Characterization of COMMD protein–protein interactions in NF-κB signalling

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COMMD [copper metabolism gene MURR1 (mouse U2af1-rs1 region 1) domain] proteins constitute a recently identified family of NF-κB (nuclear factor κB)-inhibiting proteins, characterized by the presence of the COMM domain. In the present paper, we report detailed investigation of the role of this protein family, and specifically the role of the COMM domain, in NF-κB signalling through characterization of protein–protein interactions involving COMMD proteins. The small ubiquitously expressed COMMD6 consists primarily of the COMM domain. Therefore COMMD1 and COMMD6 were analysed further as prototype members of the COMMD protein family. Using specific antisera, interaction between endogenous COMMD1 and COMMD6 is described. This interaction was verified by independent techniques, appeared to be direct and could be detected throughout the whole cell, including the nucleus. Both proteins inhibit TNF (tumour necrosis factor)-induced NF-κB activation in a non-synergistic manner. Mutation of the amino acid residues Trp^{24} and Pro^{41} in the COMM domain of COMMD6 completely abolished the inhibitory effect of COMMD6 on TNF-induced NF-κB activation, but this was not accompanied by loss of interaction with COMMD1, COMMD6 or the NF-κB subunit RelA. In contrast with COMMD1, COMMD6 does not bind to IκBα (inhibitory κBα), indicating that both proteins inhibit NF-κB in an overlapping, but not completely similar, manner. Taken together, these data support the significance of COMMD protein–protein interactions and provide new mechanistic insight into the function of this protein family in NF-κB signalling.

Key words: copper, copper metabolism gene MURR1 domain protein 6 (COMMD6), mouse U2af1-rs1 region 1 (MURR1), nuclear factor κB (NF-κB), protein–protein interaction.

INTRODUCTION

COMMD [copper metabolism gene MURR1 (mouse U2af1-rs1 region 1) domain] proteins constitute a recently described protein family initially identified as interacting partners of COMMD1 (previously known as MURR1), the prototype member of this protein family [1]. In total, ten COMMD proteins are known, of which COMMD1 remains the best characterized. Initially, COMMD1 was implicated as a regulator of copper homoeostasis by the observation that complete absence of COMMD1 protein due to a genomic deletion encompassing exon 2 of *COMMD1* causes copper toxicosis in Bedlington terriers, a severe hepatic copper overload disease in dogs [2–5]. Within the liver, copper excretion is mediated by ATP7B [6], a copper-translocating P-type ATPase mutated in Wilson disease, a hereditary copper overload disorder in man with pathophysiological similarities to copper toxicosis in Bedlington terriers [7–10]. COMMD1 interacts directly with the N-terminal copper-binding domain of ATP7B [11]. Furthermore, transient knockdown of COMMD1 in HEK-293 (human embryonic kidney) cells leads to increased cellular copper levels [12]. Taken together, these studies suggest that COMMD1 functions as a regulator of ATP7B-mediated hepatic copper excretion.

More recently, however, it was demonstrated that COMMD1 is a potent inhibitor of NF-κB (nuclear factor κB)-mediated transcription, and consequentially can inhibit HIV-1 replication in $CD4^+$ T-lymphocytes [1,13]. Interestingly, all other COMMD proteins inhibit TNF (tumour necrosis factor)-induced NF-κB activation to a similar extent as COMMD1 [1]. Although the molecular mechanisms through which COMMD proteins inhibit the NF-κB response have not yet been fully characterized, protein–protein interactions between COMMD proteins and several NF-κB subunits have been detected [1,13]. The notion that COMMD1 interacts with IκBα (inhibitory κBα), and that overexpression of COMMD1 results in decreased ubiquitination of IκBα, led to the hypothesis that COMMD1 inhibits NF-κB by regulating IκBα levels in the cell [13,14]. Additionally, it has also been observed that COMMD1 influences binding of NF-κB to its target promoter sequence [1], indicating that multiple mechanisms may be at play. Taken together, these studies implicate that COMMD proteins constitute a novel family of regulators of NF-κB activity.

In the present study, COMMD protein–protein interactions were characterized further. As COMMD6 lacks the variable extended N-terminus observed in all other COMMD proteins, this protein consists almost solely of the COMMD, making it an excellent prototype member to study the functions of this novel domain. Thus COMMD1 and COMMD6 were chosen as prototype members of this family. We show that COMMD protein–protein interactions occur endogenously, and that they are direct and can be detected throughout the whole cell, including the nucleus. In addition, we show that the COMMD plays an essential role in both COMMD protein–protein interactions and in NF-κB inhibition by COMMD proteins.

Abbreviations used: COMMD, copper metabolism gene MURR1 (mouse U2af1-rs1 region 1) domain; Diablo, direct IAP (inhibitor of apoptosis protein)-binding protein with low pI; GST, glutathione S-transferase; HA, haemagglutinin; HEK-293, human embryonic kidney; IκB, inhibitory κB; IκB S.D., superdominant IκBα; MURR1, mouse U2af1-rs1 region 1; NF-κB, nuclear factor κB; RACE, rapid amplification of cDNA ends; RT, reverse transcriptase; TNF, tumour necrosis factor; YFP, yellow fluorescent protein; C-YFP, C-terminal half of YFP; N-YFP, N-terminal half of YFP.

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EXPERIMENTAL

Constructs

Full-length and partial coding sequences of *COMMD1* and *COMMD6* were amplified from human or canine liver cDNA and cloned in pCRII vector (Invitrogen, Breda, The Netherlands). For yeast two-hybrid assays, full-length human *COMMD1* cDNA was subcloned in pDNR3 (Clontech, BD Biosciences, San Jose, CA, U.S.A.) and subsequently subcloned in pLP-GBKT7 and pLP-GADT7 using the creator cloning kit (Clontech). Partial coding sequences of human or canine COMMD1, and full-length *COMMD6*, were subcloned in pGBT9 or pGAD-GH (Clontech). For production of recombinant COMMD6, *COMMD6* coding sequence was subcloned in pQE-30 (Qiagen, Venlo, The Netherlands). For expression studies, *COMMD1* and *COMMD6* were subcloned in pEBB [15] or pZeoSV (Invitrogen) containing sequences encoding FLAG, HA (haemagglutinin), GST (glutathione S-transferase), N-YFP (N-terminal half of yellow fluorescent protein) or C-YFP (C-terminal half of yellow fluorescent protein) epitopes as indicated for each experiment. A FLAG-tagged COMMD6 isoform a expression plasmid was generated by inserting the extra coding sequence in pEBB-COMMD6-Flag using the QuikChange site-directed mutagenesis method (Stratagene, La Jolla, CA, U.S.A.). Mutations in the pEBB-COMMD6-Flag expression plasmid were also generated with the QuikChange site-directed mutagenesis method. pCDNA3.1-Flag-INCA1 was kindly provided by Dr E. Kalkhoven (Department of Metabolic and Endocrine Diseases, University Medical Centre Utrecht, Utrecht, The Netherlands). The coding sequence for a fusion protein between ubiquitin and Smac/Diablo [direct IAP (inhibitor of apoptosis protein)-binding protein with low pI] (derived from [16]) was cloned into pEBB-YFP-C to obtain pEBB-Ub-Smac/Diablo-YFP-C. 2κB–luciferase reporter, IκBα S.D. (superdominant IκBα), IκBα–HA and COMMD–GST expression plasmids have been described previously [1,17–19]. The sequence of all constructs was verified by automated sequencing.

RT (reverse transcriptase)-PCR

A human cDNA panel was obtained from Clontech. Fragments specific for *COMMD1* (sense and antisense primer sequences: 5′-ATGGCGGCGGGCGAGCTTG-3′ and 5′-TCAGTTAGGCTGGCTGATCAGTG-3′ respectively), *COMMD6* (sense and antisense primer sequences: 5′-ATGGAGGCGTCCAGCGAGCC-3′ and 5′-TCACACCGTTTCAATAACTGCAGC-3′ respectively) and *COMMD6* isoform a (sense and antisense primer sequences: 5′-GGATGCTAAGTCCGATGTCACC-3′ and 5′-AGAAGCAGAAAGGAGACTGGAGG-3′ respectively) were amplified by PCR, separated by 1.5 % agarose gel electrophoresis and visualized using the GelDoc EQ from Bio-Rad (Hercules, CA, U.S.A.). RACE (rapid amplification of cDNA ends) PCR was performed using the SMART™ RACE cDNA amplification kit (Clontech) according to the manufacturer's instructions.

Generation and purification of COMMD6 antiserum

Recombinant His_6–COMMD6 fusion protein was purified from *Escherichia coli* strain M15(pREP4) transformed with pQE-30-COMMD6 according the instructions in the QiaExpressionist manual (Qiagen). Protein purity was validated by SDS/PAGE (15 % gels), followed by Coomassie Brilliant Blue staining, or detection with alkaline-phosphatase-conjugated Ni^{2+}-nitriloacetic acid upon Western blotting. Antiserum to COMMD6 was obtained by serial immunization of rabbits with purified recombinant His_6–COMMD6 (Eurogentec, Seraing, Belgium). For affinity purification, His_6–COMMD6 was coupled to CNBr-activated Sepharose 4B (Amersham Biosciences, Uppsala, Sweden) according to the manufacturer's instructions. Crude COMMD6 antiserum was allowed to hybridize to the His_6–COMMD6 coupled Sepharose beads for 4 h at 4 °C under constant rotation. The beads were subsequently washed three times with PBS, 1 % Triton X-100, once with 10 mM Tris/HCl, pH 7.5, and once with 10 mM Tris/HCl, pH 7.5, and 0.5 M NaCl. Anti-COMMD6 antibodies were eluted from the beads with 0.1 M glycine/HCl, pH 2.5, for 30 min, after which the pH was immediately normalized to pH 7.5 using 1 M Tris/HCl, pH 8.0.

Cell culture and transfections

HEK-293 cells were obtained from A.T.C.C. (Manassas, VA, U.S.A.) and were cultured in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) foetal bovine serum, L-glutamine and penicillin/streptomycin. Calcium phosphate precipitation was used to transfect HEK-293 cells as described previously [15].

Yeast two-hybrid assay, immunoprecipitation, GST pull-down assays and immunoblotting

YGH1 yeast were transformed with yeast expression plasmids according to the SBEG (sorbitol, Bicine, ethylene glycol) method [20]. Yeast two-hybrid protein interactions were assessed according to the instructions in the Matchmaker Gal4 two-hybrid system 3 manual (Clontech).

For GSH–Sepharose precipitations, HEK-293 cells were lysed in Triton lysis buffer (1 % Triton X-100, 25 mM Hepes, 100 mM NaCl, 1 mM EDTA and 10 % glycerol), and for immunoprecipitations, HEK-293 cells were lysed in BSA-supplemented RIPA lysis buffer (1 % Nonidet P40, 0.5 % sodium deoxycholate, 0.1 % SDS and 0.5 % BSA in PBS). Both lysis buffers were supplemented with 1 mM Na_3VO_4, 1 mM PMSF, protease inhibitors (Roche, Basel, Switzerland) and 1 mM dithiothreitol. Immunoprecipitations and precipitations with GSH–Sepharose were performed as described previously [12]. Protein detection was performed by immunoblotting for COMMD1 [4], COMMD6, FLAG (Sigma), GST (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), HA (Sigma), or RelA (Santa Cruz Biotechnology) [15 % gels, except for HA (12.5 % gels) and RelA (10 % gels)] as described previously [4,12]. For *in vitro* transcription–translation, proteins were synthesized by TNT T7-coupled reticulocyte lysate (Promega, Leiden, The Netherlands) and labelled with Tran^{35}S (ICN Biomedicals, Costa Mesa, CA, U.S.A.) according to the manufacturer's instructions, using pZeo-COMMD1-HA, pZeo-COMMD6-Flag and pCDNA3.1-Flag-INCA1 as templates. Precipitations with anti-COMMD1, anti-COMMD6 or anti-FLAG were performed as described previously [12], and samples were separated by SDS/15 %-(w/v)-PAGE. Radiolabelled proteins were visualized by fluorography.

Bimolecular fluorescence complementation studies

HEK-293 cells seeded in coverglass chambers were transfected with constructs encoding COMMD1 fused to N-YFP or C-YFP and with COMMD6 fused to C-YFP or N-YFP respectively. Nuclear counterstaining was performed by adding Hoechst 33342 to Phenol Red-free culture medium to a final concentration of 5 μg/ml and incubating cells for 30 min at 37 °C. Images were obtained from living cells using a Zeiss Axiovert 100M confocal microscope equipped with a Zeiss LSM 510 Meta spectrometer.

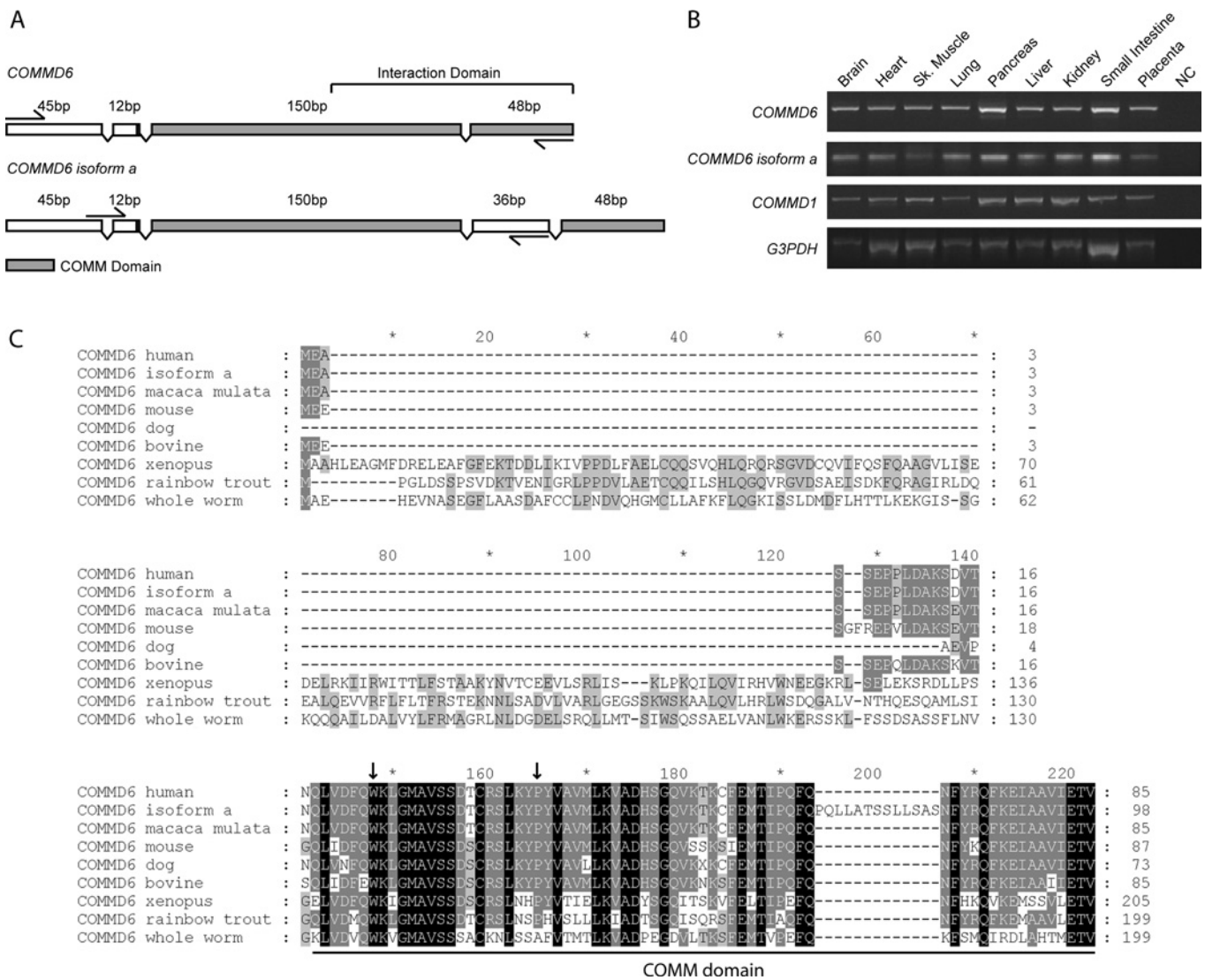

Figure 1 Genetic description of COMMD6

(**A**) Schematic representation of *COMMD6* and the *COMMD6* isoform a transcripts. Boxes indicate individual exons; exon sizes are indicated in bp. Sequences encoding the COMM domain are indicated in grey. (**B**) RT-PCR analysis of the expression of COMMD6, COMMD6 isoform a and COMMD1 in different human tissues. G3PDH (glyceraldehyde-3-phosphate dehydrogenase) RT-PCR was performed as positive control, and, as a negative control (NC), water was used as template. Sk., skeletal. (**C**) Alignment of COMMD6 amino acid sequences of different species. The COMM domain is underlined. Black, dark-grey or light-grey background colours indicate a 100%, 50% or 20% degree of conservation respectively. Arrows indicate residues mutated for experiments described in Figures 5 and 6.

Luciferase reporter assays

For luciferase assays, cells were seeded in six-well plates in triplicate for each treatment group. TNF (Roche) treatments consisted of 500 units/ml for 12 h. Luciferase activity was measured as described previously [20a] using the Luciferase assay system (Promega) and a TR717 Applied Biosystems microplate luminometer.

RESULTS

The *COMMD6* gene is highly conserved and ubiquitously expressed

The *COMMD6* gene is located on chromosome 13 of the human genome and encodes a putative 85-amino-acid protein with a predicted molecular mass of 8 kDa (depicted schematically in Figure 1A). An additional human *COMMD6* transcript, termed *COMMD6* isoform a, has been annotated in the NCBI database. Whereas *COMMD6* is transcribed from four exons, the *COMMD6* isoform a transcript contains the sequence encoded by one extra exon located between exon 3 and 4, yielding an in-frame insertion of 12 additional amino acids (Figure 1A). RT-PCR expression profiling of RNA preparations obtained from different human and mouse tissues indicated that both *COMMD6* transcript isoforms are ubiquitously expressed throughout all tissues investigated, similarly as observed for *COMMD1* and the other *COMMD* genes (Figure 1B, and results not shown) [1,3,4].

Alignment of sequences of several COMMD6 orthologues is depicted in Figure 1(C); this analysis revealed the interesting observation that COMMD6 proteins in higher vertebrates lack an extended N-terminus found in lower organisms. Repeated attempts to identify putative 5′ extensions of the human *COMMD6* transcript, by both *in silico* approaches and 5′-RACE were unsuccessful (results not shown). COMMD6 is the only member

of the COMMD protein family in which structural diversity between vertebrate and non-vertebrate COMMD is observed. As COMMD6 in higher vertebrates consists primarily of the COMMD, this indicates that the domain is important, and maybe even sufficient, for COMMD6 function.

Endogenous COMMD1 and COMMD6 interact in mammalian cells

Previously, it has been shown that most COMMD proteins, including COMMD6, associate with COMMD1. However, these data were all based on studies using overexpressed COMMD proteins. To determine whether endogenous COMMD1 and COMMD6 can be detected in complex with each other, antiserum against COMMD6 was generated. As COMMD1 and COMMD6 share a high degree of similarity in their COMM domains, it was first necessary to exclude the possibility that antibodies directed against COMMD1 cross-react with COMMD6 and *vice versa*. Using both Western blot analysis (results not shown) and immunoprecipitation of radiolabelled *in vitro* synthesized COMMD proteins (Figure 2A), it was established that the COMMD antisera reacted specifically with their cognate antigens. Immunoprecipitation using cell lysates of HEK-293 cells revealed that COMMD1 and COMMD6 interacted with each other using antisera raised against either COMMD1 or COMMD6, whereas neither protein could be detected in immunoprecipitates using pre-immune serum or antibodies directed against GST (Figure 2B). To determine whether COMMD1 can interact with both COMMD6 isoforms, COMMD1–GST fusion protein was expressed in HEK-293 cells together with COMMD6–FLAG or COMMD6 isoform a–FLAG fusion proteins. Glutathione–Sepharose precipitation revealed that COMMD1 can interact with both COMMD6 isoforms, whereas COMMD6 can only homodimerize with COMMD6, and does not bind COMMD6 isoform a (Figure 2C).

From these and previous [1] experiments, it remained unknown if interactions between COMMD proteins are direct or dependent on the presence of other proteins. To investigate whether a direct interaction between COMMD1 and COMMD6 exists, yeast two-hybrid analysis was carried out and co-immunoprecipitation studies were performed using *in vitro* synthesized [^{35}S]methionine-labelled COMMD1 and COMMD6. As depicted in Figures 3(A) and 3(B), these approaches independently revealed a direct and specific interaction between these two proteins. Homodimerization of COMMD1 or COMMD6 was not detected by yeast two-hybrid methodology (results not shown). Deletion of exon 3 of COMMD1 abolished interaction between COMMD1 and COMMD6 in our yeast two-hybrid setting, whereas the C-terminal 39 amino acids of COMMD6 are sufficient for interaction with COMMD1 (Figure 3C, and results not shown). This indicates that the direct interaction between COMMD1 and COMMD6 is mediated by the C-terminal part of the COMM domain.

To visualize the interaction between COMMD1 and COMMD6 in living cells, bimolecular fluorescence methodology was applied. In this approach, fusion proteins of COMMD1 and COMMD6 with N-YFP or C-YFP were expressed in HEK-293 cells. The two complementary portions of YFP are brought into close proximity only when the proteins to which they are fused interact, which subsequently allows for a fluorescent signal [21]. As depicted in Figure 4, this approach readily revealed interaction between COMMD1 and COMMD6 (panels C and E). This interaction was specific, as no signal was observed when the COMMD1 or COMMD6 fusion proteins were expressed with the complementary YFP fragments alone (Figures 4A and 4B), or with the complementary YFP fragments fused to the non-interacting protein Smac/Diablo (Figure 4D). The fluorescent signal, which shows the cellular location of interaction between

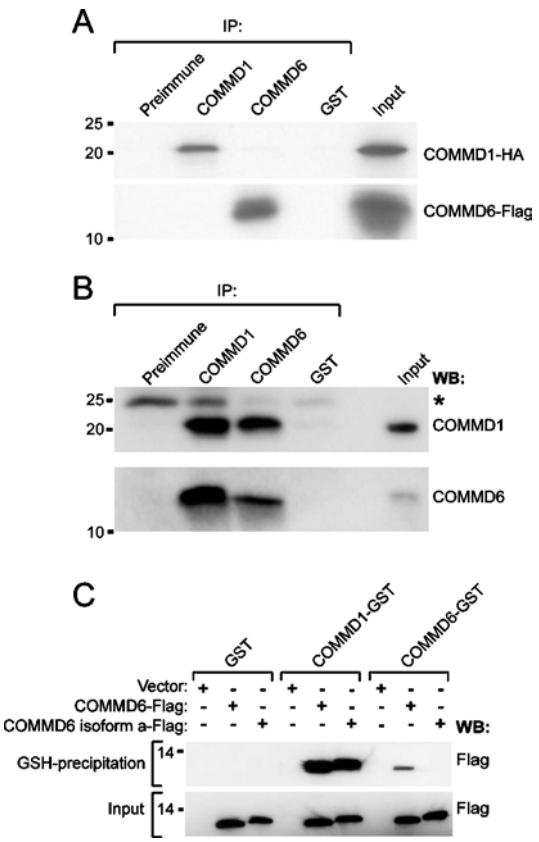

Figure 2 Detection of endogenous COMMD1- and COMMD6-containing complexes

(**A**) Constructs encoding COMMD1–HA (upper panel) or COMMD6–FLAG (lower panel) were used in coupled *in vitro* transcription–translation reactions in the presence of radiolabelled amino acids. Labelled proteins were analysed directly (Input) or immunoprecipitated (IP) using pre-immune rabbit serum, COMMD1 antiserum, affinity-purified COMMD6 antiserum or anti-GST antibody. Proteins were separated by SDS/PAGE and visualized by fluorography. Apparent molecular-mass markers are indicated in kDa on the left. (**B**) Cell lysates of untransfected HEK-293 cells were directly analysed (Input) or used for immunoprecipitation (IP) using pre-immune rabbit serum, COMMD1 antiserum, COMMD6 antiserum or anti-GST antibody. Precipitates were rinsed and separated by SDS/PAGE and immunoblotted using antibodies as indicated. IgG light chain is indicated by *. Apparent molecular-mass markers are indicated in kDa on the left. (**C**) HEK-293 cells were transfected with cDNA constructs encoding COMMD6–FLAG, COMMD6 isoform a–FLAG, GST, COMMD1–GST or COMMD6–GST as indicated. Cell lysates were made which were used for glutathione–Sepharose precipitation. Precipitates were washed and separated by SDS/PAGE and immunoblotted as indicated. Input indicates direct analysis of cell lysates. Apparent molecular-mass markers are indicated in kDa on the left. WB, Western blot.

COMMD1 and COMMD6, was distributed throughout the whole cell, with most of the signal in the cytoplasm and to a lesser extent in the nucleus. In most cells, some perinuclear aggregation of the signal was observed, the nature of which is unknown (Figure 4C).

COMMD6 inhibits TNF-induced NF-κB activation through conserved residues in the COMMD

The COMMD protein family has recently been implicated as a family of NF-κB inhibitors [1]. To investigate whether different COMMD proteins inhibit NF-κB in a synergistic manner, HEK-293 cells were transfected with a 2κB–luciferase reporter construct together with expression vectors encoding COMMD1 or COMMD6, or a combination of both. IκBα S.D. was used as a positive control. In accordance with the results described previously, COMMD1 and COMMD6 inhibit NF-κB with the same efficiency [1]. No synergism or additive effect was observed when

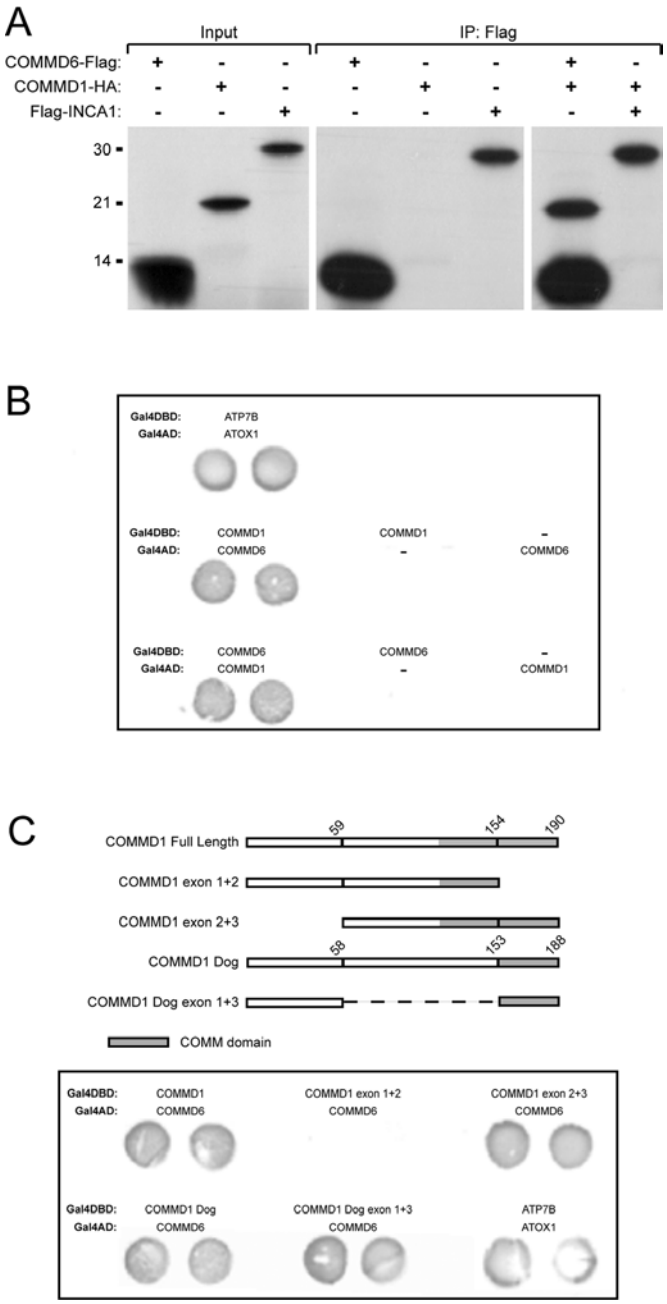

Figure 3 Interaction between COMMD1 and COMMD6 is direct and mediated through the COMM domain

(**A**) Constructs encoding the indicated proteins were used in coupled *in vitro* transcription–translation reactions in the presence of radiolabelled amino acids. Labelled proteins were analysed directly (Input; left panel) or immunoprecipitated using anti-FLAG antibody (IP: Flag; right panels). Proteins were separated by SDS/PAGE and visualized by fluorography. Apparent molecular-mass markers are indicated in kDa on the left. (**B**) *YGH1* yeast was transformed with constructs encoding COMMD1 and COMMD6, fused to Gal4DBD (GAL4 DNA-binding domain) or Gal4AD (GAL4 activation domain) as indicated. All constructs were also transfected together with empty vectors as negative controls. Strains were grown in duplicate on selective medium and were subjected to a filter lift test for β-galactosidase activity. The interaction between ATOX1 (antioxidant protein 1) and ATP7B was used as positive control for β-galactosidase activity. (**C**) Different COMMD1 deletion constructs based on COMMD1 exon boundaries are depicted schematically. Numbers indicate predicted amino acid residues. All constructs were fused to the coding region of the Gal4DBD. *YGH1* yeast were transformed with the COMMD1–Gal4DBD deletion constructs and COMMD6–Gal4AD as indicated. Strains were grown in duplicate on selective medium and were subjected to a filter lift test for β-galactosidase activity. The interaction between ATOX1 and ATP7B was used as positive control for β-galactosidase activity.

COMMD1 and COMMD6 were expressed together, whereas expression of either protein together with IκBα S.D. resulted in complete inhibition of NF-κB activation (Figure 5A). A reduction in COMMD1 and COMMD6 expression levels was observed upon co-expression with IκBα S.D. However, a similar reduction of other proteins expressed from the same vector backbone, but not of endogenous COMMD1, was also observed (results not shown). These phenomena are therefore most likely explained by the stimulatory effect of NF-κB on the exogenous promoter in the expression system, similar to that described before for other commonly used expression-system promoters [22].

Human COMMD6 consists primarily of the COMM domain. Within the COMM domain, two amino acid residues, Trp^{24} and Pro^{41} are absolutely conserved in all human COMMD proteins (Figure 1C). Mutation of either residue in COMMD6 individually to alanine did not affect NF-κB reporter activity, although a slight increase in basal activation was observed for COMMD6 P41A. Strikingly, however, when both residues were mutated simultaneously (COMMD6 W24A/P41A) the repression of TNF-induced NF-κB signalling was completely abolished. In fact, a dose-dependent increase in both basal and TNF-induced luciferase activity was observed, indicating that the COMMD6 W24A/P41A double mutant behaved as a superdominant activator of NF-κB (Figure 5B, and results not shown). Taken together, these data suggest that COMMD6, like COMMD1, is a potent endogenous repressor of NF-κB signalling, and that specific residues in the COMM domain are essential for this inhibitory effect.

W24A and P41A mutations in COMMD6 do not influence binding of COMMD6 to COMMD1 or to itself

On the basis of the observation that COMMD1 and its interacting partner COMMD6 both acted as repressors of NF-κB reporter activity, we hypothesized that the observed NF-κB repression by COMMD6 is mediated through its interaction with COMMD1. This hypothesis predicts that the superdominant activation by COMMD6 W24A/P41A is associated with a failure of this COMMD6 mutant protein to interact with COMMD1. To test this possibility, glutathione–Sepharose precipitates of COMMD1–GST were analysed for the presence of COMMD6 proteins. The results revealed that wild-type COMMD6 and all three COMMD6 mutants (COMMD6 W24A, COMMD6 P41A and COMMD6 W24A/P41A) bound to COMMD1 (Figure 6A). Homomultimerization of COMMD6 was also unaffected, as not only wild-type COMMD6, but also the double mutant, could be detected in complex with wild-type COMMD6–GST (Figure 6B).

To assess whether association of COMMD6 with NF-κB in the cell was affected by the two introduced mutations, the ability of COMMD6 and the COMMD6 mutants to bind to the NF-κB subunit RelA was determined. RelA was readily detected in complex with both COMMD1 and COMMD6. All three COMMD6 mutants were able to bind to RelA at least as strongly as wild-type COMMD6 (Figure 6C).

COMMD1 may exert its NF-κB-inhibiting effects through binding of IκBα, thereby preventing the proteasomal degradation of IκBα [16]. To investigate whether COMMD6 inhibits NF-κB by participating in this mechanism, the possibility that COMMD6 and the COMMD6 mutants could bind to IκBα was determined. Glutathione–Sepharose precipitation revealed that wild-type COMMD6 and the three COMMD6 mutants failed to bind HA–IκBα, whereas COMMD1 abundantly interacted with HA–IκBα under the same conditions (Figure 6D). These data indicate that COMMD1 and COMMD6 have overlapping, but not completely similar, functions in the NF-κB pathway.

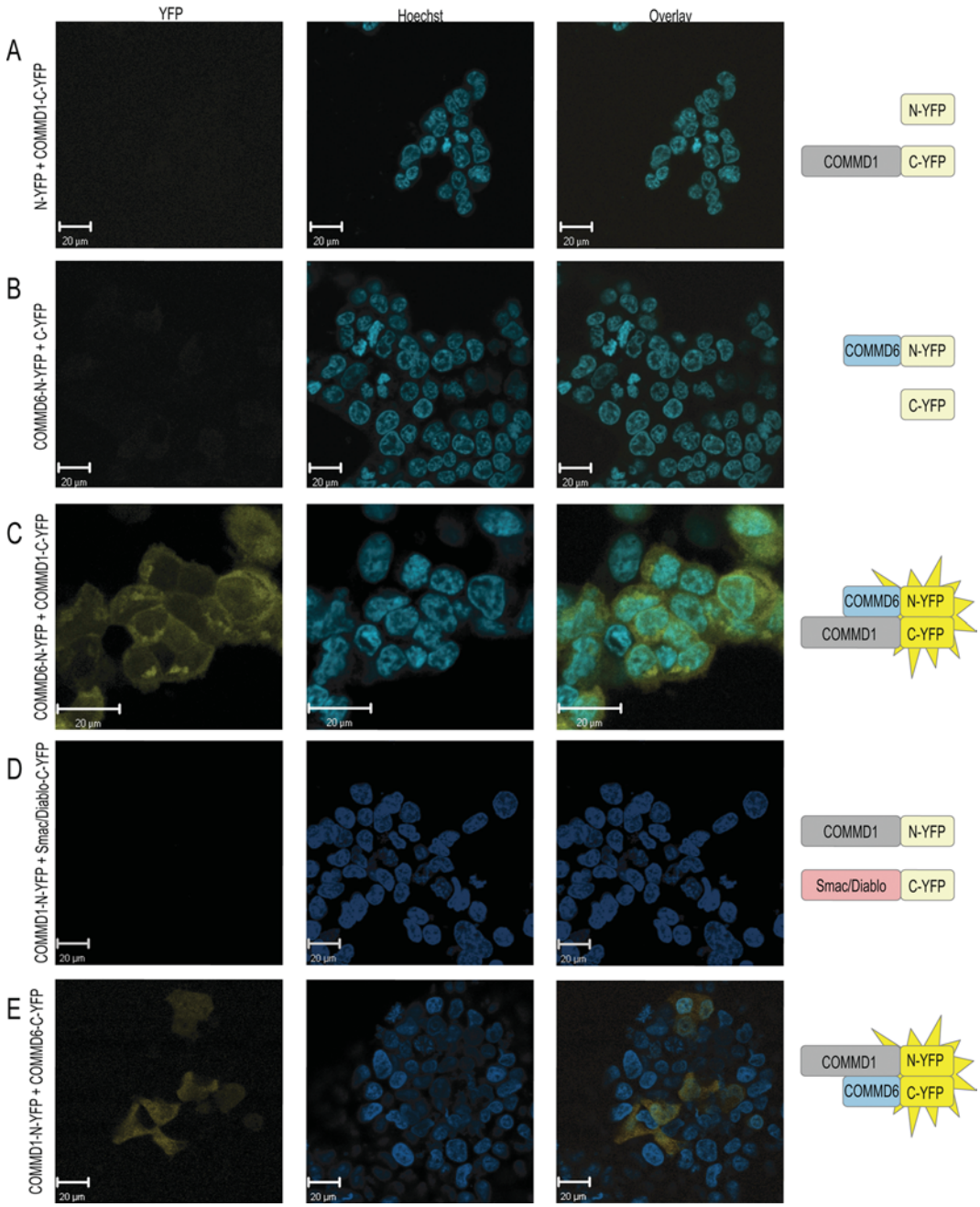

Figure 4 Bimolecular fluorescence complementation with COMMD1 and COMMD6

The principle of bimolecular fluorescence complementation is depicted schematically. HEK-293 cells were transfected with expression vectors encoding COMMD1–C-YFP and –N-YFP (**A**), COMMD6–N-YFP and –C-YFP (**B**), COMMD1-C-YFP and COMMD6-N-YFP (**C**), COMMD1–N-YFP and Smac/Diablo–C-YFP (**D**) or COMMD1–N-YFP and COMMD6–C-YFP (**E**). After overnight transfection, nuclear counterstain was performed by incubating cells with Hoechst 33342 before acquiring confocal images. The YFP images, Hoechst images and overlay images are shown as indicated.

DISCUSSION

COMMD proteins constitute a novel family of NF-κB-inhibiting proteins. As protein–protein interactions seem to be an important mechanism for these proteins to exert their function (reviewed in [5]), in the present study, we characterized further the interaction between COMMD proteins using COMMD1 and COMMD6 as prototype members. We show for the first time that COMMD protein–protein interactions occur endogenously. Moreover, these interactions are direct and are dependent on the COMM domain. The interaction between COMMD1 and COMMD6 occurs throughout the living cell, including the nucleus. In addition, by providing a dominant-negative mutant of COMMD6, unable to repress NF-κB activation, we show for the first time that the function of these proteins is dependent on conserved residues in the COMM domain.

COMMD6 is predicted to be a ubiquitously expressed small (8 kDa) soluble protein, which could potentially function as a homomultimer. *COMMD6* sequences appear to be highly conserved during evolution, although, in humans, a *COMMD6* splice

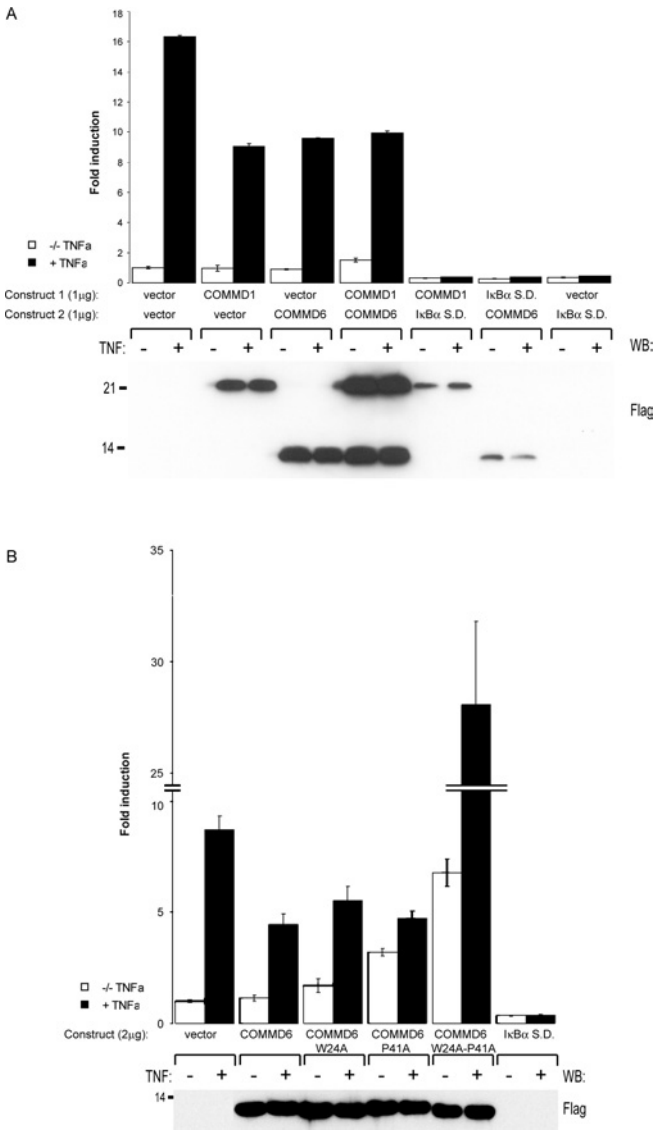

Figure 5 COMMD6 inhibits NF-κB signalling through residues in the COMMD

HEK-293 cells were transfected with empty vector (**A**, **B**), or expression vectors encoding COMMD1–FLAG (**A**), COMMD6–FLAG (**A**, **B**), COMMD6–FLAG mutants W24A, P41A, W24A/P41A (**B**) or IκBα S.D. (**A**, **B**) along with a 2κB–luciferase reporter plasmid as indicated. Cells were incubated with (open bars) or without (closed bars) TNF and were lysed after 12 h. Upper panels: luciferase activities in the lysates were measured and expressed as fold induction relative to unstimulated empty-vector-transfected cells. Results are means ± S.D. for three independent experiments performed in triplicate. Lower panels: immunoblotting of cell lysates using anti-FLAG antibodies was performed to confirm expression of COMMD1–FLAG and COMMD6–FLAG. Apparent molecular-mass markers are indicated in kDa on the left. WB, Western blot.

variant is expressed that has not been observed in other organisms, and the predicted N-termini of COMMD6 proteins in higher organisms are significantly smaller than those observed in lower organisms. In fact, COMMD6 in higher vertebrates consists primarily of the COMM domain, indicating that the COMMD is important, and maybe even sufficient, for COMMD6 function. This makes COMMD6 an excellent prototype member of the COMMD protein family to study the function and mechanism of action of the COMM domain. Using antisera directed against COMMD6, we were able to demonstrate that COMMD1–COMMD6 interactions occur endogenously in HEK-293 cells. Both COMMD1 and COMMD6 interact with almost all COMMD proteins (results not shown; [1]). It remains unclear what the exact composition is of the detected COMMD complexes, but direct interactions between COMMD proteins could indeed occur as is evident from the direct interaction between COMMD1 and COMMD6 demonstrated in the present study. In addition, homomultimerization of COMMD proteins can occur, as complexes containing multiple COMMD1 or COMMD6 molecules could be detected by GSH–Sepharose precipitation analysis (Figure 2C; [1]). Homomultimerization of COMMD1 or COMMD6 could not be detected by using yeast two-hybrid methodology. This could indicate either that homomultimerization of COMMD proteins is not direct and thus only occurs in higher-order complexes or that COMMD homomultimerization occurs with a relatively lower affinity than COMMD heteromultimerization, since the yeast two-hybrid system is known to be suitable only for the detection of high-affinity interactions [23,24]. The interaction between COMMD1 and COMMD6 was abolished by deletion of exon 3 of COMMD1. This confirms independently that interaction between COMMD proteins is mediated by the COMM domain, and that the C-terminal part of the COMM domain could be sufficient for interaction (Figure 3C, [1]). Using bimolecular fluorescence complementation methodology, the subcellular distribution of COMMD1- and COMMD6-containing complexes was determined. The signal was distributed throughout the cell, including the nucleus. Some perinuclear aggregation of the signal was observed, the nature of which remains elusive. Considering the irreversible nature of the bimolecular fluorescence complementation interaction, it cannot be excluded that this aggregation resulted from artificial accumulation of interacting complexes or from protein overexpression. However, endogenous COMMD1 and COMMD1 tagged with fluorescent proteins were also detected previously as a punctate staining pattern without overlap with lysosomes or mitochondria [4,12], suggesting that this localization might be biologically relevant.

Within the cell, different NF-κB proteins exist as homo- or hetero-dimers that are capable of binding DNA at particular sequences known as κB-sites and subsequently activate transcription [25]. IκB proteins act as inhibitors of NF-κB signalling by binding to the NF-κB dimers and preventing their nuclear translocation [26]. COMMD1 and both isoforms of COMMD6 inhibited NF-κB to a similar extent (Figure 5A, and results not shown). Co-expression of both COMMD1 and COMMD6 did not exacerbate the inhibition of NF-κB-mediated transcription, suggesting that both proteins exert their effect through the same pathway. Consistent with this observation, both COMMD1 and COMMD6 are associated with the RelA subunit of NF-κB. Previously, it has been suggested that COMMD1 regulates the proteasomal degradation of IκBα [13,14], but, whereas COMMD1 is associated with IκBα, no binding of COMMD6 to IκBα was observed. The most straightforward explanation of these data is that the COMM domain of COMMD1 acts to recruit COMMD6 to the NF-κB complex after TNF-induced dissociation of IκBα. This would eventually result in inhibition of NF-κB-mediated transcriptional activation. Recently published data suggest that this inhibition takes place in the nucleus [1]. Consistent with this observation, the observed bimolecular fluorescence signal reflecting the interaction between COMMD1 and COMMD6 was also partly detectable in the nucleus. Mutation of Trp^{24} and Pro^{41} in COMMD6 completely abolished all its ability to inhibit NF-κB signalling. This mutant behaved as a dominant inducer of NF-κB signalling in a dose-dependent manner, while retaining its ability to bind COMMD1, COMMD6 and RelA. Overexpression of COMMD6 W24A/P41A might therefore sequester COMMD1, rendering COMMD1 unavailable for interaction with

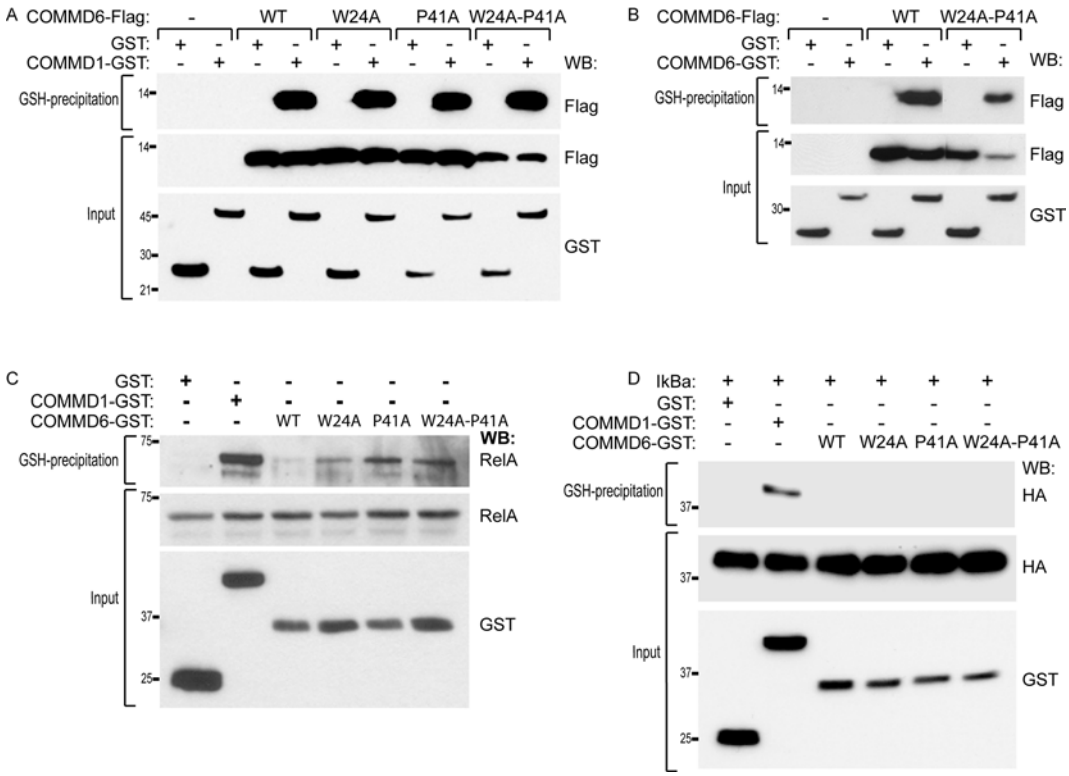

Figure 6 COMMD6–FLAG W24A/P41A mutant interacts with COMMD1, COMMD6 and RelA, but not with IκBα

(**A** and **B**) Glutathione–Sepharose precipitation using cell lysates of HEK-293 cells expressing the COMMD6–FLAG wild-type (WT) and point mutants and COMMD1–GST (**A**) or COMMD6–GST (**B**). Precipitates were washed and separated by SDS/PAGE and immunoblotted as indicated. Input indicates direct analysis of cell lysates. Apparent molecular-mass markers are indicated in kDa on the left. (**C** and **D**) Glutathione–Sepharose precipitation performed on the cell lysates of HEK-293 cells expressing COMMD1 and wild-type (WT) and mutant COMMD6 as GST fusion proteins, that were either untransfected (**C**) or transfected with an expression vector encoding HA–IκBα (**D**). Precipitates were washed and separated by SDS/PAGE and immunoblotted for RelA (**C**), HA (**D**) or GST (**C** and **D**) as indicated. Input indicates direct analysis of cell lysates. Apparent molecular-mass markers are indicated in kDa on the left. WB, Western blot.

endogenous COMMD6 or possibly other COMMD proteins, leading to disinhibition of NF-κB signalling. These data suggest that COMMD6 is an endogenous inhibitor of NF-κB, and show for the first time that specific conserved residues in the COMM domain are essential for inhibition of NF-κB-mediated transcription by COMMD proteins.

Taken together, the recent identification of the COMMD family of proteins potentially provides a complete new avenue to study the molecular mechanisms of NF-κB activation and inhibition. This could be of great interest since COMMD1 restricts HIV-1 replication in $CD4^+$ T-lymphocytes through its NF-κB-inhibiting activity [13]. Thus the COMMD protein family could potentially play an important role in the pathogenesis of HIV-1 infections. Previous studies based on RNA interference-mediated inhibition of COMMD expression revealed that COMMD1 and COMMD6 are endogenous inhibitors of NF-κB [1,13]. Consistent with these observations, the dominant-negative COMMD6 W24A/P41A mutant induces NF-κB activation, even when no exogenous stimulus was added. As all COMMD proteins are ubiquitously expressed, their activities would need to be regulated to avoid complete inhibition of NF-κB signalling. The inhibition of TNF-induced NF-κB activation involves recruitment of COMMD1 to chromatin [1]. The mechanisms that induce and terminate this recruitment might be critical in the regulation of the activity of all COMMD proteins. In addition, it has been shown previously that COMMD1 is a substrate for ubiquitination by XIAP (X-linked inhibitor of apoptosis), a potent activator of NF-κB signalling [12]. The exact signals leading to the ubiquitination and subsequent degradation of COMMD1 are unknown, but these could potentially provide a mechanism to regulate the NF-κB-inhibiting properties of COMMD1, and possibly other COMMD proteins. Further research on the mechanisms of NF-κB inhibition by COMMD proteins, and the regulation hereof, is still required, for which the COMMD6 W24A/P41A double mutant will be a valuable tool. A COMMD1-knockout mouse model will also provide an excellent model to study the regulation of both NF-κB activation and copper homoeostasis by COMMD1 *in vivo*. Finally, given the variety of cellular processes in which COMMD1 is involved [5], one might speculate that other COMMD proteins also have more functions outside the NF-κB pathway. It would be particularly interesting if these proteins would also have a function in copper metabolism. This could possibly lead to the identification of novel candidate genes for hereditary disorders of copper metabolism, because the underlying genetic mutations underlying several of such disorders still remain unknown [27].

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REFERENCES

1 Burstein, E., Hoberg, J. E., Wilkinson, A. S., Rumble, J. M., Csomos, R. A., Komarck, C. M., Maine, G. N., Wilkinson, J. C., Mayo, M. W. and Duckett, C. S. (2005) COMMD proteins, a novel family of structural and functional homologs of MURR1. J. Biol. Chem. **280**, 22222–22232

2 van de Sluis, B., Kole, S., van Wolferen, M., Holmes, N. G., Pearson, P. L., Rothuizen, J., van Oost, B. A. and Wijmenga, C. (2000) Refined genetic and comparative physical mapping of the canine copper toxicosis locus. Mamm. Genome **11**, 455–460
3 van de Sluis, B., Rothuizen, J., Pearson, P. L., van Oost, B. A. and Wijmenga, C. (2002) Identification of a new copper metabolism gene by positional cloning in a purebred dog population. Hum. Mol. Genet. **11**, 165–173
4 Klomp, A. E., van de Sluis, B., Klomp, L. W. and Wijmenga, C. (2003) The ubiquitously expressed MURR1 protein is absent in canine copper toxicosis. J. Hepatol. **39**, 703–709
5 de Bie, P., van De Sluis, B., Klomp, L. and Wijmenga, C. (2005) The many faces of the copper metabolism protein MURR1/COMMD1. J. Hered. **96**, 803–811
6 Wijmenga, C. and Klomp, L. W. (2004) Molecular regulation of copper excretion in the liver. Proc. Nutr. Soc. **63**, 31–39
7 Yamaguchi, Y., Heiny, M. E. and Gitlin, J. D. (1993) Isolation and characterization of a human liver cDNA as a candidate gene for Wilson disease. Biochem. Biophys. Res. Commun. **197**, 271–277
8 Tanzi, R. E., Petrukhin, K., Chernov, I., Pellequer, J. L., Wasco, W., Ross, B., Romano, D. M., Parano, E., Pavone, L. and Brzustowicz, L. M. (1993) The Wilson disease gene is a copper transporting ATPase with homology to the Menkes disease gene. Nat. Genet. **5**, 344–350
9 Petrukhin, K., Fischer, S. G., Pirastu, M., Tanzi, R. E., Chernov, I., Devoto, M., Brzustowicz, L. M., Cayanis, E., Vitale, E. and Russo, J. J. (1993) Mapping, cloning and genetic characterization of the region containing the Wilson disease gene. Nat. Genet. **5**, 338–343
10 Bull, P. C., Thomas, G. R., Rommens, J. M., Forbes, J. R. and Cox, D. W. (1993) The Wilson disease gene is a putative copper transporting P-type ATPase similar to the Menkes gene. Nat. Genet. **5**, 327–337
11 Tao, T. Y., Liu, F., Klomp, L., Wijmenga, C. and Gitlin, J. D. (2003) The copper toxicosis gene product Murr1 directly interacts with the Wilson disease protein. J. Biol. Chem. **278**, 41593–41596
12 Burstein, E., Ganesh, L., Dick, R. D., van de Sluis, B., Wilkinson, J. C., Klomp, L. W., Wijmenga, C., Brewer, G. J., Nabel, G. J. and Duckett, C. S. (2004) A novel role for XIAP in copper homeostasis through regulation of MURR1. EMBO J. **23**, 244–254
13 Ganesh, L., Burstein, E., Guha-Niyogi, A., Louder, M. K., Mascola, J. R., Klomp, L. W., Wijmenga, C., Duckett, C. S. and Nabel, G. J. (2003) The gene product Murr1 restricts HIV-1 replication in resting $CD4^+$ lymphocytes. Nature (London) **426**, 853–857
14 Greene, W. C. (2004) How resting T cells deMURR HIV infection. Nat. Immunol. **5**, 18–19
15 Duckett, C. S., Gedrich, R. W., Gilfillan, M. C. and Thompson, C. B. (1997) Induction of nuclear factor κB by the CD30 receptor is mediated by TRAF1 and TRAF2. Mol. Cell. Biol. **17**, 1535–1542
16 Hunter, A. M., Kottachchi, D., Lewis, J., Duckett, C. S., Korneluk, R. G. and Liston, P. (2003) A novel ubiquitin fusion system bypasses the mitochondria and generates biologically active Smac/DIABLO. J. Biol. Chem. **278**, 7494–7499
17 Duckett, C. S., Perkins, N. D., Kowalik, T. F., Schmid, R. M., Huang, E. S., Baldwin, Jr, A. S. and Nabel, G. J. (1993) Dimerization of NF-κB2 with RelA(p65) regulates DNA binding, transcriptional activation, and inhibition by an IκB-α (MAD-3). Mol. Cell. Biol. **13**, 1315–1322
18 Klug, C. A., Gerety, S. J., Shah, P. C., Chen, Y. Y., Rice, N. R., Rosenberg, N. and Singh, H. (1994) The v-abl tyrosine kinase negatively regulates NF-κB/Rel factors and blocks κ gene transcription in pre-B lymphocytes. Genes Dev. **8**, 678–687
19 Mir, S. S., Richter, B. W. and Duckett, C. S. (2000) Differential effects of CD30 activation in anaplastic large cell lymphoma and Hodgkin disease cells. Blood **96**, 4307–4312
20 Klebe, R. J., Harriss, J. V., Sharp, Z. D. and Douglas, M. G. (1983) A general method for polyethylene-glycol-induced genetic transformation of bacteria and yeast. Gene **25**, 333–341
20a Birkey Reffey, S., Wurthner, J. U., Parks, W. T., Roberts, A. B. and Duckett, C. S. (2001) X-linked inhibitor of apoptosis protein functions as a cofactor in transforming growth factor-β signaling. J. Biol. Chem. **276**, 26542–26549
21 Hu, C. D., Chinenov, Y. and Kerppola, T. K. (2002) Visualization of interactions among bZIP and Rel family proteins in living cells using bimolecular fluorescence complementation. Mol. Cell **9**, 789–798
22 Pahl, H. L. (1999) Activators and target genes of Rel/NF-κB transcription factors. Oncogene **18**, 6853–6866
23 Estojak, J., Brent, R. and Golemis, E. A. (1995) Correlation of two-hybrid affinity data with *in vitro* measurements. Mol. Cell. Biol. **15**, 5820–5829
24 Strausak, D., Howie, M. K., Firth, S. D., Schlicksupp, A., Pipkorn, R., Multhaup, G. and Mercer, J. F. (2003) Kinetic analysis of the interaction of the copper chaperone Atox1 with the metal binding sites of the Menkes protein. J. Biol. Chem. **278**, 20821–20827
25 Hayden, M. S. and Ghosh, S. (2004) Signaling to NF-κB. Genes Dev. **18**, 2195–2224
26 Yamamoto, Y. and Gaynor, R. B. (2004) IκB kinases: key regulators of the NF-κB pathway. Trends Biochem. Sci. **29**, 72–79
27 Muller, T., van de Sluis, B., Zhernakova, A., van Binsbergen, E., Janecke, A. R., Bavdekar, A., Pandit, A., Weirich-Schwaiger, H., Witt, H., Ellemunter, H. et al. (2003) The canine copper toxicosis gene MURR1 does not cause non-Wilsonian hepatic copper toxicosis. J. Hepatol. **38**, 164–168

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