

Cu,Zn Superoxide Dismutase Maturation and Activity Are Regulated by COMMD1*[§]

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The maturation and activation of the anti-oxidant Cu,Zn superoxide dismutase (SOD1) are highly regulated processes that require several post-translational modifications. The maturation of SOD1 is initiated by incorporation of zinc and copper ions followed by disulfide oxidation leading to the formation of enzymatically active homodimers. Our present data indicate that homodimer formation is a regulated final step in SOD1 maturation and implicate the recently characterized copper homeostasis protein COMMD1 in this process. COMMD1 interacts with SOD1, and this interaction requires CCS-mediated copper incorporation into SOD1. COMMD1 does not regulate disulfide oxidation of SOD1 but reduces the level of SOD1 homodimers. RNAi-mediated knockdown of COMMD1 expression results in a significant induction of SOD1 activity and a consequent decrease in superoxide anion concentrations, whereas overexpression of COMMD1 exerts exactly the opposite effects. Here, we identify COMMD1 as a novel protein regulating SOD1 activation and associate COMMD1 function with the production of free radicals.

Cu,Zn superoxide dismutase (SOD1) is a highly conserved and ubiquitously expressed anti-oxidant enzyme involved in the conversion of toxic superoxide anions into molecular oxygen and hydrogen peroxide (1). Active, mature SOD1 is a 32-kDa homodimeric protein containing two zinc (Zn^{2+}) and two copper (Cu^{2+}) ions needed for its stability and activity, respectively (2). The majority of cellular SOD1 is present as a preexisting apo-SOD1 pool, which contains zinc but lacks copper (3, 4). Consequently, apo-SOD1 lacks enzymatic activity. Although the mechanism of zinc incorporation into nascent SOD1 is elusive, its activation by copper is known to be a complex and highly regulated process. SOD1 subunits acquire copper predominantly via direct transient binding to the copper chaperone for SOD1 (CCS) and subsequent transfer of copper from CCS to SOD1 (5–7). CCS-independent activation of SOD1 has also been described but accounts for a minor fraction

of SOD1 activation (8, 9). Incorporation of copper is completed by O_2 -dependent disulfide isomerization resulting in an intra-subunit disulfide bond between SOD1^{Cys-57} and SOD1^{Cys-146}, commonly referred to as disulfide-oxidized SOD1 subunits (10, 11). The final step in SOD1 maturation is the formation of enzymatically active homodimers, composed of two oxidized SOD1 subunits (4, 12, 13). Although copper incorporation and disulfide bond formation are tightly regulated by CCS, it is unknown whether the process of SOD1 subunit dimerization is subjected to regulation.

The recently identified copper metabolism Murr1 domain containing 1 (COMMD1) protein is a member of the COMMD protein family. The COMMD family includes 10 proteins sharing a unique sequence of 50–65 amino acids referred to as the COMM domain (14). The 21-kDa COMMD1 protein is considered as the prototype of the COMMD family and is implicated in the regulation of copper homeostasis, termination of NF- κ B activity, HIF-1 α signaling, and regulation of sodium transport (15–28). COMMD1 engages in many different protein-protein interactions and acts as a scaffold protein to regulate the turnover of its interaction partners, such as the NF- κ B subunit p65, HIF-1 α , and the Wilson disease-associated protein ATP7B (15, 18, 19, 27–31). Expression of COMMD1 itself is regulated on the post-translational level by the X-linked inhibitor of apoptosis (XIAP)³ and the redox sensor protein HSCARG (20, 24, 25). Consistent with the notion that COMMD1 functions in multiple cellular pathways, COMMD1 is ubiquitously expressed and located at different subcellular sites. COMMD1 has been detected in the nucleus (23, 32) and in the cytoplasm (33), where it may be associated with intracellular membranes (29, 33). In this study, we identified COMMD1 as a novel interaction partner of SOD1, and we show that COMMD1 plays an important role in the maturation of SOD1.

EXPERIMENTAL PROCEDURES

Reagents—The following antibodies were used for immunoprecipitation and immunoblotting: rabbit anti-COMMD1 antiserum (33); monoclonal mouse anti-COMMD1 (Abnova, Taipei City, Taiwan); polyclonal rabbit anti-SOD1 (Santa Cruz Biotechnology, Santa Cruz, CA); polyclonal rabbit anti-CCS (Santa Cruz Biotechnology); polyclonal mouse anti-FLAG M2

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[§] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Figs. S1–S5 and Tables 1 and 2.

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³ The abbreviations used are: XIAP, X-linked inhibitor of apoptosis; BCS, bathocuproinedisulfonic acid; TEMED, *N,N,N',N'*-tetramethylethylenediamine; DHE, dihydroethidium; β -ME, β -mercaptoethanol.

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HRP-conjugated (Sigma); polyclonal rabbit anti-GST (Santa Cruz Biotechnology); polyclonal rabbit anti-HA (Sigma); and polyclonal rabbit anti- β -tubulin (Abcam plc, Cambridge, UK). Purified SOD1 from bovine liver was obtained from Sigma.

A nontargeting pool of siRNAs (siControl) and siRNA pools targeting hCCS, hCOMMD1, and mCommd1 (targeting sequences described in [supplemental Table 1](#)) were purchased as ON-TARGETplus SMARTpools from Dharmacon (Chicago).

Cell Culture and Transfections—Human embryonic kidney 293T cells (HEK293T), human hepatocellular liver carcinoma cells (HepG2), and mouse neuroblastoma Neuro2A (all obtained from ATCC, Manassas, VA) were cultured in high glucose Dulbecco's modified Eagle's medium GlutaMAXTM (4.5 g/liter D-glucose and pyruvate; Invitrogen) supplemented with 10% fetal bovine serum (FBS), L-glutamine, penicillin, and streptomycin at 37 °C in 5% CO₂. Monoclonal HEK293T cell lines, stably transfected with pSUPER-RETRO vector (shControl) or a plasmid encoding short hairpin RNA (shRNA) targeting COMMD1 mRNA sequence (shCOMMD1), were described previously (18) and maintained in HEK293T medium supplemented with 1 μ g/ μ l puromycin dihydrochloride (Sigma). In various experiments, cells were incubated overnight with 10–150 μ M CuCl₂ (Sigma), 150 μ M ZnCl₂ (Sigma), 10–200 μ M bathocuproinedisulfonic acid (BCS; Sigma), 50 μ M paraquat dihydrochloride (Sigma), or 50 μ M menadione sodium bisulfite (Sigma) or for 1 and 2 h with 0.1 or 1 mM CuCl₂ and 50 μ M menadione, respectively, prior to lysis.

Cells were transiently transfected by the calcium phosphate precipitation method as described elsewhere (34). Transfection of siRNA ON-TARGETplus SMARTpools was performed by means of Lipofectamine RNAiMAX (Invitrogen), according to the manufacturer's protocol. Cells were harvested 48 h after transfection for biochemical analysis.

Constructs—pEBB-HA-COMMD1, pEBB-COMMD1-FLAG, and pEBB-COMMD1-GST constructs were described previously (35). pEBB-COMMD1-GST amino acids 1–118 and 119–190 deletion constructs were kindly provided by Dr. E. Burstein (Dallas, TX) (25). Full-length coding sequences of CCS and SOD1 were amplified from human liver cDNA (Invitrogen) by using the designed primers indicated in [supplemental Table 2](#) and subcloned into pEBB-FLAG and pEBB-GST vectors. Mutations in pEBB-CCS-FLAG were introduced by the QuickChange site-directed mutagenesis method (Stratagene, primer sequences indicated in [supplemental Table 2](#)). pEBB-CCS-GST deletion constructs were kindly obtained from Dr. C. Duckett (Ann Arbor, MI) and described previously (36). A pSUPER-RETRO vector encoding short hairpin RNAs (shRNAs) against COMMD1 (shCOMMD1) was described previously (15, 18). Sequences were verified by automated sequence analysis.

GST Pulldown Assays, Co-immunoprecipitation, and Immunoblot Analyses—Precipitation of GST-tagged proteins by means of GSH-Sepharose beads was performed as described previously (20). In short, transiently transfected cells were rinsed once in PBS prior to lysis in lysis buffer A (25 mM HEPES, pH 7.9, 100 mM NaCl, 1 mM NaEDTA, 1% Triton X-100, 10% glycerol) supplemented with 1 mM Na₃VO₄, 1 mM PMSF, 10 mM DTT, and protease inhibitors (Complete; Roche Applied Science). For lysis

of copper-treated, zinc-treated, or BCS-treated cells, lysis buffer A was supplemented with 1 mM CuCl₂, ZnCl₂, or BCS, respectively (15, 37), or with variable concentrations of CuCl₂ or BCS, as indicated in [Fig. 2B](#). Protein concentrations were determined by protein assay (Bradford, Bio-Rad).

Immunoprecipitation of endogenous proteins was performed essentially as described previously (20). Antibody conjugation to protein A beads (Sigma) was performed for 1 h at room temperature. Prior to immunoprecipitation, cell lysates were precleared by incubation with 40 μ l of protein A beads.

In all interaction studies, equal amounts of proteins in lysis buffer A were used for precipitation. Input samples represent ~1% of protein amounts used for immunoprecipitation or GST-precipitation.

To study protein stability, cells were treated overnight with 15 μ g/ml cycloheximide (CHX, Sigma). Cells were lysed in sample buffer (SB: 2.5% SDS, 20% glycerol, 0.2 M Tris-HCl, pH 6.8, bromophenol blue) supplemented with 2% β -mercaptoethanol (β -ME). Prior to gel loading, protein lysates were boiled at 95 °C and sheared using a 29-gauge needle. In all experiments, SDS-PAGE was followed by protein transfer onto nitrocellulose membranes (Schleicher & Schuell) for immunoblot analysis.

Monitoring of SOD1 Disulfide Redox Status—Experiments were performed essentially as described previously (38). Briefly, cells were lysed in lysis buffer B (50 mM HEPES, pH 7.2, 2.5% SDS, 0.1 mM NaEDTA) supplemented with 100 mM 2-iodoacetamide (Sigma) and incubated for 30 min at 37 °C. SDS-PAGE was performed under nonreducing conditions preceding boiling of the samples at 95 °C. After SDS-PAGE and prior to blotting, proteins were subjected to in-gel reduction by shaking in protein transfer buffer containing 2% β -ME. Input samples were prepared in sample buffer supplemented with 2% β -ME. Percentages of reduced SOD1 were quantified using densitometry (GS-700; Bio-Rad).

Visualization of SOD1 Monomers and Dimers—Visualization of SOD1 monomers and dimers was performed as described previously (39) with slight modifications. In short, cells were rinsed once in PBS prior to lysis in lysis buffer A supplemented with 1 mM Na₃VO₄, 1 mM PMSF, and protease inhibitors. 30 μ g of protein was incubated for 30 min at 37 °C in sample buffer without β -ME and analyzed by SDS-PAGE and immunoblotting under nonreducing conditions. In the case of purified SOD1, samples were preincubated in PBS without sample buffer for 1 h at 37 °C. Samples were not boiled prior to gel loading.

SOD1 Activity Assay—For the in-gel SOD1 activity assay, cells were washed once in PBS prior to lysis in lysis buffer A supplemented with 1 mM Na₃VO₄, 1 mM PMSF, and protease inhibitors. 50 μ g of protein lysates were analyzed by PAGE under native conditions, without boiling of the samples. Gels were incubated for 1 h in the dark on a shaking platform in 50 mM potassium phosphate buffer, pH 7.8, supplemented with 0.34 mM nitro blue tetrazolium chloride (Sigma), 14 mM riboflavin (Sigma), and TEMED (Bio-Rad). Subsequently, yellow-stained gels were transferred into deionized H₂O and exposed to bright light to induce a color change by the generation of free radicals, which is inversely correlated to the SOD1 activity (1).

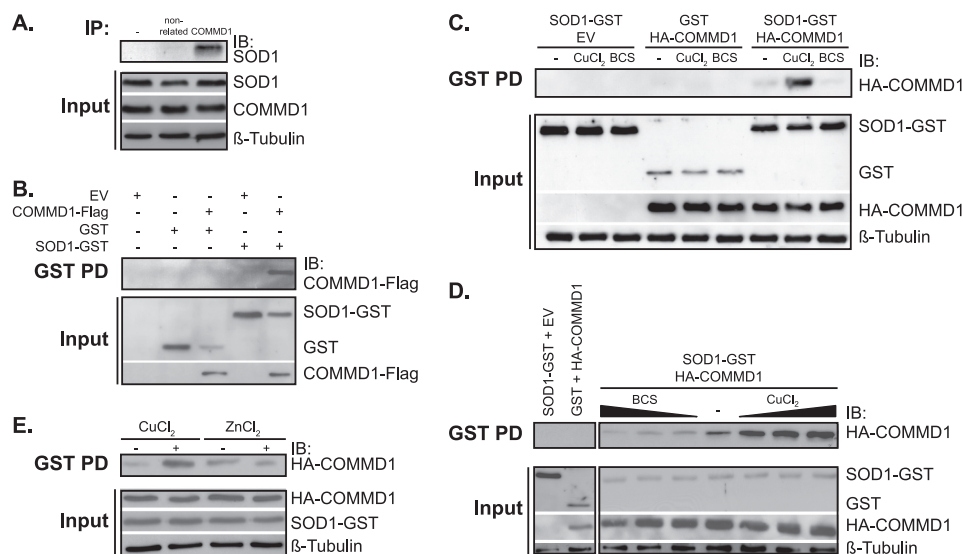


FIGURE 1. COMMD1 interacts with SOD1 in a copper-dependent manner. *A*, endogenous COMMD1 proteins were immunoprecipitated from HepG2 cell lysates. Immunoprecipitation was performed using protein A-agarose beads coupled to antibodies against COMMD1, isotype-control antibodies (nonrelated), or no antibodies (–). Precipitates (IP) or total cell lysates (Input; 30 μ g) were analyzed by SDS-PAGE and immunoblotting (IB) using antibodies directed against SOD1 as indicated on the right side. *B*, HEK293T cells were transiently transfected with pEBB (empty vector, EV) or with constructs encoding COMMD1-FLAG, GST only, or SOD1-GST as indicated. GST fusion proteins were precipitated using glutathione-Sepharose beads. Precipitates (GST PD) or total cell lysates (Input; 20 μ g) were analyzed by SDS-PAGE and immunoblotting (IB) using antibodies directed against the FLAG tag or GST as indicated on the right side. *C*, HEK293T cells were transiently transfected with empty vector or constructs encoding HA-COMMD1, GST only, or SOD1-GST as indicated above the figure. Cells were incubated overnight under basal conditions (–), with 150 μ M CuCl₂ or 200 μ M BCS. Cells were lysed (lysis buffer supplemented with 1 mM CuCl₂ or BCS, respectively), and GST fusion proteins were precipitated using GSH beads and analyzed as in *B* using antibodies directed against the HA tag, GST, or β -tubulin as indicated on the right side. *D*, HEK293T cells were transiently transfected with empty vector or constructs encoding HA-COMMD1, GST only, or SOD1-GST and incubated overnight under basal conditions (–), with increasing concentrations of CuCl₂ (10, 50, and 150 μ M) or BCS (10, 50, and 200 μ M) as indicated. Cells were lysed in lysis buffer supplemented with similar concentrations of CuCl₂ or BCS as incubation. GST fusion proteins were precipitated using GSH beads and analyzed as in *C* using antibodies directed against the HA tag, GST, or β -tubulin. Negative control samples (SOD1-GST + EV and GST + HA-COMMD1) were analyzed on a separate membrane. *E*, HEK293T cells were transiently transfected with constructs encoding HA-COMMD1 and SOD1-GST. Cells were incubated overnight under basal conditions (–), with 150 μ M CuCl₂ or 150 μ M ZnCl₂. Cells were lysed, and GST fusion proteins were precipitated and analyzed as in *C*.

A quantitative SOD1 activity assay, based on the formation of formazan by the tetrazolium salt WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt) upon reduction with a superoxide anion, was performed according to manufacturer's product sheet (Sigma). 1 mM KCN (Sigma) was used as a specific inhibitor of SOD1 activity, and measured activities were corrected for protein concentration.

Assessment of Intracellular Superoxide Anions by Flow Cytometry—Cells were incubated with 3 μ M dihydroethidium (hydroethidine, DHE; Invitrogen) in PBS for 30 min at 37 °C. Cells were incubated with menadione sodium bisulfite prior to DHE incubation. Cells were washed once with PBS, trypsinized, and immediately analyzed on a FACSCalibur flow cytometer (BD Biosciences; software CellQuest Pro).

Statistical Analysis—The quantitative data in this paper are represented as means \pm S.D. Statistical evaluation was made using one-way analysis of variance, followed by the post hoc Bonferroni test. Differences were considered to be significant at $p < 0.05$.

RESULTS

COMMD1 Interacts with SOD1 in a Copper-dependent Manner—To investigate a putative role for COMMD1 in regulating SOD1 maturation, the potential binding of COMMD1 to

SOD1 was examined. Endogenous COMMD1 was immunoprecipitated from HepG2 cells, followed by immunoblot analysis using SOD1 antibodies. This experiment revealed a specific interaction of endogenous COMMD1 with SOD1 (Fig. 1*A*). Transient expression of COMMD1-FLAG and SOD1-GST, followed by GST pull-down of the SOD1-GST fusion protein and immunoblot analysis of the presence of COMMD1, confirmed the interaction between these proteins in HEK293T cells (Fig. 1*B*) and in the neuroblastoma cell line Neuro2A (data not shown). These experiments indicate that COMMD1 interacts with SOD1 and validate the use of GST pull-down analysis for subsequent interaction studies in our cellular model systems.

To examine whether the interaction between COMMD1 and SOD1 was dependent on copper, GST pull-down assays were performed after incubation of HEK293T cells with CuCl₂ or the copper chelator BCS, respectively. These studies showed that the interaction was markedly enhanced upon addition of copper, whereas copper depletion decreased the binding to almost undetectable amounts (Fig. 1*C*). Incubation with various concentrations

of CuCl₂ or BCS confirmed the copper dependence of the interaction, as binding of COMMD1 to SOD1 was enhanced by copper in a concentration-dependent manner (Fig. 1*D*). In contrast to copper, addition of ZnCl₂ had no significant effect on the interaction (Fig. 1*E*).

Copper-dependent Binding of COMMD1 to SOD1 Is Mediated by CCS—Because we were unable to identify a direct interaction between recombinant purified SOD1 and COMMD1 (data not shown) and because SOD1 acquires its copper predominantly via its metallochaperone CCS (5, 6), we determined whether the interaction between COMMD1 and SOD1 was affected by siRNA-mediated depletion of CCS protein expression in HEK293T cells. GST pull-down analysis revealed a reduced interaction between COMMD1 and SOD1 after CCS depletion, which could be partially restored by addition of excess copper (Fig. 2*A*). Similar to the effects shown in Fig. 1, *C* and *D*, copper depletion resulted in a diminished interaction of COMMD1 with SOD1, and this was not further inhibited by depletion of CCS. Conversely, overexpression of CCS enhanced the interaction between COMMD1 and SOD1 in a CCS concentration-dependent manner (Fig. 2*B*). Incubation of cells with high copper concentrations in addition to CCS overexpression did not result in further enhancement of binding

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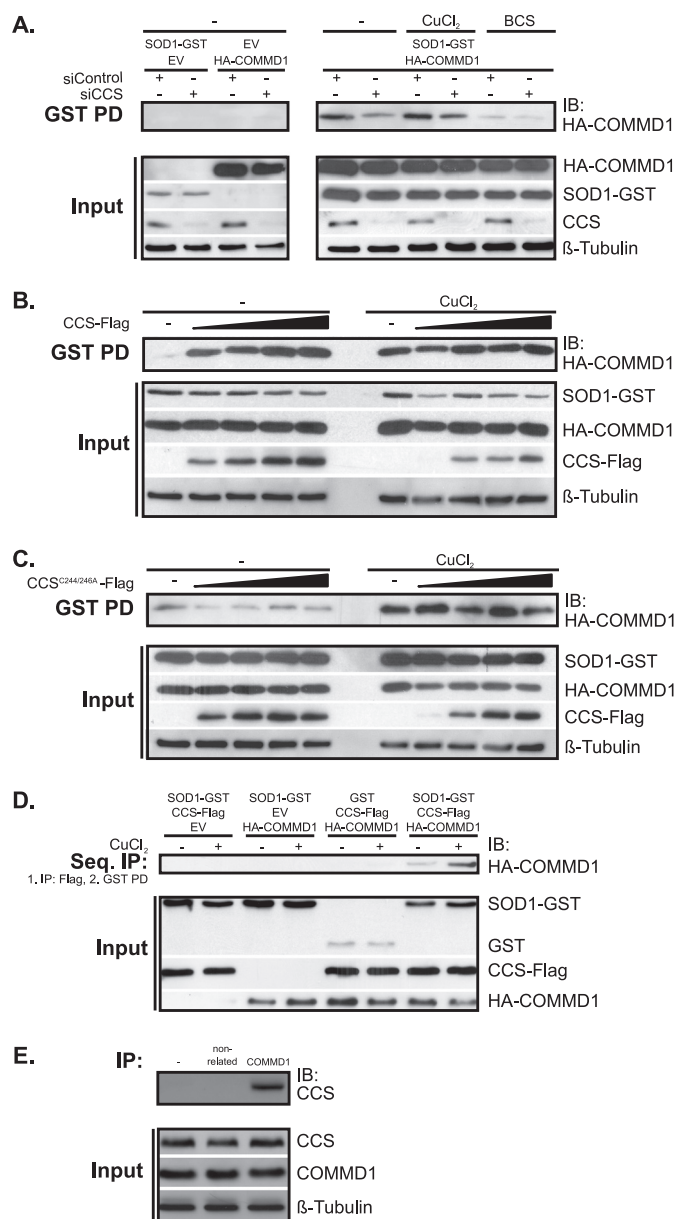


FIGURE 2. Interaction between SOD1 and COMMD1 is mediated by CCS. A, HEK293T cells were transiently transfected with nontargeting siRNA ON-TARGETplus SMARTpools (*siControl*) or with siRNA ON-TARGETplus SMARTpools targeting CCS (*siCCS*). Cells were additionally transiently transfected with empty vector (EV) or constructs encoding HA-COMMD1 and SOD1-GST as indicated above the figure. Copper incubation, lysis, and GST precipitation were performed as in Fig. 1C. Immunoblot (IB) analysis was done using antibodies directed against the HA tag, CCS, GST, or β -tubulin as indicated on the right side. Negative control samples (*SOD1-GST + EV* and *EV + HA-COMMD1*) were analyzed on a separate membrane. B and C, HEK293T cells were transiently transfected with constructs encoding HA-COMMD1 and SOD1-GST, and variable amounts of constructs encoding CCS-FLAG (*B*) or $CCS^{C244A/C246A}$ -FLAG (*C*) as indicated above the figures. Cells were incubated overnight under basal conditions (–) or with $150 \mu\text{M}$ CuCl_2 . Cells were lysed, and GST fusion proteins were precipitated and analyzed as in Fig. 1B using antibodies directed against the HA tag, the FLAG tag, GST, or β -tubulin as indicated on the right side. D, HEK293T cells were transiently transfected with empty vector or constructs encoding CCS-FLAG, SOD1-GST, and HA-COMMD1. Cells were incubated overnight under basal conditions (–) or with $150 \mu\text{M}$ CuCl_2 . First, FLAG fusion proteins were precipitated using FLAG beads followed by protein elution from beads using FLAG peptides. Second, GST fusion proteins present in the eluates were precipitated using GSH-Sepharose beads and analyzed using antibodies directed against the HA tag, FLAG tag, or GST as indicated on the right side. E, endogenous COMMD1 proteins were immunoprecipitated from HepG2 cell lysates. Immunoprecipitation

between COMMD1 and SOD1. This suggests that the copper-dependent binding of COMMD1 to SOD1 was predominantly mediated by CCS.

To assess if this effect of CCS on the COMMD1-SOD1 interaction could be attributed to its function in copper incorporation into SOD1, a $CCS^{C244A/C246A}$ mutant was used. In this mutant, the CXC copper-binding motif located in CCS domain III, which is essential for the metal transfer activity of CCS toward SOD1 (supplemental Fig. S1A) (40, 41), was mutated to AXA, resulting in a failure to load SOD1 with copper (see Refs. 40, 42 and confirmed by our own data not shown). Although this $CCS^{C244A/C246A}$ mutant interacted with SOD1 (supplemental Fig. S1B), $CCS^{C244A/C246A}$ could not increase the binding between COMMD1 and SOD1 as seen with the wild-type CCS protein (Fig. 2C). Taken together, the observation that the interaction SOD1-COMMD1 is dependent on CCS or excess copper implies that binding of COMMD1 to SOD1 requires CCS-mediated copper incorporation into SOD1.

Next, we set out to investigate whether COMMD1 is present in a complex with both SOD1 and CCS. A sequential immunoprecipitation was performed in HEK293T cells in which CCS-FLAG proteins were precipitated prior to the precipitation of SOD1-GST. Immunoblot analysis of HA-COMMD1 revealed that COMMD1 is in the complex with CCS and SOD1. In line with previous observations, the formation of this heterocomplex (COMMD1-CCS-SOD1) depended on copper (Fig. 2D). In addition, as shown in Fig. 2E, endogenous COMMD1 was able to physically interact with endogenous CCS. In more detail, domain 2 of the CCS protein, a region that shows high homology to SOD1, is essential for this interaction (supplemental Fig. S2A). The only identified domain in the COMMD1 sequence thus far, the COMM domain, was shown to bind to CCS (supplemental Fig. S2B). In contrast to SOD1-COMMD1 interaction, binding of COMMD1 to CCS was copper-independent (data not shown). Although CCS enhanced the interaction between SOD1 and COMMD1, COMMD1 did not affect the binding of SOD1 to CCS (supplemental Fig. S3).

COMMD1 Does Not Affect Stability and Disulfide Oxidation of SOD1 Subunits but Reduces the Level of SOD1 Homodimers—Because COMMD1 reduces the protein stability of its interaction partners by promoting their proteasomal degradation (15, 19, 27, 28, 31), we determined its effect on SOD1 stability. SOD1 turnover in HEK293T cells was monitored after inhibition of protein biosynthesis by cycloheximide. Incubation with cycloheximide resulted in a marked decline of immunodetectable SOD1 compared with vehicle-treated cells within 16 h. The expression of exogenous COMMD1 did not enhance SOD1 turnover rates, either under basal or high copper conditions (Fig. 3).

Incorporation of copper is completed by disulfide oxidation of the SOD1 subunits (10, 11). To investigate if COMMD1 influenced holo-SOD1 biogenesis downstream of copper incor-

tation was performed using protein A-agarose beads coupled to antibodies against COMMD1, isotype-control antibodies (nonrelated), or no antibodies (–). Precipitates (IP) or total cell lysates (Input; 30 μg) were analyzed by SDS-PAGE and immunoblotting (IB) using antibodies directed against CCS as indicated on the right side.

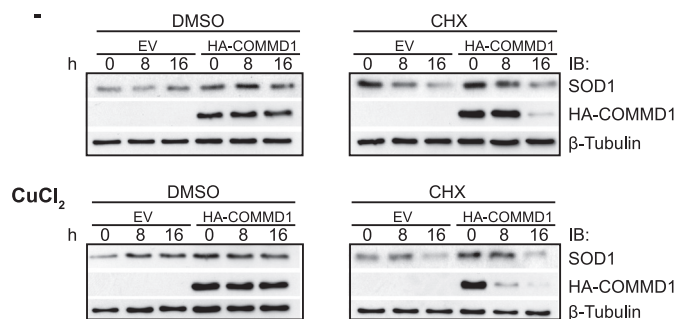


FIGURE 3. SOD1 protein stability is not affected by COMMD1. HEK293T cells were transiently transfected with empty vector (EV) or a construct encoding HA-COMMD1 as indicated above the figure and incubated overnight under basal conditions (–) or with 150 μ M CuCl₂. Cells were additionally incubated for 0, 8, and 16 h with vehicle-control DMSO (left panel) or with 15 μ g/ml cycloheximide (CHX, right panel). Cells were lysed as described under “Experimental Procedures.” Antibodies directed against endogenous SOD1, the HA tag, or β -tubulin as indicated on the right side were used for immunoblotting (IB).

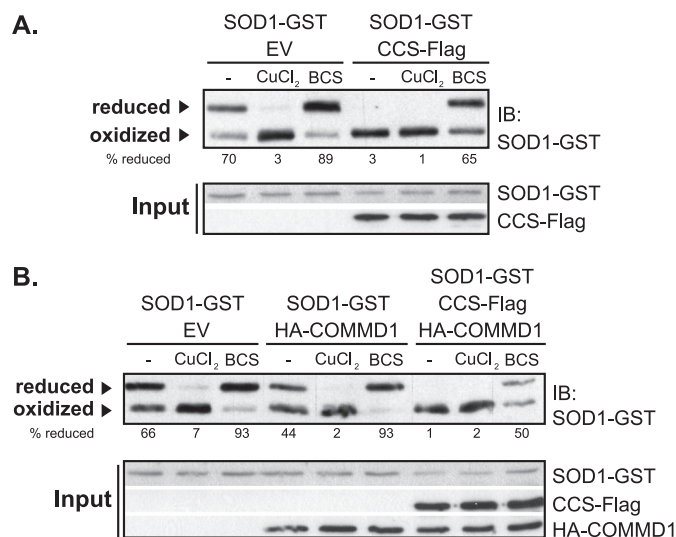


FIGURE 4. Effect of COMMD1 on disulfide oxidation of SOD1 subunits. *A*, HEK293T cells were transiently transfected with SOD1-GST in combination with empty vector (EV) and CCS-FLAG and incubated overnight under basal conditions (–), with 150 μ M CuCl₂ or 200 μ M BCS. Samples were prepared and analyzed as described under “Experimental Procedures.” Input samples (20 μ g) were prepared in sample buffer supplemented with β -ME. For immunoblotting (IB), an antibody directed against the FLAG-tag was used as indicated on the right side. The migration of the disulfide-reduced and disulfide-oxidized forms of SOD1 is indicated. The indicated percentages represent the relative amount of reduced SOD1 in each sample. *B*, HEK293T cells were transiently transfected with constructs encoding SOD1-GST in combination with empty vector, HA-COMMD1, or HA-COMMD1 and CCS-FLAG and incubated overnight under basal conditions (–), with 150 μ M CuCl₂ or 200 μ M BCS. Samples were prepared and analyzed as described in *A* using antibodies against GST, the HA tag, or the FLAG tag as indicated on the right side.

poration into SOD1, the effect of COMMD1 on SOD1 disulfide oxidation was monitored in HEK293T cells transiently transfected with COMMD1 and incubated with varying copper concentrations. As described previously (38), SOD1 disulfide oxidation was promoted by overexpression of CCS or excess copper, whereas copper depletion by BCS promoted the reduced state of the SOD1 monomer (Fig. 4*A*). Overexpression of COMMD1 did not interfere with the oxidation of SOD1 subunits (Fig. 4*B*). In addition, exogenous COMMD1 did not alter CCS-induced oxidation of SOD1, as the ratio between reduced and oxidized SOD1 was comparable in the

presence and absence of COMMD1 (Fig. 4*B* and data not shown).

Because homodimerization is an essential step in SOD1 activation subsequent to SOD1 oxidation, the effect of COMMD1 on this process in SOD1 maturation was examined. First, we determined whether COMMD1-SOD1 binding was dependent on SOD1 homodimers. Protein lysates of HEK293T cells transfected with SOD1-FLAG and COMMD1-GST were incubated with increasing concentrations of dithiothreitol (DTT), which reduces the intramolecular disulfide bonds of SOD1 dimers and favors the monomeric form of SOD1 (13). As predicted, the amount of SOD1 dimers decreased upon DTT treatment. However, the interaction between COMMD1 and SOD1 was unaffected as determined by GST pulldown analysis (Fig. 5*A*). This demonstrates that COMMD1-SOD1 interaction does not depend on the dimerization state of SOD1.

Second, the effect of COMMD1 on SOD1 dimerization was studied. COMMD1 overexpression resulted in a decrease of detectable SOD1 dimers in HEK293T cells, whereas knockdown of COMMD1 resulted in an increased level of SOD1 dimers (Fig. 5*B*). Normally, excess copper promotes the formation of SOD1 dimers (supplemental Fig. S4). However, the SOD1 dimerization in COMMD1-overexpressing cells upon copper incubation could not be enhanced to a similar extent compared with control cells (Fig. 5*C*). Together, these data indicate that COMMD1 impairs the expression of SOD1 dimers, although the COMMD1-SOD1 interaction seems to be independent of SOD1 homodimers.

COMMD1 Expression Impairs SOD1 Activity—Next, we set out to determine the relevance of this interaction for the enzymatic activity of SOD1, and we examined the effect of COMMD1 on SOD1 activity by an in-gel SOD1 activity assay. Expression of exogenous COMMD1 in HEK293T cells resulted in reduced SOD1 activity (Fig. 6*A*), consistent with the observation that COMMD1 expression resulted in impaired SOD1 homodimerization (Fig. 5, *B* and *C*). The repression of SOD1 activity by COMMD1 under basal conditions was confirmed in Neuro2A cells (Fig. 6*B*). As shown previously, a rise in intracellular copper concentrations readily induced SOD1 activity (Fig. 6*B*) (43), but the COMMD1-mediated inhibition of SOD1 activity was not abrogated by copper incubation.

We next considered it important to investigate whether endogenous COMMD1 modulates SOD1 activity. We hypothesized that a reduction of COMMD1 expression would lead to an increase in SOD1 activity. To address this hypothesis, endogenous COMMD1 expression was knocked down by transient transfection of shRNA directed against COMMD1 mRNA (shCOMMD1). Consistent with our expectations, knockdown of COMMD1 resulted in enhanced SOD1 activity (Fig. 6, *C* and *D*) and further enlarged the copper-dependent increase in SOD1 activity (Fig. 6*D*).

The effect of COMMD1 expression on SOD1 activity was further investigated using a quantitative SOD1 activity assay based on the superoxide anion-responsive production of formazan. As a control for these experiments, we demonstrated that the amount of detectable SOD1 was not affected by overexpression of COMMD1 and copper overload (Fig. 6, *E* and *F*, lower panels). Overexpression of COMMD1 in HEK293T cells

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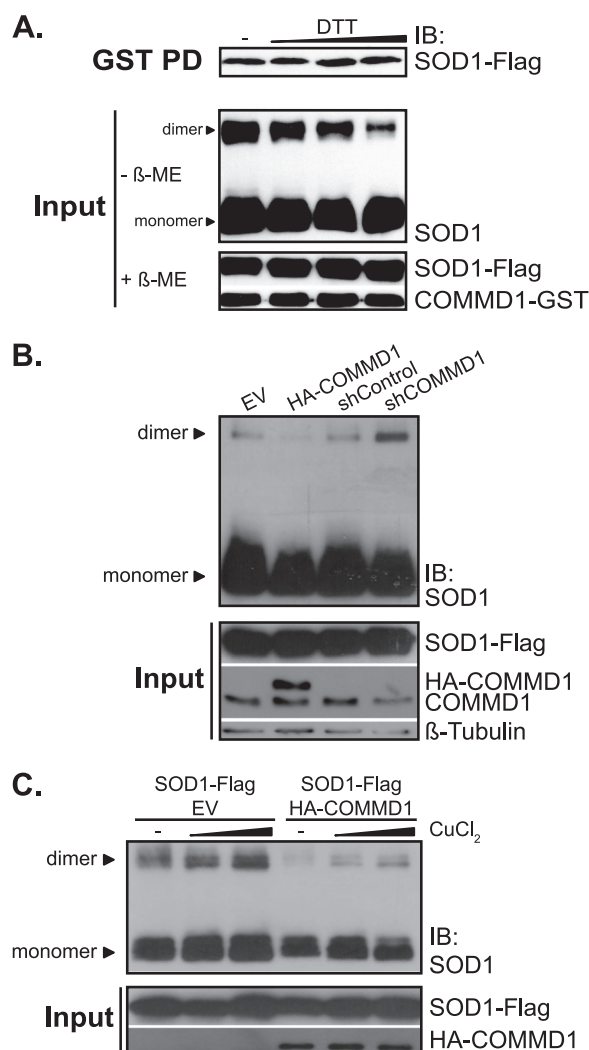


FIGURE 5. COMMD1 expression reduces the level of SOD1 homodimers. A, HEK293T cells were transiently transfected with constructs encoding SOD1-FLAG and COMMD1-GST. Cells were lysed in lysis buffer containing varying concentrations of DTT (0, 1, 10, and 100 mM, respectively). GST fusion proteins were precipitated from these same cell lysates and analyzed as in Fig. 1B using antibodies directed against the FLAG tag (*upper panel*; GST PD). SOD1 monomers and dimers were visualized by SDS-PAGE under nonreducing conditions and without boiling (*Input*; $-\beta$ -ME) or after reduction with β -ME and boiling at 95 °C (*Input*; $+\beta$ -ME). Expression of SOD1-FLAG and COMMD1-GST was monitored by immunoblot (IB) analysis using anti-FLAG and anti-GST antibodies. B, HEK293T cells were transiently transfected with constructs encoding SOD1-FLAG in combination with empty vector (EV) and HA-COMMD1 or in combination with pSUPER-RETRO vectors encoding nonspecific shRNA (*shControl*) or shRNA against COMMD1 (*shCOMMD1*). Samples (30 μ g) were prepared and analyzed as described under "Experimental Procedures." SDS-PAGE of SOD1 monomers and dimers was performed as described in A. Immunoblot analysis (IB) was performed using antibodies directed against endogenous SOD1, the FLAG tag, endogenous COMMD1, or β -tubulin as indicated on the *right side*. C, HEK293T cells were transiently transfected with constructs encoding SOD1-FLAG and empty vector or HA-COMMD1 and incubated for 1 h with CuCl_2 (0, 0.1, or 1 mM, respectively). Visualization of SOD1 monomers and dimers by SDS-PAGE under nonreducing conditions and immunoblot analysis were performed as described under B.

resulted in a markedly decreased SOD1 activity under basal conditions (Fig. 6E), confirming that the COMMD1 mediated inhibition on SOD1 activity as seen in Fig. 6, A and B. Overexpression of CCS resulted in slightly enhanced SOD1 activity, but simultaneous overexpression of COMMD1 overruled this CCS-mediated increase of SOD1 activity. SOD1 activity was

clearly and significantly decreased in these cells in a COMMD1 concentration-dependent manner. In line with the observation that binding of COMMD1 to SOD1 is mediated by CCS, the inhibitory effect of COMMD1 on SOD1 activity was more pronounced when CCS was co-expressed (Fig. 6E). Conversely, knockdown of COMMD1 expression in HEK293T cells resulted in a significant increase in SOD1 activity (~ 2.5 -fold) on top of the induction of SOD1 function upon copper incubation (Fig. 6F). Together, these data demonstrate that endogenous COMMD1 represses cellular SOD1 activity. More specifically, these findings indicate an inverse correlation between COMMD1 expression and SOD1 activity in different cell lines and suggest that COMMD1 might help regulate cellular superoxide anion concentrations.

COMMD1 Induces an Increase in Intracellular Superoxide Anion Concentration—As readout for intracellular superoxide anion concentrations, the fluorescence of the superoxide-specific probe DHE was measured in cells. HEK293T cells stably transfected with shControl or shCOMMD1 were incubated with the pro-oxidant menadione prior to DHE labeling and analyzed by flow cytometry. Upon menadione incubation, the DHE fluorescence was markedly increased in shControl-expressing cells (Fig. 7, A and B). In COMMD1 knockdown cells, a significant decline in DHE fluorescence was detected both under basal and menadione-induced conditions (Fig. 7, A and B) corresponding to the observed increase in SOD1 activity (Fig. 6 and data not shown). The interaction between COMMD1 and SOD1 remained unaffected under these conditions (supplemental Fig. S5).

These data were confirmed in Neuro2A cells in which Commd1 expression was transiently decreased by siRNA targeting of Commd1 mRNA (siCommd1). Cells transfected with a nontargeting siRNA pool were used as control (siControl). Commd1 depletion did not result in differences in DHE fluorescence under basal conditions. Incubation with menadione showed a clear induction of DHE fluorescence in siControl transfected cells. However, siRNA-mediated decrease in Commd1 expression revealed a significant reduction of DHE fluorescence under these conditions (Fig. 7, C and D), which is in accordance with the enhanced SOD1 activity observed in COMMD1 knockdown cells (Fig. 6 and data not shown). In both cell lines, the SOD1 protein expression was not changed by a decrease in Commd1 expression (Fig. 7, B and D, *lower panels*). However, the differences in DHE fluorescence decline upon COMMD1 knockdown in untreated cell lines might be explained by variation in the efficiency of COMMD1 knockdown. Collectively, these data demonstrate that endogenous COMMD1 expression significantly induces intracellular superoxide anion concentrations in different cell lines, likely in response to its repression of SOD1.

DISCUSSION

The post-translational events required for activation of human SOD1 provide a unique and elegant mechanism to tailor the activity of this abundant anti-oxidant enzyme to the availability of oxygen and the transition metal copper (7, 12). Here, we identify COMMD1 as a novel interaction partner of SOD1. This interaction between COMMD1 and SOD1 is genuine

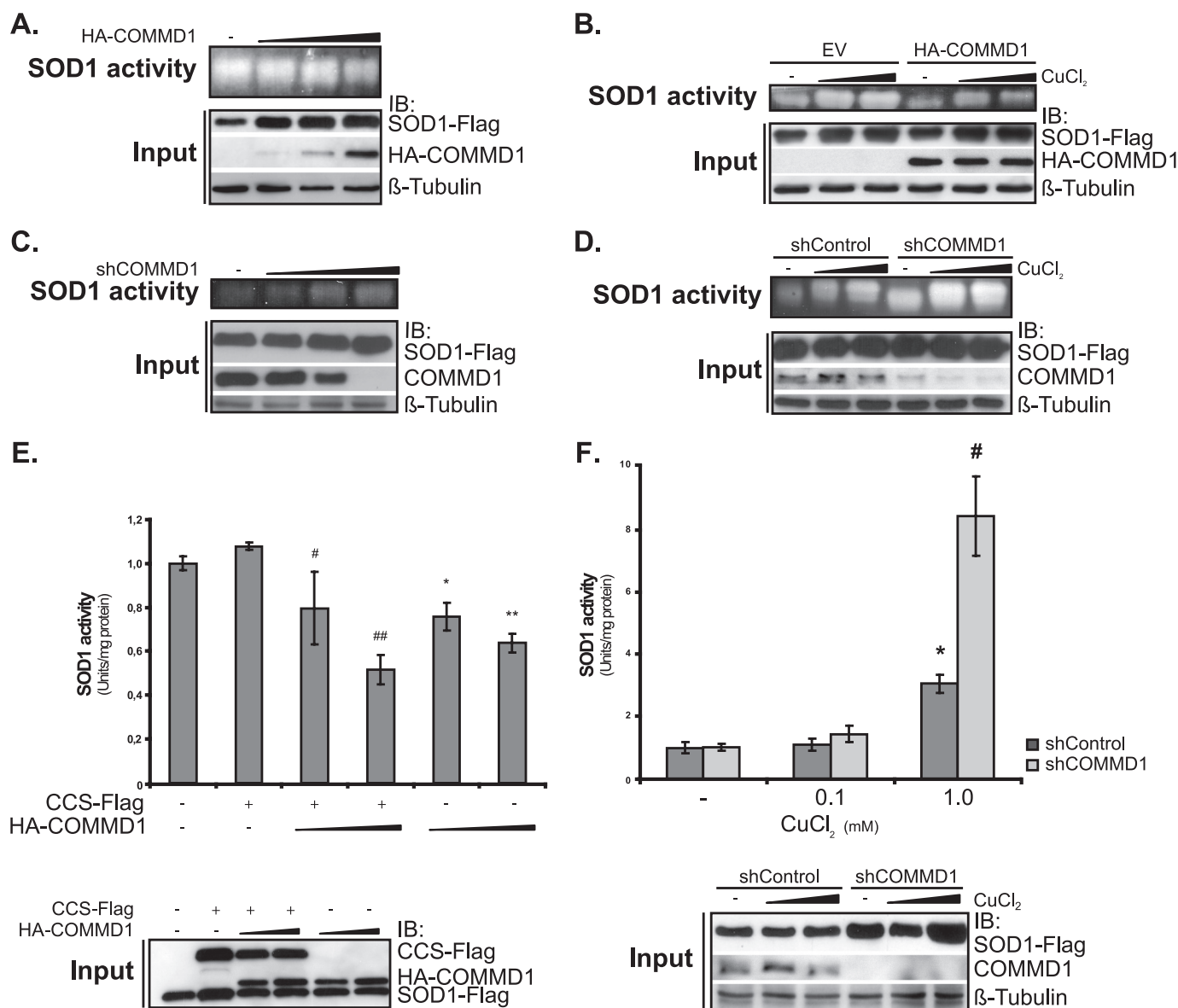


FIGURE 6. COMMD1 impairs SOD1 activity. A, HEK293T cells were transiently transfected with constructs encoding SOD1-FLAG in combination with varying amounts of HA-COMMD1. Cells were lysed, and SOD1 activity was determined in 50 μg of cell lysates by the in-gel activity assay as described under "Experimental Procedures" (upper panels). Expression of SOD1-FLAG, COMMD1, and β -tubulin was monitored in 20 μg of the cell lysates by reducing, denaturing SDS-PAGE followed by immunoblot analysis (IB) using FLAG, HA, or β -tubulin antibodies as indicated (lower panels; Input). B, Neuro2A cells were transiently transfected with SOD1-FLAG in combination with constructs encoding empty vector (EV) or HA-COMMD1. Cells were incubated for 1 h with CuCl_2 (0, 0.1, or 1 mM, respectively). Visualization of SOD1 activity (upper panels) and immunoblot analysis (lower panels; Input) were performed as described in A. C, HEK293T cells were transiently transfected with SOD1-FLAG in combination with increasing concentrations of shCOMMD1. Cells were lysed and analyzed as described in A. D, HEK293T cells were transiently transfected with SOD1-FLAG in combination with shControl or shCOMMD1. Sample preparation and analysis was performed as described for B. E, HEK293T cells were transiently transfected with SOD1-FLAG in combination with empty vector or CCS-FLAG and varying amounts of HA-COMMD1. Cells were lysed, and SOD1 activity was measured by formation of formazan upon reduction with superoxide anion. Obtained values were corrected for protein concentration and normalized for basal conditions, which was set at 1. For each sample, mean values \pm S.D. are shown ($n = 4$). Data represent three independent experiments. * and ** indicate significantly different values compared with SOD1-FLAG-transfected cells (*, $p < 0.05$; **, $p < 0.001$). # and ## indicate significantly different values compared with SOD1-FLAG and CCS-FLAG co-transfected cells (# $p < 0.05$, ## $p < 0.005$). Expression of SOD1-FLAG, CCS-FLAG, and HA-COMMD1 in cell lysates (20 μg of protein) was monitored by immunoblot analysis using the indicated antibodies (Input; panel below graph). F, HEK293T cells were transiently transfected with constructs encoding SOD1-FLAG in combination with shControl and shCOMMD1 plasmids and incubated under basal conditions (-) or for 1 h with CuCl_2 (0.1 and 1 mM, respectively). Cells were grown under basal conditions. SOD1 activity was analyzed as described in E. For each sample, mean values \pm S.D. are shown ($n = 3$). Data represent three independent experiments. * indicates significantly different values compared with untreated and 0.1 mM CuCl_2 -treated cells; $p < 0.001$. # indicates significantly different values compared with shControl-transfected cells treated with 1.0 mM CuCl_2 ; $p < 0.001$. Expression of SOD1-FLAG, COMMD1, and β -tubulin in cell lysates (20 μg of protein) was monitored by immunoblot analysis using the indicated antibodies (Input; panel below graph).

because it was detected in multiple different cell types, using overexpressed as well as endogenous proteins. In addition, the interaction displayed specificity with respect to varying amounts of copper and the metallochaperone CCS. Moreover,

COMMD1 impaired SOD1 activity by reducing the expression levels of enzymatically active SOD1 homodimers late in the post-translational maturation process of SOD1. Intriguingly, COMMD1 expression was correlated with an increase in intra-

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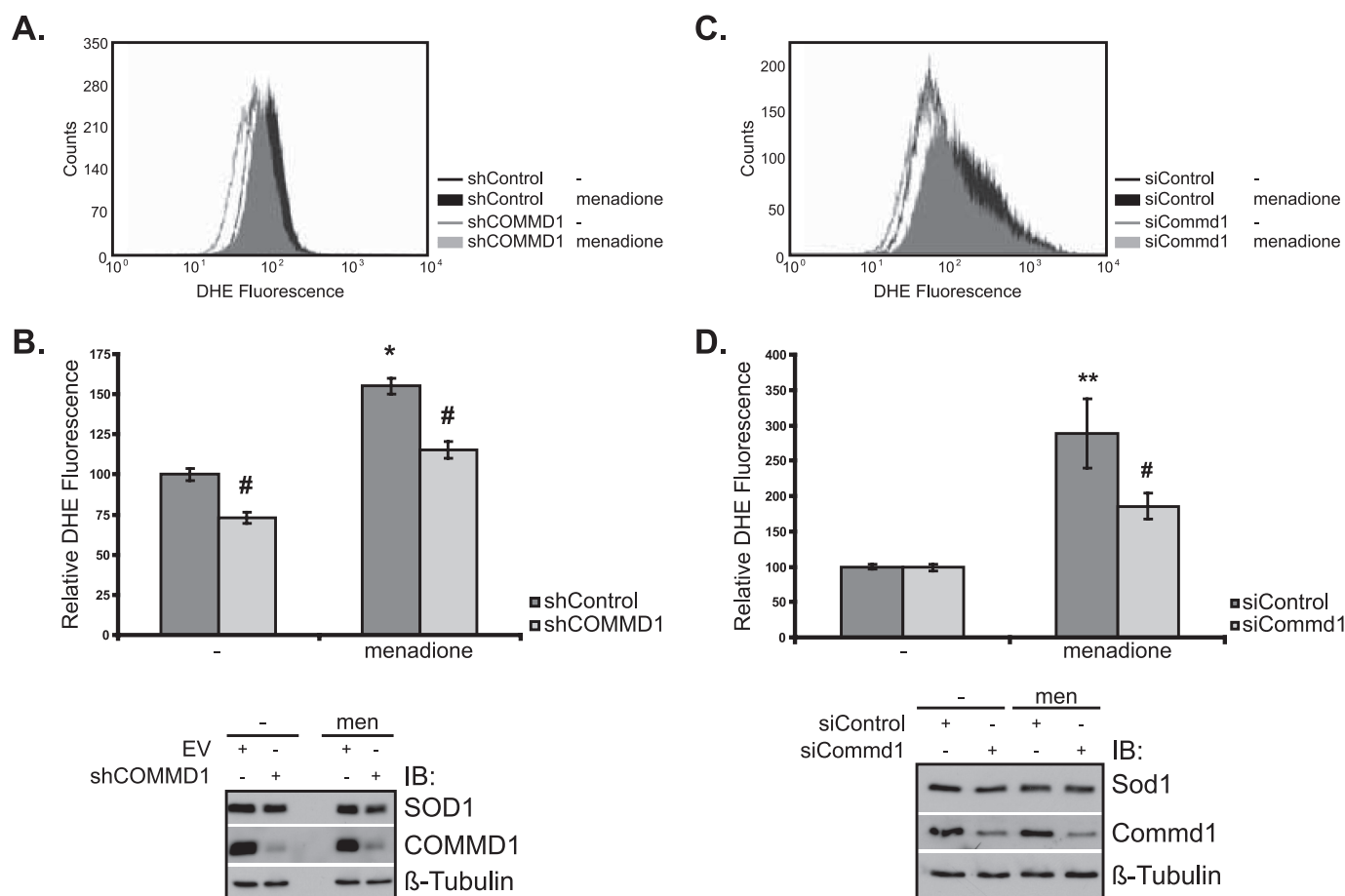


FIGURE 7. COMMD1 expression induces intracellular superoxide anion concentrations. *A* and *B*, intracellular superoxide anion concentrations were assessed by DHE fluorescence using FACS analysis in HEK293T cells stably transfected with shControl or shCOMMD1. Cells were incubated under basal conditions (–) or with 50 μM menadione for 2 h prior to DHE loading (3 μM for 30 min, 37 $^{\circ}\text{C}$) and analyzed on a FACSCalibur flow cytometer. Data represent DHE fluorescence of 25,000 cells. *A*, FACSCalibur fluorescence histogram representing shControl (black) and COMMD1 knockdown cells (gray), loaded with DHE, after incubation without (open curves) or with menadione (filled curves). *B*, relative DHE fluorescence. Values obtained in *A* were normalized for shControl basal conditions, which was set at 100%. For each sample, mean values \pm S.D. are shown ($n = 3$). Data are representative of three experiments ($n = 3$). * indicates significant increase in DHE fluorescence of cells grown under basal conditions or after incubation with menadione; $p < 0.001$. # indicates significant decline in measured DHE fluorescence of shControl cells compared with shCOMMD1 expressing HEK293T; $p < 0.001$. Expression of SOD1, COMMD1, and β -tubulin in 20 μg of cell lysates was monitored by immunoblot analysis using the indicated antibodies (panel below graph; –, basal conditions; men, incubation with 50 μM menadione). *C* and *D*, Neuro2A cells were transiently transfected with nontargeting siRNA ON-TARGETplus SMARTpools (siControl) or with siRNA ON-TARGETplus SMARTpools targeting mouse Commd1 (siCommd1). Cells were treated with menadione and DHE, and DHE fluorescence was analyzed as described in *A* and *B*. *C*, FACSCalibur fluorescence histogram representing siControl (black) and Commd1 knock-down cells (gray), loaded with DHE, after incubation without (open curves) or with menadione (filled curves). *D*, relative DHE fluorescence. Values obtained in *C* were normalized for siControl basal conditions, which were set at 100%. For each sample, mean values \pm S.D. are shown ($n = 3$). Data are representative of three experiments ($n = 3$). ** indicates significant increase in measured DHE fluorescence of cells grown under basal conditions or after incubation with menadione; $p < 0.001$. # indicates significant decline in measured DHE fluorescence of siControl cells compared with siCommd1 expressing cells; $p < 0.01$. Immunoblot analysis was performed as in *B* (panel below graph).

cellular superoxide anions. These findings extend the already impressive COMMD1 protein-protein interaction network and support previous suggestions that COMMD1 exerts its function through physical interactions with proteins that are structurally and functionally unrelated (15, 19–21, 27). More importantly, our results provide the first indication that SOD1 dimer formation is a regulated final step in SOD1 activation and implicate COMMD1 in this process.

The current findings are integrated in a model that describes the effect of COMMD1 on CCS-mediated SOD1 maturation and activation (summarized in Fig. 8). The majority of cellular SOD1 is present as a preexisting apo-SOD1 pool, which contains zinc but lacks copper (E,Zn-SOD1 subunit (3, 4)). Consequently, the inactive apo-SOD1 requires copper insertion, disulfide oxidation, and homodimerization for its activation (Fig. 8, upper panel).

Several observations are consistent with the conclusion that COMMD1 acts downstream of copper incorporation and intramolecular disulfide oxidation. First, COMMD1 expression did not reduce the half-life time of SOD1, suggesting that it does not interfere with the stability of the E,Zn-SOD1 apo-pool. Second, COMMD1 expression had no effect on the ratio of disulfide oxidized to disulfide-reduced SOD1. As described previously, CCS overexpression or elevated copper concentrations favored transition of SOD1 to the disulfide-oxidized form, and COMMD1 did not affect this process.

Our data demonstrate that COMMD1 impairs the expression levels of SOD1 homodimers and activity in conditions of excess copper (Fig. 8, lower panel). Copper incorporation into SOD1 and disulfide isomerization are strictly coupled events (4, 13). Both excess copper and CCS expression lead to enhanced copper incorporation into apo-SOD1, thus increasing the

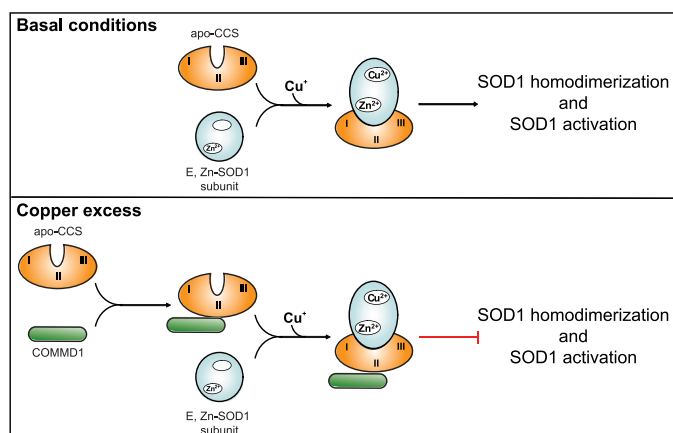


FIGURE 8. Hypothetical model, COMMD1 reduces CCS-mediated activation of SOD1 by impairment of SOD1 dimerization. Schematic representation of SOD1 post-translational activation, adapted from Refs. 9, 13. Under basal conditions (*upper panel*), CCS delivers copper to monomeric zinc-containing apo-SOD1 (*E,Zn-SOD1 subunit*) by transient interaction. This transfer of copper ions involves the formation of an intermolecular disulfide bond between SOD1 and CCS. Copper incorporation is followed by dissociation of CCS from SOD1 prior to disulfide oxidation of cysteine residues 57 and 146 of SOD1. This results in SOD1 homodimerization and induction of the superoxide scavenging activity of SOD1. In excess copper (*lower panel*), COMMD1-bound CCS interacts with SOD1 leading to formation of COMMD1-CCS-SOD1 heterocomplex. Consequently, SOD1 homodimerization is impaired resulting in decreased level of SOD1 homodimers and subsequently reduced SOD1 anti-oxidant activity.

amount of active holo-SOD1 (7, 43). We showed that excess copper and overexpression of CCS, but not a copper incorporation-deficient mutant of CCS (CCS^{C244A/C246A}), induced COMMD1 binding to SOD1. In contrast, RNAi-mediated depletion of CCS diminished COMMD1-SOD1 interaction and demonstrated that CCS indeed predominantly mediated that copper-dependent binding of COMMD1 to SOD1. At the same time, COMMD1 binds to CCS copper independently, but in excess copper, the two proteins are found in a complex together with SOD1. Collectively, our data suggest that COMMD1 binding requires CCS-mediated copper incorporation in SOD1 to establish its inhibitory effect on SOD1 dimerization.

Perhaps the most striking finding presented here indicates that siRNA-mediated depletion of endogenous COMMD1 results in increased formation of enzymatically active SOD1 dimers. COMMD1 can therefore be considered as a novel endogenous regulator of the final step in SOD1 activation, which is the formation of active dimers. This COMMD1-mediated reduction in SOD1 dimers and activity is most abundant in conditions of excess copper and overrules the CCS-mediated copper-responsive maturation of SOD1. The biological relevance of these observations is reflected by the repression of intracellular superoxide anion concentrations upon depletion of endogenous COMMD1. It has previously been described that copper incorporation into SOD1 and disulfide oxidation results in a conformational change of the SOD1 subunit necessary for homodimerization (reviewed in Ref. 12). This conformational change may expose a binding site for COMMD1 that had previously been buried within the SOD1 structure. It might be possible that COMMD1 prevents SOD1 dimerization by directly competing for the same SOD1-SOD1 interaction interface. Structural studies should provide detailed mechanistic answers into the many

unsolved questions raised by the findings presented in this paper.

Interestingly, XIAP has also recently been identified as a novel protein involved in the regulation of SOD1 activation. Brady *et al.* (36) showed that, depending on the intracellular copper status, CCS delivers copper either to SOD1 or to XIAP. Under basal conditions, XIAP enhances copper acquisition and delivery to SOD1 by promoting the nondegradative ubiquitination of CCS. However, in excess copper, CCS delivers copper to XIAP resulting in XIAP degradation, induction of caspases, and eventually apoptosis. Because XIAP also binds to COMMD1 and enhances the degradative ubiquitination of COMMD1 (20, 25), it might be possible that COMMD1 function is related to this CCS- and XIAP-mediated dynamic regulation of copper delivery and SOD1 activation. However, additional studies are needed to clarify the possible role of COMMD1 in this new association of copper metabolism and apoptosis.

Taken together, COMMD1 regulates SOD1 maturation and is a novel regulator of SOD1 activity. These findings will be important for further understanding of the molecular mechanism of post-translational modifications of SOD1 maturation and activation in its defense against toxic superoxide anions.

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