

Identification of COMMD1 as a novel lamin A binding partner

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Abstract. Lamin A, which is encoded by the *LMNA* gene, regulates gene expression and genome stability through interactions with a variety of proteins. Mutations in *LMNA* lead to a diverse set of inherited human diseases, collectively referred to as laminopathies. To gain insight into the protein interactions of lamin A, a yeast two-hybrid screen was conducted using the carboxy-terminus of lamin A. The screen identified copper metabolism MURR1 domain-containing 1 (COMMD1) as a novel lamin A binding partner. Colocalization experiments using fluorescent confocal microscopy revealed that COMMD1 colocalized with lamin A in 293 cells. Furthermore, the COMMD1-lamin A protein interaction was also demonstrated in co-immunoprecipitation experiments. Collectively, the present study demonstrated a physical interaction between COMMD1 and lamin A, which may aid to elucidate the mechanisms of lamin A in the aging process.

Introduction

Lamin A, which is encoded by the *LMNA* gene, is a major component of the nuclear lamina and nuclear skeleton (1). Lamin A is expressed in most adult tissues (2,3), with the expression of lamin A increasing with age in somatic cells (4). Although studies have revealed that lamin A serves a structural role during interphase (5), lamin A is increasingly recognized as a mediator, and possibly a regulator, of nuclear

processes through its interactions with a variety of nuclear factors, including double-stranded DNA, transcriptional regulators, nuclear membrane-associated proteins and nuclear pore complexes (6,7). Dysfunction of lamin A interrupts chromatin organization, the DNA damage response, telomere maintenance, cellular senescence and apoptosis (8,9). Mutations in the *LMNA* gene cause a heterogeneous group of human diseases that are collectively termed laminopathies, including progeroid syndromes and premature aging disorders that primarily affect striated muscle, adipose, bone and neuronal tissues, such as Hutchinson-Gilford progeria syndrome (HGPS) (6,10,11). Mutations leading to laminopathies are distributed throughout the *LMNA* gene and show a high degree of tissue specificity (3,12). How mutations in *LMNA* cause disease and why laminopathies are highly tissue-specific remain unclear (3). Nuclear envelope proteomes are highly variable among tissues (13,14). Additionally, variants of lamin A may interact differently with proteins that are themselves expressed in a tissue-specific manner, which could explain the tissue specificity of laminopathies (15). However, determining the molecular mechanisms underlying changes in lamin A protein interactions remains a clinical challenge.

The G608G mutation in the *LMNA* gene causes a truncation of lamin A, with a 50-residue region lost that includes a second proteolytic site for zinc metallopeptidase STE24 (ZMPSTE24), resulting in an unprocessed prelamins A termed progerin in patients with HGPS (16). The accumulation of truncated lamin A in HGPS impedes the release of proteins from the nuclear membrane and disrupts their regulatory functions, thereby accelerating a subset of pathological changes that contribute to the aging processes (17,18). Notably, mice carrying lamin A mutations also exhibit symptoms consistent with HGPS, including the thinning of skin, hypoplasia, the degeneration of cardiac and skeletal muscles, and osteoporosis (19). Increased levels of wild-type lamin A in normal human cells result in a decreased replicative lifespan and nuclear membrane alterations that lead to phenotypic changes similar to those observed in HGPS fibroblasts (13,14). These studies suggest that wild-type lamin A, similar to mutated lamin A, is also involved in the aging processes.

To improve understanding of the pathological mechanisms involved in laminopathies and the aging process, the present

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Abbreviations: HGPS, Hutchinson-Gilford progeria syndrome; COMMD1, copper metabolism MURR1 domain-containing 1; GFP, green fluorescent protein; RT-PCR, reverse transcription polymerase chain reaction; NF- κ B, nuclear factor- κ B; ARF, alternate reading frame protein product of the CDKN2A locus

Key words: lamin A, aging, interaction, COMMD1

study sought to systematically identify lamin A-interacting proteins in an unbiased manner. A yeast two-hybrid screen of a human skeletal muscle cDNA library was performed using the carboxy (C)-terminus of lamin A as bait to search for novel lamin A-interacting factors. This screening identified copper metabolism MURR1 domain-containing 1 (COMMD1, formerly known as MURR1) as a novel binding partner of lamin A. Their binding affinity was further validated using confocal colocalization and co-immunoprecipitation experiments.

Materials and methods

Yeast two-hybrid analysis. Yeast two-hybrid analysis was conducted using a GAL4-based system to screen a human skeletal muscle complementary DNA (cDNA) library (Matchmaker GAL4 two-hybrid system; Clontech Laboratories, Inc.). Briefly, a bait protein was constructed by cloning the C-terminus of lamin A (mRNA sequence 1,413-2,241) in frame with the GAL4 binding domain using the *EcoRI* and *BamHI* restriction sites of the pGBKT7 vector (Clontech Laboratories, Inc.). The yeast *Saccharomyces cerevisiae* strain AH109 (Clontech Laboratories, Inc.) was sequentially transformed with the C-terminal lamin A bait vector (pGBKT7-LA-C; Clontech Laboratories, Inc.) and the Matchmaker human skeletal muscle cDNA library (Clontech Laboratories, Inc.) cloned into pACT2 (Clontech Laboratories, Inc.) according to the manufacturer's protocol (Clontech Laboratories, Inc.). *Saccharomyces cerevisiae* strain AH109 transformed with pCL1 (encodes the full-length and wild-type GAL4 protein) vector was provided as positive control. Transformants were plated on synthetic defined (SD)/histidine/leucine/tryptophan (TDO) medium (Clontech Laboratories, Inc.) (low-stringency protocol); a total of 2×10^7 colonies were screened. Colonies were transferred to SD/adenine/histidine/leucine/tryptophan (QDO) plates (Clontech Laboratories, Inc.) containing 5-bromo-4-chloro-3-indolyl- α -D-galactopyranoside (X- α -Gal) following two rounds of selection. Positive clones were identified under high-stringency conditions and were defined as clones that exhibited growth on the QDO plates that were strongly positive for galactosidase activity. The selected clones were further analyzed by Sanger sequencing (Sangon Biotech Co., Ltd.) and compared with known sequences in GenBank using a BLAST search (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Cell culture. The 293 cell line (Stem Cell Bank; Chinese Academy of Sciences) was cultivated in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal calf serum (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 μ g/ml streptomycin. Serial passaging was performed when the cells reached a confluence of 80%.

Western blotting. Whole cell extracts were prepared using RIPA buffer [50 mM Tris-HCl (pH 7.4), 150 mM sodium chloride, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 1 μ g/ml leupeptin and 1 μ g/ml pepstatin]. Protein was quantified using a bicinchoninic protein assay kit (Pierce; Thermo Fisher Scientific, Inc.). Subsequently, 10-20 μ g protein was loaded into each lane. Proteins were separated by 12% SDS-PAGE. Subsequently,

gels were blotted onto PVDF membranes. The PVDF membranes were blocked for 1 h at room temperature (25°C) in Tris-buffered saline containing 0.1% Tween 20 and 5% non-fat milk. Primary antibodies were diluted in blocking solution and incubated with the membranes overnight at 4°C. Anti-COMMD1 antibody (1:1,000; cat. no. sc-166248; Santa Cruz Biotechnology, Inc.), anti-lamin A/C antibody (1:2,000; cat. no. sc-20681; Santa Cruz Biotechnology, Inc.), anti-hemagglutinin (HA) antibody (1:1,000; cat. no. 11867423001; Roche Diagnostics) and anti-Flag antibody (1:1,000; cat. no. F3165; Sigma-Aldrich; Merck KGaA) were used as primary antibodies. Anti-GAPDH antibody (1:1,000; cat. no. 60004-1-Ig; ProteinTech Group, Inc.) was used to detect the protein expression level of the loading control GAPDH. Secondary antibodies horseradish peroxidase (HRP)-conjugated goat anti-rabbit (cat. no. A120-101P; Bethyl Laboratories, Inc.), goat anti-rat (cat. no. A110-143P; Bethyl Laboratories, Inc.) and goat anti-mouse (cat. no. AP308P; Merck KGaA) were diluted at 1:5,000 and incubations were performed for 1 h at room temperature (25°C). The proteins were detected using a western chemiluminescent HRP substrate (EMD Millipore).

Fluorescence confocal microscopy. Total RNA was extracted from 293 cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The first strand cDNA was synthesized using the PrimeScript 1st Strand cDNA Synthesis kit (Takara Bio, Inc.) following the manufacturer's protocol. COMMD1 coding sequence (CDS) and Lamin A CDS were synthesized using the same first strand cDNA templates. COMMD1 CDS was synthesized using the primers forward, 5'-CCCTCGAGATGGCGGGCGAGCTTGAG-3' and reverse, 5'-CGGAATTCGGTTAGGCTGGCTGATC-3'. PCRs were performed using the rTaq DNA Polymerase (Takara Bio, Inc.) in a total volume of 20 μ l and amplification protocol consisted of an initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation for 45 sec at 94°C, annealing for 45 sec at 60°C and extension for 1 min at 72°C, followed by a final extension at 72°C for 10 min. The green fluorescent protein (GFP)-COMMD1 vector was generated by inserting COMMD1 CDS into the pEGFP-N1 vector (Clontech Laboratories, Inc.), which contains a GFP expression cassette, with *XhoI* and *EcoRI* restriction sites. Lamin A CDS was generated with the following thermocycling conditions: Initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation for 45 sec at 94°C, annealing for 1 min sec at 60°C and extension for 90 sec at 72°C, followed by a final extension at 72°C for 10 min. Lamin A CDS was amplified using the primers forward 5'-ACGCTCGAGATGGAGACC CCGTCCCAGCGGC-3' and reverse 5'-CGGGATCCGCGG GCTCTGGGTTTCGGGGCT-3'. The red fluorescent protein (RFP)-lamin A vector was generated by inserting lamin A CDS into the pDsRed2-N1 vector (Clontech Laboratories, Inc.), which contains an RFP expression cassette, using the *XhoI* and *BamHI* restriction sites. All sequences from all plasmids were confirmed by DNA sequencing following cloning.

The day prior to transfection, 293 cells (2×10^5) were seeded into confocal dishes. When the cells reached ~80% confluence, 2.5 μ g pEGFP-N1-COMMD1 and 2.5 μ g pDsRed2-N1 or 2.5 μ g pDsRed2-N1-lamin A were transfected into cells using Lipofectamine[®] 2000 transfection reagent (Invitrogen;

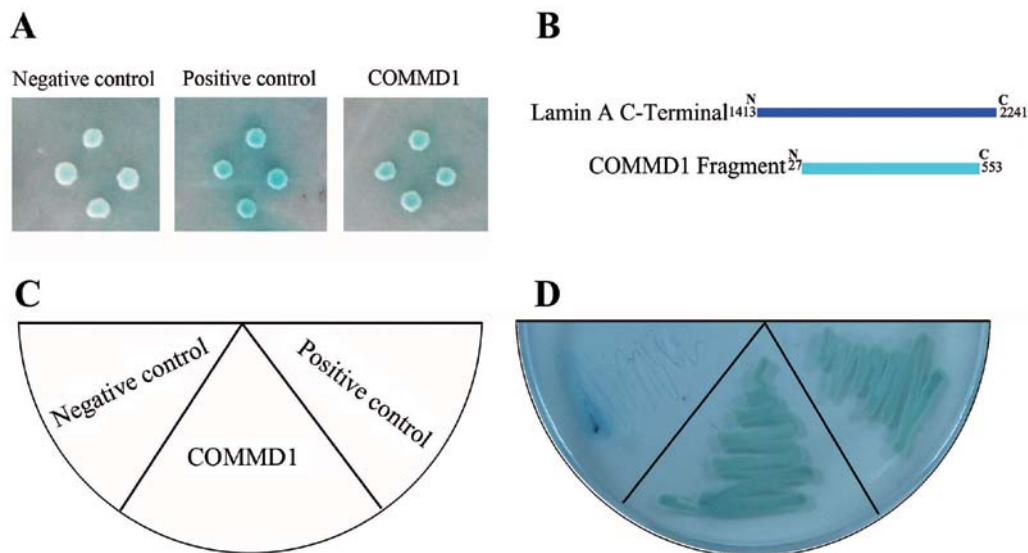


Figure 1. Analysis of the COMMD1-lamin A interaction using a yeast two-hybrid assay. (A) Positive clones interacting with the C-terminus of lamin A on low-stringency synthetic defined/histidine/leucine/tryptophan plates containing X- α -gal (blue). (B) Schematic diagram of the interaction between the COMMD1 sequence and the C-terminus of lamin A. (C) Schematic diagram of the second round selection using a high stringency protocol. (D) Diploid AH109 yeast were re-streaked onto synthetic defined/adenine/histidine/leucine/tryptophan plates containing X- α -gal to test for an interaction. AH109 yeast transformed with the C-terminus of lamin A in the pGBKT7 vector. Empty pGBKT7-LA-C was used as a negative control. AH109 yeast transformed with pCL1 was used as positive control. A blue signal indicates the activation of the reporter genes. COMMD1, copper metabolism MURR1 domain-containing 1; C-terminus, carboxy-terminus; X- α -gal, 5-bromo-4-chloro-3-indolyl- α -D-galactopyranoside.

Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. At 48 h after transfection, co-localization of COMMD1 and lamin A in transfected cells was observed using a Leica TCS SP5 confocal microscope (Leica Microsystems, Inc.; magnification, x63) operated using Leica confocal software (LAS AF lite; version 2.0; Leica Microsystems, Inc.).

Co-immunoprecipitation. The pCMV-HA-COMMD1 vector was generated by inserting COMMD1 cDNA into the pCMV-HA vector (Clontech Laboratories, Inc.) with the *EcoRI* and *BglII* restriction sites. Lamin A cDNA was inserted into the pCMV-Flag vector (Clontech Laboratories, Inc.) with the *HindIII* and *NaeI* restriction sites. The pCMV-Flag-lamin A, and pCMV-HA-COMMD1 or pCMV-HA plasmids were co-transfected into 293 cells using Lipofectamine[®] 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. For immunoprecipitation, whole cell extracts of non-transfected or co-transfected cells were lysed with RIPA buffer (Thermo Fisher Scientific, Inc.). Equal amounts (500 μ g) of protein samples were incubated with 2 μ g antibody. The antibodies used in the co-immunoprecipitation experiment were as follows: Anti-HA and anti-Flag antibody for the overexpression plasmid and anti-COMMD1 or anti-lamin A/C for endogenous co-immunoprecipitation. Normal mouse IgG (cat. no. sc-2025; Santa Cruz Biotechnology, Inc.) and normal rabbit IgG (cat. no. sc-2027; Santa Cruz Biotechnology, Inc.) were used as control. Antibodies were incubated for 2 h at 4°C. The antibody-protein complex was mixed with 100 μ l of a protein A/G-agarose suspension (cat. nos. 1134515 and 1243233; Roche Diagnostics, Inc.) for 24 h at 4°C. After centrifugation at 12,000 x g for 20 sec at 4°C, the pellet was washed twice with wash buffer 1 [50 mM Tris-HCl (pH 7.5), 150 mM sodium chloride, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 1 tablet complete protein

inhibitor cocktail/50 ml] and once each with wash buffer 2 (50 mM Tris-HCl, pH 7.5, 500 mM sodium chloride, 0.1% Nonidet P-40, and 0.05% sodium deoxycholate) and wash buffer 3 (50 mM Tris-HCl, pH 7.5, 0.1% Nonidet P-40 and 0.05% sodium deoxycholate) prior to resuspension in loading buffer. The suspension was denatured by heating for 5 min at 95-100°C and centrifuged at 12,000 x g for 10 min at 4°C. The supernatant fraction was used for the identification of the co-precipitated proteins via western blotting, using the aforementioned procedure.

Results

Identification of COMMD1 as a novel lamin A-binding protein using yeast two-hybrid screening. To identify lamin A-binding proteins, a human skeletal muscle cDNA library was screened using the yeast two-hybrid system. Yeast cells expressing the C-terminus of lamin A (mRNA sequence 1,413-2,241) as bait were mated with yeast cells expressing the appropriate prey to make diploid yeast cells that were then streaked onto low-stringency TDO medium and high-stringency QDO medium to test for an interaction. At lower stringency, the colonies displayed X- α -Gal activity similar to that of the positive control (Fig. 1A). Following three rounds of selection, 9 colonies survived and were subjected to Sanger sequencing (Table SI). A fragment (Data S1) of the full sequence of COMMD1 (Data S2) gene was identified as a potential binding partner of lamin A via sequencing and a BLAST search (Fig. 1B). To validate this interaction, the COMMD1 prey was reintroduced into yeast cells and streaked onto QDO medium (Fig. 1C). Colonies growing on the QDO medium displayed X- α -Gal activity similar to that of the positive control (Fig. 1D). These results suggested that COMMD1 may interact with lamin A.

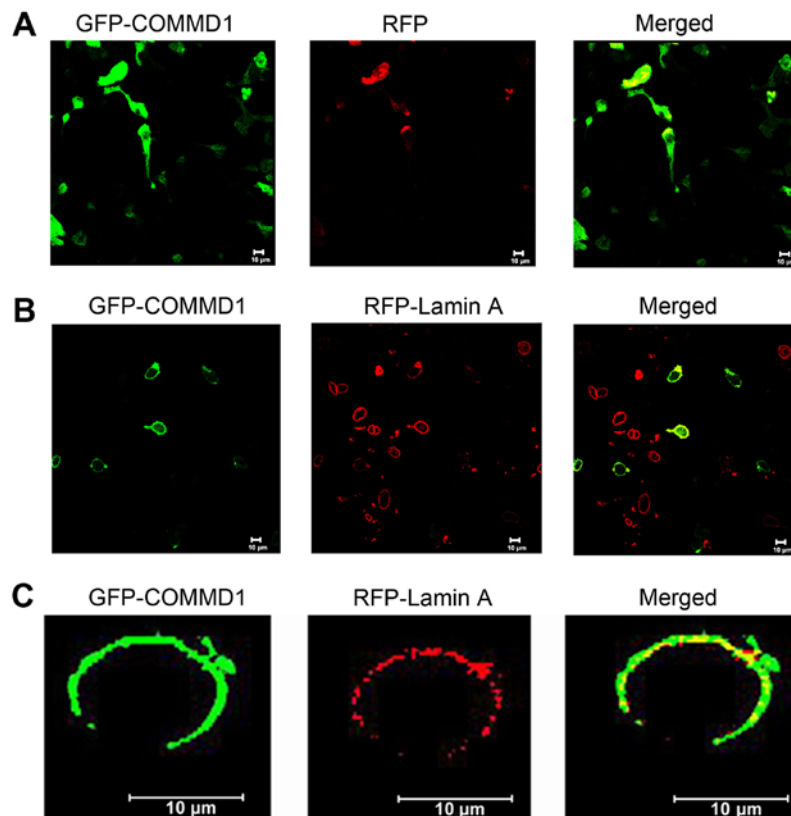


Figure 2. Co-localization of COMMD1 and lamin A in 293 cells. Co-transfection of 293 cells with (A) GFP-COMMD1 and pDsRed1-N1 (RFP) or (B) GFP-COMMD1 and RFP-lamin A. (C) Higher magnification view of cells co-transfected with GFP-COMMD1 and pDsRed1-N1-lamin A (RFP-lamin A). Cells were examined 24 h following transfection using confocal scanning laser microscopy. COMMD1, copper metabolism MURR1 domain-containing 1; GFP, green fluorescent protein; RFP, red fluorescent protein; RFP-lamin A, pDsRed1-N1-lamin A.

COMMD1 colocalizes with lamin A. To further verify the interaction between COMMD1 and lamin A, the subcellular localization of COMMD1 and lamin A was analyzed using fluorescence confocal microscopy. As shown in Fig. 2A, COMMD1 was predominantly cytoplasmic. The results of the co-transfection of 293 cells with GFP-tagged COMMD1 and RFP-tagged lamin A plasmids demonstrated that COMMD1 co-localized with lamin A in these cells (Fig. 2B and C).

COMMD1 physically interacts with lamin A. A co-immunoprecipitation assay was conducted to further test the interaction between COMMD1 and lamin A. Fig. 3A shows that COMMD1 interacted with lamin A. The interaction between endogenous COMMD1 and lamin A in 293 cells was evaluated by immunoprecipitation using anti-COMMD1 and anti-lamin A/C antibodies followed by western blotting with an anti-lamin A/C or anti-COMMD1 antibody. Following the co-transfection of 293 cells with the pCMV-Flag-lamin A plasmid, and the pCMV-HA-COMMD1 or pCMV-HA plasmid, immunoprecipitation was performed using an anti-HA antibody, followed by western blotting using an anti-Flag and anti-HA antibody. As shown in Fig. 3B, HA-COMMD1 was present in cell lysates from 293 cells transfected with both the pCMV-Flag-lamin A plasmid and the pCMV-HA-COMMD1 plasmid but not in cell lysates from 293 cells cotransfected with the pCMV-Flag-lamin A and pCMV-HA plasmids. Collectively, these results suggested that COMMD1 interacts with lamin A *in vivo*.

Discussion

In the present study, lamin A binding proteins were screened using the yeast two-hybrid system, and a novel interaction was identified between COMMD1 and lamin A. In addition to interacting with lamin A, COMMD1 was also observed to interact with prelamin A in other yeast two-hybrid screens (data not shown). The yeast two-hybrid system is a powerful method for the identification of proteins that interact with one another; however, it exhibits high false positive rates and involves non-physiological conditions (20). To overcome these drawbacks, the interaction between COMMD1 and lamin A was further validated using co-localization and co-immunoprecipitation experiments. Co-localization experiments using fluorescent confocal microscopy revealed that COMMD1 and lamin A co-localized to the nuclear envelope, which indicated the potential functional importance of their interaction. The interaction between COMMD1 and lamin A was also tested in endogenous and exogenous co-immunoprecipitation experiments. Collectively, the results of the present study revealed that COMMD1 physically interacts with lamin A.

COMMD1 is the best characterized member of the COMMD protein family, which consists of 10 subgroups (COMMD1-COMMD10) (21). A mutation in COMMD1 was originally identified as the genetic cause of canine copper toxicosis (22). Following that report, COMMD1 was found to be a pleiotropic protein that participates in several cellular processes, ranging from copper homeostasis and sodium

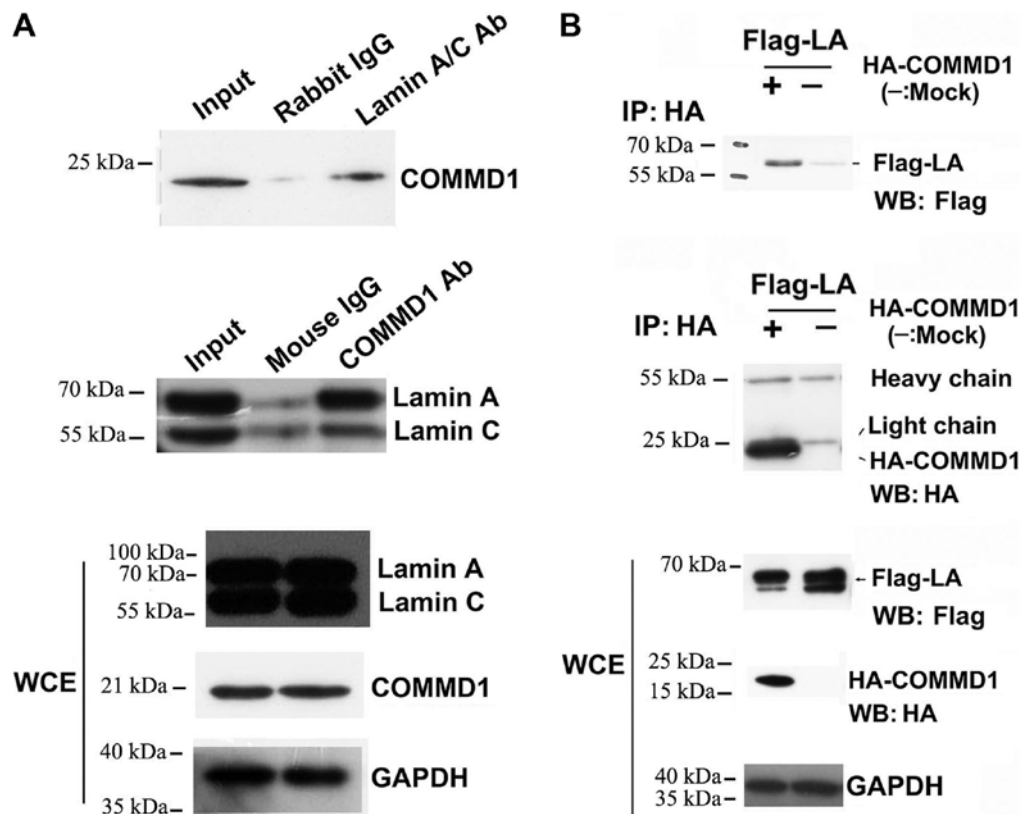


Figure 3. COMMD1 interacts with lamin A. (A) IP of 293 cell extracts with anti-lamin A/C or anti-COMMD1 antibodies, followed by western blotting with anti-COMMD1 and anti-lamin A/C antibodies. (B) Following co-transfection of 293 cells with pCMV-HA or HA-COMMD1 and Flag-lamin A, IP was conducted with an anti-Flag antibody, followed by western blotting using an anti-Flag antibody. COMMD1, copper metabolism MURR1 domain-containing 1; HA, hemagglutinin; Ab, antibody; IgG, immunoglobulin G; WCE, whole cell extract; IP, immunoprecipitation; WB, western blotting.

transport, to the regulation of the NF- κ B pathway and the expression of hypoxia-inducible factor-1 α (HIF-1 α)-mediated genes (23). COMMD1 suppresses NF- κ B activity; however, the activation of NF- κ B in *Zmpste24*-deficient mice resulted in premature aging (24). COMMD1 was found to regulate HIF-1 α activity during the adaptation to hypoxia by controlling HIF-1 α protein stability (25). Furthermore, the stabilization of HIF-1 α increased the lifespan of *Caenorhabditis elegans* (26,27). Another COMMD1 binding partner, superoxide dismutase 1, which is regulated by COMMD1 (28,29), has also been shown to regulate normal cellular lifespan (30,31). A G608G mutation in the *LMNA* gene or the loss of *ZMPSTE24* activity resulted in the accumulation of unprocessed prelamin A in cells, which induced DNA damage and genomic instability, and led to premature aging (8,9). DNA damage stimulated the relocalization of COMMD1 into the nucleoplasm and its interaction with alternate reading frame p14ARF (ARF) in humans and p19ARF in mice (32). ARF stabilizes the basal level of COMMD1 through K63-dependent polyubiquitination (32), which also promotes cellular senescence in mouse and human cells by regulating the p53 pathway (33,34), and mediates resistance to cell cycle arrest in *LMNA*^{-/-} cells (35).

Lamin A is first synthesized as a prelamin A precursor with a conserved CAAX domain that is proteolytically processed by *ZMPSTE24*, removing the final 15 amino acids (36). The mature lamin A protein comprises a 28-residue positively charged globular head domain, a central α -helical

rod-shaped domain, a conserved nuclear localization signal and one immunoglobulin (Ig)-like structure domain formed of 116 residues at the C-terminus (37). The Ig-like domain was identified as an interaction hotspot that regulates interactions between lamin A and its binding partners (37,38), its destabilization was found to affect most lamin A protein-protein interactions (3). The yeast two-hybrid results from the present study suggested that COMMD1 may interact with the C-terminus of lamin A. The interaction between COMMD1 and lamin A was verified using co-localization and co-immunoprecipitation assays. COMMD1 is highly conserved in vertebrates (39) and ubiquitously expressed in diverse eukaryotic tissues (40). COMMD1 predominantly localizes in the cytoplasm surrounding the nucleus (41,42); however, a small fraction of COMMD1 can be found in the nucleus (43,44). COMMD1 contains a COMM domain near its C-terminus, which serves as a scaffold for protein-protein interactions (21), and has two highly conserved nuclear export signals, which are necessary and sufficient to induce nuclear export (44). In addition, in nucleocytoplasmic shuttling, COMMD1 may interact with lamin A at the nuclear envelope (44). The nuclear export of COMMD1 is important in the regulation of NF- κ B and HIF-1 activity in response to cellular stress (44). The interaction between COMMD1 and lamin A may also be involved in the regulation of the translocation of COMMD1 to the nucleoplasm from the perinuclear regions following DNA damage (32). In the present study, it was found that COMMD1 interacts with the C-terminus of

lamin A; however, these results do not indicate if the interaction between COMMD1 and lamin A is direct or indirect. Co-immunoprecipitation and confocal fluorescence microscopy assays could not exclude the fact that other mediators are involved in this protein-protein interaction complex. As such, how and where COMMD1 interacts with lamin A requires further study.

In conclusion, to the best of our knowledge, the present study provided the first evidence that COMMD1 physically interacts with lamin A, suggesting possible roles for COMMD1 in cellular senescence and nucleocytoplasmic transport. Further studies are required to elucidate the precise mechanisms underlying the lamin A-COMMD1 interaction with regard to the aging process and laminopathies.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XL, WC and ZZ designed the study. ZJ drafted the manuscript. ZJ, JZ, QP, HZ and YY performed and analyzed the experiments. HC, WZ and XS analyzed the data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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