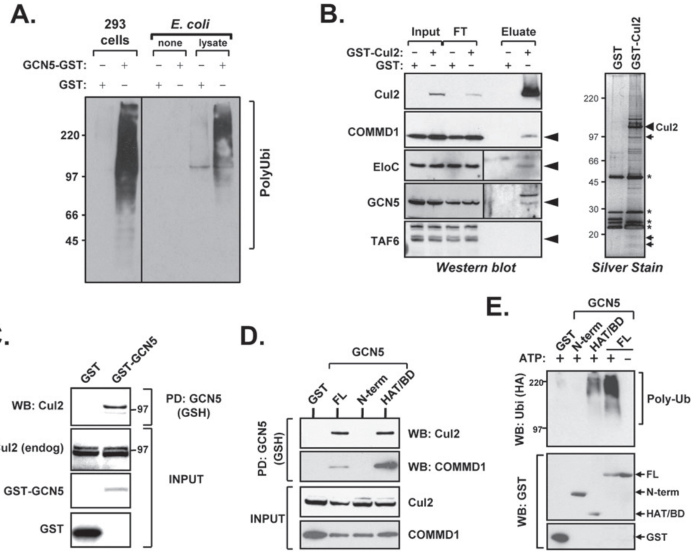

Figure 3. GCN5 interacts with the COMMD1-containing ligase. (*A*) GCN5 precipitates ubiquitin ligase activity. GCN5–GST or GST precipitated from transfected HEK293 cells, or recombinant (r) GST, or GCN5–GST prepared in *E. coli* were added to an in vitro ubiquitination reaction. In addition, rGST or rGCN5–GST were mixed with an HEK293 cell lysate, incubated at 4°C for 2 h, and then extensively washed prior to being used as a source for ubiquitin ligase activity. Formation of polyubiquitin chains in the reaction was determined by SDS-PAGE and immunoblotting for ubiquitin. (*B*) GCN5 was copurified as a Cul2-associated factor. Lysates were prepared from HEK293 cells overexpressing a GST-Cul2 fusion protein (Input) and were applied to a GSH-Sepharose affinity column, from which Cul2-containing complexes were subsequently eluted and concentrated by filtration (Eluate). The input, the flow-through material not bound to the column (FT), and eluate were subjected to immunoblotting for Cul2, COMMD1, Elongin C (EloC), GCN5, and TAF6. Silver stain of the eluate is shown in the *right* panel with the bands corresponding to Cul2, and the immunoblotted subunits noted by small arrows. Nonspecific bands are indicated by an asterisk (*). (*C*) GCN5 binds to Cul2, the main scaffold protein of the COMMD1-containing ligase. HEK293 cells were transfected with GST or GST–GCN5, which were precipitated from cell lysates. The presence of coprecipitated endogenous Cul2 was determined by Western blot. The position of the 97-kDa molecular weight marker is indicated. (*D*) The C terminus of GCN5 binds to COMMD1 and Cul2. Cells were transfected with GCN5 full-length (FL) fused to GST or the indicated truncation mutants along with Flag-Cul2 or COMMD1-Flag. GCN5 was precipitated by GSH Sepharose beads, and the presence of Cul2 or COMMD1 in the precipitated material was determined by Western blot analysis. (*E*) The C terminus of GCN5 containing the HAT and Bromo domains precipitates E3 ligase activity. GCN5 full-length (FL) fused to GST or truncation mutants spanning its N terminus (N-term, amino acids 1–491) or C terminus (HAT/Bromo, amino acids 492–837) were expressed in mammalian cells. These proteins were precipitated from cell lysates, added to in vitro ubiquitination reactions, and polyubiquitin chain formation was determined as before.

presence of ATP (Fig. 4H). This was particularly evident when the cells had been treated with Calyculin A prior to lysis, with the caveat that this treatment also increased the amount of RelA that coprecipitates with GCN5. Altogether, the data indicated that GCN5-associated ligase activity can ubiquitinate RelA, particularly when it is phosphorylated. This is consistent with the observed propensity of phosphorylated RelA to bind to GCN5 and to be ubiquitinated and degraded in cells.

RelA S468 phosphorylation promotes GCN5 binding

We next turned our attention to the mechanism responsible for RelA–GCN5 complex formation by examining the domain in RelA responsible for GCN5 binding. In vitro translated radiolabeled GCN5 bound avidly to the transactivation domain of RelA (TAD, spanning amino acids 305–551) (Fig. 5A), in contrast to COMMD1, which binds to the N-terminal end of the RHD (Burstein et al. 2005; Maine et al. 2007). Similar results were observed when precipitating RelA truncation mutants expressed in human embryonic kidney (HEK) 293 cells (Supplemental Fig. S8A). In addition, binding between GCN5 and the TAD of RelA was similarly inducible by RelA phosphorylation and reversible by dephosphorylation with λ-PPase (Fig. 5B), indicating that the phosphorylation site(s) responsible for inducible binding might be contained within the TAD of RelA.

Further in vitro binding experiments indicated that the last 21 amino acids that constitute the first transactivation domain (TA1) within the TAD (Schmitz and Baeuerle 1991) are critical for GCN5 binding (Fig. 5C, RelA 1–530),

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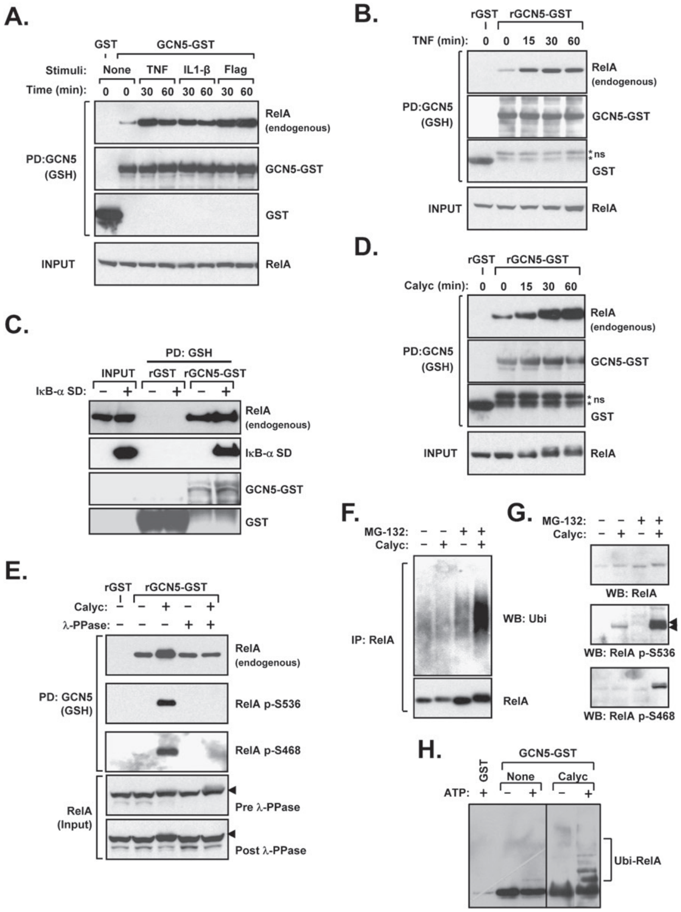

Figure 4. RelA phosphorylation enhances GCN5-RelA interactions. (*A*) Various IKK-activating stimuli promote GCN5–RelA binding. HEK293 cells were transfected with GCN5–GST or GST and subsequently treated with TNF, IL-1β, or Flagellin prior to lysis and GSH precipitation. The presence of coprecipitated endogenous RelA was determined by immunoblotting. (*B*) TNF treatment prior to lysis promotes RelA–GCN5 binding in vitro. HEK293 cells were treated with TNF or Calyculin A, and the obtained lysates were mixed with bacterially made rGST or rGCN5–GST, followed by precipitation. The presence of endogenous RelA in the recovered material was determined by immunoblotting. (*C*) IκB does not impair the GCN5–RelA interaction. HEK293 cells were transfected with IκB-α superdominant (SD), and the ability of recombinant GCN5 to precipitate endogenous RelA post-lysis was examined as in *B*. (*D*) The phosphatase inhibitor Calyculin A promotes RelA–GCN5 binding in vitro. HEK293 cells were treated with Calyculin A and the obtained lysates were mixed with rGST or rGCN5–GST, followed by precipitation. The presence of endogenous RelA in the recovered material was determined by immunoblotting. (*E*) Dephosphorylation of RelA abrogates its induced binding to GCN5. HEK293 cells were treated with Calyculin A and subsequently lysed in a buffer without phosphatase inhibitors. The lysate was incubated with λ-protein phosphatase (λ-PPase) as indicated, and in vitro binding of RelA to recombinant GCN5 was performed as in *D*. The recovered material was immunoblotted to detect the coprecipitation of endogenous RelA and phosphorylated RelA. (*F*) Phosphorylation promotes RelA ubiquitination. Wild-type MEFs were treated with MG-132 (30 min) and Calyculin A as indicated. RelA was subsequently immunoprecipitated from cell lysates and the presence of ubiquitinated RelA was determined by immunoblotting for ubiquitin. (*G*) Phosphorylated RelA is labile and is stabilized by proteasomal blockade. Wild-type MEFs were treated with Calyculin A and MG-132 as indicated. Phosphorylated RelA levels were determined by Western blot using two phospho-specific antibodies. (*H*) GCN5-associated ligase activity ubiquitinates RelA in vitro. HEK293 cells transfected with GCN5–GST or GST were treated without or with Calyculin A as shown. Material precipitated by GSH beads was applied to an in vitro ubqitination reaction as in Figure 3A. Coprecipitated RelA and its ubiquitination were shown by immunoblotting.

and similar results were obtained in coprecipitation experiments using mammalian cell lysates (Supplemental Fig. S8B). Additionally, a recombinant peptide prepared in *E. coli* and spanning most of the TA1 region (amino acids 531–547) was capable of completely disrupting the RelA–GCN5 complex (Fig. 5D), consistent with the notion that TA1 is a major interface for RelA–GCN5 binding. Interestingly, TA1 contains Ser 536, a major phosphorylation site in RelA that has been implicated previously in the stability of this protein (Lawrence et al. 2005). Therefore, we initially speculated that perhaps Ser 536 might be responsible for phosphorylation-dependent binding between RelA and GCN5. Indeed, loss of the last 21 amino acids of RelA abrogated all inducible binding between RelA–TAD and GCN5 (Fig. 5E, Δ21). However, point mutations of any of the six serines located in this region, including Ser 536, did not have a substantial effect on binding (data not shown). Surprisingly, even a combined mutation of all six serines in TA1 did not abrogate the inducible binding between RelA–TAD and GCN5 (Fig. 5E, 6S/A), suggesting that the phosphorylation site might be located elsewhere in the TAD. Indeed, examination

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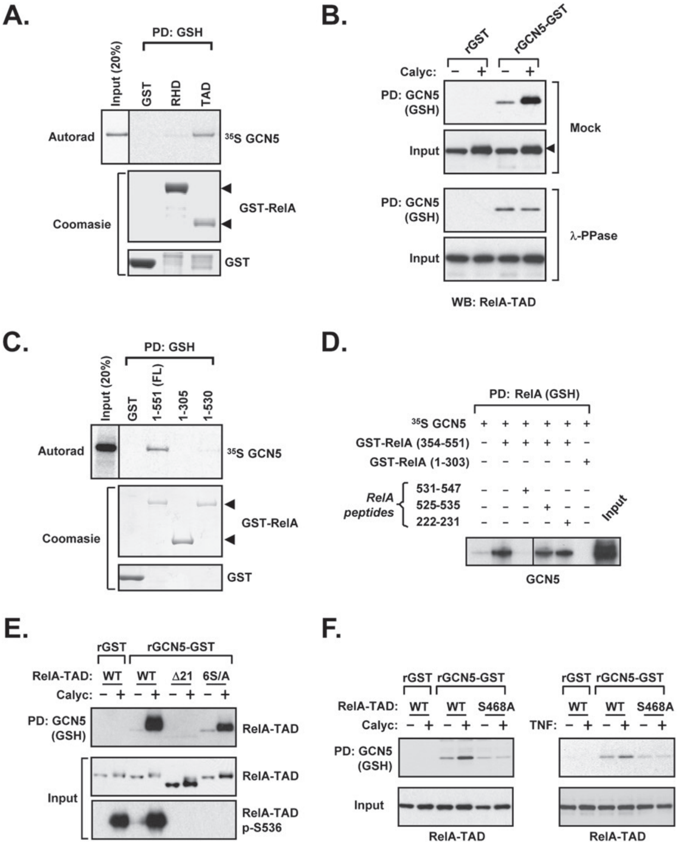
A.
PD: GSH
Input (20%)
GST
RHD
TAD
Autorad
³⁵S GCN5
Coomasie
GST-RelA
GST
B.
rGST
rGCN5-GST
Calyc: – + – +
PD: GCN5 (GSH)
Input
Mock
PD: GCN5 (GSH)
Input
λ-PPase
WB: RelA-TAD
C.
PD: GSH
Input (20%)
GST
1-551 (FL)
1-305
1-530
Autorad
³⁵S GCN5
Coomasie
GST-RelA
GST
D.
PD: RelA (GSH)
³⁵S GCN5
GST-RelA (354-551)
GST-RelA (1-303)
RelA peptides
531-547
525-535
222-231
Input
GCN5
E.
rGST
rGCN5-GST
RelA-TAD: WT WT Δ21 6S/A
Calyc: – + – + – + – +
PD: GCN5 (GSH)
RelA-TAD
Input
RelA-TAD
RelA-TAD p-S536
F.
rGST rGCN5-GST
RelA-TAD: WT WT S468A
Calyc: – + – + – +
TNF: – + – + – +
PD: GCN5 (GSH)
Input
RelA-TAD
RelA-TAD

Figure 5. The GCN5–RelA interaction requires the C terminus of RelA and is enhanced by phosphorylation at Ser 468. (*A*) GCN5 interacts with the transactivation domain of RelA. Recombinant RelA fragments fused to GST were purified from *E. coli* and their ability to precipitate radiolabeled in vitro translated GCN5 was examined by autoradiography. Coomasie staining demonstrates the amount of recombinant protein used. (*B*) The TAD of RelA is responsible for phosphorylation-inducible binding. The same experiment as in *B* was performed, but this time using lysates from cells transfected with RelA–TAD (306–551). (*Bottom* panel) In addition, λ-PPase treatment of the lysate was also performed prior to binding. (*C*) GCN5 interacts with TA1, encompassed by the last 21 amino acids of RelA. The indicated recombinant RelA fragments fused to GST were purified from HEK293 cells, and their ability to precipitate radiolabeled in vitro translated GCN5 was examined by autoradiography. Coomasie staining demonstrates the amount of recombinant protein used. (*D*) A peptide from TA1 is capable of disrupting GCN5–RelA binding in vitro. Recombinant RelA fragments fused to GST were purified from *E. coli*, and their ability to precipitate radiolabeled in vitro translated GCN5 was examined as in *A*. In addition, peptides spanning the indicated amino acid residues in RelA were added to the in vitro binding reaction as potential competitive inhibitors. (*E*) The phosphorylation residue that mediates RelA–GCN5 inducible binding is not contained in the last 21 amino acids of RelA. The same experiment as in Figure 4E, but this time using two mutations in the TAD: deletion of the last 21 amino acids (306–530), Δ21; or mutation of the last six serines in RelA (S535, 536, 543, 547, 550, 551A), 6S/A. (*F*) Ser 468 phosphorylation is required for RelA–GCN5-inducible binding. The same experiment as in *B* and *E*, but using either Calyculin A treatment (*left*) of TNF stimulation (*right*). Wild-type RelA or a point mutant at Ser 468 are compared.

of other serine phosphorylation sites within RelA indicated that mutation of Ser 468 abrogated the inducible binding between RelA and GCN5 in response to either Calyculin A or TNF treatments (Fig. 5F). Collectively, these data suggested that the last 21 amino acids of RelA provide the main domain for GCN5 binding, and that upon phosphorylation of RelA at Ser 468, additional conformational changes take place that increase its binding to GCN5.

IKK is required for RelA–GCN5 complex formation

Previous reports indicate that IKKβ or IKKε can phosphorylate RelA on Ser 468 (Schwabe and Sakurai 2005; Mattioli et al. 2006), while other studies have linked IKKα-mediated phosphorylation of RelA to its proteasomal degradation (Lawrence et al. 2005). Hence, we examined the possible participation of the IKK complex as the kinase for RelA that promotes GCN5 binding. To address this question, we used *Ikk*-deficient mouse embryo fibroblasts (MEFs) to examine the binding between RelA and GNC5 in vitro (Fig. 6A). TNF treatment prior to lysis induced RelA–GCN5 binding when lysates were prepared from wild-type fibroblasts, while loss of *Ikkα* or *Ikkβ* completely abrogated the effect of TNF. These data indicated that an intact Ikk complex is required for inducing the binding between RelA and GCN5, likely because of the role of this kinase complex in Ser 468 phosphorylation.

IKK and GCN5 are required for inducible RelA degradation

If the IKK complex promotes RelA–GCN5 binding, one predicted outcome would be that IKK activation should

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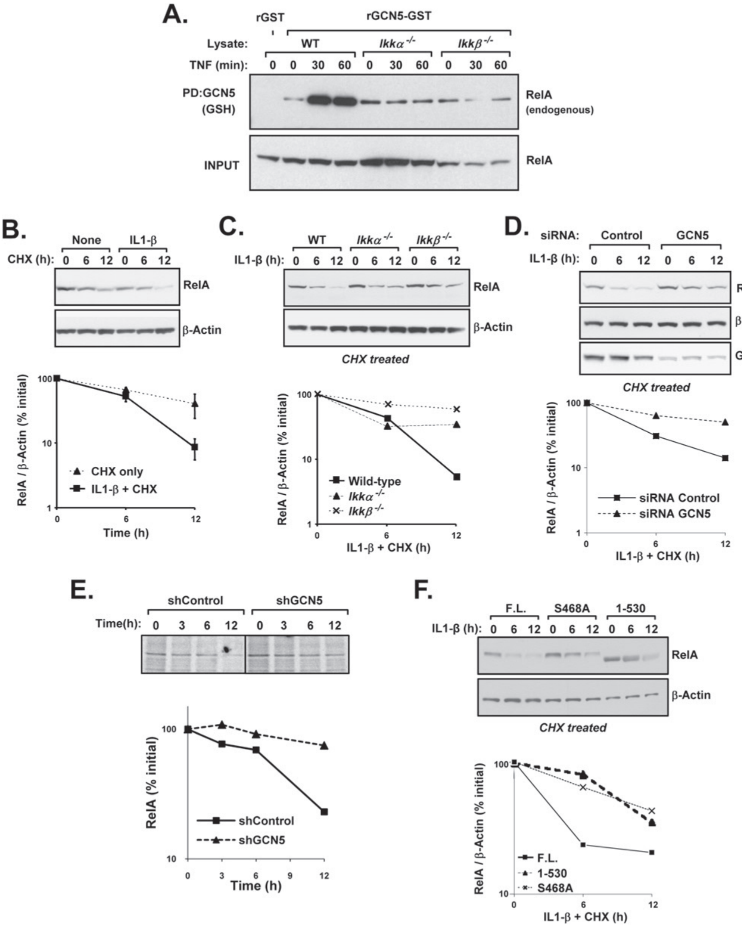

Figure 6. IKK and GCN5 promote RelA degradation. (*A*) IKK is required for TNF-induced RelA–GCN5 binding. Wild-type (WT), *Ikkα* $^{-/-}$, or *Ikkβ* $^{-/-}$ MEFs were stimulated with TNF and subsequently lysed. This material was applied to rGCN5–GST for in vitro binding as before. The coprecipitation of endogenous RelA was determined by immunoblotting. (*B*) IL1-β stimulation decreases RelA stability. Cycloheximide (CHX) was used to determine the stability of RelA in wild-type MEFs that were untreated or stimulated with IL1-β. RelA levels were determined by immunoblotting and densitometry analysis. (*C*) *Ikk* deficiency stabilizes RelA in IL1-β treated cells. Similar to *B*, but the effects of IL1-β treatment were compared between wild type, *Ikkα* $^{-/-}$, or *Ikkβ* $^{-/-}$ MEFs. (*D*) GCN5 deficiency prevents IL1-β-promoted degradation. HEK293 cells were transfected with the indicated siRNA and subsequently treated with IL1-β. The stability of RelA after cycloheximide (CHX) treatment was examined by immunoblotting and densitometry analysis. (*E*) GCN5 deficiency stabilizes RelA in U2OS cells. GCN5-deficient U2OS cells and the corresponding control cells were metabolically labeled by ^{35}S-methionine and ^{35}S-cysteine. At the indicated time points after labeling, cells were lysed for a denatured immunoprecipitation for RelA. The recovered material was examined by autoradiography after SDS-PAGE separation. (*F*) RelA 1–530 and RelA S468A are more stable than full-length (FL) RelA. The indicated human RelA constructs were introduced into *rela*-deficient MEFs through lentiviral infection and selection. Protein stability was examined as before.

destabilize RelA. Using IL1-β as a means of activating the kinase, we found that this led to decreased stability of RelA in wild-type fibroblasts compared with unstimulated cells (Fig. 6B). In addition, and consistent with the role of IKK in RelA phosphorylation, the protein was stabilized in *Ikk*-deficient fibroblasts treated with IL1-β (Fig. 6C). Similar to MEFs, cytokine stimulation resulted in a shorter half-life of RelA in 293 cells (data not shown), and most importantly, GCN5 deficiency greatly stabilized RelA in this context (Fig. 6D), akin to the effect of

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IKK deficiency. The stabilization of RelA was similarly observed in pulse-chase metabolic labeling studies examining GCN5-deficient U2OS cells (Fig. 6E). Finally, the stability of mutations in RelA that affect its binding to GCN5, namely a truncation of its TA1 (RelA 1–530) or loss of Ser 468 (S468A), was examined after stably reintroducing these proteins into *rela*-deficient MEFs. As shown in Figure 6F, these mutants were stabilized compared with the wild-type protein. Altogether, these findings are consistent with the role of IKK-mediated phosphorylation in promoting the RelA–GCN5 interaction, which in turn leads to RelA ubiquitination.

Discussion

An important phase in the regulation of κB-dependent transcription is the termination of NF-κB activity, which has been largely ascribed to NF-κB-mediated resynthesis of IκB proteins (Hoffmann et al. 2002). Recently, ubiquitination of NF-κB has been found to be an additional mechanism by which transcriptionally active NF-κB is suppressed (Saccani et al. 2004). One of the ligases responsible for this effect contains COMMD1 in association with a multimeric complex that contains Cul2 (Ryo et al. 2003; Maine et al. 2007), while others have identified PDLIM2 in a similar role (Tanaka et al. 2007). Additionally, phosphorylation of RelA by IKKα has been reported to destabilize the protein, and similar to ubiquitination, this controls the chromatin association of RelA (Lawrence et al. 2005). However, since Cul2-containing complexes are not known to engage phospho-serine substrates (Petroski and Deshaies 2005), a link between IKK-mediated phosphorylation of RelA and its ubiquitination by this ligase was not evident prior to this study.

This report indicates that GCN5, a HAT best known as an activator of transcription, functions in an unexpected manner in the NF-κB pathway, where it represses transcription by providing a critical link between serine phosphorylation of RelA and its ubiquitination. Various data presented here support a model where GCN5 promotes RelA ubiquitination by interacting directly with both the ubiquitin ligase and RelA in a manner akin to a substrate adaptor protein. Similarly, we conclude that in addition to the well-known core components associated with Cul2 (Ganoth et al. 2001), the ligase uses accessory factors such as COMMD1 and GCN5. Interestingly, the HAT activity of GCN5 is dispensable in this setting, as the point mutant GCN5 E575Q retains the ability to promote RelA ubiquitination and repress NF-κB-dependent transcription. Additionally, the sequential purification of the GCN5/COMMD1 complex suggests that only a small fraction of GCN5 interacts with COMMD1, leading us to speculate that most cellular GCN5 is probably engaged in its well-recognized role in coactivator complexes.

The most interesting property of GCN5 in this pathway is its ability to interact with RelA, preferentially after IKK activation. Our data indicate that phosphorylation of RelA itself is responsible for promoting GCN5–RelA binding, as the increased binding between these proteins can be reversed in vitro by treating the lysates with λ-PPase. This finding is corroborated by mutational analysis, which indicates that phosphorylation of RelA at Ser 468 is required for its inducible binding with GCN5. Interestingly, this phosphorylation event has been reported to have an inhibitory effect on transcription (Buss et al. 2004; Schwabe and Sakurai 2005; Mattioli et al. 2006), and, indeed, RelA S468A promotes greater expression of NF-κB target genes such *Il6* and *Tnf* (Supplemental Fig. S9). This finding is consistent with our observation that phosphorylation of Ser 468 facilitates RelA binding to GCN5, a preamble to the ubiquitination and degradation of the protein. In addition, the data also demonstrate that TA1, encompassed in the last 21 amino acids, is critically required for basal GCN5 binding. We speculate that upon Ser 468 phosphorylation, a conformational change in RelA likely takes place that possibly cooperates with TA1 to promote the GCN5–RelA interaction. Given that the structure of the TAD of RelA has not been solved, the nature of such conformational change remains unclear. Finally, the data presented here is consistent with prior reports that place these events in the nucleus, particularly on gene promoter sites. Indeed, GCN5, as well as COMMD1, are inducibly recruited to NF-κB-responsive promoters, and their deficiency results in prolonged occupancy of the promoter by RelA, an event that presumably explains the increased transcriptional response.

The participation of GCN5 in the ubiquitination pathway is consistent with genetic evidence that GCN5 has HAT-independent functions. Deficiency of *Gcn5* in mice prevents the formation of somites, a neural tube or a notochord, while a HAT-inactivating mutation in the *Gcn5* gene results in a milder phenotype (Bu et al. 2007). Finally, our study provides a mechanism to link IKK activation, RelA phosphorylation, and the degradation of DNA-bound, transcriptionally active RelA. In this model, activation of the IKK complex not only initiates NF-κB-mediated transcription, but sets in motion a process that ultimately inhibits active NF-κB through the targeting of RelA for GCN5 binding and ubiquitination.

Materials and methods

Plasmids and siRNA

The plasmids pEBB, pEBG, pEBB-COMMD1-Flag, pEBB-COMMD1-GST FL, N-term (amino acids 1–118), COMM domain (amino acids 119–190), pEBB-Flag-Cul2, pEBG-Cul2 FL pEBG-RelA FL and its truncations 1–305, 1–180, 306–551, pEBB-HA-RelA, pEBB-T7-IκB-α SD, pCW7-His$_6$-Myc-Ubiquitin, 3κB-luc, 2κB-luc, and SBE-JONK have been described previously (Hay et al. 2001; Burstein et al. 2004, 2005; Lewis et al. 2004; Maine et al. 2007). The full-length coding sequence for human GCN5 was obtained from an EST clone (IMAGE clone 5575574) to generate pEBB-GCN5 (untagged). Using this EST clone as template, PCR was used to generate the following plasmids: pcDNA3.1-GCN5, pEBB-Flag-GCN5, pET21b-GCN5–GST, pEBB-GCN5–GST and pEBG-GCN5 FL. Similarly, PCR was used to generate pEBG-GCN5 N-term (amino acids 1–491), pEBG-GCN5 HAT/Bromo domain (amino acids 492–837), pEBG RelA

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1–530, pEBB HA- RelA 1–530, pEBG RelA 1–450. pcDNA-Flag-p300 was a gift from Dr. Colin Duckett. Expression vectors for point mutants of GCN5 and RelA (pEBB-Flag GCN5 E575Q, Y814A, GCN5 RNAi-resistant, pEBG-RelA S536A, pEBB HA-RelA TAD S468A, pEBB HA-RelA 6S/A) were created by site directed mutagenesis (Stratagene). The constructs pGEX RelA RHD (1–303) and pGEX RelA TAD (354–551) were used for recombinant protein preparation in *E. coli* as described previously (Ramsey et al. 2008). The plasmid pEBB-TB was generated by amplifying the coding sequence for TEV cleavage site and Biotinylation signal from pFA6a HTB kanMX6 as template, kindly provided by Dr. Kaiser (Tagwerker et al. 2006). The COMMD1 coding sequence was subcloned into this plasmid to generate pEBB-COMMD1-TB. Synthetic siRNA oligonucleotides against Chloramphenicol acetyl transferase (a control target), human GCN5 and human RelA were used, and detailed information about targeting sequences are available upon request. The plasmids pHCMV-VSV-G, pMDLg/pRRE, pRSV Rev, and FG12 (Lois et al. 2002) were used for the generation of recombinant lentiviruses (kindly provided by Dr. David Baltimore). For stable RNAi using lentiviral delivery, we introduced a cassette containing the Histone 1 promoter and a shRNA from pSUPER into the XbaI and XhoI sites of FG12.

Cell culture, transfection, and luciferase assays

HEK293 cells, HEK 293T cells, and U2OS cells were obtained from American Type Culture Collection and cultured in DMEM supplemented with 10% FBS and L-glutamine (2mM). *Ikkα*$^{-/-}$, *Ikkβ* $^{-/-}$, and wild-type MEFs were kindly provided by Dr. Inder Verma (Li et al. 1999a, b). All transient transfection experiments were performed in HEK293 cells using a standard calcium phosphate transfection protocol to transfect plasmids and siRNA (Burstein et al. 2004). In certain experiments, the following agents were applied at the indicated final concentration to the growth media: TNF (Roche, 1000 U/mL), IL-1β (Roche, 10 ng/mL), Flagellin (Inotek, 50 μg/mL), Calyculin A (Cell Signaling, 50 nM), Cycloheximide (BioVision, 60 μg/mL), and MG-132 (Boston Biochem, 40 μM). Luciferase assays were performed as described previously (Burstein et al. 2005).

TAP screening

The TAP screening performed here has been reported previously (Burstein et al. 2005). Briefly, 293 cells seeded in 15-cm plates were transiently transfected with pEBB-COMMD1-TAP (15 μg of plasmid per plate). Cell lysates were subsequently prepared and applied to a chromatography column containing IgG sepharose beads (GE Healthcare). After a 2-h incubation at 4°C, the column was drained and washed prior to addition of TEV protease. The eluted material was applied to a chromatography column containing calmodulin 4B beads Healthcare) and incubated for 1 h at 4°C. The column was then drained and washed prior to addition of calmodulin elution buffer containing EGTA. The final eluate collected was mixed with cold 10% Trichloroacetic acid in acetone; after overnight incubation at –20°C, precipitated proteins were collected by centrifugation, rinsed in 100% acetone, and allowed to air dry. These samples were then submitted to the Proteomics Center at the University of Victoria for further processing, including tryptic digestion, HPLC separation, and tandem mass spectrometry (MS/MS) to determine peptide sequences.

Quantitative RT–PCR (qRT–PCR)

Total RNA was extracted from cells using the RNeasy kit (Qiagen) according to the manufacturer's instructions. An RT reaction with 500 ng of total RNA in 25 μL was performed using random hexamers and TaqMan Reverse Transcription Reagents (Applied Biosystems). This was followed by quantitative PCR performed in the 7500 Real-Time PCR system (Applied Biosystems). Oligonucleotides and internal probes for *NFKBIA*, *ICAM1*, *TNF*, and *IL8* transcripts were obtained from Applied Biosystems, and a TaqMan PCR Master Mix with *GAPDH* mRNA quantitation was duplexed in the same well as an internal control. To quantify *CX3CL1* mRNA, specific primers spanning over an intron were designed (sequences available upon request). These were combined with SYBERGreen PCR Master Mix for mRNA quantitation and parallel reactions with primers targeting *GAPDH* were performed as a control for mRNA abundance.

Immunoblotting, immunoprecipitation, and ChIP

WCLs were prepared by adding Triton lysis buffer (25 mM HEPES, 100 mM NaCl, 10 mM DTT, 1 mM EDTA, 10% Glycerol, 1% Triton X-100), RIPA buffer (PBS, 1% NP-40, 0.5% Deoxycholate, 0.1% SDS, 10 mM DTT), or an 8-M urea buffer (8 M urea, 50 mM Tris at pH 8.0, 300 mM NaCl, 50 mM $NaPO_4$, 0.5% NP-40) supplemented with 1 mM sodium orthovanadate and protease inhibitors (Roche) as indicated in each experiment. Preparation of cytosolic and nuclear extracts was performed as described previously (Burstein et al. 2005). Immunoprecipitations, GSH precipitations, Ni-NTA precipitations, and immunoblotting were performed as previously described (Burstein et al. 2004, 2005). Densitometry analysis consisted in measurements of integrated density using the Scion Image software. For denatured immunoprecipitation, cells were harvested and lysed initially in Triton X-100 Buffer (75 μL). The lysate was then mixed with an equal volume of TSD buffer (50 mM Tris at pH7.5, 2% SDS, 5 mM DTT) and sonicated for 10 sec. Subsequently, the lysate was heated for 5 min at 80°C and Triton X-100 buffer was then added to a final volume of 1500 μL (or a final concentration of 0.1% SDS) prior to conventional immunoprecipitation. For in vitro binding assays, *E. coli*-expressed GST fusion proteins were loaded onto glutathinone sepharose beads (Burstein et al. 2005) and 2–5 μg of recombinant protein was mixed with mammalian cell lysates to coprecipitate its binding partner. The following antibodies were used in our studies: acetylated lysine (Cell Signaling, 9441), β-Actin (Sigma, A5441), COMMD1 (Burstein et al. 2005), Cul2 (Zymed, 51-1800), Elongin C (BioLegend, 613101), Flag (Sigma, A8592), GST (Santa Cruz Biotechnologies, sc-459), GCN5 (Santa Cruz Biotechnologies, sc-20698, sc-6306), HA (CoVance, MMS101R), IκB-α (Upstate Biotechnologies, 06-494), IKKα (Cell Signaling, 2682), IKKβ (Cell Signaling, 2684), Rbx1 (LabVision, 127-075-160), RelA (Santa Cruz Biotechnologies, sc-372 and sc-8008), RelA phospho-S-536 (Cell Signaling, 3031), RelA phospho-S-468 (Cell Signaling, 3039), ubiquitin (Stressgen, SPA-205), PCAF (Santa Cruz Biotechnologies, sc-13124). The TAF6 antibody was a kind gift from Dr. Robert Roeder. ChIP was performed as described previously (Burstein et al. 2005). Details regarding primer sequences are available upon request.

In vitro binding experiments

GST fusion proteins bound to GSH sepharose were incubated at room temperature with BSA (1 mg/mL) in 200 μL of Z-buffer (25 mM HEPES at pH 7.5, 12.5 mM $MgCl_2$, 150 mM KCl, 0.1% NP-40, 20% glycerol). After 15 min, in vitro translated proteins were added and incubated for another 45 min at room temperature. Peptides used for competition experiments were prepared as previously as GST-PTD fusion proteins, followed by thrombin

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cleavage as described by the manufacturer (GE Healthcare). The prepared peptides were applied to in vitro binding reactions at a final concentration of 50 μM. 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 at pH 8.0). Bound proteins were resolved by SDS-PAGE and subjected to autoradiography. Protein dephosphorylation was performed by incubating cell lysates with λ-PPase (New England Biolabs) for 30 min at 30°C after addition of the recommended reaction buffer and Mn.

In vitro ubiquitination assays

Each reaction mixture consisted of recombinant ubiquitin (2.5 μg), E1 (Uba1, 50 ng), E2 (UbcH5a, 100 ng), and ATP regenerating buffer (all obtained from Boston Biochem). These were mixed in reaction buffer (40 mM HEPES at pH 7.9, 60 mM potassium acetate, 2 mM DTT, 5 mM $MgCl_2$, 10% glycerol) and incubated for 90 min at 30°C (Maine et al. 2007).

Metabolic labeling

This was performed as described previously (Maine et al. 2007). Briefly, cells were seeded 24 h prior to the experiment and then placed for 30 min in a cysteine and methionine-deficient medium. At the end of this period, ^{35}S-radiolabeled methionine and cysteine was added to the medium for 1 h and then replaced with regular growth medium supplemented with excess nonradiolabeled methionine (2 mM) and cysteine (2 mM). WCLs were then prepared at the indicated time points and the samples were subjected to denatured immunoprecipitation of RelA (Santa Cruz Biotechnologies, sc-372). The recovered material at the end of the immunoprecipitation was resolved by SDS-PAGE and RelA was detected by autoradiography.

Acknowledgments

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Figure S1

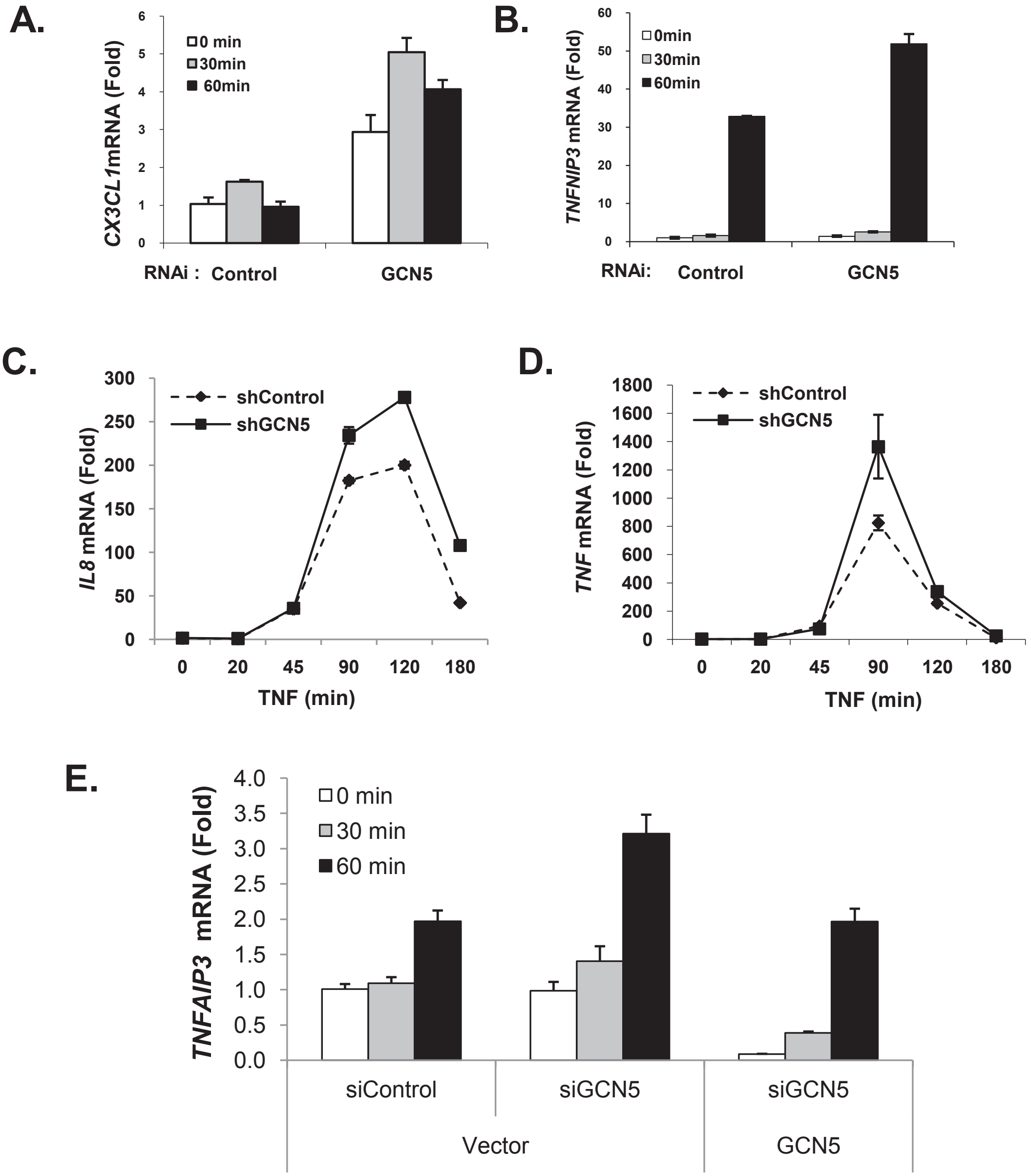

Figure S1. (A-D) GCN5 expression was decreased by transient transfection of small interfereing RNA in HEK293 cells (A,B) or by the small hairpin RNA expression delivered by a lentivirus vector in U2OS cells (C,D). Expression of the indicated NF-κB responsive genes after TNF stimulation were assayed by quantitative real-time RT-PCR. **(E)** GCN5 siRNA was performed as before, and in addition, GCN5 was re-expressed during siRNA by introducing silent point mutations over the siRNA targeting sequence. *TNFAIP3* transcript levels after TNF stimulation were measured by quantitative real-time RT-PCR as before.

Figure S2

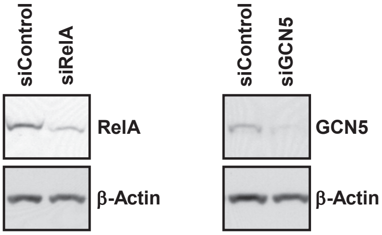
siControl
siRelA
RelA
β-Actin
siControl
siGCN5
GCN5
β-Actin

Figure S2. The effect of siRNA against RelA and GCN5 in the experiment shown on Figure 1H was confirmed by western blot analysis of protein extracts obtained from representative wells.

Figure S3

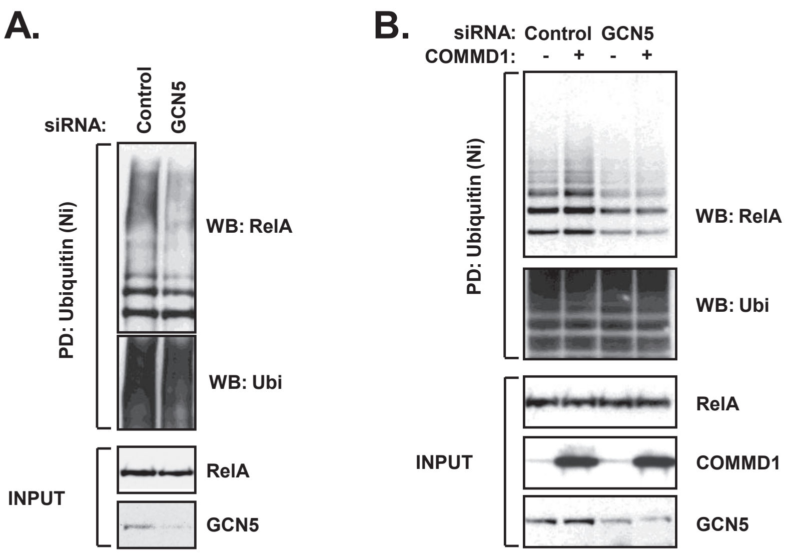
A.
siRNA:
Control
GCN5
PD: Ubiquitin (Ni)
WB: RelA
WB: Ubi
INPUT
RelA
GCN5
B.
siRNA: Control GCN5
COMMD1: - + - +
PD: Ubiquitin (Ni)
WB: RelA
WB: Ubi
INPUT
RelA
COMMD1
GCN5

Figure S3. (A) GCN5 promotes RelA ubiquitination. HEK293 cells were transfected with HA-RelA and His6-tagged ubiquitin, along with siRNA against GCN5. Ubiquitinated proteins were precipitated from denatured lysates by nickel beads and the presence of ubiquitinated RelA was determined by immunobloting. **(B) GCN5 deficiency impairs COMMD1 promoted RelA ubiquitination.** HEK293 cells were transfected with HA-RelA and His6-tagged ubiquitin, as well as COMMD1 as indicated. Endogenous GCN5 levels were suppressed by transfection of siRNA. Ubiquitinated proteins were precipitated from denatured lysates by nickel beads and the presence of ubiquitinated RelA was determined by immunobloting.

Figure S4

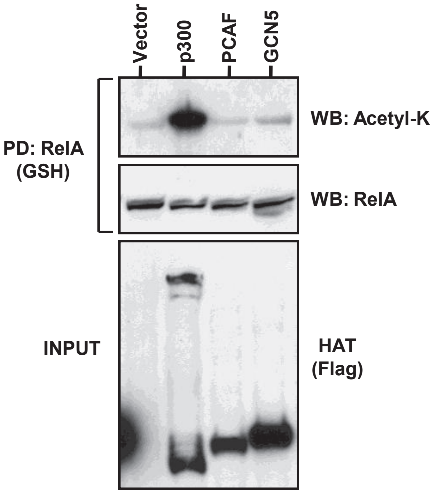
Vector
p300
PCAF
GCN5
PD: RelA
(GSH)
WB: Acetyl-K
WB: RelA
INPUT
HAT
(Flag)

Figure S4. GCN5 does not acetylate RelA. HEK293 cells were transfected with RelA fused to GST along with Flag-p300, PCAF or GCN5. RelA was precipitated by GSH sepharose beads and subjected to immnoblotting for acetyled lysine (top panel) and RelA (middle panel). Expression of the transfected acetyltransferases were determined by western blot analysis in the input lysate (bottom panel).

Figure S5

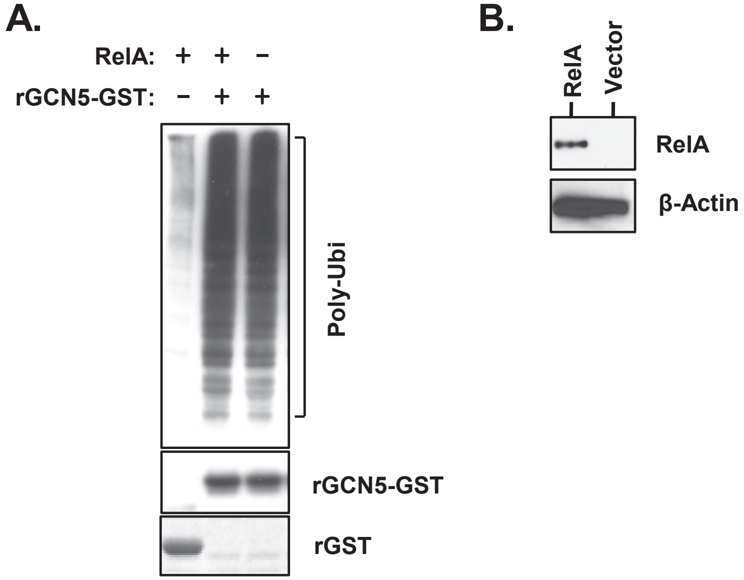

Figure S5. GCN5 precipitated ligase activity does not depend on RelA expression. Recombinant GCN5 protein (or GST control) was mixed with cellular lysates, thoroughly washed and subsequently offered as a source for ligase activity in an *in vitro* ubiquitination reaction. In this case, the lysates utilized were from *rela* deficient mouse embryo fibroblasts reconstituted with wild type human RelA or transfected with an empty vector control. E3 ligase activity precipitated by recombinant GCN5 was shown by immunoblotting for ubiquitin (A). RelA expression was confirmed by western blotting (B).

Figure S6

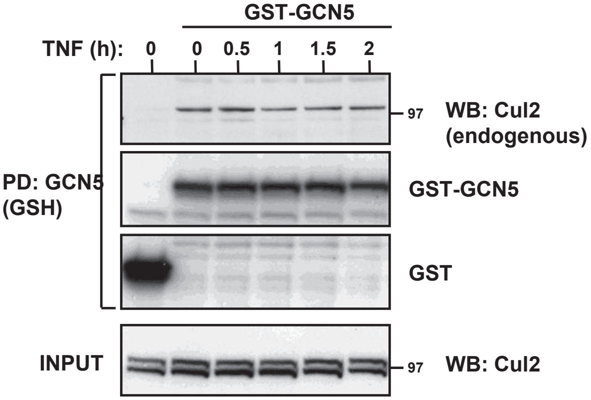
GST-GCN5
TNF (h): 0 0 0.5 1 1.5 2
97
WB: Cul2 (endogenous)
PD: GCN5 (GSH)
GST-GCN5
GST
INPUT
97
WB: Cul2

Figure S6. The Cul2-GCN5 interaction is not affected by TNF. HEK293 cells transfected with GCN5 fused to GST were treated with TNF for variable amounts of time as indicated. Subsequently, lysates were prepared from the cells and utilized for GSH precipitation. The precipitated materials were immnoblotted for GCN5 and endogenous Cul2.

Figure S7

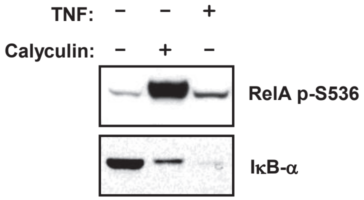
TNF: − − +
Calyculin: − + −
RelA p-S536
IκB-α

Figure S7. Calyculin A promotes RelA phosphorylation. HEK293 cells were treated with Calyculin A or TNF for 30 min as indicated. Total levels of levels of phosphorylated RelA and IκB-α and were determined by immunoblotting.

Figure S8

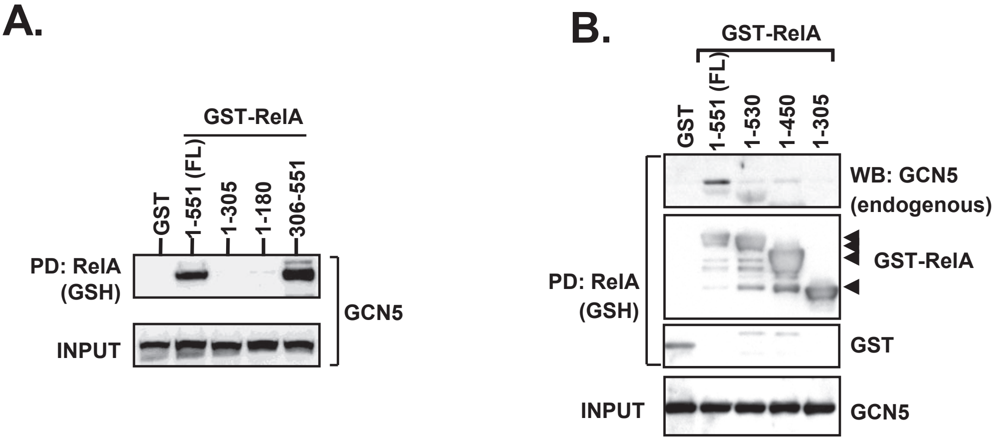
A.
GST-RelA
GST
1-551 (FL)
1-305
1-180
306-551
PD: RelA (GSH)
INPUT
GCN5
B.
GST-RelA
GST
1-551 (FL)
1-530
1-450
1-305
WB: GCN5 (endogenous)
GST-RelA
PD: RelA (GSH)
GST
INPUT
GCN5

Figure S8. GCN5 interact primarily with the TAD of RelA (306-551). (A) HEK293 cells were co-transfected with GCN5 and RelA full-length (FL), or truncation mutants spanning the indicated amino acids, fused to GST. Subsequently, GST proteins were precipitated from cell lysates and the recovered material was immunoblotted for GCN5. **(B)** Similar to (A), the indicated RelA truncations were transfected, and subsequently precipitated from cell lysates. Co-precipitation of endogenous GCN5 was determined by immunoblotting.

Figure S9

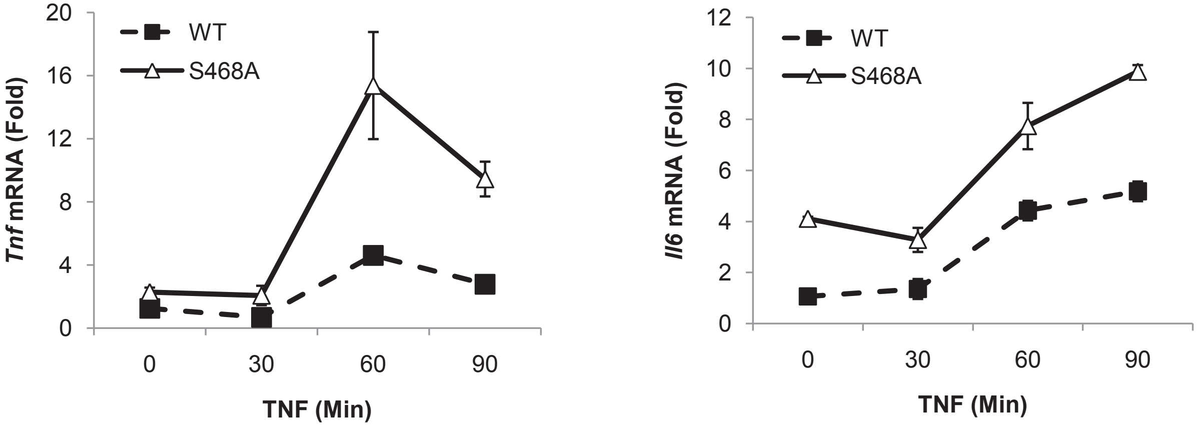

Figure S9. Wild type RelA or RelA S468A were introduced into *rela* deficient mouse embryo fibroblasts by lentivirus mediated transduction. Expression of the *Tnf* and *Il6* genes was determined by quantitative real-time RT-PCR at the indicated time points following stimulation with TNF for 10 min. No difference of RelA level was observed between these two cell lines by western blot (data not shown) .

Chapter 7

COMMD1 prevents inflammation and inhibits NF-κB mediated gene expression

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submitted

COMMD1 prevents inflammation and inhibits NF-κB mediated gene expression

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Running Title: COMMD1 and inflammation

ABSTRACT

The transcription factor NF-κB is a master regulator of inflammation. Its activity is controlled by various mechanisms, including the degradation of chromatin-bound NF-κB subunits. This latter event requires a protein termed COMMD1, which is involved in NF-κB ubiquitination. However, the physiologic importance of COMMD1 in the control of inflammation has not been previously studied. Using tissue-specific deletion of *Commd1* in mice, we report that deficiency of this gene in myeloid cells leads to greater activation of NF-κB and exaggerated inflammatory responses. Moreover, in models of chronic colitis these animals demonstrate a greater propensity for cancer progression. The importance of these events in human disease is highlighted by the fact that inflammatory conditions such as inflammatory bowel disease can lead to *COMMD1* suppression, a process that we found can be mediated by interferon-γ.

INTRODUCTION

The transcription factor NF-κB controls many aspects of both innate and adaptive immunity[1]. NF-κB is comprised of homo- and heterodimers of Rel family proteins, which in mammals are encoded by 5 genes: *RELA* (encoding RelA, also known as p65), *REL* (encoding c-Rel), *RELB* (encoding RelB), *NFKB1* (encoding p50 and its precursor polypeptide p105) and *NFKB2* (encoding p52 and its precursor p100). The so-called 'canonical pathway' is mediated primarily by RelA or c-Rel containing dimers that are kept in the cytosol through interactions with the inhibitory IκB proteins (primarily 'classical' IκB proteins, namely IκB-α, -β and -ε). Activation of canonical NF-κB promotes pro-inflammatory innate immune responses and requires the degradation of IκB, an event triggered by a critical kinase complex known as IKK[2]. This kinase in turn, sits at the crossroads of numerous signaling pathways, and can lead to NF-κB activation in a variety of settings.

Following its activation a number of mechanisms restore homeostasis and terminate canonical NF-κB activity. NF-κB is known to induce negative feedback loops, such as the induction of IκB gene expression[3, 4], or the induction of proteins that inhibit IKK activity[5-7], such as A20 or CYLD. In addition, it has been recognized that ubiquitination and proteasomal degradation of RelA is critical to terminate transcription of a variety of genes[8-14]. One ligase responsible for these effects contains the scaffold protein Cul2, in association with the co-factor COMMD1[10, 13], or in other settings SOCS1[12, 15] (or potentially both). In addition, another report has also implicated the RING containing protein PDLIM2 as a ligase for RelA[11].

COMMD1 is the founding member of a conserved family of factors whose function is largely unexplored at this point[16-18]. Most of what is known about COMMD proteins is restricted to COMMD1, which has been implicated in a variety of cellular processes, including NF-κB regulation[13, 19] as well as copper transport[20], electrolyte balance[21], and hypoxia responses[22]. In most instances, these effects have been ascribed to increased ubiquitination and/or degradation of a cellular factor, a mechanism that is in agreement with the ability of this protein to activate ubiquitin ligases containing Cullin proteins[23].

While the effects of COMMD1 on RelA ubiquitination have been well characterized, it remains unclear whether this factor plays a physiologically important in the control of inflammation. Testing this hypothesis has been complicated by the embryonic lethality that results from complete *Commd1* deficiency in mice[24]. With the recent development of a conditional *Commd1* allele[25], tissue specific deletion became an attractive option to test the contribution of *Commd1* to inflammation. Here we report that myeloid deficiency of *Commd1* leads to more severe inflammation in a variety of models including sepsis, acute colitis and chronic colitis, and is accompanied by exaggerated NF-κB activation. Moreover, we find that *COMMD1* is repressed in the setting of inflammation through a mechanism that involves IFN-γ production. These events are observed in various conditions, including inflammatory bowel disease, suggesting an important role for COMMD1 in human disease.

RESULTS

Commd1 deficient fibroblasts demonstrate decreased RelA ubiquitination

In order to study the contribution of *Commd1* to the control of inflammation *in vivo*, we generated *Commd1* deficient mice using a recently described *Commd1* conditional allele[25] (Figure S1A). Using these 'floxed' mice, we generated murine embryonic fibroblasts (MEFs) which were infected with an adenovirus expressing Cre (or a LacZ as a negative control). The isogenic lines derived demonstrated gene deletion (Figure S1B) and complete loss of Commd1 protein expression (Figure 1A). In these cells, *Commd1* deficiency led to decreased ubiquitination of nuclear RelA (Figure 1B) and exaggerated expression of pro-inflammatory NF-κB target genes such as *Il6* and *Tnf* (Figure 1C) in agreement with previously published data[10, 13].

Generation of myeloid-specific Commd1 deficient mice

Commd1 'floxed' mice were crossed with LysM-Cre 'knockin' mice, which express Cre in the myeloid lineage[26], a critical arm of the innate immune system. LysM-Cre led to the expected loss of Commd1 expression in macrophage populations derived from bone marrow cells or splenocytes of *Commd1* floxed mice but not wild-type controls (Figure 1D). These myeloid specific Commd1 deficient mice (Mye-K/O) did not demonstrate any overt phenotype at baseline, except for a small increase in leukocyte counts in blood, still within the normal range for mice (Figure 1E, left panel), which was not due to significant alterations in any particular leukocyte lineage (Figure 1E, right panel). In addition, B lymphocytes ($B220^+$) and T lymphocyte populations ($CD3^+$ and CD4/CD8 subpopulations) were not significantly different (Figure 1F, left) in the spleen or mesenteric lymph nodes (MLN). Similarly, myeloid populations, including granulocytes ($Ly6G^+$), monocytes and macrophages ($CD11b^+$, $Ly6C^+$), macrophages ($F4/80^+$), and dendritic cells ($CD11c^+$, both high and intermediate) were not significantly different in frequency between these two mouse strains (Figure 1F, right, and Figure S2).

Myeloid deficiency of Commd1 sensitizes mice to sepsis

Next, we compared the sepsis response of Mye-K/O mice and littermate WT controls (floxed mice lacking the LysM-Cre gene). Animals were challenged with LPS, and in a blinded manner, morbidity and mortality (or need for euthanasia) were assessed in the ensuing 30 hours. Mye-K/O mice demonstrated increased mortality after LPS administration (Figure 2A), as well as greater morbidity (Figure 2B). Tissue injury, manifested by enzyme release, was also more pronounced in Mye-K/O mice (Figure 2C). Similarly, plasma levels of pro-inflammatory cytokines such as TNF, IL-6 and CCL5, were significantly elevated in Mye-K/O, while the anti-inflammatory cytokine IL-10 was unchanged (Figure 2D). At the tissue level, expression of pro-inflammatory genes in various organs was more pronounced in Mye-K/O mice, as exemplified by *Tnf* (Figure 2E). Several other pro-inflammatory genes, such as *Ccl5*, *Il6*, *Il1b*, *Cxcl1*, and *Saa3*, were more robustly induced in Mye-K/O mice (Figure 2F), and the same was true of other NF-κB target genes such as *Nfkbia* and *Tnfaip3* (Figure 2G). Altogether, these data indicated that *Commd1* deficiency in the myeloid lineage leads to more profound inflammatory responses in this sepsis model.

Commd1 deficiency enhances NF-κB dependent gene expression in myeloid cells

Given the observed phenotype, we examined the effect of *Commd1* on NF-κB dependent gene expression in bone marrow derived myeloid (BMDM) cells. GM-CSF driven differentiation, reported to induce primarily immature dendritic cells, led to very significant deletion of *Commd1*. These cells demonstrated comparable kinetics of IκB-α degradation and phosphorylation in response to LPS (Figure 3A), indicating that IKK activation downstream of Tlr4 was not significantly affected. Similar results were obtained when stimulating these cells with TNF (not shown here) and were also confirmed in RAW 264.7 murine macrophages with Commd1 deficiency secondary to stable shRNA (Figure S3). However, *Commd1* deficiency in BMDM cells led to increased expression of several NF-κB inducible genes (Figure 3B, left). Interestingly, while Commd1 can inhibit hypoxia-dependent gene expression during development and in cancer cell lines[22, 24], BMDM cells did not demonstrate significant differences in hypoxia-induced expression of several genes (Figure 3B, right). Similarly, expression of IFN-γ inducible genes seemed unaffected in *Commd1* deficient BMDM cells (Figure S4). Altogether, these data indicate that Commd1 deficiency leads to increased NF-κB dependent gene expression, which in turn probably explains the increased inflammatory response seen in mice after LPS exposure.

Commd1 deficiency leads to more severe mucosal inflammation

We decided to extend these observations to other models of inflammation where innate immunity plays an important role. Therefore, we examined mucosal inflammation in response to dextran sodium sulfate (DSS), a chemical agent that triggers mucosal inflammation that is driven by an innate immune response to luminal bacteria[27]. The experiments were performed in a blinded fashion and disease severity was determined over a 10-day period while mice were receiving DSS. Mye-K/O mice demonstrated a more severe disease course (Figure 4A), with excess mortality, more pronounced weight loss, and more severe disease activity index. In agreement with this finding, the colon length in these animals, a macroscopic marker of tissue injury, was significantly shorter, and blinded histologic evaluation for tissue injury also indicated more severe damage (Figures 4B and 4C). Myeloid and lymphoid populations in the MLN (Figure 4D) and the spleen (not shown), were not significantly different between the groups. However, immunohistochemistry for the macrophage marker CD68 demonstrated higher numbers of myeloid cells in the mucosa of Mye-K/O mice, consistent with the more severe disease course and greater tissue injury (Figure 4E).

Mye-K/O mice did not display preferential M1 activation

Macrophage differentiation can lead to a classically activated phenotype (M1) characterized by robust production of pro-inflammatory cytokines and reactive oxygen species. In addition, macrophages can differentiate into various alternatively activated phenotypes (M2) that generally promote tissue repair, and in specific contexts, can promote tumor growth or participate in anti-parasitic responses[28]. Therefore, as a potential explanation for the differences in mucosal inflammation, we examined whether Mye-K/O mice displayed alterations in the balance of the M1/M2 polarity *in vivo*. To that end, the expression of M1 and M2 markers in the inflamed colonic mucosa DSS-treated mice was examined by qRT-PCR. Consistent with the more severe injury noted in Mye-K/O mice, these animals displayed increased expression of pro-inflammatory genes, such as *Il6*, *Il1b* and *Cxcr4* (Figure 4F). Next, we observed increased expression of the pan-macrophage marker *Emr1* (also referred to as *F4/80*), in agreement with the tissue immunohistochemistry demonstrating greater numbers of CD68+ cells (Figure 4F). The M1 differentiation marker *Nos2* was also significantly elevated in Mye-K/O animals. On the other hand, M2 differentiation markers such as *Renlta* (also referred to as *Fizz1*) and *Mrc1* (also known as *Cd206*) were significantly elevated as well (Figure 4F). Altogether, the increase in both M1 and M2 differentiation markers suggested that the balance between M1 and M2 differentiation was not dramatically perturbed in Mye-K/O mice, but that the increased expression of these genes is likely due to the overall increase in macrophages in the mucosa (demonstrated by both *F4/80* mRNA expression and CD68 staining).

Loss of Commd1 in myeloid cells promotes tumor development

Persistent colitis can promote cancer development in patients with inflammatory bowel disease[29], which can be modeled in rodents by repeated bouts of DSS-induced colitis[30]. This leads to dysplasia and locally invasive tumors, particularly if the animals are pre-treated with low doses of the carcinogen azoxymethane (AOM). Myeloid activation of NF-κB plays a critical role in this model of colitis-associated cancer[31] through the expression of a variety of factors that work in a paracrine fashion to promote tumor growth[32]. Therefore, we examined the propensity of Mye-K/O mice to develop colonic dysplasia after a 50 day treatment course (Figure 5A and 5B). Macroscopically, the animals had shorter colons and larger spleens, both gross markers of chronic colitis in rodent models (Figure 5C and 5D). Moreover, blinded histologic evaluation of the colonic tissue demonstrated greater chronic inflammatory injury (glandular distortion, Figure 5C), as well as greater dysplasia, high grade dysplasia and multifocal dysplasia in Mye-K/O mice (Figure 5C and 5E). Altogether, these results indicate that *Commd1* not only restricts pro-inflammatory myeloid responses, but also limits the tumor promoting capabilities of this cell population.

Inflammatory disorders are associated with decreased COMMD1 expression

To examine the relevance of our findings to the pathogenesis of human disease we decided to evaluate *COMMD1* gene expression in various inflammatory states. First, we observed that the expression of *COMMD1* is decreased in circulating leukocytes from patients with inflammatory bowel disease (IBD) when compared to unaffected control individuals, both at the mRNA level (Figure 6A) and at the protein level (Figure 6B). Moreover, consistent trends were observed in colonic mucosal samples from patients with IBD (Figure S5A) and in ileal mucosal samples from patients with Crohn's disease (Figure S5B), where *COMMD1* and other *COMMD* genes were suppressed in the setting of inflammation. Other mucosal inflammatory conditions also revealed a similar pattern; for example, inflammation in the small intestine in patients with celiac disease also led to decreased *COMMD* gene expression (Figure S5C) where the degree of suppression correlated with histological severity according to the Marsh score. Altogether, these findings indicated that various inflammatory disorders in humans are associated with decreased *COMMD* gene expression.

Given the heterogeneity of these processes, we reasoned that *COMMD* gene suppression was probably secondary to inflammation itself, rather than an initiating event. To test this possibility, we examined the effect of acute colitis on *Commd* gene expression in normal C57BL6 mice. Murine colitis induced by DSS led to decreased *Commd* gene expression in the colon (Figure 6C), which correlated with reduced Commd1 and Commd9 protein levels in the colon at the end of the 10-day course of DSS treatment (Figure 6D). These data indicate that inflammation can lead to *COMMD* gene suppression, a process observed in clinical situations.

Interferon-γ suppresses COMMD gene expression

Next, we examined the mechanism by which *COMMD* gene expression is repressed in the setting of inflammation. Similar to the results observed in patients with inflammatory disorders, we found that LPS could induce *COMMD* gene suppression in whole blood of normal individuals (Figure S6). This was recapitulated in mouse splenocyte cultures (Figure 6E, left), where the effect was most pronounced for *Commd10* and was dependent on Myd88, as splenocytes isolated from *Myd88*$^{-/-}$ mice did not display *Commd10* gene suppression (Figure 6E, right). As expected, *Myd88*$^{-/-}$ splenocytes lacked induction of *Il6* and *Infg* (Figure S7).

Despite its effect on whole blood or splenocytes, LPS and other stimuli such as TNF, IL-1β, or TGF-β1 did not cause *COMMD* gene suppression in a variety of cell lines, including

RAW 264.7 macrophages (Figure S8A). This suggested that perhaps a product produced in response to LPS by blood cells or splenocytes could be responsible for *COMMD* gene suppression in an indirect paracrine fashion. In this regard, careful evaluation of the timing required for *Commd* suppression in splenocytes indicated that *Commd1* and *Commd10* suppression is a delayed phenomenon that coincides with the induction of IFN-γ in these cells (Figure S9). Notably, LPS stimulation of RAW macrophages did not result in *Ifng* induction (Figure S8B), but direct stimulation of splenocytes or RAW macrophages with IFN-γ led to repression of *Commd* genes that was comparable to the effects of LPS (Figure 6F). Altogether, these studies suggest that IFN-γ production is likely responsible for *COMMD* gene suppression in the setting of inflammation.

DISCUSSION

In this study we demonstrate that COMMD1 plays an important role in the regulation of inflammatory responses *in vivo*. Given that diverse functions[21, 33-37] and *in vivo* phenotypes[20, 22, 24, 25] have been reported for this gene, our studies fill a critical void by underscoring that COMMD1 also participates in immune regulation. The data also support the role for COMMD1 as a negative regulator of NF-κB through RelA ubiquitination[10, 13, 14, 17, 19]. The increased propensity to colitis that results from *Commd1* deficiency coupled with the decreased expression of this gene in IBD patients suggests a potential role for *COMMD1* in the pathogenesis of this disorder. Although COMMD1 itself has not been implicated in IBD pathogenesis so far, its associated co-factor for RelA ubiquitination, Cul2 (encoded by the *CUL2* gene), has been recently reported as a risk allele in genetic studies[38].

An important question to be resolved is why seemingly diverse cellular functions have been assigned to a single protein, and whether such convergence has deeper biological meaning. While the molecular mechanism that links these different activities has not been completely unveiled, protein ubiquitination leading to degradation or trafficking along the endocytic pathway has been demonstrated in a number of instances[23, 34, 36]. With this in mind, it is interesting to note that other factors that participate in protein ubiquitination are also at the convergence point of seemingly unrelated cellular pathways, as is the case for β-TrCP and other ligase cofactors[39]. Hence, the convergence of diverse targets and cellular functions on COMMD1 might represent part of larger paradigm that still needs to be better understood.

Our studies also identified that inflammation can lead to suppressed expression of *COMMD1* and other *COMMD* genes. Similar alterations in gene expression were evident when examining the primary data from a large study of differentially regulated genes in leukocytes of patients with IBD[40], where decreases in *COMMD3* and *COMMD4* were noted.

The data indicate that this is likely the consequence rather than the cause of inflammation in human disease, because this suppression can be induced by inflammation itself in animal studies. Moreover, the disparate nature of the disorders in which this phenomenon was observed suggests that this is not likely to be a primary genetic alteration, but rather a consequence of inflammation. This decrease in *COMMD1* expression probably plays a physiologic role in the initiation of an appropriate inflammatory response. Nevertheless, in view of the more severe inflammatory responses seen in *Commd1* deficient mice, persistent *COMMD1* suppression might promote more severe inflammatory responses in human disease. Moreover, polymorphisms in the *COMMD1* gene that could affect its expression in the setting of inflammation might play a role in the genetic susceptibility to IBD and other inflammatory disorders.

Our studies demonstrate that *COMMD* gene suppression can be induced by IFN-γ, a cytokine that is potently induced in many inflammatory disorders. Interestingly, decreased expression of *COMMD1* has also been found in a variety of tumors, where the degree of suppression correlates with poor outcomes, including decreased survival[22]. Those studies also demonstrated that neoplastic and non-neoplastic cells in the tumor display decreased *COMMD1* expression, suggesting that signals present in the microenvironment are responsible. Therefore inflammation, which is regarded as one of the cardinal characteristics of cancer[41], could be playing a role in *COMMD1* repression in this setting. Moreover, the data here indicate that decreased *COMMD1* expression in non-neoplastic cells, specifically in myeloid cells, may be similarly important in promoting tumor progression in the setting of colitis-associated cancer. Thus, decreased *COMMD1* expression in the setting of cancer can have cell-autonomous and non-cell autonomous effects that promote tumor growth and progression. Finally, our work highlights the need to gain a refined understanding of the molecular events that control *COMMD1* gene expression in the setting of inflammation. With this knowledge we may begin to envision potential interventions that might restore gene expression. If such an approach were available, it could be of therapeutic potential in chronic inflammatory disorders and cancer.

ONLINE METHODS

Mouse strains: Mice were maintained in ventilated microisolator cages in barrier facilities with irradiated standard diet. All animal procedures were approved by the UT Southwestern Medical Center Animal Care and Use Committee. To generate myeloid specific *Commd1* deficient mice (Mye-K/O), animals carrying the conditional Commd1 allele[25] ('floxed' *Commd1* or *Commd1*$^{F/F}$) were mated with the LysM-Cre mice[26] (obtained from the Jackson laboratory, stock no: 004781).

Cell lines and tissue culture: RAW 264.7 macrophages were obtained from ATCC and maintained in high-glucose DMEM containing 10% FCS. Mouse embryo fibroblasts (MEFs) obtained at E13.5 were immortalized by lentiviral delivery of E1A and Ras, as previously described[42]. These cells were maintained in high-glucose DMEM containing 10% FCS and subsequently infected with an adenovirus encoding Cre recombinase or a control virus expressing LacZ (Vector Core, University of Michigan Medical Center). Bone marrow cells were harvested from long bones and cultured in RPMI media supplemented with 10% FCS, antibiotics (penicillin G 100μg/mL and streptomycin 100μg/mL) and Fungizone (Fisher), in the presence of GM-CSF (20ng/ml, Peprotech). After myeloid differentiation for 10 days, the cells were treated with LPS (10ng/mL, *Escherichia coli* 026:B6, Sigma), mouse IFN-γ (100U/mL, Roche), or TNF (10ng/mL, Invitrogen), as indicated in each experiment. The cells were also cultured under 3% oxygen in HeraCell incubators for specific experiments. Mouse splenocytes and thymocytes were placed in RPMI containing 10% FCS prior to stimulation with LPS or murine IFN-γ.

Lentiviral mediated shRNA of Commd1: Recombinant lentiviral particles were prepared as previously described[43, 44]. The target sequences in the *Commd1* mRNA used for RNA interference are the following: shRNA *Commd1-1*: ATGAAGTTAAAGTCAAGCAA, and shRNA *Commd1-2*: GTCTATTGCATCTGCAGACAT.

Sepsis model: LPS (*Escherichia coli* 055:B5, Sigma) was diluted in water to 0.2mg/mL and placed in a water bath sonicator for 15-30 min at room temperature (RT) prior to use (Model FS20, Fisher). After sonication, LPS was filtered (0.2 m pore) and injected intraperitoneally (0.1mg/mouse). After injection, the mice were individually housed and monitored for morbidity every hour for the first 4-6 hours, then every 2 hours for the next 6 hours, and finally every 4-6 hours until the conclusion of the experiment (Table S1). The investigators were blinded to the genotype of individual mice until data analysis. Mice were monitored hourly if they developed moderate distress (morbidity score of 5). At the end of the experiment, or at any point when the animals demonstrated severe morbidity (score of 3 in any individual category of the morbidity index), they were euthanized and various organs (spleen, kidney and lung) were harvested for RNA extraction as described below. Blood was also collected through orbital puncture, and plasma was separated using EDTA (5mM) and stored at -80ºC.

Acute and chronic DSS-induced colitis: For the acute colitis model, dextran sulfate sodium salt (DSS, Fisher) was administered in the drinking water at a concentration of 3g/dL (3%). Freshly prepared DSS drinking solution was replaced every 5 days. Mice were housed in individual cages and evaluated daily for their weight and presence of diarrhea or bleeding to calculate a disease activity index (Table S2), which was based on the scoring system reported by Cooper and colleagues[45]. Evaluation for occult bleeding included Hemoccult® testing (Beckman Coulter). Age- and sex-matched littermates receiving water without DSS served as controls. Treatment was given for up to 10 days and animals demonstrating severe distress were euthanized at any point prior to the conclusion of the experiment. At the end of the experiment or at time of euthanasia, the colon was dissected and opened along the mesenteric side. The open colon was transected longitudinally with one half rolled onto itself as a 'swiss roll' and fixed in 4% PFA (in PBS). After paraffin embedding and sectioning, the tissue was stained with hematoxylin and eosin (HE). The second half was used for RNA extraction as described below. For the chronic colitis and cancer model, the animals were given azoxymethane (AOM, 10mg/kg) intraperitoneally (Sigma). After 7 days, DSS was given in the drinking water over 5 days at a concentration of 2.5% followed by 10 days of regular water. This was repeated for 3 cycles and at the end of 49 days, the animals were euthanized, and the spleen and colon were harvested. The organ length and weight were determined and the colon was opened along the mesenteric side, rolled onto itself as a 'swiss roll', and stained as before with HE for histology.

Histology and immunohistochemistry: A histologic severity index was utilized[45] to score the HE stained colonic sections from mice with acute DSS colitis (Table S3). For specimens from the chronic colitis and cancer model, the presence of glandular distortion, low-grade or high-grade dysplasia, multifocal dysplasia, and invasion was determined by standard pathological criteria. All of this analysis was performed by a gastrointestinal pathologist in a blinded fashion. For CD68 staining, we first generated tissue microarrays. For the acute colitis model, random colonic tissue cores were extracted for each mouse from prior paraffin blocks. After constructing the microarrays, 5 m sections were subjected to CD68 staining at a 1:25 dilution (Clone F8-11, Biolegend) by the immunohistochemistry core laboratory at UT Southwestern. Images were then analyzed using the ImageJ software.

Protein extraction, immunoblotting and immunoprecipitation: Cell lysate preparation, immunoprecipitations and immunoblotting were performed as previously described[16, 46]. Mouse tissues were harvested and snap frozen in liquid nitrogen. After mincing with a razor blade, tissue pieces were submerged in lysis buffer (50mM Tris-Hcl pH^+ 7.5, 250mM NaCl, 3mM EDTA, 3mM EGTA, 1% Triton X-100, 0.5% NP40, 10% glycerol, 25mM Na-pyrophosphate) supplemented with protease inhibitor tablets (Roche). Thereafter, tissue homogenization in lysis buffer was performed using a 2mL Douncer and a cleared lysate (supernatant after 10,000*g* centrifugation), was used for further analysis.

RNA extraction and quantitative real-time RT-PCR: RNA was extracted from cells and tissues using Trizol (Invitrogen) according to the manufacturer’s instructions. In the case of colonic RNA from colitic mice we obtained much better results by first stabilizing the freshly isolated tissue in RNAlater (Qiagen), followed by RNA extraction using the RNeasy chromatography method (Qiagen), according to the manufacturer’s instructions. RNA (5μg) was used for cDNA synthesis using the Superscript III strand synthesis system (Invitrogen). Real-time PCR was performed in an Eppendorf real time PCR system (Eppendorf). Most assays were based on gene specific primers and SYBR Green Mix (Invitrogen), as noted in Table S4. Experiments were performed in triplicate, data were normalized to housekeeping genes and the relative abundance of transcripts was calculated by the comparative *ΔΔCt* method.

Flow cytometry: Spleen or mesenteric lymph nodes (MLN) were freshly isolated and placed on a sterile tissue culture dish containing RPMI. The tissue was manually ground and strained through a 40 m tissue strainer. After centrifugation (300*g* for 5 minutes at 4ºC), the cell pellet was resuspended in 2mL of ACK buffer (150mM NH_4Cl, 10mM $KHCO_3$, 0.1mM EDTA, pH^+ 7.2) to lyze red blood cells. After 3 minutes at RT, the ACK lysis was neutralized by adding 10mL of FACS media buffer (0.01% Sodium azide, 2.5% FCS in PBS). This cell suspension was strained again through the tissue strainer to obtain a single cell suspension, which was pelleted by centrifugation. The splenocytes were resuspended in 2mL of FACS media buffer and the cells from the MLN were resuspended in 600 L. For antibody staining, 100 L of each cell suspension was incubated on ice with the following primary antibodies: anti-B220-PE (BD Pharmingen), anti-CD3-PE (BD Pharmingen), anti-CD4-perCP

(BD Pharmingen), anti-CD8a-APC (BD Pharmingen), anti-Ly6G-APC (eBioscience), anti-Ly6C- PerCP-Cy5.5 (eBioscience), anti-CD11C-APC (BD Pharmingen), anti-CD11b-PE (eBioscience), anti-F4/80-FITC (eBioscience) and corresponding isotype controls. After 30 minutes, the cells were pelleted by centrifugation (300*g* for 5 minutes at 4ºC) and washed twice in 500 L of FACS media buffer. Flow cytometric analysis was performed using FACSCalibur (BD Biosciences, Mountain View, CA) and analyzed using FlowJo software.

Multiplex cytokine measurements. EDTA-Plasma was separated from blood samples by centrifugation and stored at -80 C until analysis. These samples were then processed for cytokine measurements using Milliplex MAP beads (Millipore) for quantitation of IL-10, IL-6, CCL5 and TNF, according to the manufacturer's instructions.

Human studies: All procedures involving human subjects were reviewed and approved by the respective Institutional Review Boards. The analysis of *COMMD* gene expression in the setting of sepsis was performed at UT Southwestern, while the examination of *COMMD* gene expression in inflammatory bowel disease was performed at Tel Aviv Sourasky Medical Center. The analysis of *COMMD* gene expression in celiac disease was performed at UT Southwestern Medical Center from samples obtained at UMC Groningen. Circulating leukocytes were isolated from patients with IBD and RNA was isolated using the Trizol method as described above. In the case of intestinal tissue, this was obtained during endoscopy which was performed as part of medical care.

Statistical analysis: In all graphs, the mean is presented and the error bars correspond to the standard error of the mean (SEM). All statistical comparisons between values in the study populations were performed using one-tailed, homoscedastic, Student's *t* test.

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CONFLICT OF INTEREST

The Authors have no conflicts of interest to report in connection to this work.

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FIGURE LEGENDS

Figure 1: Commd1 deficiency impairs RelA ubiquitination. **(A)** Commd1 deficiency in MEFs after adenoviral delivery of Cre. MEFs from Commd1 floxed embryos were isolated, immortalized and then transiently infected with adenoviral particles expressing Cre recombinase or a LacZ control virus. A western blot for Commd1 is shown here. **(B)** RelA ubiquitination in Commd1 deficient MEFs. After preparation of nuclear extracts, the lysate was denatured by boiling and RelA was immunoprecipitated. The material was immunoblotted for ubiquitin to detect ubiquitinated RelA. **(C)** Exaggerated gene induction after TNF. MEFs were treated with TNF and expression of *Il6* and *Tnf* was determined by qRT-PCR. **(D)** Commd1 deficiency in myeloid cells. Commd1 levels were determined by western blot in lymphocyte populations (primary thymocytes and non-adherent splenocytes) as well as in bone marrow cells or splenocyte-derived myeloid cells. Floxed Commd1 mice that also carried the LysM-Cre gene demonstrated myeloid specific Commd1 deficiency (Mye-K/O). **(E)** Myeloid deficiency of Commd1 induced a minimal elevation of WBC (left), with no specific skewing of leukocyte populations (right). **(F)** Spleen and MLN populations were not affected in Mye-K/O mice. Various cell populations were determined using flow cytometry for the indicated surface markers.

Figure 2: Mye-K/O mice have a more severe sepsis response. **(A)** and **(B)** Mye-K/O mice (*Commd1* $^{F/F}$, LysM-Cre) have greater morbidity and mortality compared to wild-type (WT) littermate controls (*Commd1* $^{F/F}$) after intraperitoneal LPS administration. **(C)** Plasma levels of tissue enzymes were elevated in Mye-K/O mice. **(D)** Plasma levels of cytokines were similarly elevated. **(E)**, **(F)** and **(G)** Expression of various NF-κB target genes in tissues was determined by qRT-PCR. In (E), expression of *Tnf* was determined in 3 different organs. The rest of the analysis was performed in kidney tissue.

Figure 3: Commd1 deficiency leads to exaggerated pro-inflammatory gene expression. **(A)** Bone marrow derived myeloid (BMDM) cells did not exhibit alterations of NF-κB activation or IκB turnover. These cells were stimulated with LPS and the kinetics of IκB-α phosphorylation and degradation were examined by western blotting. **(B)** BMDM cells were stimulated with LPS and gene expression was determined by qRT-PCR (left column). Similarly, gene induction by hypoxia was also examined (right column).

Figure 4: Myeloid deficiency of *Commd1* predisposes to more severe acute colitis. **(A)** Worse survival, weight loss and disease activity index (DAI) were noted in Mye-K/O mice (*Commd1* $^{F/F}$, LysM-Cre) compared to wild-type littermate controls (*Commd1* $^{F/F}$). **(B)** Colon length was significantly shorter in Mye-K/O mice after acute colitis. **(C)** Worse tissue injury was also evident histologically. Using a severity score, colonic sections were examined in a blinded fashion. **(D)** Lymphocyte and myeloid populations were not substantially different in the MLN of Mye-K/O mice compared to controls. Flow cytometry was utilized to identify the indicated cell populations as before. **(E)** Expansion of macrophage populations in the colonic mucosa of Mye-K/O mice. A tissue microarray containing colonic samples from experimental animals was constructed, and subsequently stained for CD68 by immunohistochemistry. The number of CD68+ cells present in the mucosa was assessed in a blinded fashion in microscopic images and the numbers were normalized to the surface area (expressed as microscopic fields). **(F)** RNA extracted from colonic tissue was utilized for qRT-PCR analysis for the genes indicated.

Figure 5: Mye-K/O mice show greater progression to colitis-associated dysplasia. **(A)** Schematic representation of the experimental design. **(B)** Weight during the course of the experiment is presented. **(C)** Greater severity of colitis and dysplasia was noted in Mye-K/O mice. Colon length, spleen weight, and glandular distortion histologically indicated more

pronounced chronic inflammation in Mye-K/O mice. Similarly, blinded evaluation of dysplasia also indicated more severe disease in Mye-K/O mice. **(D)** and **(E)** Images of the spleen are shown in (D), and representative microphotographs of dysplasia are shown in (E).

Figure 6: Inflammation and IFN-γ repress COMMD1 expression. **(A)** *COMMD1* gene expression was decreased in circulating leukocytes from patients with IBD (n=13, 10 with Crohn's disease and 3 with ulcerative colitis) compared to normal controls (n=8). **(B)** Similar reduction in COMMD1 protein levels was noted in western blots from leukocytes of IBD patients (n=5, 3 with Crohn's disease and 2 with ulcerative colitis), compared to healthy controls (n=4) **(C)** and **(D)** Commd gene expression was similarly decreased in colonic tissue after DSS-induced acute colitis as shown in (C), and this was demonstrated at the protein level for Commd1 and Commd9 in (D). **(E)** LPS induces *Commd* gene suppression in a *Myd88* dependent manner. Freshly isolated splenocytes were treated with LPS for 24 hours and the expression of *Commd* genes was determined by qRT-PCR (left). A similar experiment comparing the effects of LPS on *Commd10* expression was performed using WT or *Myd88*$^{-/-}$ splenocytes (right). **(F)** IFN-γ stimulation of splenocytes or RAW 264.7 macrophages induced similar repression of *Commd* genes.

Figure 1

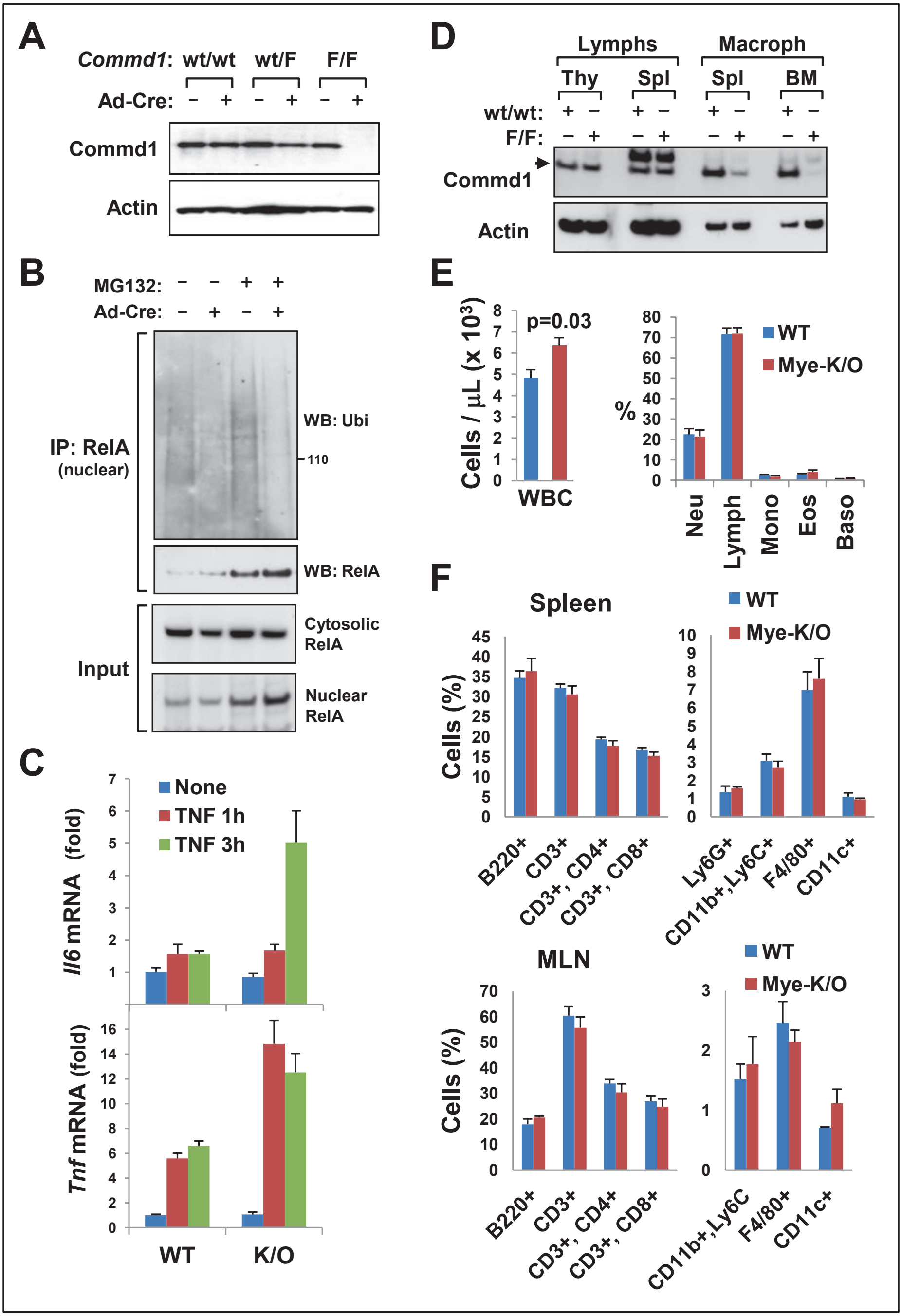
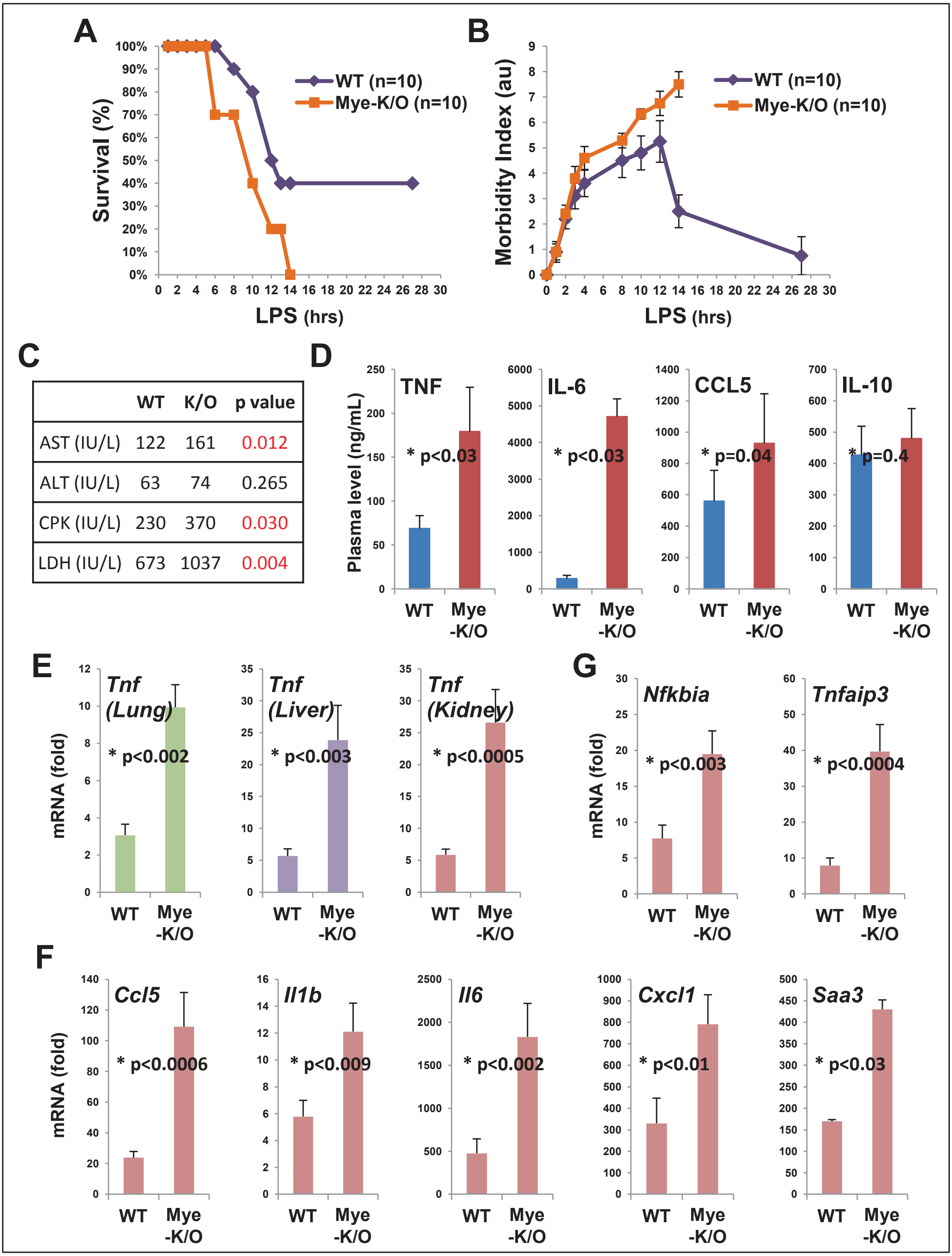

A
Commd1: wt/wt wt/F F/F
Ad-Cre: – + – + – +
Commd1
Actin
B
MG132: – – + +
Ad-Cre: – + – +
IP: RelA
(nuclear)
WB: Ubi
110
WB: RelA
Input
Cytosolic RelA
Nuclear RelA
C
Il6 mRNA (fold)
None
TNF 1h
TNF 3h
0 1 2 3 4 5 6 7
Tnf mRNA (fold)
0 2 4 6 8 10 12 14 16
WT
K/O
D
Lymphs
Macroph
Thy
Spl
Spl
BM
wt/wt: + – + – + – + –
F/F: – + – + – + – +
Commd1
Actin
E
Cells / µL (x 10^3)
p=0.03
0 1 2 3 4 5 6 7 8
WBC
%
0 10 20 30 40 50 60 70 80
WT
Mye-K/O
Neu
Lymph
Mono
Eos
Baso
F
Spleen
WT
Mye-K/O
Cells (%)
0 5 10 15 20 25 30 35 40 45
0 1 2 3 4 5 6 7 8 9 10
B220+
CD3+
CD3+, CD4+
CD3+, CD8+
Ly6G+
CD11b+,Ly6C+
F4/80+
CD11c+
MLN
WT
Mye-K/O
Cells (%)
0 10 20 30 40 50 60 70
0 1 2 3
B220+
CD3+
CD3+, CD4+
CD3+, CD8+
CD11b+,Ly6C
F4/80+
CD11c+

Figure 2



	WT	K/O	p value
AST (IU/L)	122	161	0.012
ALT (IU/L)	63	74	0.265
CPK (IU/L)	230	370	0.030
LDH (IU/L)	673	1037	0.004

Figure 3

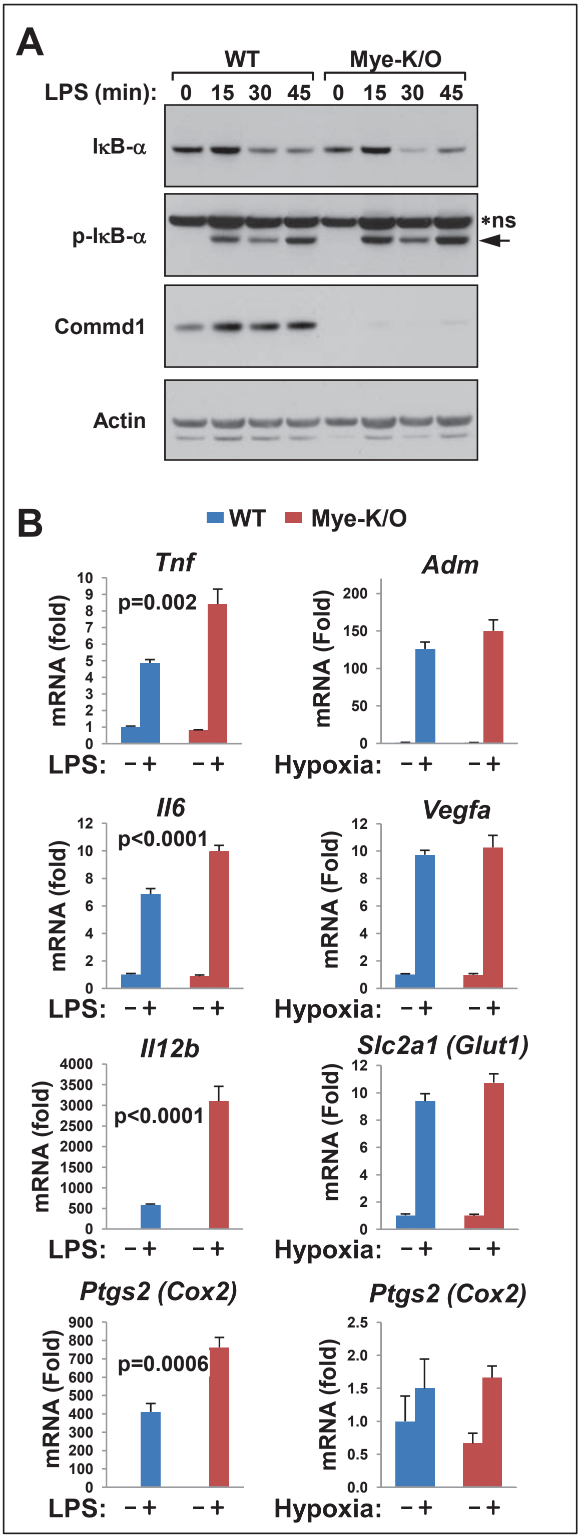

A
WT
Mye-K/O
LPS (min):
0 15 30 45 0 15 30 45
IκB-α
p-IκB-α
*ns
Commd1
Actin
B
WT
Mye-K/O
Tnf
p=0.002
mRNA (fold)
0 1 2 3 4 5 6 7 8 9 10
LPS: − + − +
Adm
mRNA (Fold)
0 50 100 150 200
Hypoxia: − + − +
Il6
p<0.0001
mRNA (fold)
0 2 4 6 8 10 12
LPS: − + − +
Vegfa
mRNA (Fold)
0 2 4 6 8 10 12
Hypoxia: − + − +
Il12b
p<0.0001
mRNA (fold)
0 500 1000 1500 2000 2500 3000 3500 4000
LPS: − + − +
Slc2a1 (Glut1)
mRNA (Fold)
0 2 4 6 8 10 12
Hypoxia: − + − +
Ptgs2 (Cox2)
p=0.0006
mRNA (Fold)
0 100 200 300 400 500 600 700 800 900
LPS: − + − +
Ptgs2 (Cox2)
mRNA (fold)
0.0 0.5 1.0 1.5 2.0 2.5
Hypoxia: − + − +

Figure 4

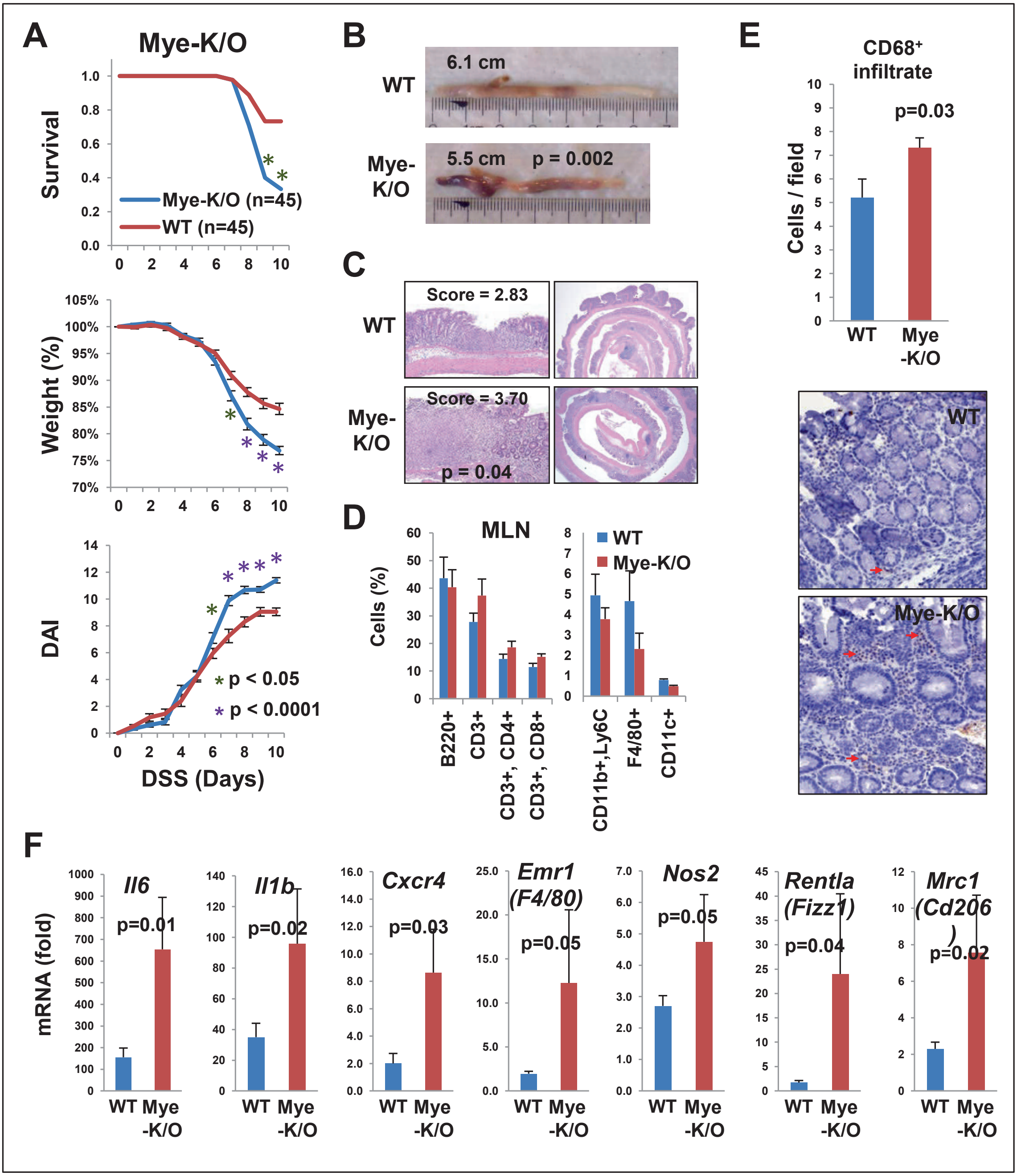
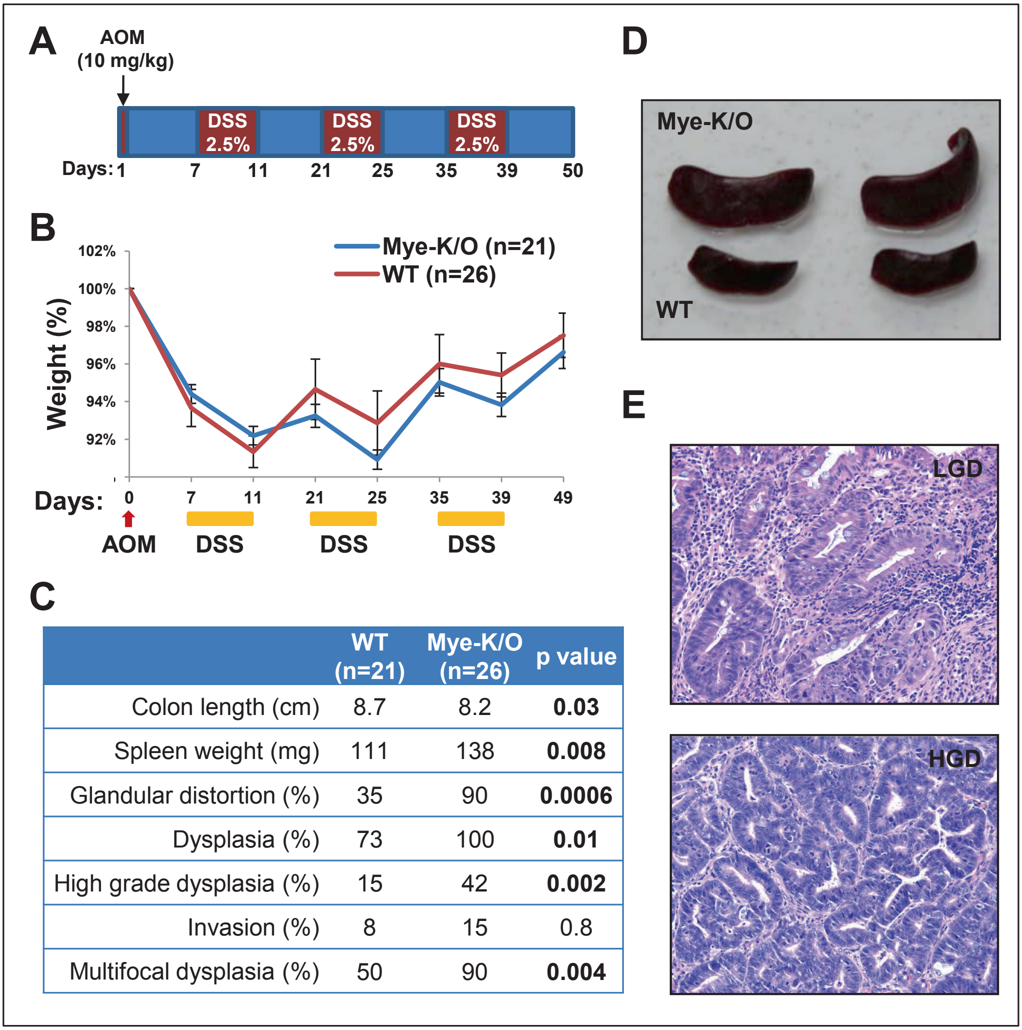
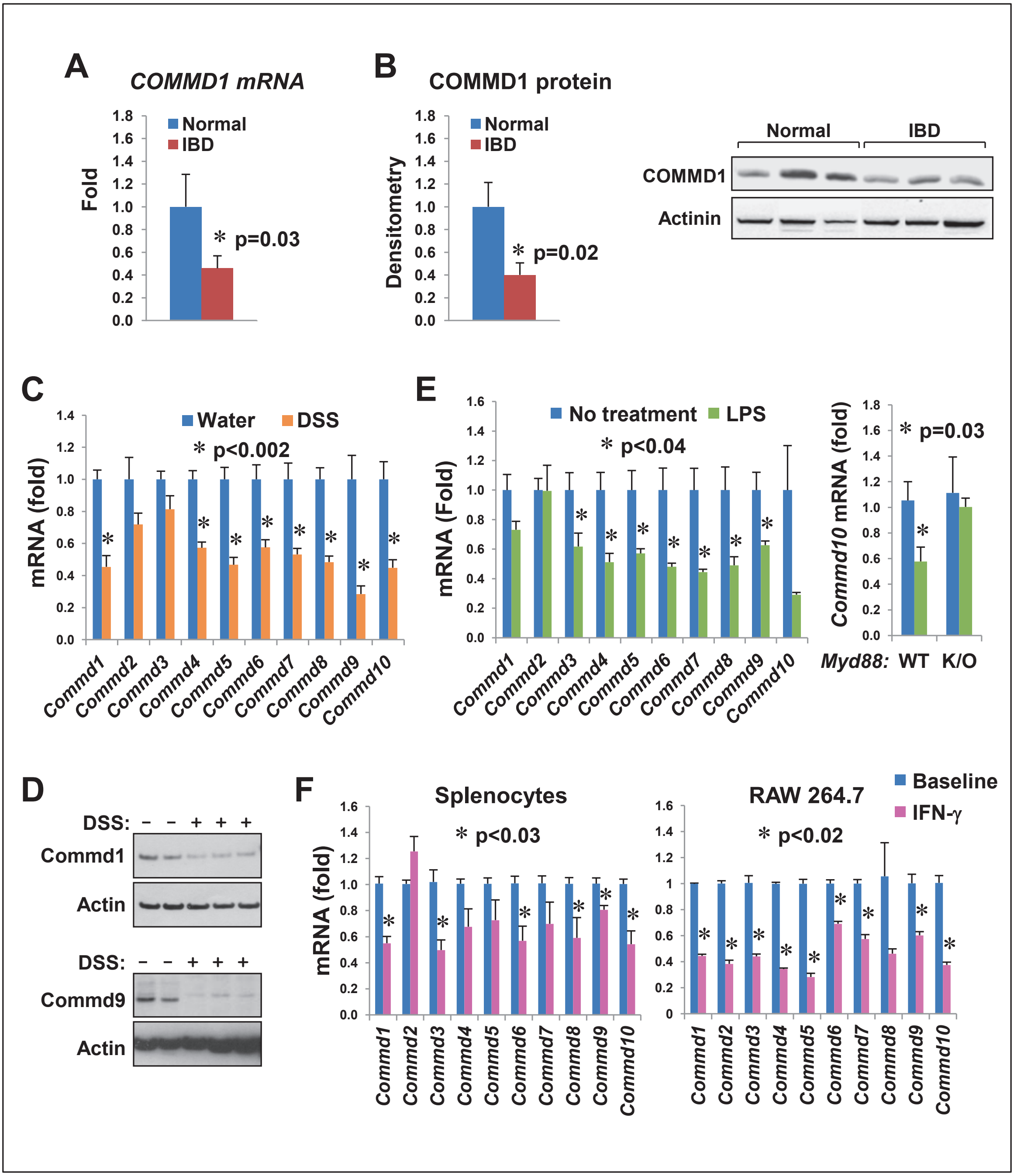

A
Mye-K/O
Survival
1.0
0.8
0.6
0.4
0.2
0.0
0 2 4 6 8 10
Mye-K/O (n=45)
WT (n=45)
*
Weight (%)
105%
100%
95%
90%
85%
80%
75%
70%
0 2 4 6 8 10
DAI
14
12
10
8
6
4
2
0
0 2 4 6 8 10
* p < 0.05
* p < 0.0001
DSS (Days)
B
WT
6.1 cm
Mye-K/O
5.5 cm
p = 0.002
C
WT
Score = 2.83
Mye-K/O
Score = 3.70
p = 0.04
D
MLN
Cells (%)
60
50
40
30
20
10
0
8
7
6
5
4
3
2
1
0
WT
Mye-K/O
B220+
CD3+
CD3+, CD4+
CD3+, CD8+
CD11b+,Ly6C
F4/80+
CD11c+
E
CD68+ infiltrate
p=0.03
Cells / field
10
9
8
7
6
5
4
3
2
1
0
WT
Mye-K/O
WT
Mye-K/O
F
mRNA (fold)
Il6
p=0.01
Il1b
p=0.02
Cxcr4
p=0.03
Emr1 (F4/80)
p=0.05
Nos2
p=0.05
Rentla (Fizz1)
p=0.04
Mrc1 (Cd206)
p=0.02
WT
Mye-K/O

Figure 5



	WT (n=21)	Mye-K/O (n=26)	p value
Colon length (cm)	8.7	8.2	**0.03**
Spleen weight (mg)	111	138	**0.008**
Glandular distortion (%)	35	90	**0.0006**
Dysplasia (%)	73	100	**0.01**
High grade dysplasia (%)	15	42	**0.002**
Invasion (%)	8	15	0.8
Multifocal dysplasia (%)	50	90	**0.004**

Figure 6


A
COMMD1 mRNA
Fold
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Normal
IBD
* p=0.03
B
COMMD1 protein
Densitometry
Normal
IBD
* p=0.02
Normal
IBD
COMMD1
Actinin
C
mRNA (fold)
Water
DSS
* p<0.002
Commd1
Commd2
Commd3
Commd4
Commd5
Commd6
Commd7
Commd8
Commd9
Commd10
E
mRNA (Fold)
No treatment
LPS
* p<0.04
Commd10 mRNA (fold)
* p=0.03
Myd88: WT K/O
D
DSS: – – + + +
Commd1
Actin
DSS: – – + + +
Commd9
Actin
F
Splenocytes
mRNA (fold)
* p<0.03
RAW 264.7
* p<0.02
Baseline
IFN-γ

Supplemental Figures

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Figure S1

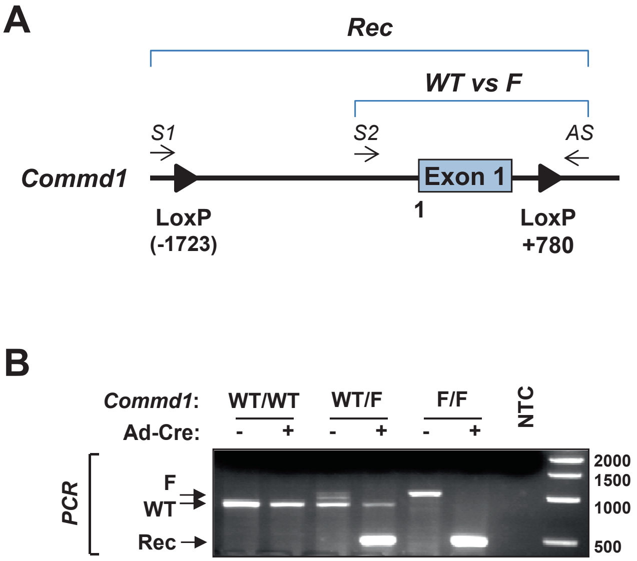
A
Rec
WT vs F
S1
S2
AS
Commd1
Exon 1
1
LoxP
(-1723)
LoxP
+780
B
Commd1:
WT/WT
WT/F
F/F
NTC
Ad-Cre:
-
+
-
+
-
+
PCR
F
WT
Rec
2000
1500
1000
500

Figure S1. Generation of *Commd1* deficient fibroblasts. **(A)** Schematic representation of the position of LoxP sites in the *Commd1* gene, and the position of primers used to detect recombination of this allele. **(B)** Murine embryonic fibroblasts from *Commd1*$^{WT/WT}$, *Commd1*$^{WT/F}$ and *Commd1*$^{F/F}$ mice were infected by an adenovirus expressing Cre (+) or a LacZ control virus (-). Recombination in the *Commd1* gene was detected by PCR using primers depicted in (A).

Figure S2

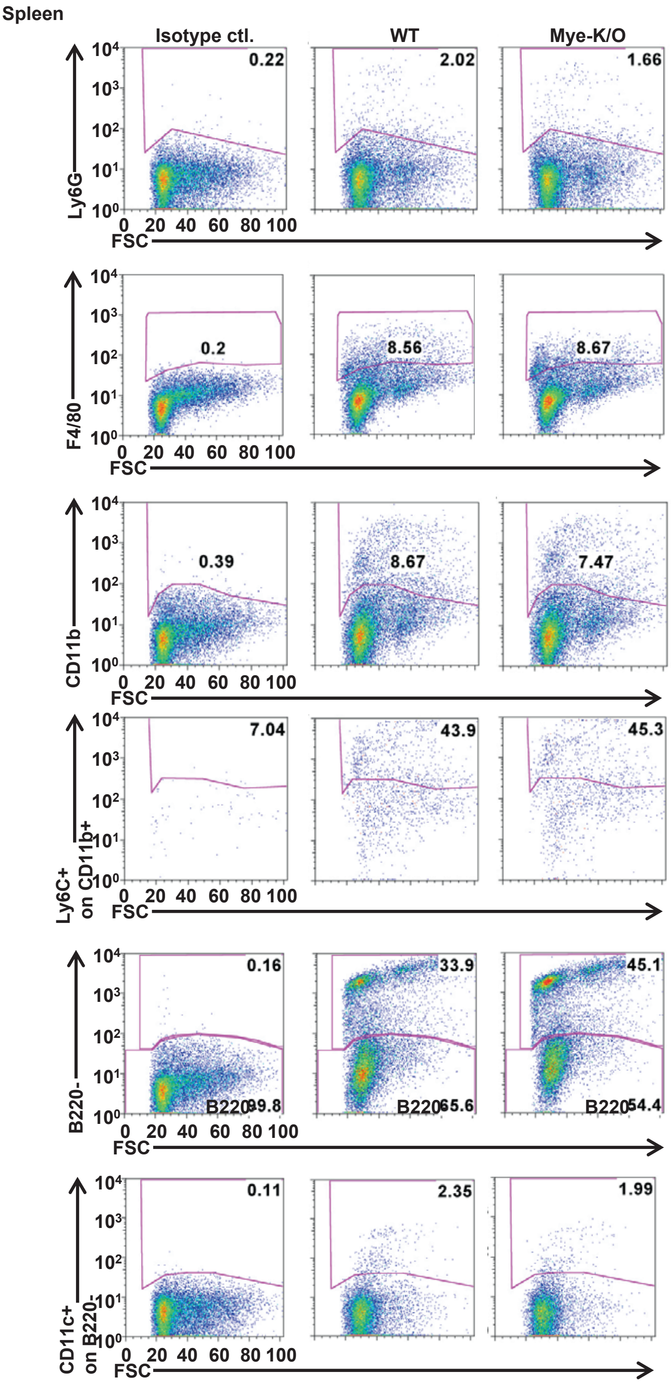

Figure S2. Immune cell populations were not significantly different in Mye-K/O mice compared to WT controls. Flow cytometry immunophenotypic analysis of splenocytes was performed using markers for granulocytes (Ly6G+), macrophages (CD11b+ and Ly6C+), activated phagocytes (F4/80), dendritic cells (CD11c+) and B lymphocytes (B220+). Representative plots of cells expressing the indicated markers are shown.

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Figure S3

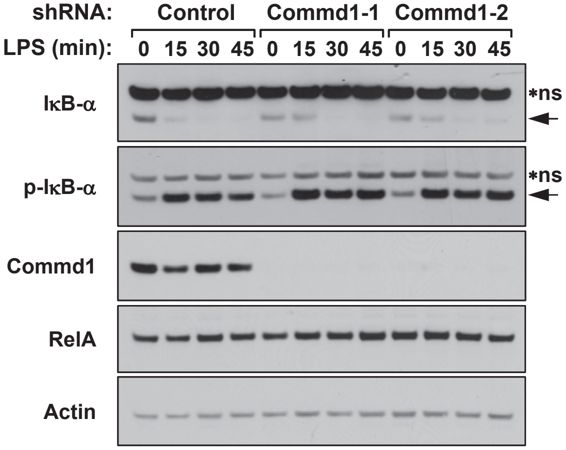
shRNA:
Control
Commd1-1
Commd1-2
LPS (min):
0 15 30 45 0 15 30 45 0 15 30 45
IκB-α
*ns
p-IκB-α
*ns
Commd1
RelA
Actin

Figure S3. Commd1 deficiency did not significantly affect Tlr4 dependent activation of the NF-κB pathway. RAW264.7 cells were infected with lentiviral particles to stably express 2 different shRNA constructs targeting Commd1. These cells were treated with LPS and cell lysates were subjected to western blot analysis. Comparable kinetics of IκB-α degradation and phosphorylation were found in control and Commd1 deficient cells.

Figure S4

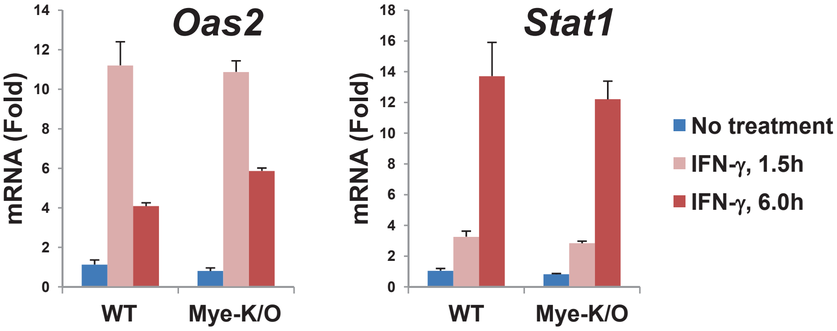

Figure S4. Commd1 deficiency did not substantially affect interferon inducible gene expression. Bone-marrow derived myeloid cells were stimulated with IFN-γ and expression of Oas2 and Stat1 was determined by qRT-PCR. *Commd1* deficiency did not significantly affect gene expression upon IFN-γ stimulation.

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Figure S5

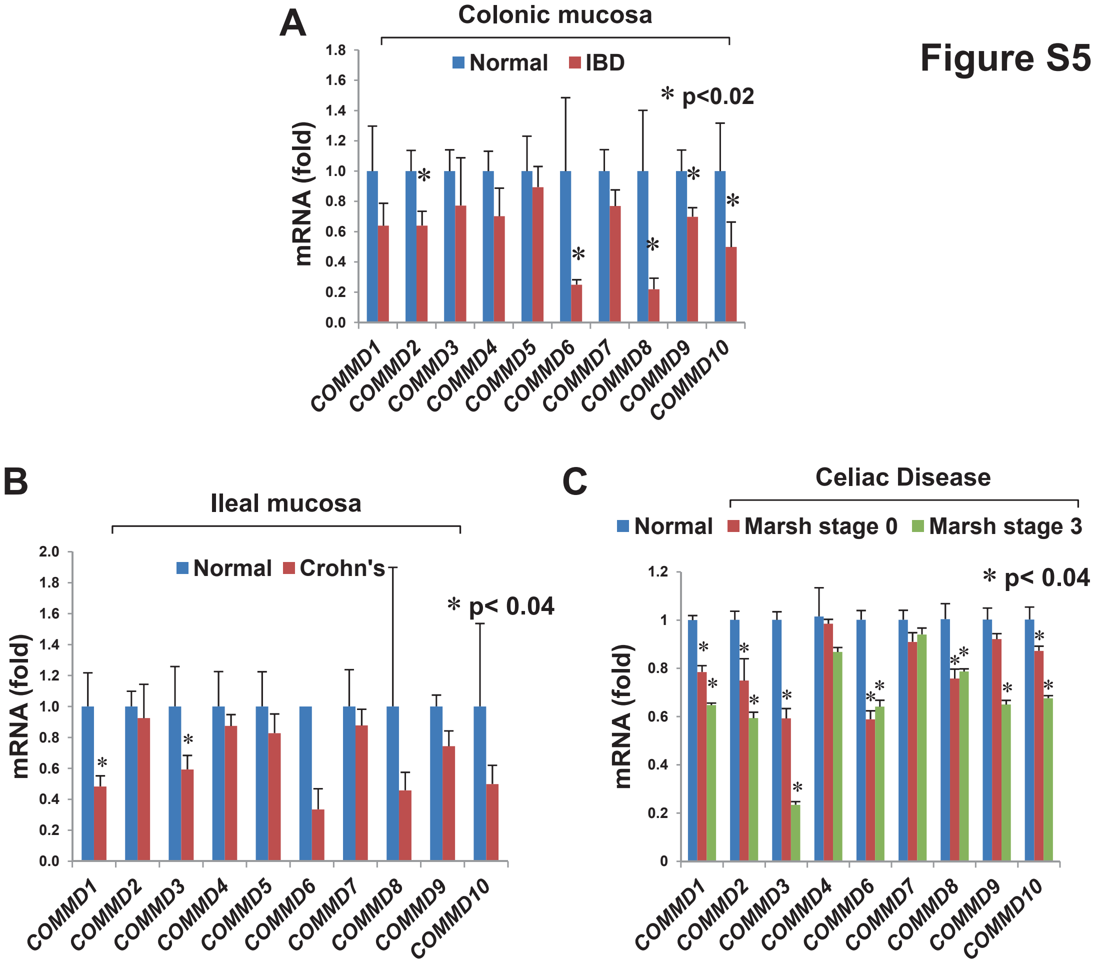

Figure S5. *COMMD* gene suppression was found in inflammatory diseases of the gastrointestinal tract. (**A**) Expression of *COMMD* genes in endoscopic biopsies of the colon was determined by qRT-PCR in patients with IBD (n=28, 17 with Crohn's disease and 11 with ulcerative colitis) and compared to normal individuals (n=13). **(B)** Expression of *COMMD* genes in endoscopic biopsies of the ileum was determined by qRT-PCR in patients with Crohn's disease (n=7) and compared to normal individuals (n=4). **(C)** Decreased *COMMD* gene expression was also noted in small intestinal biopsies from patients with celiac disease compared to controls (pools of 5 patient samples per group). Histologic severity according to the Marsh score is also indicated.

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Figure S6

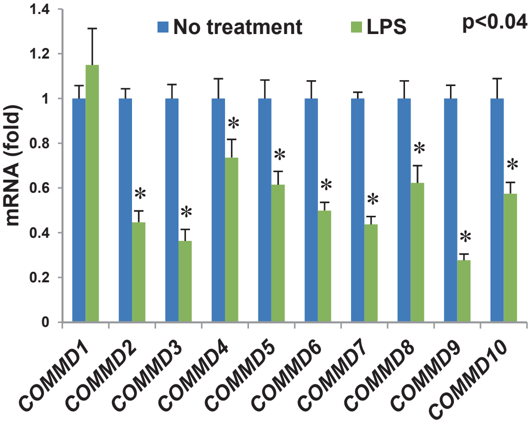

Figure S6. LPS induce *COMMD* gene suppression. LPS stimulation of blood cells from normal volunteers (n=5) induced a similar repression of *COMMD* mRNA expression.

Figure S7

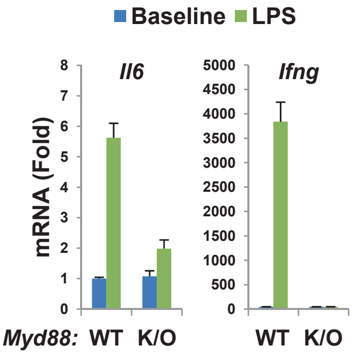

Figure S7. Splenocytes from *Myd88*$^{-/-}$ mice failed to respond to LPS stimulation. LPS-induced *Il6* and *Ifng* expression, determined by qRT-PCR, was abolished in *Myd88*$^{-/-}$ splenocytes.

Figure S8

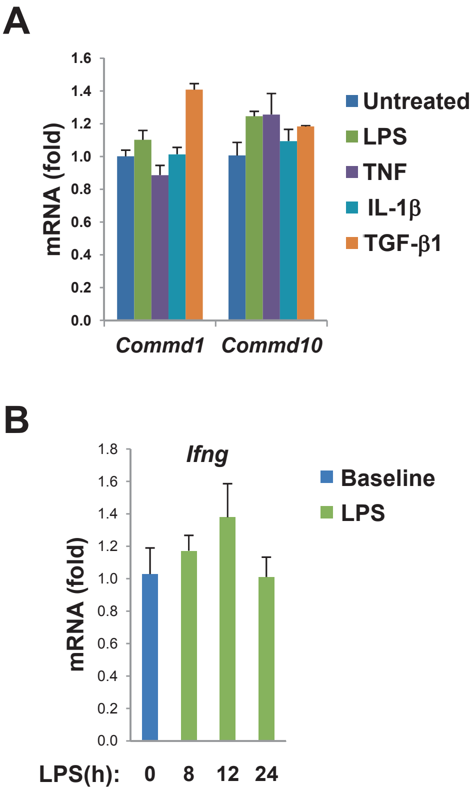

Figure S8. LPS fails to induce *Commd* gene suppression in the absence of *Ifng* induction. **(A)** Stimulation of RAW264.7 macrophages with LPS, TNF, IL-1β, or TGF-β1 failed to induce suppression of 2 representative *Commd* genes. **(B)** These cells do not induce *Ifng* expression in response to LPS stimulation.

Figure S9

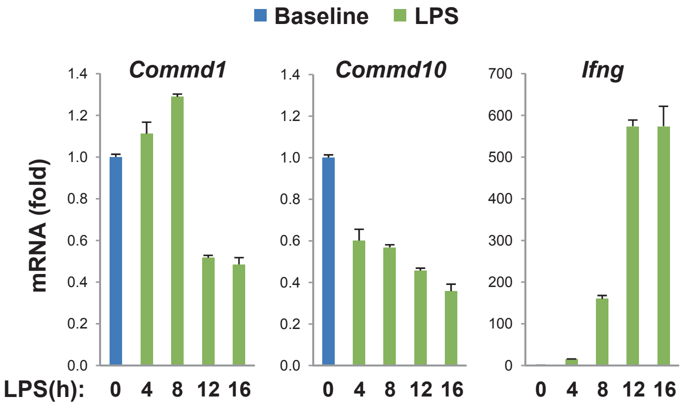

Figure S9. *Ifng* induction is temporally correlated with *Commd* gene suppression in splenocytes. LPS-induced *Commd1* and *Commd10* suppression , along with *Ifng* induction, were determined over a time course by qRT-PCR.

Table S1: Morbidity score for the LPS model of sepsis

APPEARANCE	
0	Normal
1	Lack of grooming
2	Coat rough, possible nasal or ocular discharge
3	Coat very rough, abnormal posture, eyes sunken and glazed
CLINICAL SIGNS	
0	Normal
1	Diarrhea, constipation
2	Respiratory rate altered, respiratory depth altered, skin tents
3	Cyanotic extremities, labored breathing
UNPROVOKED BEHAVIOR	
0	Normal
1	Minor changes
2	Abnormal behavior, less mobile, less alert, inactive when activity expected
3	Paralysis, inability to remain upright, shivering, convulsion
Total score: Sum of (Domain scores) for each domain in the scoring system	

Table S2: Disease activity index in the acute DSS colitis model

WEIGHT	
0	Unchanged
1	Loss of 1-5%
2	Loss of 6-10%
3	Loss of 11-20%
4	Greater than 20% weight loss
STOOL CONSISTENCY	
0	Normal
2	Loose stools (not watery)
4	Diarrhea (liquid stool)
BLEEDING	
0	None
2	Hemoccult positive stools
3	Visible blood on stool
4	Gross bleeding per rectum
Total score:	Sum of (Domain scores) for each domain in the scoring system

Table S3: Histologic score for the acute DSS colitis model

DOMAIN	INVOLVEMENT
A) CRYPT LOSS 0 Intact 1 Loss of basal one-third of crypt 2 Loss of basal two-thirds of crypt 3 Loss of entire crypt 4 Erosion (loss of entire crypt and surface epithelium)	1 1-25% of surface 2 26-50% of surface 3 51-75% of surface 4 76-100% of surface
B) CRYPT DISTORTION 0 None 1 Basal one-third of crypt 2 Basal two-thirds of crypt 3 Entire crypt 4 Entire crypt and surface epithelium	
C) INFLAMMATION 0 None 1 Mild 2 Moderate 3 Severe	
D) HYPERPLASTIC EPITHELIUM 0 None 1 Basal one-third of crypt 2 Basal two-thirds of crypt 3 Entire crypt 4 Entire crypt and surface epithelium	
Total score: Sum of (Domain score x involvement score) for each domain in the scoring system	

Table S4: Primer sequences used in quantitative PCR analysis

Mouse genes

Gene target	*Sense primer*	*Antisense primer*	*Reference*
Actb	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT	This study
Adm	TCCGAAAGAAGTGGAATAAGTGG	GTCCCGTAGGGTAGCTGCT	PrimerBank ID 6752987b2
Ccl5	GTGCCCACGTCAAGGAGTAT	CCCACTTCTTCTCTGGGTTG	Ramirez-Carrozzi, et al[6]
Cd40	TCACCATTTTCGGGGTGTTTC	CCGCAGGGGGTAACATCTC	PrimerBank ID 6755829a1
Chi3l3	ATCTATGCCTTTGCTGGAATGC	TGAATGAATATCTGACGGTTCTGAG	Zhao, et al[7]
Commd1	TGGCGAAGATGAGAGGACTT	GTGATGCCACCTTGCTTTTT	This study
Commd2	Taqman primer and probe set from Applied Biosystems		
Commd3	Taqman primer and probe set from Applied Biosystems		
Commd4	Taqman primer and probe set from Applied Biosystems		
Commd5	Taqman primer and probe set from Applied Biosystems		
Commd6	Taqman primer and probe set from Applied Biosystems		
Commd7	Taqman primer and probe set from Applied Biosystems		
Commd8	Taqman primer and probe set from Applied Biosystems		
Commd9	CCTCCTCTGACAACATCAGC	GGAGGGTTTCTCTCCACAC	This study
Commd10	TGAGAAGTTCCGCCAGAG	TTGCTCACTCCCAGTTGC	This study
Cxcl1	TGTCAGTGCCTGCAGACCAT	GTGGCTATGACTTCGGTTTGG	Ramirez-Carrozzi, et al[6]
Cxcr4	GACTGGCATAGTCGGCAATG	AGAAGGGGAGTGTGATGACAAA	PrimerBank ID 16268122b1
Emr1	AAAGACTGGATTCTGGGAAGTTTGG	CGAGAGTGTTGTGGCAGGTTG	Zhao, et al[7]
Gapdh	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA	This study
Ifng	ACTGGCAAAAGGATGGTGAC	TGAGCTCATTGAATGCTTGG	Benson et al[8]
Il1b	GCTGAAAGCTCTCCACCTCA	AGGCCACAGGTATTTTGTCG	Ramirez-Carrozzi, et al[6]
Il6	TGGATGCTACCAAACTGGAT	GGACTCTGGCTTTGTCTTTC	Moreno, et al[9]
Il12b	AGCCACTCACATCTGCTGCT	AACCGTCCGGAGTAATTTGG	Ramirez-Carrozzi, et al[6]
Mrc1	TTTGGAATCAAGGGCACAGAG	TGCTCCACAATCCCGAACC	Zhao, et al[7]
Nfkbia	CGCAGACCTGCACACCCCAG	GGAGGGCTGTCCGGCCATTG	Li, et al[10]
Nos2	CAGCTGGGCTGTACAAACCTT	CATTGGAAGTGAAGCGTTTCG	Ramirez-Carrozzi, et al[6]
Oas2	AAACCTCACACCCAACGAAAA	CACCGAGGACACCGCAATC	PrimerBank ID 29576888b1
Ptgs2	TGTGACTGTACCCGGACTGG	TGCACATTGTAAGTAGGTGGAC	PrimerBank ID 18130137b2
Rentla	CCTCCACTGTAACGAAGACTCTC	GCAAAGCCACAAGCACACC	Zhao, et al[7]
Saa3	CTGTTCAGAAGTTCACGGGAC	AGCAGGTCGGAAGTGGTT	Moreno, et al[9]
Slc2a1	GGGCATGTGCTTCCAGTATGT	ACGAGGAGCACCGTGAAGAT	Mastrogiannaki, et al[11]
Stat1	TCACAGTGGTTCGAGCTTCAG	CGAGACATCATAGGCAGCGTG	PrimerBank ID 14326481b1
Tnf	CCCCAAAGGGATGAGAAGTT	TGGGCTACAGGCTTGTCACT	Ramirez-Carrozzi, et al[6]
Tnfaip3	GGCAGCTGGAATCTCTGAAA	CTGCAGGTGTGTCTGCTGAT	Ramirez-Carrozzi, et al[6]
Vegfa	CTTGTTCAGAGCGGAGAAAGC	ACATCTGCAAGTACGTTCGTT	PrimerBank ID 6678563a2

Human genes

Gene target	*Sense primer*	*Antisense primer*	*Reference*
ACTB	GCGGGAAATCGTGCGTGACATT	GATGGAGTTGAAGGTAGTTTCGTG	This study
COMMD1	GCAAATATGGACAGGAATC	CAGAATTTGGTTGACTTTGAC	Burstein, et al[5]
COMMD2	CTCAAAGCTCATGATTTC	TGTCCAGATAAAGCTGAAG	Burstein, et al[5]
COMMD3	TGACAGAGAGCGAATAGAAC	TGCCTATACTTCCCAGTAG	Burstein, et al[5]
COMMD4	CCAAGATGTCCTCTGTGAAG	ACATCGCCTGACTCAAAC	Burstein, et al[5]
COMMD5	ATACTGCGGGGCTTTTCC	ATTGCTGCTGCTTCCTCTTT	This study
COMMD6	CATTCAGGCCAAGTAAAG	TAGAAATTCTGAAACTGTGG	Burstein, et al[5]
COMMD7	GGCGCGCAGCAGTTCTCA	CGATGCTTCTGAGGGAGCCAAG	Burstein, et al[5]
COMMD8	GTTTGGGAATCAGAAGAATG	GCTGAAATATCTCTTCATCAG	Burstein, et al[5]
COMMD9	GTCGATCTGGACTGGAGAG	TCTTCTTGGATCTTCATCTGG	Burstein, et al[5]
COMMD10	TCTATGGGTCAAGAAACAG	CGGTCTCTAGCTTACAGG	Burstein, et al[5]
GAPDH	CCCACTCCTCCACCTTTGAC	TGTTGCTGTAGCCAAATTCGT	This study

Chapter 8

General Discussion

General Discussion

Inflammation is a complex multicellular response to tissue injury that is genetically programmed and highly regulated (*1, 2*). Its importance in physiology and disease pathogenesis cannot be overstated. In fact, a large number of human diseases involve an inflammatory process either as the driving force that leads to tissue injury, or as a necessary contributor to the disease process. As such, today it is recognized that inflammation plays a critical role in such disorders as the metabolic syndrome (*3*), atherosclerosis (*2*), and neurodegeneration (*4*) which have not been traditionally viewed as primary inflammatory disorders. In addition, inflammation is now regarded as a cardinal characteristic of cancer, where it can play a critical role in initiating the neoplastic process or in promoting tumor growth and metastasis (*1, 5, 6*).

Among the various mechanisms that orchestrate the inflammatory response, activation of the transcription factor NF-κB is a critically important event (*6-8*). This transcription factor, initially identified through homology with the viral oncoprotein v-Rel (encoded by the avian reticuloendotheliosis virus), consists of a family of polypeptides that form dimers and bind DNA through a homology domain termed the Rel homology domain or RHD (*9*). In mammals, 7 polypeptides encoded by 5 genes (indicated in brackets) are recognized as the main NF-κB subunits: RelA, also referred to as p65 (*RELA*), c-Rel (*REL*), RelB (*RELB*), p50 and its precursor p105 (*NFKB1*), and p52 and its precursor p100 (*NFKB2*). While multiple heterodimer combinations have been described, the Rel proteins are the only ones that contain a transactivation domain capable of mediating gene expression.

The classical or canonical NF-κB pathway is mediated primarily by RelA or c-Rel containing dimers, with the RelA/p50 heterodimer being particularly abundant (*9*). Under basal conditions, this pathway is inhibited by the so-called classical Inhibitors of kB (IκB-α, -β, and -ε), which bind to NF-κB dimers and prevent DNA binding and nuclear accumulation (*10*). Canonical NF-κB activation can occur downstream of a variety of upstream signaling pathways that converge on the IκB kinase complex (IKK), resulting in phosphorylation and degradation of IkB proteins (*11-13*). A related activation pathway for RelB containing complexes has been elucidated and termed the non-canonical NF-κB pathway in which RelB/p100 heterodimers are activated by IKKα-mediated phosphorylation of p100 and its proteolytic processing to p52 (*14-17*).

Given its central role in NF-κB activation, the intricate signaling pathways and biochemical events that control IKK function have received great attention. On the other hand, the events responsible for terminating NF-κB dependent transcription have not been as extensively studied. In this regard, it is known that NF-κB activates transcription of IkB encoding genes, thus resulting in expression of its own inhibitors (*9*). Similarly, the IKK inhibitors A20 and CYLD are also encoded by NF-κB responsive genes (*18, 19*). Therefore, NF-κB dependent transcription sets off a negative feedback loop that promotes a return to basal conditions. In addition to this, work over the past decade has uncovered that DNA-bound active NF-κB subunits are actively removed by a ubiquitin dependent mechanism (*20-25*). The identification of the ubiquitin ligase(s) responsible for this step in the pathway and its regulation have been the main areas of interest of my laboratory. The cumulative evidence presented in this thesis indicates that COMMD1 is an essential cofactor for the ubiquitin ligase responsible for NF-κB termination, and is physiologically important for inflammation and human disease.

Ubiquitination, CRLs and the NF-κB pathway

Ubiquitin is a highly conserved 76 amino acid polypeptide that can be conjugated to substrate proteins through an enzymatic machinery present in all eukaryotic cells. A sequential reaction involving 3 enzymes culminates in the transfer of the ubiquitin moiety to the target protein by an E3 enzyme, also referred to as a ubiquitin ligase. Through an isopeptide bond, a lysine residue on the target protein is linked to the carboxyl-terminal glycine of ubiquitin. In a similar way, additional ubiquitin residues can be added onto ubiquitin itself to form polyubiquitin chains. Polyubiquitin chains formed onto Lysine 48 of ubiquitin direct the substrate for degradation by the proteasome, a multimeric protease complex (*26*). However, other lysine linkages result in different effects such as signaling activation or transcriptional regulation.

A particularly prolific group of ubiquitin ligases are multimeric complexes containing at their core a protein of the Cullin family and the RING domain containing proteins Rbx1 or Rbx2 (*27-29*). These complexes are referred to as Cullin RING ligases (CRL). A substrate binding complex (SBC) composed of 1 to 3 polypeptides is recruited to the amino-terminus of the Cullin protein. Each of the Cullin proteins present in mammals (Cul1, Cul2, Cul3, Cul4A, Cul4B, Cul5, and Cul7) has a specific preference for particular SBCs (*27*). For example, Cul1 and Cul7 interact with complexes containing the adaptor protein Skp1 and any of a multitude of F box proteins, while Cul2 and Cul5 interact with complexes containing the Elongin B and C polypeptides and any of several factors containing a BC box.

CRLs regulate multiple transcription factors including NF-κB. In fact, the NF-κB pathway is regulated at various levels by ubiquitination steps and in particular, two CRLs have been linked to this pathway. A Cul1-containing CRL, the CRL1-bTrCP complex is responsible for ubiquitinating phosphorylated IkB proteins (*30-34*). This results in their proteasomal degradation, leading to the nuclear translocation of NF-kB complexes. As alluded before, ubiquitination of the NF-κB subunits themselves has been recognized as a mechanism of regulation in this pathway, as it is required for proper transcriptional termination. Work from our group and other laboratories has identified a Cul2-containing CRL as the ubiquitin ligase responsible for NF-κB/RelA degradation (*21, 23, 24*).

COMMD1 is a required factor for NF-kB ubiquitination and signal termination

In 2003, we reported that COMMD1, referred to at the time as Murr1, is an inhibitor of NF-κB dependent gene expression (*35*). These studies were the result of a yeast two-hybrid screen for interacting proteins that bind to the anti-apoptotic factor XIAP (*36*). Later work identified that COMMD1 binds to all NF-κB subunits through a conserved motif in the RHD (*37*). The effects of COMMD1 on NF-κB activity lead to decreased pro-inflammatory gene expression (*23, 35, 37*) and have been similarly implicated in the ability of COMMD1 to inhibit HIV-1 replication (*35*).

We demonstrated that binding of COMMD1 to the NF-κB subunit RelA decreases the duration of RelA recruitment to promoter sites. Interestingly, COMMD1 itself is also recruited to promoter sites in an inducible manner alongside with NF-κB (*37*). Based on this observation and reports at the time that ubiquitination controls chromatin occupancy, we investigated whether COMMD1 plays a role in RelA ubiquitination. Through a series of studies, we demonstrated that COMMD1 promotes the ubiquitination of RelA, decreasing its half-life, particularly in the nuclear compartment (*23*).

The mechanism by which COMMD1 promotes RelA ubiquitination is through its association with a Cul2-containing CRL. Work from Ryo and colleagues had identified that SOCS1 promotes the ubiquitination of RelA (*21*). This factor is devoid of intrinsic E3 activity but serves as substrate binding subunit for a Cul2-containing CRL. Therefore, we investigated whether COMMD1 might interact with Cul2, as well as the other components of the complex. We found that to be the case, and moreover, found that COMMD1-mediated ubiquitination of RelA requires the expression of ligase subunits such as Cul2 (*23*). Conversely, deficiency of COMMD1 greatly impaired the ubiquitination of RelA and promoted NF-κB mediated transcription in transformed cell lines, and more recently, in cells derived from *Commd1* deficient mice (Li et al, *submitted*). Interestingly, others have reported that certain viruses of the herpes family encode proteins that can associate with a Cul2 or Cul5 complex to form an active ligase targeting RelA for ubiquitination (*38, 39*). This mechanism seems to provide the virus with the means to attenuate NF-kB activation and promotes completion of the viral cycle.

The ability of COMMD1 to inhibit NF-κB mediated transcription has been independently replicated by other groups (*24, 40, 41*). Moreover, the critical events that we described, namely the recruitment of COMMD1 to chromatin, its ability to promote RelA ubiquitination and its release from promoter sites have been similarly re-demonstrated by other laboratories. Interestingly, there seems to be a correlation between the effects of COMMD1 on gene expression and its ability to be recruited to promoter sites, whereby genes that are not affected by COMMD1 levels do not demonstrate the presence of this factor in the promoter region, and vice versa (*24*). However, the mechanisms responsible for COMMD1 recruitment to chromatin, particularly in a gene selective manner, remain unknown.

Identification of the COMMD gene family

The discovery of COMMD1 as a regulator of NF-κB mediated transcription was nearly coincidental to the identification of this gene as a regulator of copper metabolism in mammals (*42-47*). An effort by van de Sluis and colleagues to identify the gene responsible for an autosomal recessive form of copper toxicosis in dogs culminated in 2002 with the discovery of a large deletion in the *MURR1* gene -today referred to as *COMMD1*- as the cause of this syndrome (*42*). In parallel to those studies, we had already identified a family of 10 homologous factors present in a range of organisms from unicellular protozoa to humans (*37*). These factors otherwise had no known function and were largely known only as ESTs. In consultation HGNC, these genes were named "*Co*pper *m*etabolism *M*URR1 *d*omain containing", or COMMD, with MURR1 being renamed as COMMD1.

All members in this family possess a carboxyl-terminal homology domain that is unique to this protein family (*48*). This region is necessary and often sufficient to mediate critical protein-protein interactions. For example, members of this protein family can bind to each other via their COMM domains (*37*). The precise stoichiometry of these complexes is not known, and although the structure of the amino-terminus of COMMD1 has been solved, the COMM domain has been more difficult to investigate due to its tendency to form insoluble aggregates when purified at high concentrations (*49*).

The precise biological roles of most COMMD proteins remain largely unknown. Only five reports regarding COMMDs other than COMMD1 have been published to date and concern COMMD5 and COMMD6 function (*41, 50-52*). We reported that other COMMD proteins share some properties with COMMD1, at least with regards to NF-κB regulation (*37*). However, despite these similarities, the conservation of all 10 members of this family along a great evolutionary canvas suggests that individual *COMMD* genes have unique and non-redundant functions. Consistent with this notion, *Commd1* deficiency in mice is embryonic lethal by ~ E8.5 indicating that its deficiency cannot be compensated by other family members (*53*). Mutant embryos display abnormal placental vasculogenesis and inappropriate activation of the HIF pathway. In preliminary studies, we have found that deficiency of *Commd9* or *Commd10* are also lethal during embryogenesis. In each case, the defect is different and does not match the reported phenotype of the *Commd1* knockout, highlighting their unique developmental functions.

Post-translational modifications of NF-kB as triggers for the ubiquitination pathway

One critical mechanistic aspect of the ubiquitination of NF-kB is how this process is targeted to active NF-κB subunits and how this is triggered at the right stage in the transcriptional response. While the answer to this question is not complete, we and others have demonstrated that NF-κB/RelA phosphorylation plays a central role in this process. Specifically, IKK dependent phosphorylation and modification of Serine 468 are required to trigger RelA ubiquitination (*24, 25*). Interestingly, Serine 536 phosphorylation has been similarly implicated in myeloid cells (*54*), but whether this is linked to COMMD1-depedent ubiquitination remains unclear.

The link between RelA phosphorylation on Serine 468 and its subsequent ubiquitination by a COMMD1-containing ligase was not initially evident. In this regard, a tandem affinity purification scheme identified an interaction between COMMD1 and the histone acetylase GCN5. Interestingly, GCN5 proved to be a suppressor of NF-κB dependent gene expression and it is able to promote RelA ubiquitination (*25*). In this role, its acetylase activity is completely dispensable, but its interaction with COMMD1 and the CRL2 ligase seems critical. One unique feature of GCN5 is its preferential binding to phosphorylated RelA, particularly at Serine 468. Thus, GCN5 serves a role similar to a substrate adaptor, being able to bind to the phosphorylated form of the substrate and thus bringing it into contact with the ligase for subsequent ubiquitination. Altogether, this work highlighted the intricacy of the ubiquitination mechanism and demonstrated for the first time an acetylation-independent role for GCN5. In addition, more recent work highlights the fact that other post-translational modifications of lysine residues, such as acetylation, can compete with ubiquitination for the same residues and thus modulate protein stability (*55*).

Despite these advances, a number of questions still remain. For example, the gene specific nature of the regulation that this pathway offers, suggests that it is highly targeted. We speculate that RelA ubiquitination is selectively induced on specific gene promoters where COMMD1 is recruited. However, the mechanism that mediates this selective recruitment is currently unknown and the post-translational modifications at play remain to be discovered. Similarly, while IKK and Serine 468 phosphorylation are clearly required for GCN5-RelA interactions *in vivo*, these are not sufficient to induce the above biochemical events in vitro, thus suggesting that additional post-translational modification may be at play. Finally, up to this point all of our attention has been focused on RelA, the most abundant NF-κB subunit. Nevertheless, there is evidence that the same ligase components can trigger the ubiquitination of other NF-κB family members (*23*), suggesting other potential roles for COMMD1 in the regulation of NF-κB function.

COMMD1 mediated regulation of CRLs

Our work has also uncovered critical details of the molecular mechanism of action of COMMD proteins. In this regard, the specific role of COMMD1 binding to CRLs remained unclear until recently. Our initial studies suggested that COMMD1 can improve SOCS1-RelA interactions, and perhaps facilitate substrate recruitment (*23*). However, this failed to explain the broad interactions of COMMD1 with other Cullin family members. In this regard, our most recent studies implicate COMMD1 and other COMMD family members in the activation cycle of CRL complexes (*56*). CRL complexes undergo a cycle of activation characterized by sequential changes in complex composition and post-translational modifications. In its most active form, CRLs are bound to the substrate binding complex (SBC) and the Cullin protein is modified by neddylation, a post-translational modification with the ubiquitin-like modifier Nedd8 which promotes ligase activity (*57*). In addition, neddylation prevents binding of CAND1, a key CRL inhibitor that prevents recruitment of the SBC to the CRL (*58-60*). The transition from the neddylated active state to the deneddylated and CAND1-bound state is mediated by the COP9 signalosome, a large protein complex that deneddylates Cullin proteins.

Upon formation of a CAND1-Cullin complex, its dissociation and the initiation of the activation cycle are not well understood. It is known that this event requires energy (ATP) and is promoted by the SBC, but the specific events involved are still poorly understood (*59, 61*). Our recent work identified that COMMD1 is involved in this process (*56*). COMMD1 and CAND1 compete for binding to a common and conserved domain among Cullin proteins. Moreover, COMMD1 can displace CAND1 from CRLs, thus promoting the initiation of the activation cycle.

This work suggests a model in which COMMDs play a role in the activation of possibly hundreds of CRLs and therefore it predicts that these proteins can play vast biological roles beyond regulation of the immune response. Indeed, this notion is in agreement with the diverse function already ascribed to COMMD1 (*62*) and the increasing complexity of roles that other COMMD genes likely play based on their unique developmental roles.

Role of COMMD1 in inflammation and tumor progression

A critically important question is to what extent COMMD1 plays a role in regulating inflammation *in vivo*. Our studies using tissue-specific *Commd1* deletion in mice are beginning to address this question. The evidence indicates that this gene plays a critical anti-inflammatory role in the innate immune system. In particular, we find that deficiency of this factor in myeloid cells leads to a pro-inflammatory phenotype characterized by exaggerated cytokine induction in response to bacterial dissemination.

Moreover, these mice also display a propensity to intestinal inflammation and its progression to dysplasia and cancer. In this regard it is striking to note that individuals with inflammatory bowel disease (IBD), an idiopathic disorder characterized by chronic intestinal inflammation, display reduced COMMD1 expression in peripheral leukocytes and in the intestinal mucosa. Thus, IBD is a state of partial COMMD1 deficiency and the evidence from the myeloid knockout model would argue that this change in turn would foment the inflammatory process. Genetic evidence linking *COMMD1* to IBD risk is still being explored but the 2p15 region where COMMD1 resides has already been linked to the risk of IBD (*63*) and also ankylosing spondylitis (*64-67*), a chronic inflammatory process affecting mostly the spine that share genetic determinants with IBD. In addition, firm data already links *CUL2* to IBD risk (*68*), and as mentioned above, this gene encodes the main ligase component that works together with COMMD1 to control NF-κB activity. Therefore, we believe that these data altogether point to a role of this pathway in inflammatory disorders in humans, but additional work to cement its role is still ahead of us.

In addition, the underlying mechanism by which IBD is associated with reduced COMMD1 expression remains incompletely understood. However, the bulk of our data indicate that inflammatory mediators acting in the local environment probably mediate the changes in gene expression that we have observed. In particular, our data indicate that IFN-γ can suppress *COMMD1* expression. In addition, similar changes in *COMMD1* expression have been noted in a variety of malignancies, where reduced levels of *COMMD1* mRNA correlated with a more invasive and aggressive tumor phenotype (*69*). It is tempting to speculate that inflammation in the tumor microenvironment, a cardinal feature of cancer, may lead through a similar mechanism to reduced *COMMD1* expression. This is turn would facilitate the activation of NF-κB as well as HIF-1, and can have a variety of effects on cancer cells that increase their invasive potential (*69*). Therefore, understanding the mechanisms that control *COMMD1* expression and how to manipulate them for therapeutic purpose could one day have various beneficial applications.

Future perspective

COMMD1 is a multifunctional protein that participates in a variety of cellular process, all apparently linked at some level to protein ubiquitination, probably through CRL enzymes. The aggregate of the data presented in this thesis clearly demonstrate the role of this gene in controlling inflammatory responses. In this setting, COMMD1 plays an interesting role as a factor that facilitates termination of an initiated signal, by promoting the removal of active NF-κB dimers from chromatin. Thus, alterations in this mechanism would be expected to lead to a pathologic prolongation and accentuation of inflammatory responses, and our data suggests that indeed this is the case using animal models.

Despite the progress that we have made, a number of critical questions remain unanswered and should be the focus of additional investigation in years to come. I will delineate the questions that I view as most pressing for this field in the few lines that follow:

1) How is COMMD1 recruited to chromatin and is this required for its specific effects of certain NF-κB target genes? At the present time, it is clear that the binding to chromatin by COMMD1 is induced by NF-kB activating stimuli with similar kinetics to that of NF-κB dimer binding. In addition, we know that its recruitment to gene promoters is limited to certain targets and not others, but the basis for such specificity is not known. Moreover, while there is limited data supporting the notion that COMMD1 recruitment to chromatin sites is a prerequisite for gene regulation by this factor (*24*), this is far from proven at a genomic scale. An ongoing project in our lab is exploring the possibility that chromatin modification with SUMO marks might be the signal that brings COMMD1 to certain promoters. This hypothesis is based on our recent identification of SUMO interacting motif in COMMD1, and we are currently examining this hypothesis using a variety of biochemical and genetic approaches.

2) What mechanisms control COMMD1 expression in the context of inflammation and cancer? I view this as a central question in the horizon, one that may be of significant translational potential. Although there is still a lot to be explored here, we are examining transcriptional and post-transcriptional mechanisms that may operate downstream of IFN-γ and may control COMMD1 expression. Validating those findings in vivo in the context of inflammatory and cancer models will be an important next step as well.

3) Is there genetic evidence for a role for COMMD1 and its associated factors in inflammatory disorders? As mentioned above, the general locus containing the COMMD1 gene is linked to inflammatory disorders, but additional work needs to be done to ascertain if COMMD1 itself is responsible for such associations. Moreover, gene variants in *CUL2* are linked to IBD risk, but whether they participate in IBD pathogenesis though effects on COMMD1 or on an unrelated pathway will need to be elucidated.

4) What is the role of other COMMD family members, if any, in the inflammatory response? Despite our identification of this gene family 7 years ago, nearly nothing has been published on other family members and little is known about their function. Nevertheless, their strong conservation through evolution argues that each one of these family members probably plays non-redundant function. In fact, we have generated mouse line deficient in *Commd9* or *Commd10* and in both instances, these genes proved essential for embryonic development. In addition, recent studies from our laboratory have identified that COMMD1 and other COMMD family members bind to a common co-factor termed CCDC22 (Gluck et al, *submitted*). Mutations in this gene have been identified in a large Australian family with X-linked intellectual disability (*70*). Interestingly, a collaborative effort between our group and the geneticists that identified this mutation, indicates that these patients have alterations in NF-κB signaling. In particular, we have identified that CCDC22, acting in concert with another COMMD family member - COMMD8 -, is required for the CRL-dependent degradation of IκB. Thus, the hypomorphic mutation in this family leads to impaired IkB degradation and defective activation of pro-inflammatory gene expression, and these findings can be recapitulated by RNAi-mediated silencing of

CCDC22. These findings highlight the potential for additional roles for members of this family in the control of inflammatory responses.

Finally, as these questions are tackled, we hope to gain a more complete understanding of how this factor plays a role in inflammation during physiologic responses and in disease states. We hope that such knowledge will provide clues to devise potential strategies that might leverage this pathway for therapeutic purposes.

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Summaries

Summary

Inflammation is a genetically programmed cellular response to injury and pathogen invasion. The NF-κB transcription factor is a central regulator of this response and plays a critical role in immunity. As such, pathways that control its activity are intensely studied. Most of the work in the field has focused on activation steps that initiate NF-κB dependent gene expression, primarily by activation of the IKK complex downstream of various signal transduction pathways. Thereafter, homeostatic pathways that inactivate NF-κB and restore back the basal state are important to avert prolonged NF-κB responses and chronic inflammation. However, this has received less attention in the field.

Over the past few years it has been appreciated that ubiquitin-mediated degradation of active, DNA-bound NF-κB subunits plays an important role in controlling pro-inflammatory gene expression. This pathway constitutes a major mechanism to restore the basal state in the NF-κB system, but the mechanism responsible for mediating and regulating NF-κB ubiquitination was not initially apparent. ***The work presented here has elucidated that COMMD1 is essential in this process and plays a regulatory role in inflammation in vivo.***

1) COMMD1 inhibits NF-κB dependent gene expression. The gene repertoire under the control of COMMD1 includes a substantial number of pro-inflammatory genes. In addition, COMMD1-dependent effects also regulate HIV-1 replication, a process that is linked to host cell NF-κB activity given the presence of kB sites in the HIV-1 viral genome. As such, COMMD1 serves as a factor that mediates the resistance of naïve CD4+ cells to HIV-1 replication.

2) The mechanism of COMMD1's inhibitory activity on NF-κB can be ascribed to its ability to promote the degradation of nuclear and chromatin bound NF-κB/RelA. This occurs through the binding of COMMD1 to NF-κB itself and to Cul2, a central scaffold subunit of a ubiquitin ligase belonging to the Cullin-RING ligase (CRL) family. These events are initiated at the promoter level, where COMMD1 is recruited. However, the mechanism responsible for COMMD1 recruitment to chromatin remains unclear.

3) COMMD1 in turn is the founding member of a large family of highly conserved factors present in a variety of organisms. Despite their shared carboxyl-terminal COMM domain, individual members in this gene family appear to have non-redundant functions. However, the spectrum of activities of the various COMMD proteins in mammals, including any potential role in regulating NF-κB and the inflammatory response, remains an area for future investigation.

4) The mechanism by which COMMD1 promotes ubiquitination of NF-κB/RelA can be traced to its ability to activate the Cul2-containing ligase. COMMD1 binds to a region in Cul2 that is conserved among Cullin family members. This region in turn is also involved in binding an inhibitory factor known as CAND1. This large protein promotes the dissociation of the substrate binding components of the Cullin-RING ligase complex and therefore inactivates these complexes. The overlapping binding of COMMD1 and CAND1 on the same domain in Cullin proteins leads to CAND1 displacement upon COMMD1 binding. As a result, COMMD1 promotes the assembly

of a more active Cul2 containing ligase, and this is likely the mechanism by which it promotes NF-κB ubiquitination.

5) The ubiquitination of NF-κB/RelA is additionally regulated by specific post-translational modifications that target this protein for degradation in the right physical and temporal context. In particular, the phosphorylation of this protein at residue Ser468 seems quite important. This step occurs downstream of the IKK complex, the same kinase responsible for IκB degradation. As such, IKK activation not only promotes pathway activation but sets the stage for subsequent inactivation and a return to basal conditions.

6) The link between RelA phosphorylation and subsequent ubiquitination is provided by the chromatin regulator GCN5. Our studies indicate that the phosphorylated form of RelA is recognized by GCN5. This factor in turn binds to COMMD1 and promotes the ubiquitin dependent degradation of RelA. This effect of GCN5 is independent of its histone acetylase activity and is in sharp contrast with its general transcriptional promoting activities.

7) Consistent with its ability to regulate NF-κB activity, COMMD1 plays an anti-inflammatory role in vivo. Using a tissue specific knockout mouse model, our studies indicate that Commd1 deficiency in myeloid cells renders mice prone to more exaggerated inflammatory responses, including in sepsis models. These studies also confirm that Commd1 deficiency results in exaggerated expression of NF-κB target genes in vivo, through impaired NF-κB/RelA ubiquitination.

8) Inflammatory states in humans, such as inflammatory bowel disease, are associated with reductions in COMMD1 expression. Importantly, correlative experiments using the mouse knockout model indicate that Commd1 deficiency worsens the course of colitis and its progression towards cancer in these animals. Thus, the observed reduction in COMMD1 expression seen in patients with inflammatory bowel disease is most likely maladaptive and could serve to perpetuate chronic inflammation.

9) Finally, our studies indicate that suppressed COMMD1 expression in the setting of inflammation can be recapitulated in culture conditions by IFN-γ stimulation, suggesting a potential paracrine effect of this cytokine in inflammatory states. As such, these effects of IFN-γ would serve as a novel cross-talk mechanism between this cytokine and the NF-κB pathway.

Nederlandse samenvatting

Ontsteking is een belangrijk mechanisme van ons lichaam om het te beschermen tegen infecties en beschadiging van weefsels. Deze ontstekingsreactie heeft als doel lichaamsvreemde en beschadigde cellen te verwijderen en te herstellen. Daarentegen kan ongecontroleerde chronische ontsteking veel schade veroorzaken aan weefsel. Om dit te voorkomen is het van belang dat deze ontstekingsreacties op tijd worden stopgezet. De transcriptie factor NF-κB heeft in dit proces een cruciale rol, naast zijn functie in de regulatie van het immuunsysteem. Vanwege zijn centrale rol is de regulatie van deze transcriptie factor zeer intens bestudeerd. De afgelopen jaren is er veel aandacht besteed aan het verkrijgen van inzicht rond het aanzetten van ontstekingsreacties via NF-κB maar er is nog maar weinig bekend over de cellulaire mechanismen en de factoren die betrokken zijn bij het stoppen van ontstekingsreacties.

De laatste jaren is men steeds meer te weten gekomen over de rol van ubiquitine-gecontroleerde degradatie van DNA-gebonden NF-κB subunits. Uit dit onderzoek is gebleken dat ubiquitine-gecontroleerde eiwit afbraak een belangrijke rol speelt in het controleren van de expressie van genen tijdens ontstekingsreacties. Het blijkt dat dit mechanisme belangrijk is om de activiteit van NF-κB terug te brengen tot een basaal niveau. Echter was het mechanisme van deze specifieke regulatie nog vrij onbekend. In dit proefschrift tonen we het belang van COMMD1 in dit specifieke proces aan en hebben we de betrokkenheid van COMMD1 in de regulatie van ontstekingsreacties *in vivo* bestudeerd. Hieronder worden de belangrijkste bevindingen samengevat die beschreven zijn in dit proefschrift.

1) Naast de remming van COMMD1 op NF-κB activiteit, blijkt COMMD1 ook een effect te hebben op de regulatie van HIV-1 replicatie, een proces dat is gekoppeld aan de activiteit van NF-κB in zijn gastheer. Het HIF-1 viraal genoom bevat verschillende κB-sites en via deze sites kan COMMD1 optreden als een factor om de resistentie van naïeve CD4+ cellen tegen HIV-1 replicatie te beïnvloeden.

2) Het mechanisme waarmee COMMD1 de activiteit van NF-κB remt kan toegeschreven worden aan de mogelijkheid om eiwit degradatie van de NF-κB subunit RelA te bevorderen. Dit vindt plaats via de binding van COMMD1 aan NF-κB zelf en aan Cul2. Cul2 is een familielid van de Cullin-RING ligase (CRL) familie en treedt op als een soort “scaffold subunit” binnen dit eiwit-complex. De initiatie van COMMD1-gecontroleerde RelA degradatie vindt plaats op promoter sites van verschillende NF-κB target genen. Het mechanisme hoe COMMD1 naar deze specifieke promoter sites wordt gerekruteerd is nog onbekend.

3) COMMD1 vormde de basis voor de ontdekking van de hele COMMD eiwit familie. Deze familie is zeer sterk geconserveerd tussen verschillende organismen. Ondanks hun gemeenschappelijk carboxyl-terminale COMMD domein, blijkt elke individuele familie lid een specifieke functie te hebben die niet overgenomen kan worden door een ander lid. De functie van de meeste leden, waaronder hun rol in NF-κB regulatie, is nog onduidelijk en moet nog verder worden onderzocht.

4) COMMD1 vormt samen met Cullin een ubiquitine-ligase complex om de mate van RelA ubiquitinatie te bevorderen. COMMD1 bindt aan een gebied binnen het Cullin eiwit dat zeer sterk geconserveerd is binnen de Cullin familie leden. Dit gedeelte van het Cullin eiwit is ook betrokken bij de binding aan CAND1. CAND1 remt op zijn buurt de functie van Cullin-ubiquitine ligase door de binding tussen het substraat (in dit geval RelA) en het Cullin-ubiquitine ligase te verbreken. COMMD1 expressie voorkomt de CAND1 gecontroleerde remming door een competitie aan te gaan met CAND1. Deze gegevens suggereren dat de

ubiquitinatie van RelA via COMMD1 wordt bevordert doordat COMMD1 voorkomt dat CAND1 de activiteit van het Cullin-ubiquitne ligase complex remt.

5) Ubiquitinatie van RelA wordt niet alleen via COMMD1 gereguleerd, maar ook via post-translationele modificaties. Met name de fosforylatie van RelA op het aminozuurresidu serine 468 blijkt een belangrijke rol te spelen. Deze post-translationele modificatie wordt gereguleerd via het IKK complex. Dit is het complex dat zorgt voor IκB degradatie en hiermee NF-κB activeert. Dus, het blijkt nu dat via dit mechanisme IKK niet alleen NF-κB activiteit bevordert maar ook NF-κB deactiveert om zo NF-κB terug te brengen naar zijn basale activiteit.

6) De histon acetyltransferase GCN5 zorgt voor een link tussen RelA fosforylatie en ubiquitinatie. In onze studies tonen we aan dat fosforylatie van RelA wordt herkend door GCN5. GCN5 zorgt ervoor dat COMMD1 bindt aan RelA en hiermee de ubiquitinatie gecontroleerde RelA degradatie. Dit effect van GCN is onafhankelijk van zijn histon acetylase activiteit, en heeft dus niks te maken met zijn functie als transcriptie activator.

7) In lijn met zijn functie als NF-κB remmer blijkt COMMD1 een belangrijk rol te hebben als ontstekingsremmer *in vivo*. Door gebruik te maken van een weefsel specifieke knock-out muis tonen we aan dat muizen deficiënt voor Commd1 in myeloide cellen veel gevoeliger zijn voor ontstekingsreacties, waaronder sepsis. Deze studies laten ook zien dat Commd1 deficiëntie een verhoging in de expressie van NF-κB gecontroleerde genen veroorzaakt, waarbij de mate van RelA ubiquitinatie verlaagd is. Deze studie bevestigt onze bevindingen in cellijnen en laten voor het eerst zien dat COMMD1 ook *in vivo* belangrijk is om de mate van NF-κB activiteit te controleren.

8) Interessant is dat de mens met chronische ontsteking, zoals patiënten met de ziekte van Crohn, geassocieerd wordt met verlaagde COMMD1 expressie. Met onze knock-out muis modellen laten we een duidelijk correlatie zien tussen verlaagde Commd1 expressie en verhoogde gevoeligheid voor darmontstekingen en geassocieerde darmkanker. Dus onze waarneming dat COMMD1 expressie is verlaagd in patiënten met darmontstekingen is waarschijnlijk maladaptatie en zorgt voor de continuering van de chronische ontstekingen in de darm van deze patiënten.

9) Tot slot, onze studies laten zien dat de reductie in COMMD1 expressie tijdens inflammatoire condities kunnen worden herhaald in cellulaire condities waarbij we het IFNγ route activeren. Hiermee laten we zien dat er een mogelijke link is tussen het paracrine effect van deze cytokine en inflammatoire condities. Daarmee, tonen we een mogelijke “cross-talk” aan tussen de effecten van IFNγ en NF-κB signalering.

Samenvattend, dit proefschrift beschrijft de eerste bevindingen en het mechanisme waarmee COMMD1 de mate van NF-κB activiteit controleert. Daarnaast, door middel van muismodellen, bevestigen we de rol van COMMD1 in NF-κB signalering, en dat in de afwezigheid van COMMD1 de inflammatoire status in de verschillende ziektemodellen zeer sterk toeneemt en hierdoor het ziekteproces verslechtert.

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Curriculum Vitae

Curriculum Vitae

Ezra Burstein was born and raised in Lima, Peru. In 1994, he completed Medical School at Cayetano Heredia University in Lima, Peru as the Valedictorian of his class. Thereafter, he continued his training in Internal Medicine at UT Southwestern Medical School, where he was the recipient of the John Miller Award for outstanding performance as a House Officer. Dr. Burstein then completed a Gastroenterology fellowship at the University of Michigan Medical School and joined the laboratory of Gary Nabel as a research fellow at the Vaccine Research Center at the National Institutes of Health (1999-2001). During this period, he studied the regulation of XIAP and the activation of the control of the NF-κB pathway. These studies led to the identification of COMMD1 as a regulator of NF-κB activity. After completion of his training with Dr. Nabel, he joined the Faculty at University of Michigan where he initially worked in the laboratory of Dr. Colin Duckett (2001-2004) studying the role of XIAP and COMMD1 on copper metabolism and inflammatory signals. After establishing an independent laboratory in 2004, he was subsequently recruited to UT Southwestern in 2008, joining the Departments of Internal Medicine (Division of Digestive and Liver Diseases) and Molecular Biology. Dr. Burstein′s research interests include the molecular regulation of the inflammatory response, particularly in the intestinal tract, and ubiquitination events targeting the NF-κB transcription factor.

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