Published in final edited form as:

Hum Mutat. 2012 August ; 33(8): 1207–1215. doi:10.1002/humu.22099.

Molecular and biochemical characterization of a unique mutation in CCS, the human copper chaperone to superoxide dismutase

Peter Huppke^{1,*}, Cornelia Brendel¹, Georg Christoph Korenke², Iris Marquardt², Anthony Donsante³, Ling Yi³, Julia D. Hicks³, Peter J. Steinbach⁴, Callum Wilson⁵, Orly Elpeleg⁶, Lisbeth Birk Møller⁷, John Christodoulou⁸, Stephen G. Kaler^{3,†}, and Jutta Gärtner^{1,†}

¹Department of Pediatrics and Pediatric Neurology, Georg August University, Göttingen, Germany ²Department of Pediatric Neurology, Children's Hospital, Oldenburg, Germany ³Unit on Human Copper Metabolism, Molecular Medicine Program, The Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland, USA ⁴Center for Molecular Modeling, Center for Information Technology, National Institutes of Health, Bethesda, Maryland, USA ⁵National Metabolic Service, Starship Children's Hospital, 1142 Auckland, New Zealand ⁶Monique and Jacques Roboh Department of Genetic Research, Department of Genetic and Metabolic Diseases, Hadassah, Hebrew University Medical Center, Jerusalem, Israel ⁷Center for Applied Human Molecular Genetics, Kennedy Center, DK-2600 Glostrup, Denmark ⁸Western Sydney Genetics Program, The Children's Hospital at Westmead, Hawkesbury Rd, Westmead, and Disciplines of Paediatrics and Child Health and Genetic Medicine, University of Sydney, Sydney, Australia

Abstract

Copper is a trace metal that readily gains and donates electrons, a property that renders it desirable as an enzyme cofactor but dangerous as a source of free radicals. To regulate cellular copper metabolism, an elaborate system of chaperones and transporters has evolved, although no human copper chaperone mutations have been described to date. We describe a child from a consanguineous family who inherited a homozygous mutations in the *SLC33A1*, encoding an acetyl CoA transporter, and in *CCS*, encoding the copper chaperone for superoxide dismutase. The *CCS* mutation, p.Arg163Trp, predicts substitution of a highly conserved arginine residue at position 163 with tryptophan in domain II of CCS, which interacts directly with SOD1. Biochemical analyses of the patient's fibroblasts, mammalian cell transfections, immunoprecipitation assays, and Lys7Δ (*CCS* homolog) yeast complementation support the pathogenicity of the mutation. Expression of CCS was reduced and binding of CCS to SOD1 impaired. As a result this mutation causes reduced SOD1 activity and may impair other mechanisms important for normal copper homeostasis. *CCS-Arg163Trp* represents the primary example of a human mutation in a gene coding for a copper chaperone.

Keywords

CCS; SOD1; copper; chaperone

*Address correspondence to Peter Huppke, M.D., Department of Pediatrics and Pediatric Neurology, Georg August University, Faculty of Medicine, Robert-Koch-Strasse 40, D-37075 Göttingen, Germany, Phone: +49-551-398035, Fax: +49-551-396252, phuppke@med.uni-goettingen.de.

†Both authors contributed equally

Conflicts of interest: There are no conflicts of interest.

Supporting Information for this preprint is available from the *Human Mutation* editorial office upon request (humu@wiley.com)

Introduction

Copper is an essential metal that facilitates electron transfer reactions (Festa and Thiele, 2011) and is required by enzymes involved in mitochondrial respiration, antioxidant defense, connective tissue formation, neurotransmitter and melanin biosynthesis and peptide amidation (Kaler, 2011). Excess amounts of copper, however, are toxic due to the production of reactive oxygen species (Rae et al., 1999). Following absorption in the duodenum, copper is taken up into blood and circulated to tissues, including the kidney and liver, the central organ in copper homeostasis. In the liver, copper is incorporated into ceruloplasmin (EC 1.16.3.1), a secreted glycoprotein which functions as an iron oxidase and accounts for approximately 95% of copper measureable in serum (de Bie et al., 2007). Alternatively, hepatic copper is incorporated by other cuproenzymes and excess copper is excreted into the bile.

Several other copper transport proteins are specifically involved in human copper metabolism (Lutsenko, 2010). Copper is taken up into cells via copper transporter 1 (CTR1). Intracellular copper chaperones (Robinson and Winge, 2010) distribute copper to various organelles and protein targets: ATOX1 delivers copper to ATP7A and ATP7B at the *trans*-Golgi network; COX17, SCO1 and SCO2 ferry copper within mitochondria to the copper-dependent enzyme, cytochrome C oxidase (EC 1.9.3.1); the copper chaperone for superoxide dismutase (CCS) delivers copper to Cu/Zn superoxide dismutase 1 (SOD1, EC 1.15.1.1).

Human disorders affecting copper metabolism with a known molecular pathogenesis include Menkes disease (MIM# 309400), occipital horn syndrome (MIM# 304150), ATP7A-related distal motor neuropathy (MIM# 300489), Wilson disease (MIM# 277900), and aceruloplasminemia (MIM# 604290). These conditions are caused by mutations in *ATP7A*, *ATP7B*, or ceruloplasmin (CP) (Das et al., 1994; Ferenci, 2004; Harris et al., 1995; Kaler et al., 1994; Kennerson et al., 2010; Moller et al., 2009). Mutations in human copper uptake or copper chaperone genes have not yet been reported.

CCS is a ubiquitously expressed cytosolic protein responsible for delivery of copper to SOD1 (Culotta et al., 1997; Rothstein et al., 1999). The delivery requires a direct protein-protein interaction involving a CCS-SOD1 heterodimeric intermediate in which SOD1 binds to a highly homologous (SOD1-like) domain of CCS (domain II) (Casareno et al., 1998; Culotta et al., 1997; Schmidt et al., 1999). Studies in liver and erythrocytes of rodents fed copper-deficient diets have shown high concentrations of CCS; thus, CCS levels provide a useful biomarker for copper deficiency (Bertinato et al., 2003; Lassi and Prohaska, 2011; Prohaska et al., 2003a). Conversely, CCS concentrations are reduced in conditions of cellular copper excess, presumably due to increased proteasomal degradation of CCS (Bertinato and L'Abbe, 2003). In a *Drosophila melanogaster* model, CCS enhanced CTR1-mediated Cu uptake (Hua et al., 2010) supporting the concept that CCS may normally interact with CTR1 in the process of copper uptake.

CCS may also act as a chaperone to another copper-binding protein, XIAP (X-linked inhibitor of apoptosis) (Brady et al., 2010; Mufti et al., 2006). Metallation of XIAP with copper provokes a conformational change or covalent modification which may mediate XIAP degradation, providing a possible link between copper metabolism and apoptosis. XIAP also appears to be implicated in copper homeostasis by influencing hepatic copper excretion through its regulation of MURR1 (also named COMMD1), the protein defective in a dog model of hepatic copper toxicosis (Burstein et al., 2004; van De Sluis et al., 2002). Investigation of a mouse knockout of CCS disclosed no apparent effects on copper uptake and distribution or incorporation into cuproenzymes other than SOD1 (Wong et al., 2000).

Here, we describe an infant, born to consanguineous parents, in whom we documented a homozygous mutation in domain II of the copper chaperone to SOD1. Although this individual's predominant clinical phenotype (cataracts, deafness, severe developmental delay) was presumed due mutation of *SLC33A1* (MIN# 614482) (Huppke et al., 2012), we characterized the biochemical and molecular effects of his *CCS* mutation, given additional symptoms not evident in other infants with *SLC33A1* mutations.

Material and Methods

Homozygosity mapping

Genetic investigations were performed with the written informed consent of the patient's parents. This study was performed according to the guidelines for genetic tests approved by Georg August University. Homozygous regions were searched for in the patient's DNA sample using GeneChip Human Mapping 250K Nsp Array from Affymetrix. The chip allowed genotyping of SNPs with an average distance of 10 kb between the markers. Digestion with Nsp, ligation of the adaptor, and amplification with a generic primer that recognizes the adaptor sequence were followed by fragmentation, end labeling, and hybridization to the chip in accordance with the manufacturer's instructions. Homozygous regions > 5 Mb were manually detected.

Mutation analysis

Candidate genes were identified in the critical region using the NCBI (<http://www.ncbi.nlm.nih.gov>) and UCSC (<http://www.genome.ucsc.edu>) human genome databases. Total genomic DNA was prepared from peripheral blood samples. Oligonucleotides were designed to amplify the eight exons including the intron-exon boundaries of *CCS* using the published genomic sequence (GenBank accession number: NM_005125.1) (Supp. Table S1). After purification, PCR products were analyzed using the DNASTAR 5.07 software after direct sequencing on an ABI 377 semi-automated sequencer using the DNA Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Darmstadt, Germany) according to the manufacturer's instructions. Mutalyzer 2.0.beta-12 and PolyPhen-2 (Polymorphism Phenotyping v2) were used as tools to predict the possible impact of amino acid substitutions on the structure and function of a human protein using physical and comparative considerations.

Restriction digestion

For mutation analysis in 100 normal control chromosomes, restriction enzyme *MspI* was used which has a cut site 5'-C[^]CGG-3' at codon 486, and is lost as a consequence of through the C to T substitution at position 487. The normal 330 bp PCR product of exon 5 is cut into two fragments, resulting in 222 bp and 108 bp digestion products. PCR products and restriction digestion products were analysed on 2% agarose gels.

Generation of a structural model of *CCS* domain 2

Wall-eyed stereo views of domain II of hCCS were generated based on the previously described crystal structure of *CCS*, 1DO5.pdb, chain A (Lamb et al., 2000).

Cell culture

Fibroblasts from the affected patient and apparently healthy controls, as well as from patients with Menkes disease, occipital horn syndrome, and ATP7A-related distal motor neuropathy, *CCS*^{-/-} knock-out mouse fibroblasts (generous gift from Phillip Wong), *CCS*^{+/+} mouse fibroblasts, and HeLa cells were grown in Dulbecco's modified Eagle's medium

supplemented with 10% fetal bovine serum and 0.1% L-glutamine. Cells were incubated at 37°C in an atmosphere of 5% CO₂.

Yeast Complementation Assay

Yeast Lys7Δ/F945, (a CCS null strain) transformation was accomplished with the S.c. EasyComp Transformation Kit (Invitrogen Catalog No. K5050-01). A YPD plate was streaked with Lys7Δ (F945) strain such that isolated, single colonies grew up after incubation at 28–30°C for 2 days. Single clones were picked and incubated in 10 ml of YPD and grown overnight at 28–30°C in a shaking incubator (250–300 rpm). Cells from the overnight culture were diluted to OD₆₀₀ between 0.200 and 0.400 in a total volume of 10 ml of YPD. These cultures were grown at 28–30°C in a shaking incubator for 3–6 hrs until the OD₆₀₀ reached 0.600 to 1.000. The cells were pelleted by centrifugation at 500g (1500rpm) for 5 min at room temperature and the supernatant discarded. The cell pellet was resuspended in 10 ml of Solution I and re-centrifuged. The cell pellet was resuspended in 1 ml of Solution II, and these competent cells used immediately for transformation. For each transformation, 50 μl of fresh competent cells were transferred into 15 ml Falcon tubes. DNA (2–5 μg) was added to the competent cells representing pYES6/CT constructs containing the wildtype *CCS* allele, the *CCS-p.Arg163Tyr* allele, or no additional DNA. Solution III (500 μl) was added to the DNA/cell mixture, mixed by vortexing, and incubated for 1 hr at 30°C, vortexing the mixture every 15 min. YPD media (1 ml) was added to each tube, and the cells incubated in a 30°C shaker for 1 hr to allow expression of blasticidin resistance. The cells were pelleted by centrifugation at 3000g for 5 min at RT, and resuspended in 100–150 μl YPD medium and spread on YPD plates containing blasticidin (300 μg/ml). The plates were incubated at 30°C for 2 to 4 days. Six colonies per transformation were isolated and streaked onto fresh YPD + 100μg/ml Blasticidin plates at 30°C for two days. Individual colonies from these plates were grown in 5 ml YPD + 100μg/ml Blasticidin at 30 C shaker overnight. These cells were spun and washed thrice with sterile dH₂O and resuspended in 5 ml induction medium (6.7g YNB Sunrise Scientific, Cat#1501-500, with ammonium sulfate without amino acids; 0.77g CSM-URA (complete supplement mixture minus uracil); 0.02g uracil; 20g galactose; 10g raffinose, ddH₂O to 950 ml, 50ml MES, pH6.1), and shaken at 30°C for 16 hrs. The transformed cells were spun and washed thrice with sterile ddH₂O and resuspended in medium without lysine (CSM-lys complete supplement mixture minus lysine; Sunrise Scientific, Cat #1032-010) at 30°C for 16 hrs. Cells were spun and washed thrice with sterile ddH₂O, resuspended in media without lysine and diluted to an OD₆₀₀ of 0.1 in 50 ml Falcon tubes containing 10 ml total volume. Cultures were grown in a shaking 30 C incubator and 800 μl aliquots withdrawn after 2, 4 and 6 hrs of growth for measurement of OD₆₀₀ absorbance. All strain/media combinations were set up in triplicate.

SOD1 Activity Assay

SOD1 activity in cells was measured using the colorimetric based Superoxide Dismutase Activity Assay (Assay Designs, Stressgen, Michigan, USA) following the manufacturer's instructions. Standards, controls, and samples were assayed in triplicate. Cell pellets were suspended in 5–10 volumes of Cell Extraction Buffer and incubated on ice with periodic vortexing for 30 minutes. Insoluble material was cleared by centrifugation at 10,000 g for 10 minutes at 4°C, and the supernatant transferred to a clean tube. Protein concentrations were determined by a Commassie Blue dye-binding assay (BioRad, Hercules, CA, USA). To inactivate SOD2, 500 μl of each sample was treated with 800 μl of chloroform: ethanol (3:5), shaken for 30 seconds, and then centrifuged at 2,500 g for 10 minutes. The aqueous phase was transferred to a fresh tube. A set of SOD standards was generated by serial two-fold dilution of the standard included with the kit. 25 μl of each sample and standard was assayed at room temperature on a Powerwave x340 (Bio-Tek Instruments, Inc., Winooski,

Vermont, USA) with the wavelength set to 450 nm and plate reads performed every minute for ten minutes. SOD1 units were determined for each sample from a standard curve generated by plotting SOD standard units versus $\Delta A_{450}/\text{min}$, and normalized to total protein. Unpaired two-tailed t-tests were performed to compare SOD1 activity between CCS mutations (p.Arg163Trp in human fibroblasts, Ex1_Ex2Ddel exon1-2 in mouse fibroblasts) and normal controls.

Quantitative RT-PCR

Total RNA was extracted from primary fibroblast cultures (Qiagen RNeasy Kit, Qiagen) in conjunction with Qias shredder columns according to the manufacturer's instructions. First strand cDNA synthesis was performed with Enhanced Avian RT First Strand Kit (Sigma) using the anchored oligo (dT) primer. Quantitative RT-PCR was carried out using SYBR Green JumpStart Taq Ready-Mix (Sigma) in an Opticon instrument (MJ Research) with primers for *CCS* (forward: 5'-ACGGGGACCTTACAAACAACACTGC-3'; reverse: 5'-AGCATCAGCACGGACATTGCC-3') and β -actin (forward: 5'-CGGCCAGGTCATCACCATT-3'; reverse: 5'-TGGAAGAGTGCCTCAGGGC-3'). Samples were measured twice in triplicate and CCS values were normalized to β -actin.

X-box binding protein 1 (XBP1) transcript analysis (Misfolded Protein Response Assay)

Total RNA was extracted from primary fibroblast cultures (Qiagen RNeasy Kit, Qiagen, Valencia, CA) in conjunction with Qias shredder columns according to the manufacturer's instructions. First strand cDNA synthesis was performed with Enhanced Avian RT First Strand Kit (Sigma). *XBP1* cDNA was amplified using primers (forward 5'-AAACAGAGTAGCAGCTCAGACTGC-3', rev: 5'-TCCTTCTGGGTAGACCTCTGGGAG-3'), and digested with *Pst*I as previously described (Zhang and Kaufman, 2006). The digested products were electrophoresed through a 2.5% agarose gel, and the transcript fragments were digitized and quantitated by densitometry using ImageJ (<http://rsb.info.nih.gov/ij>).

Construction of eukaryotic expression vectors

Expression vectors, encoding wild type human CCS and CCS carrying c.487C>T mutation, were constructed by amplification of full length wild type CCS cDNA via PCR with cDNA generated from human fibroblasts and oligonucleotides containing restriction enzyme sites (FLAG-CCS forward, 5'-ATAGAAATTCAATGGCTTCGGATTCGGGG-3'; FLAG-CCS reverse, 5'-ATAGGATCCTCAAAGGTGGGCAGGGGGCTG-3'). CCS mutant cDNA was generated by PCR with cDNA generated from patients RNA. Synthesized wild type and mutant cDNAs were cloned into the eukaryotic expression vector p3xFLAG-CMV 7.1 (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Sequence fidelity for all constructs was verified by sequencing.

Transfection

HeLa cells were transfected with the eukaryotic expression vectors using Effectene reagent (QIAGEN, Hilden, Germany) following manufacturer's protocols. Briefly, 1×10^6 HeLa cells were plated onto 10cm culture plates and grown for 24 hours. Transfection was performed with 2 μg of plasmid DNA for 8 hours in fresh media. After transfection, cells were washed once with PBS (PAA Laboratories GmbH, Pasching Austria), and incubation in fresh media continued for 24 hours.

Preparation of cell lysates

Fibroblasts and HeLa cells were rinsed twice with PBS and lysed on ice in 500 μl RIPA buffer ([150mM NaCl; 50mM Tris/HCl (pH 8.0); 5 mM EDTA (pH 8.0); 0.8% sodium

deoxycholate; 1% Nonidet P-40; 0.1% SDS] containing 0.1% (v/v) of a stock solution of the proteinase inhibitor cocktail Complete™ (Roche Diagnostics, Heidelberg, Germany)) per plate for 15 min. Lysates were centrifuged at 4°C (20.000 × *g* for 15 min) to remove insoluble matter. The concentration of protein in the supernatant fractions was determined using the BC Assay (Interchim, Montluçon Cedex, France), following the manufacturer's protocols.

Antibodies, FLAG-Immunoprecipitation and Western blotting analysis

Commercially available antibodies were used for the detection of CCS (mouse monoclonal CCS (H-7) antibody; Santa Cruz Biotechnology Inc., Heidelberg, Germany), SOD1 (rabbit polyclonal SOD1 (FL-154) antibody; Santa Cruz Biotechnology Inc., Heidelberg, Germany) and GAPDH (mouse monoclonal (6C5) to GAPDH; Abcam, Cambridge, UK). Recombinant proteins were detected with mouse monoclonal anti-FLAG M2 antibody (Stratagene GmbH, La Jolla, CA, USA). For immunoprecipitation, HeLa cells were lysed 24h after transfection in IP buffer (50 mM Tris-HCl pH 7.5, 200 mM NaCl, 1 mM EDTA, 1% [v/v] Triton X-100, 0,1% [v/v] NP-40 containing 0.1% (v/v) of a stock solution of the proteinase inhibitor cocktail Complete™ (Roche Diagnostics, Heidelberg, Germany) kept on ice for 15 min and centrifuged at 4°C for 10 min (20000 *g*) to remove insoluble matter. Supernatant fractions were incubated at 4°C for 4h with 40 µl of EZviewRed anti-FLAG M2 Affinity Gel (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) previously equilibrated with TBS (50 mM Tris HCl, 150 mM NaCl, pH 7.4). After incubation samples were centrifuged (8200 *g* for 30 sec at RT) and washed thrice with TBS. For western blots samples were prepared by addition of sample buffer (4x concentrated: 160 mM Tris-HCl pH 6.8, 140 mM SDS, 20% [v/v] Glycerol, 40mg/ml DTT, Bromophenol blue indicator) and heating to 95°C for five minutes. Protein samples were separated on 15% SDS-polyacrylamide gels, and transferred onto nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol). Non-specific binding sites were blocked by incubation with blocking solution (5% non-fat milk powder in TBST [1 × TBS/ 0,1% Tween 20]) for 1h at RT, following an overnight incubation with specific primary antibodies diluted in blocking solution at 4°C. As secondary antibodies, horseradish-peroxidase-conjugated donkey anti-mouse IgG and goat anti-rabbit IgG (Jackson Immuno-research Laboratories, West Grove, PA, USA) were used diluted in TBST. Immunoreactive bands were visualized using enhanced chemiluminescence (Lumi-Light Western Blotting Substrate, Roche Diagnostics, Heidelberg, Germany).

Statistical analyses—Fibroblast SOD1 enzyme activity data were analyzed by unpaired Student's *t*-tests. *P* values < 0.05 (two-tailed) were considered statistically significant.

Results

Case Report

The patient's clinical phenotype was described in brief previously (Huppke et al., 2012). He was the firstborn offspring to first cousin parents of Turkish descent. The family history was unremarkable as was the pregnancy and birth (weight 3,480g, length 51 cm, OFC 33.5 cm). On the first day of life, the infant presented with hypoglycemia (serum glucose = 1.8mmol/L) and was diagnosed with a mild pericardial effusion. Both conditions resolved. He was noted to have muscular hypotonia, congenital cataracts that required surgery at age 4 weeks, horizontal nystagmus and severe bilateral sensorineural hearing loss. A diagnostic evaluation showed normal results for karyotype, acylcarnitines, lactate, pyruvate and liver enzymes. A brain ultrasound showed no abnormalities. His early psychomotor development was delayed; he was able to roll by age 8 months but never learned to sit, walk or speak. Cranial MRI at 11 months of age was abnormal with delayed myelination and mild

ventricular enlargement. At age 18 months, following hospitalization for a severe pneumonia, developmental regression was noted; he lost the ability to roll and developed swallowing difficulties. Partial and generalized seizures were noted. A muscle biopsy showed low normal cytochrome C oxidase activity and no other abnormalities. Copper and ceruloplasmin, first measured at age 21 months, were profoundly reduced (copper = 0.5 $\mu\text{mol/l}$; normal range 12.6–23.6 $\mu\text{mol/l}$ and ceruloplasmin = 0.02 g/l; normal range 0.2–0.6 g/l). Treatment with daily subcutaneous copper-histidine injections failed to increase serum copper or ceruloplasmin levels although urinary copper excretion increased during therapy (333 $\mu\text{g/g}$ creatinine; normal <67 $\mu\text{g/g}$). Fibroblast Cu^{64} transport studies showed normal copper egress (11.9%; normal range: 9.7–22.7%), excluding Menkes disease. He also showed anemia (Hgb 80g/l) with low serum iron (2.33 $\mu\text{mol/l}$; normal range 1.5–32.2 $\mu\text{mol/l}$) low transferrin saturation (3%; normal range 7–46%) but normal serum ferritin (121 pmol/l; normal range 67–674pmol/l). Iron supplementation resulted in a rise of serum ferritin to 3.121 pmol/l but did not alter the serum hemoglobin level, iron concentration, or ferritin saturation.

By 32 months of age, pronounced cerebellar hypoplasia and generalized brain atrophy, hypomyelination, subdural hygroma and symmetrical thalamic lesions were seen on cranial MRI (Fig. 1). At age 3½ years, he showed microcephaly, macular degeneration, severe generalized muscular hypotonia, was unable to sit or stand and did not play or communicate. Skin and hair were normal and ultrasound examination did not reveal any bladder diverticuli. He had abnormal liver function tests; alanine aminotransferase (ALT) and aspartate aminotransferase were elevated to 134 U/L (normal <49 U/L) and 152 U/L (normal <45 U/L) and cholinesterase was reduced to 1,014 U/L (normal range: 5,320–12,920 U/L). Three months later, he developed rapidly progressive liver and kidney failure and died at age 45 months. His parents declined postmortem examination.

Genetic Analysis

Homozygosity mapping in the family revealed 14 regions of homozygosity spanning approximately 200Mb, including the copper chaperone to SOD1 *CCS* (Gene bank accession number: NM_005125.1). Sequencing of all exons and exon-intron junctions of *CCS* in the patient revealed a homozygous transition c.487C->T in exon 5 (Fig. 2A) that was not present in 100 normal chromosomes as determined by restriction digestion by *MspI*. His parents were each found to be heterozygous for the mutation (Fig. 2A, B). According to Mutalyzer 2.0.beta-12 analysis, the transition results in substitution of a highly conserved arginine residue at position 163 with tryptophan (Fig. 2C). *In silico* analysis (PolyPhen2) of the p.Arg163Trp mutation indicated that this alteration was “probably damaging,” with a score of 1.000 (sensitivity: 0.00; specificity: 1.00). Structural modeling demonstrated the crucial importance of the arginine residue at position 163, which forms a salt bridge with Asp 185, one of several electrostatic interactions that stabilize the protein (Fig. 3).

Characterization of Arg163Trp in CCS

To evaluate the pathogenicity of the p.Arg163Trp amino acid substitution, we also performed yeast complementation assays using the *S. cerevisiae* deletion strain *Lys7Δ*, the *CCS* homolog. Enzymes involved in lysine biosynthesis are sensitive to reactive oxygen species, thus when lysine is unavailable and yeast SOD cannot receive copper, growth of strain *Lys7Δ* is impaired. Transformation experiments showed approximately 50% complementation by pArg163Trp compared to the wildtype *CCS* allele (Fig. 4A). SOD1 enzyme activity, which depends on the delivery of copper by *CCS* was measured in p.Arg163Trp fibroblasts and found to be significantly reduced compared to normal controls ($p=0.007$) and a panel of patients with diverse mutations in the copper-transporting ATPase, ATP7A (Fig. 4B). Measureable SOD1 activity for p.Arg163Trp was not significantly

different ($p=0.249$) than that in mouse fibroblasts with deletion of exons 1 and 2 in murine CCS (Fig. 4B).

To further investigate the biochemical and molecular functional consequences of the c.487C->T transition in *CCS*, we performed Western blot analyses of the patient's fibroblasts which showed a CCS protein level approximately 40% compared to that in control fibroblasts (Fig. 5A, top panel). In contrast, SOD1 protein levels were unaltered (Fig. 5A, middle panel). Quantitative RT-PCR demonstrated slightly increased (140% of normal) CCS mRNA levels (Fig. 5B). To assess the molecular basis for reduced levels of mutant CCS protein, we evaluated the unfolded protein response in the patient's fibroblasts by quantitation of an XBP-1 splice variant known to be associated with this adaptive stress signaling pathway (Zhang and Kaufman, 2006). Results showed that the XBP-1 splice variant level in the patient's cells was 20% compared to 4 to 9% in three normal controls (data not shown), and 15–19% in a positive control (p.Gly727Arg in *ATP7A*) (Tang et al., 2008). Immunoprecipitation experiments using HeLa cells transfected with either wildtype *CCS* or c.487C->T *CCS* demonstrated that binding of the mutant CCS to SOD1 was drastically diminished, while the CCS-XIAP interaction appeared to be unaffected (Fig. 5C).

We also evaluated DNA from 35 unrelated patients from the USA, Europe, New Zealand and Australia who had been investigated for developmental delay and found to have low serum copper and ceruloplasmin but no mutations in *ATP7A* or *ATP7B*. No abnormalities in the *CCS* gene, including the exon-intron boundaries, were detected on direct DNA sequencing.

Discussion

We recently described five children with a lethal autosomal recessive disorder featuring congenital cataracts, bilateral hearing loss, developmental delay and very low serum copper and ceruloplasmin (Huppke et al., 2012). Following homozygosity mapping, we screened candidate genes that are involved in copper metabolism and found in the patient described here a homozygous mutation in *CCS*. However, the analysis of the *CCS* gene in the other patients did not show any mutations and the genetic analysis revealed mutations in *SLC33A1*, a gene encoding an acetyl CoA transporter-1, in all patients including the one presented in this article. Comparing the clinical details of all patients we became aware that the patient with the concurrent *CCS* mutation had additional symptoms not evident in the other patients. As a neonate, he showed severe muscular hypotonia, hypoglycemia and a pericardial effusion. At age 18 month he showed rapid developmental regression and epilepsy following a bout of pneumonia. Cranial MRI performed after this crisis showed persistent bilateral thalamic lesions that were not present in the other patients (Fig. 1). Given these findings and since no human disorder has been linked to mutations in *CCS*, we investigated the biochemical and molecular effects of the p.Arg163Trp *CCS* mutation.

The pathogenicity of the CCS mutation, c.478C->T, detected in our patient was supported by yeast complementation assays using the *S. cerevisiae* deletion strain Lys7 Δ , the CCS homolog, which showed reduced complementation by p.Arg163Trp compared to wild-type human CCS, indicating impaired Cu delivery to yeast SOD. CCS has three functional domains. Domain I receives incoming copper from Ctr1 and moves it to domain III, which mediates insertion of copper into apo-SOD1. Domain II, which contains residue 163 that is mutated in the patient, is highly homologous to SOD1, and has no catalytic activity but facilitates the recognition and binding of CCS to SOD1 (Schmidt et al., 1999). Other CCS mutations created in domain II have been shown to disrupt binding to SOD1 (Schmidt et al., 2000). Immunoprecipitation experiments showed clearly that the p.Arg163Trp CCS mutation disrupted *in vitro* binding to SOD1 (Fig. 5C).

The structural importance of Arg 163 was also evident from the crystal structure of CCS domain II (Lamb et al., 2000). Domain II of CCS has over 50% amino acid identity to Cu/Zn SOD1 (Endo et al., 2000). As seen in Fig. 3, Arg163 forms a salt bridge with Asp185 that spans the beta sheets of CCS domain II. This salt bridge is one of several electrostatic interactions among neighboring residues that stabilize the protein fold in this region, including those in the zinc-binding site, the bridge between nearby Asp160 and Arg209, and the bridges between nearby Arg165 and Asp151 and Asp192. Arg163 and Asp185 are conserved in human SOD1 and yeast SOD1, as are Asp 160 and the four residues that bind the zinc ion: His147, His155, His164, and Asp167. In addition, both human and yeast SOD1 have a lysine in the position corresponding to Arg209, retaining the positive charge at this site. The p.Arg163Trp mutation deletes the salt bridge with Asp185 and presumably destabilizes other conserved interactions in this region.

In addition, we documented low CCS protein levels despite normal SOD levels (Fig. 5A) and normal copper egress mechanisms. In *CCS*^{-/-} mouse fibroblasts transfected with CCS mutated in domain II, turnover of the mutant protein was increased, leading to decreased CCS protein levels (Caruano-Yzermans et al., 2006). The abundance of CCS is normally in inverse proportion to dietary intake of copper, a regulation that is post-translational. In a low copper state, copper is exchanged rapidly from CCS to SOD1 and CCS concentrations become high. In an excess copper state, CCS with copper bound is degraded, resulting in lowering of CCS levels (Bertinato and L'Abbe, 2003). However, in the context of the p.Arg163Trp CCS mutation, these normal physiologic responses would likely be perturbed.

SOD1, representing 1–2% of total cellular protein, is a ubiquitous cytoplasmic enzyme involved in antioxidant defense by converting superoxide to hydrogen peroxide. In mice with targeted disruption of the *CCS* alleles, SOD1 protein levels were reported to be normal in the original study (Wong et al. 2000) while a subsequent analysis (Prohaska et al 2003) found that SOD1 protein levels in *CCS*^{-/-} liver were reduced 79% compared to *CCS*^{+/+}. Both studies showed that SOD1 enzymatic activity was markedly reduced in nearly all tissues (Prohaska et al., 2003b; Wong et al., 2000).

Analyses of hepatic copper metabolism in the *CCS*^{-/-} knockout mice showed normal copper distribution and incorporation into ceruloplasmin, leading the authors to conclude that CCS was not crucial for proper copper homeostasis (Wong et al., 2000). However, since liver from the *CCS*^{-/-} mouse showed significantly higher SOD1 activity than all other tissues (about 30% compared to normal), the presence of some functional CCS in this tissue seemed possible. Liver tissue from wildtype, *CCS*^{+/+} and *CCS*^{-/-} mouse showed immunoreactive bands on Western blots probed with an anti-CCS antibody (Wong et al., 2000), which suggested presence of a truncated version of CCS. Since the *CCS* knockout mouse was generated by deletion of *CCS* exons 1 and 2 (Wong et al., 2000), an in-frame *CCS* transcript beginning in exon 3, perhaps related to a liver-specific *CCS* promoter, could explain the additional band visible. The Reference Sequence (RefSeq) collection annotates a representative transcript for *Mus musculus CCS* and cDNA sequences, co-aligned on the genome and clustered in a minimal non-redundant way by the manually supervised *AceView* program, support at least seven splice variants (Thierry-Mieg and Thierry-Mieg, 2006). One splice variant in fact lacks *CCS* exons 1 and 2. Thus, the clinical and biochemical phenotypes in that mouse model may not reflect complete hepatic knockout of *CCS*.

In the patient's fibroblasts, we consistently found reduced SOD1 activity despite normal SOD1 protein levels indicating that the p.Arg163Trp CCS mutation disrupts normal SOD1 metallation in this tissue. Our combined data indicate that the mutant CCS cannot deliver copper to SOD1, which could enhance oxidative stress. Since CCS is a cytosolic, rather than

endoplasmic reticulum–associated protein, the misfolded protein response we documented may represent a consequence of general cellular oxidative stress.

The p.Arg163Trp CCS mutation may have more global effects on intracellular copper homeostasis through potential interactions with two other proteins, XIAP and COMMD1 (Brady et al., 2010; Burstein et al., 2004; Mufti et al., 2006). CCS is felt to normally deliver copper to XIAP as well as to SOD1. Our results indicate that the p.Arg163Trp mutation disrupts the binding of CCS to SOD1, but does not seem to impair interaction with XIAP (Fig. 4C). It has been proposed that copper delivery to XIAP via CCS leads to autodegradation of XIAP with concomitant upregulation of COMMD1 (Mufti et al., 2006) and enhanced hepatic copper excretion. Alternatively, impaired metallation of XIAP due to CCS mutation could lead to unbridled degradation of COMMD1 and hepatic copper overload. These potential effects could be formally evaluated through *in vitro* experiments by over-expressing mutant CCS in HepG2 cells, or through study of a mutant CCS-R163W mouse model.

It is unclear whether the signs and symptoms present in this patient but not in the other patients with *SLC33A1* mutations namely neonatal hypotonia and hypoglycemia, pericardial effusion, dramatic neurodevelopmental regression after infection, thalamic signal hyperintensity on T2-weighted brain MRI, were related to the CCS mutation with concomitant reduced SOD1 activity. Mutations in *SOD1*, causing misfolding of the protein and a gain in toxicity, have been found in about 25% of patients with familial amyotrophic lateral sclerosis emphasizing that CCS mutations could act as modifying factors in various neurological diseases (Boillee et al., 2006). To date no human disease directly caused by SOD1 deficiency has been reported. Mice deficient in SOD1 show slow progressive motor loss and enhanced vulnerability to motor neuron loss after axonal injury under stressful conditions (Reaume et al., 1996; Shefner et al., 1999).

Our studies on the biochemical effects of CCS-Arg163Trp, the first human mutation reported in CCS, highlight the importance of this intracellular copper chaperone. Identification of other individuals with homozygous CCS mutations would help clarify whether such defects have pathogenic health consequences in humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding: This work was supported by the Intramural Research Program of the U.S. National Institutes of Health (Project #HD008768-08) and the German Research Foundation (GA 354/9-1).

References

- Bertinato J, Iskandar M, L'Abbe MR. Copper deficiency induces the upregulation of the copper chaperone for Cu/Zn superoxide dismutase in weanling male rats. *The Journal of nutrition*. 2003; 133:28–31. [PubMed: 12514262]
- Bertinato J, L'Abbe MR. Copper modulates the degradation of copper chaperone for Cu, Zn superoxide dismutase by the 26 S proteasome. *The Journal of biological chemistry*. 2003; 278:35071–8. [PubMed: 12832419]
- Boillee S, Vande Velde C, Cleveland DW. ALS: a disease of motor neurons and their nonneuronal neighbors. *Neuron*. 2006; 52:39–59. [PubMed: 17015226]

- Brady GF, Galban S, Liu X, Basrur V, Gitlin JD, Elenitoba-Johnson KS, Wilson TE, Duckett CS. Regulation of the copper chaperone CCS by XIAP-mediated ubiquitination. *Molecular and cellular biology*. 2010; 30:1923–36. [PubMed: 20154138]
- Burstein E, Ganesh L, Dick RD, van De Sluis B, Wilkinson JC, Klomp LW, Wijmenga C, Brewer GJ, Nabel GJ, Duckett CS. A novel role for XIAP in copper homeostasis through regulation of MURR1. *The EMBO journal*. 2004; 23:244–54. [PubMed: 14685266]
- Caruano-Yzermans AL, Bartnikas TB, Gitlin JD. Mechanisms of the copper-dependent turnover of the copper chaperone for superoxide dismutase. *The Journal of biological chemistry*. 2006; 281:13581–7. [PubMed: 16531609]
- Casareno RL, Waggoner D, Gitlin JD. The copper chaperone CCS directly interacts with copper/zinc superoxide dismutase. *The Journal of biological chemistry*. 1998; 273:23625–8. [PubMed: 9726962]
- Culotta VC, Klomp LW, Strain J, Casareno RL, Krems B, Gitlin JD. The copper chaperone for superoxide dismutase. *The Journal of biological chemistry*. 1997; 272:23469–72. [PubMed: 9295278]
- Das S, Levinson B, Whitney S, Vulpe C, Packman S, Gitschier J. Diverse mutations in patients with Menkes disease often lead to exon skipping. *Am J Hum Genet*. 1994; 55:883–9. [PubMed: 7977350]
- de Bie P, Muller P, Wijmenga C, Klomp LW. Molecular pathogenesis of Wilson and Menkes disease: correlation of mutations with molecular defects and disease phenotypes. *Journal of medical genetics*. 2007; 44:673–88. [PubMed: 17717039]
- Endo T, Fujii T, Sato K, Taniguchi N, Fujii J. A pivotal role of Zn-binding residues in the function of the copper chaperone for SOD1. *Biochemical and biophysical research communications*. 2000; 276:999–1004. [PubMed: 11027581]
- Ferenci P. Pathophysiology and clinical features of Wilson disease. *Metabolic brain disease*. 2004; 19:229–39. [PubMed: 15554419]
- Festa RA, Thiele DJ. Copper: an essential metal in biology. *Curr Biol*. 2011; 21:R877–83. [PubMed: 22075424]
- Harris ZL, Takahashi Y, Miyajima H, Serizawa M, MacGillivray RT, Gitlin JD. Aceruloplasminemia: molecular characterization of this disorder of iron metabolism. *Proceedings of the National Academy of Sciences of the United States of America*. 1995; 92:2539–43. [PubMed: 7708681]
- Hua H, Georgiev O, Schaffner W, Steiger D. Human copper transporter Ctr1 is functional in *Drosophila*, revealing a high degree of conservation between mammals and insects. *J Biol Inorg Chem*. 2010; 15:107–13. [PubMed: 19856191]
- Huppke P, Brendel C, Kalscheuer V, Korenke GC, Marquardt I, Freisinger P, Christodoulou J, Hillebrand M, Pitelet G, Wilson C, et al. Mutations in SLC33A1 Cause a Lethal Autosomal-Recessive Disorder with Congenital Cataracts, Hearing Loss, and Low Serum Copper and Ceruloplasmin. *Am J Hum Genet*. 2012; 90:61–8. [PubMed: 22243965]
- Kaler SG. ATP7A-related copper transport diseases-emerging concepts and future trends. *Nature reviews*. 2011; 7:15–29.
- Kaler SG, Gallo LK, Proud VK, Percy AK, Mark Y, Segal NA, Goldstein DS, Holmes CS, Gahl WA. Occipital horn syndrome and a mild Menkes phenotype associated with splice site mutations at the MNK locus. *Nature genetics*. 1994; 8:195–202. [PubMed: 7842019]
- Kennerson ML, Nicholson GA, Kaler SG, Kowalski B, Mercer JF, Tang J, Llanos RM, Chu S, Takata RI, Speck-Martins CE, et al. Missense mutations in the copper transporter gene ATP7A cause X-linked distal hereditary motor neuropathy. *Am J Hum Genet*. 2010; 86:343–52. [PubMed: 20170900]
- Kraulis PJ. Similarity of protein G and ubiquitin. *Science (New York, NY)*. 1991; 254:581–2.
- Lamb AL, Wernimont AK, Pufahl RA, O'Halloran TV, Rosenzweig AC. Crystal structure of the second domain of the human copper chaperone for superoxide dismutase. *Biochemistry*. 2000; 39:1589–95. [PubMed: 10677207]
- Lassi KC, Prohaska JR. Erythrocyte Copper Chaperone for Superoxide Dismutase Is Increased following Marginal Copper Deficiency in Adult and Postweanling Mice. *The Journal of nutrition*. 2011

- Lutsenko S. Human copper homeostasis: a network of interconnected pathways. *Current opinion in chemical biology*. 2010; 14:211–7. [PubMed: 20117961]
- Merritt EA, Bacon DJ. Raster3D: photorealistic molecular graphics. *Methods in enzymology*. 1997; 277:505–24. [PubMed: 18488322]
- Moller LB, Mogensen M, Horn N. Molecular diagnosis of Menkes disease: genotype-phenotype correlation. *Biochimie*. 2009; 91:1273–7. [PubMed: 19501626]
- Mufti AR, Burstein E, Csomos RA, Graf PC, Wilkinson JC, Dick RD, Challa M, Son JK, Bratton SB, Su GL, et al. XIAP Is a copper binding protein deregulated in Wilson's disease and other copper toxicosis disorders. *Molecular cell*. 2006; 21:775–85. [PubMed: 16543147]
- Prohaska JR, Broderius M, Brokate B. Metallochaperone for Cu, Zn-superoxide dismutase (CCS) protein but not mRNA is higher in organs from copper-deficient mice and rats. *Archives of biochemistry and biophysics*. 2003a; 417:227–34. [PubMed: 12941305]
- Prohaska JR, Geissler J, Brokate B, Broderius M. Copper, zinc-superoxide dismutase protein but not mRNA is lower in copper-deficient mice and mice lacking the copper chaperone for superoxide dismutase. *Experimental biology and medicine* (Maywood, NJ). 2003b; 228:959–66.
- Rae TD, Schmidt PJ, Pufahl RA, Culotta VC, O'Halloran TV. Undetectable intracellular free copper: the requirement of a copper chaperone for superoxide dismutase. *Science* (New York, NY). 1999; 284:805–8.
- Reaume AG, Elliott JL, Hoffman EK, Kowall NW, Ferrante RJ, Siwek DF, Wilcox HM, Flood DG, Beal MF, Brown RH Jr, et al. Motor neurons in Cu/Zn superoxide dismutase-deficient mice develop normally but exhibit enhanced cell death after axonal injury. *Nature genetics*. 1996; 13:43–7. [PubMed: 8673102]
- Robinson NJ, Winge DR. Copper metallochaperones. *Annu Rev Biochem*. 2010; 79:537–62. [PubMed: 20205585]
- Rothstein JD, Dykes-Hoberg M, Corson LB, Becker M, Cleveland DW, Price DL, Culotta VC, Wong PC. The copper chaperone CCS is abundant in neurons and astrocytes in human and rodent brain. *Journal of neurochemistry*. 1999; 72:422–9. [PubMed: 9886096]
- Schmidt PJ, Kunst C, Culotta VC. Copper activation of superoxide dismutase 1 (SOD1) in vivo. Role for protein-protein interactions with the copper chaperone for SOD1. *The Journal of biological chemistry*. 2000; 275:33771–6. [PubMed: 10944535]
- Schmidt PJ, Rae TD, Pufahl RA, Hama T, Strain J, O'Halloran TV, Culotta VC. Multiple protein domains contribute to the action of the copper chaperone for superoxide dismutase. *The Journal of biological chemistry*. 1999; 274:23719–25. [PubMed: 10446130]
- Shefner JM, Reaume AG, Flood DG, Scott RW, Kowall NW, Ferrante RJ, Siwek DF, Upton-Rice M, Brown RH Jr. Mice lacking cytosolic copper/zinc superoxide dismutase display a distinctive motor axonopathy. *Neurology*. 1999; 53:1239–46. [PubMed: 10522879]
- Tang J, Donsante A, Desai V, Patronas N, Kaler SG. Clinical outcomes in Menkes disease patients with a copper-responsive ATP7A mutation, G727R. *Molecular genetics and metabolism*. 2008; 95:174–81. [PubMed: 18752978]
- Thierry-Mieg D, Thierry-Mieg J. AceView: a comprehensive cDNA-supported gene and transcripts annotation. *Genome Biol*. 2006; 7(Suppl 1):S12, 1–14. [PubMed: 16925834]
- van De Sluis B, Rothuizen J, Pearson PL, van Oost BA, Wijmenga C. Identification of a new copper metabolism gene by positional cloning in a purebred dog population. *Hum Mol Genet*. 2002; 11:165–73. [PubMed: 11809725]
- Wong PC, Waggoner D, Subramaniam JR, Tessarollo L, Bartnikas TB, Culotta VC, Price DL, Rothstein J, Gitlin JD. Copper chaperone for superoxide dismutase is essential to activate mammalian Cu/Zn superoxide dismutase. *Proceedings of the National Academy of Sciences of the United States of America*. 2000; 97:2886–91. [PubMed: 10694572]
- Zhang K, Kaufman RJ. The unfolded protein response: a stress signaling pathway critical for health and disease. *Neurology*. 2006; 66:S102–9. [PubMed: 16432136]

Figure 1.

T2 weighted cranial MRI images at age 11 months (A), 23 month (B) and 32 months (C) showing mildly delayed myelination and ventricular enlargement at age 11 months and progressive brain atrophy, hypomyelination and symmetrical lesions of the thalami at 23 and 32 months (white arrows). D) T2 weighted cranial MRI images of another child with mutations in *SLC33A1* but no mutations in *CCS* at age 24 month showing hypomyelination but no thalamic lesions.

ASHGGPQDSD R HRGDLGNVR	172	CCS	Homo sapiens
ASHGGPKDSD R HRGDLGNVH	172	CCS	Canis lupus
ASHGGPQDTD R HRGDLGNVR	172	CCS	Mus musculus
ASHGGPQDTD R HRGDLGNVH	172	CCS	Rattus norvegicus
KQHGA PQDSD R HV GDLGNIS	166	CCS	Danio rerio
HGSPAAGAEER R HAGDLGNIR	168	CCS	Drosophila melangogaster
RKHGGPKDEER R HV GDLGNVT	88	SOD1	Homo sapiens

Figure 2.

A. Sequencing analysis of exon 5 from the patient and his parents showing a C→T substitution at nucleotide 487 (c.487C>T, p.Arg163Trp), homozygous in the patient and heterozygous in the parents. B. Restriction enzyme analysis by *Msp* I. The recognition site (CCGG) for *Msp* I is abolished by substitution of C to T. PCR fragments and restriction digestion products were analyzed in the patient (P), his mother (Mo), his father (F), and normal control subjects (C1-5). M, molecular size markers. C. ClustalW analysis of CCS homologues from various species and human SOD1. The p.Arg163Trp substitution affects a residue highly conserved in evolution and located in the homologous region of SOD1 (highlighted in bold).

Figure 3.

Wall-eyed stereo views of domain II of hCCS (crystal structure 1DO5.pdb, chain A). The protein backbone is colored by secondary structure: beta strands are yellow; alpha helices light blue; loops light green. The side chain of Arg 163 is shown as space filling. The side chain of Asp 185 is shown as sticks, as are the conserved side chains of His 147, His 155, His 164, and Asp 167, which coordinate the Zn (II) ion, shown as a small purple sphere. Upper panel: The salt bridge between Arg 163 and Asp 185 links the two beta sheets of domain II and is proximal to the zinc binding site. Lower panel: View rotated by 90 degrees about the horizontal axis in the plane of the page. The side chains of Arg 209, Asp 160, Asp 151, Arg 165, and Asp 192 are shown as sticks. This figure was prepared using the programs Mol Script and Raster3D (Kraulis, 1991; Merritt and Bacon, 1997).

Figure 4.

Results of biochemical analyses. A. Transformation experiments with the *S. cerevisiae* deletion strain *Lys7Δlys7Δ S. cerevisiae* transformed with wildtype CCS and *CCSR163W*. B) Analysis of SOD activity in fibroblasts of the patient with the *R163W-CCS* mutation and control fibroblasts from subjects with Menkes disease (ATP7A mutations: IVS21, DS, +1g>a; IVS9, DS, -1, G>A; del exon 21-23; p.Gly666Arg), occipital horn syndrome (ATP7A mutation (p.Ser833Gly), and ATP7A-related distal motor neuropathy (ATP7A mutations: p.Thr994Ile, p.Pro1386Ser). Asterisks denote statistically significant results for SOD activity in CCS mutant cells compared to wild-type ($p < 0.05$).

Figure 5.

A. Western blot analysis of endogenous CCS and SOD1 detected in cell lysates generated from normal control fibroblasts and fibroblasts from the patient. GAPDH was used as a loading control. B. Quantification of CCS mRNA by RT PCR in fibroblast of the patient and a healthy control. C. Western blots of anti-FLAG immunoprecipitates obtained from transiently transfected HeLa cells expressing either recombinant FLAG-CCS-wildtype or FLAG-R163W-CCS fusion proteins. Endogenous SOD1 and XIAP fusion proteins were detected with either rabbit polyclonal anti-SOD1, anti XIAP, or mouse monoclonal anti-FLAG antibodies. I: input; IP: pellet fraction after immunoprecipitation.