THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 287, NO. 41, pp. 34189–34201, October 5, 2012

Lanthionine Synthetase C-like Protein 1 Interacts with and Inhibits Cystathionine β-Synthase

*A TARGET FOR NEURONAL ANTIOXIDANT DEFENSE**

Received for publication, May 19, 2012, and in revised form, August 10, 2012 Published, JBC Papers in Press, August 13, 2012, DOI 10.1074/jbc.M112.383646

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Background: The function of LanCL1 remains unknown. Cystathionine β-synthase (CBS) is important for GSH synthesis.
Results: LanCL1 directly binds and inhibits CBS. Oxidative stress down-regulates the binding and increases CBS activity. Intervention in the binding exerts neuronal antioxidant defense.
Conclusion: LanCL1 is a negative regulator of CBS and a sensor of oxidative stress.
Significance: This study reveals a novel function of LanCL1 in CBS-mediated neuronal redox homeostasis.

The finding that eukaryotic lanthionine synthetase C-like protein 1 (LanCL1) is a glutathione-binding protein prompted us to investigate the potential relationship between LanCL1 and cystathionine β-synthase (CBS). CBS is a trans-sulfuration enzyme critical for the reduced glutathione (GSH) synthesis and GSH-dependent defense against oxidative stress. In this study we found that LanCL1 bound to CBS in mouse cortex and HEK293 cells. Mapping studies revealed that the binding region in LanCL1 spans amino acids 158–169, and that in CBS contains N-terminal and C-terminal regulatory domains. Recombinant His-LanCL1 directly bound endogenous CBS from mouse cortical lysates and inhibited its activity. Overexpression of LanCL1 inhibited CBS activity in HEK293 cells. CBS activity is reported to be regulated by oxidative stress. Here we found that oxidative stress induced by H_2O_2 or glutamate lowered the GSH/GSSG ratio, dissociated LanCL1 from CBS, and elevated CBS activity in primary rat cortical neurons. Decreasing the GSH/GSSG ratio by adding GSSG to cellular extracts also dissociated LanCL1 from CBS. Either lentiviral knockdown of LanCL1 or specific disruption of the LanCL1-CBS interaction using the peptide Tat-LanCL1$_{153–173}$ released CBS activity in neurons but occluded CBS activation in response to oxidative stress, indicating the major contribution of the LanCL1-CBS interaction to the regulation of CBS activity. Furthermore, LanCL1 knockdown or Tat-LanCL1$_{153–173}$ treatment reduced H_2O_2 or glutamate-induced neuronal damage. This study implies potential therapeutic value in targeting the LanCL1-CBS interaction for neuronal oxidative stress-related diseases.

Eukaryotic lanthionine synthetase C-like protein 1 (LanCL1),[3] a member of the LanC-like protein family, is homologous to prokaryotic lanthionine synthetase component C (lanthionine cyclases) and is highly expressed in brain (1). Lanthionine cyclase catalyzes thioether cross-link reactions between cysteine and the dehydrated serine or threonine residues of particular precursor polypeptides, yielding potent antibiotics or "lantibiotics," which are not present in mammals (2). It has been reported that LanCL1 is a reduced glutathione (GSH)-binding protein in brain; however, unlike other GSH-binding proteins such as glutathione peroxidase and glutathione transferase (GST), LanCL1 is reported to have no enzymatic activity (3, 4). Furthermore, increased levels of LanCL1 are found in the spinal cord of a mouse model of amyotrophic lateral sclerosis (3, 4). These previous studies suggest that LanCL1 is important in the GSH-dependent antioxidant system. However, the primary function of LanCL1 in the brain remains unknown.

Cystathionine β-synthase (CBS) is a trans-sulfuration enzyme catalyzing the condensation of serine with homocysteine to form cystathionine. Cystathionine is needed to synthesize cysteine (5), the rate-limiting substrate for GSH synthesis (6, 7). In mammalian brain, lanthionine, a thioether analog of cysteine, is thought to be produced by CBS (8, 9) and converted to the unusual cyclic thioether lanthionine ketimine, which also binds to LanCL1 (10), suggesting that LanCL1 may be involved in a CBS-mediated trans-sulfuration pathway.

Glutathione exists in reduced (GSH) and oxidized (GSSG) forms, and the ratio of GSH to GSSG is an indicator of the cellular redox state (11, 12). GSH is an antioxidant, protecting cells from injury caused by reactive oxygen species such as free radicals and peroxides (13). The brain is susceptible to oxidative stress, and a GSH deficit is associated with cell damage and

* This work was supported by grants from the National Basic Research Program of China (2010CB912002), the Natural Science Foundation of China (81221003), and the Natural Science Foundation of Zhejiang, China (Y2100494).

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[3] The abbreviations used are: LanCL1, lanthionine synthetase C-like protein 1; CBS, cystathionine β-synthase; IP, immunoprecipitation; AdoMet, *S*-adenosylmethionine; Ni-NTA, nickel-nitrilotriacetic acid.

neurodegenerative diseases (14). CBS activity is up-regulated by oxidative stress (15, 16). Thus, by increasing GSH synthesis, CBS is closely associated with GSH-dependent redox homeostasis and the capacity of brain to resist oxidative stress.

In this study we investigated the physical and functional relationships between LanCL1 and CBS. We revealed a direct interaction and an inhibitory effect of LanCL1 on CBS and demonstrated that the LanCL1-CBS dissociation under oxidative stress elevated the CBS activity. LanCL1-CBS binding was sensitive to the GSH/GSSG ratio, and the sensitivity was dependent on the LanCL1-GSH binding. Furthermore, we clarified the neuroprotective effect of LanCL1 knockdown or uncoupling between LanCL1 and CBS in primary cultured rat cortical neurons under oxidative stress.

EXPERIMENTAL PRECEDURES

Antibodies—Anti-LanCL1 rabbit polyclonal antibody was generated in our laboratory. It was raised against His-LanCL1 (human full-length coding sequence) fusion protein in New Zealand White rabbits. The immune serum was purified by GST-LanCL1 fusion protein immobilized on AminoLink Plus Resin (Pierce 44894). Anti-His antibody was from Abcam (Ab1187). Anti-YFP or GFP rabbit polyclonal antibody was also developed in our laboratory. Anti-FLAG antibody was from Sigma (F3165). Anti-LanCL1 mouse polyclonal antibody was from Abnova (H00010314-A01). Anti-CBS antibodies were from Santa Cruz Biotechnology (SC-133208, SC-67154) and Abnova (133312–351, 08218). HRP-conjugated secondary antibodies were from Pierce. The secondary antibodies for immunocytochemistry were Alexa Fluor 488 anti-mouse IgG and Alexa Fluor 546 anti-rabbit IgG (Invitrogen).

Construction of CBS and LanCL1 Variants—Human CBS cDNA was kindly provided by Warren D. Kruger, and human LanCL1 cDNA was provided by Dr. Bo Xiao. CBS and its truncated variants (CBS_{1-413}, CBS_{70-413}, and CBS_{70-551}) were amplified by PCR using human CBS cDNA as template and the respective set of primers in which EcoRI and XhoI sites were introduced at the 5′ and 3′ ends. Amplified products were cloned in p3*FLAG-CMV-10 (Sigma). The FLAG-CBS_{S466L} single-site mutation was designed by the Primer X website and constructed using a KOD Plus mutagenesis kit (Toyobo). LanCL1 and its truncated variants ($LanCL1_{1-131}$, $LanCL1_{1-143}$, $LanCL1_{1-157}$, $LanCL1_{1-169}$, $LanCL1_{1-183}$, $LanCL1_{1-197}$, $LanCL1_{1-263}$, and $LanCL1_{1-334}$) were amplified by PCR using human LanCL1 cDNA as template and the respective set of primers, in which EcoRI and BamHI sites were introduced at the 5′ and 3′ ends. Amplified products were cloned in pEYFP-C1 (Clontech). The YFP-$LanCL1_{\Delta 158-169}$ mutant was produced by two-step construction.

Primary Culture of Rat Cortical Neurons—All animal experiments were carried out with the approval of the Animal Care Committee of Zhejiang University. Primary cortical neuron cultures were prepared from brains of Sprague-Dawley rats of either sex at embryonic day 18. Cortex was digested in 0.5% trypsin for 15 min at 37 °C in a 5% CO_2 incubator. For immunostaining, dissociated neurons were plated in 35-mm dishes with poly-L-lysine-coated coverslips in neurobasal medium containing 5% horse serum, 0.5 mM glutamine, 1% antibiotic, and 2% B27 (Invitrogen) at 37 °C under 5% CO_2. For biochemical studies, neurons were seeded in 60-mm poly-L-lysine-coated dishes. To assess neuron viability, 96-well culture plates were used. At 5 days *in vitro*, cytosine arabinofuranoside was added to a final concentration of 2.5 μM. After 3 days the medium was replaced with neurobasal medium containing 0.5 mM glutamine, 1% antibiotic, and 2% B27. Subsequently, 50% of the medium was replaced with fresh medium every 4 days. Neurons at 12–14 days *in vitro* were used for experiments.

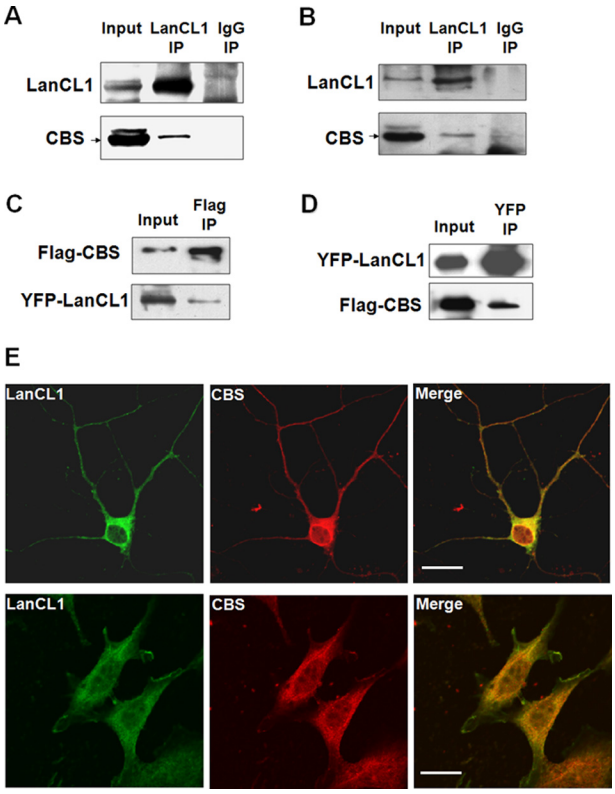

FIGURE 1. **LanCL1 is a CBS-interacting protein.** *A*, LanCL1 interacted with CBS in mouse brain. Mouse cortical lysates were precipitated with anti-LanCL1 rabbit antibody or nonspecific IgG and probed with anti-LanCL1 antibody and anti-CBS antibody. *B*, endogenous LanCL1 co-precipitated CBS in HEK293 cells. *C*, co-IP of heterogeneously expressed YFP-LanCL1, and FLAG-CBS was performed in HEK293 cells. Anti-YFP antibody precipitated YFP-LanCL1 and co-precipitated FLAG-CBS. *D*, in reverse co-IP, anti-FLAG antibody precipitated FLAG-CBS and co-precipitated YFP-LanCL1. *E*, immunocytochemistry with anti-LanCL1 mouse antibody and anti-CBS rabbit antibody in primary cultured rat cortical neurons (*upper panels*) and HEK293 cells (*lower panels*) showed LanCL1 and CBS were cytosolic proteins. *Scale bar*, 10 μm.

Culture of HEK293 Cells—HEK293 cells were grown at 37 °C under 5% CO_2 in DMEM supplemented with 10% FCS. Cells were transfected with appropriate plasmids (1 μg/60-mm dish) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The transfection mixture was replaced 3 h after transfection with fresh medium. After 24–48 h, cells were used for experiments.

H_2O_2 or Glutamate Treatment—A dose of 100 μM hydrogen peroxide (H_2O_2) or 100 μM glutamate was applied to cells for 2 h. Cells were collected and used for CBS activity measurement and co-immunoprecipitation (IP) analysis. To analyze neuronal viability and apoptosis, neurons were replaced in culture medium for another 24 h.

Treatment with the Cell-permeable Peptide—We designed a cell membrane-permeable peptide (Tat-$LanCL1_{153-173}$) by fusing

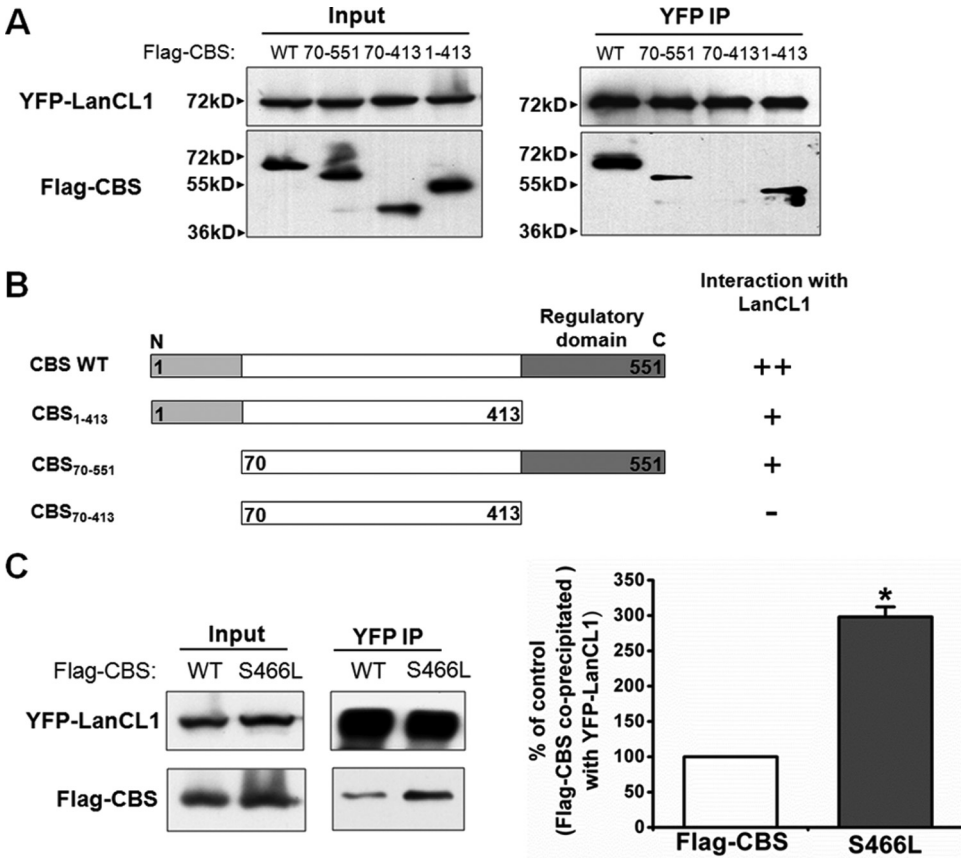

FIGURE 2. **N- and C-terminal domains of CBS are regions of LanCL1 interaction.** *A*, co-IP of FLAG-CBS_{WT} with YFP-LanCL1 was stronger than that of FLAG-CBS_{1-413} or FLAG-CBS_{70-551}. FLAG-CBS_{70-41} did not co-IP with YFP-LanCL1. *B*, shown is a schematic illustration of wild-type and truncated forms of CBS used in *panel A*. ++, +, and − indicate the positive and negative interactions with LanCL1. *C*, *left*, FLAG-CBS_{S466L} (S466L) had a stronger interaction with YFP-LanCL1 than the wild-type FLAG-CBS (FLAG-CBS). *Right*, shown is quantification of three independent co-IP tests. The ratio of the band intensity of co-precipitated FLAG-CBS to precipitated YFP-LanCL1 in the wild-type FLAG-CBS group (FLAG-CBS) was normalized to 100% (mean ± S.E.; *, $p < 0.05$; unpaired *t* test; $n = 3$).

the 21 residues (^{153}YGRIGYIFALLFVNKNFGVEK^{173}) of LanCL1 containing the binding region (158–169; identified below) of LanCL1 to the cell-membrane transduction domain of the HIV-1 Tat protein (YGRKKRRQRRR) (17). A peptide (Tat-s$LanCL1_{153-173}$) with a scrambled 153–173 sequence of LanCL1 (FIKVLEKFGGAIVGNFLYNRY) fused to Tat protein was also constructed as the negative control. The peptides were synthesized by GL Biochem Ltd (Shanghai, China). For peptide blocking, 2.5 μM Tat-$LanCL1_{153-173}$ or Tat-s$LanCL1_{153-173}$ was added to primary cultures of rat cortical neurons for 1 h before H_2O_2 or glutamate treatment and washed out after the treatment. In the case of co-transfected HEK293 cells, 5 μM peptide was used.

Lentivirus-mediated RNAi in Primary Rat Cortical Neurons—LanCL1 shRNA (rat) lentiviral particles (SC-270327-V) and control shRNA lentiviral particles were from Santa Cruz. Cultured cortical neurons at days *in vitro* 6 were infected by lentivirus. Six days later, infected neurons were used for experiments.

Co-immunoprecipitation and Immunoblotting—Briefly, cultured rat cortical neurons, HEK293 cells, or adult mouse cortex were lysed in buffer containing 20 mM Tris, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM PMSF, 1 mM Na_3VO_4, 1 mM NaF, 10 μg/ml aprotinin, 5 μg/ml leupeptin, and 5 μg/ml pepstatin. Protein concentration was determined by a BCA protein assay kit (Pierce). Immunoprecipitation antibody was added to the lysates and incubated for 2 h or overnight at 4 °C. Protein A-Sepharose beads (GE Healthcare) were then added. After incubation for 1 h at 4 °C, the beads were washed 4 times with lysis buffer, and the immunoprecipitates were eluted with 2×SDS-PAGE loading buffer by boiling at 100 °C for 5 min and loaded onto SDS-PAGE gels for immunoblots. Each experiment was repeated at least three times. For quantification, the x-ray films were scanned and analyzed using Quantity-One software (Bio-Rad).

Immunocytochemistry—Cortical neurons (days *in vitro* 12–14) or HEK293 cells were fixed in 4% paraformaldehyde at room temperature. Fixed cells were rinsed with PBS, permeabilized, and blocked in PBS with 0.1% Triton X-100 and 5% bovine serum albumin for 1 h and then labeled with anti-LanCL1 mouse antibody and anti-CBS rabbit antibody for 2 h at room temperature. After washing 3 times in PBS, cells were incubated with Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 546 goat anti-rabbit IgG for 1 h at room temperature. After washing, cells were mounted with SlowFade Light Antifade reagent (Invitrogen). Images were acquired with a confocal microscope (Fluoview FV1000, Olympus).

Assay of CBS Activity—CBS activity was measured by cystathionine formation through an *in vitro* enzymatic reaction with minor modifications (18). Briefly, crude extracts from homogenates of cortex from adult male mice or cell lysates were prepared in 0.05 M potassium phosphate buffer (pH 6.9) followed

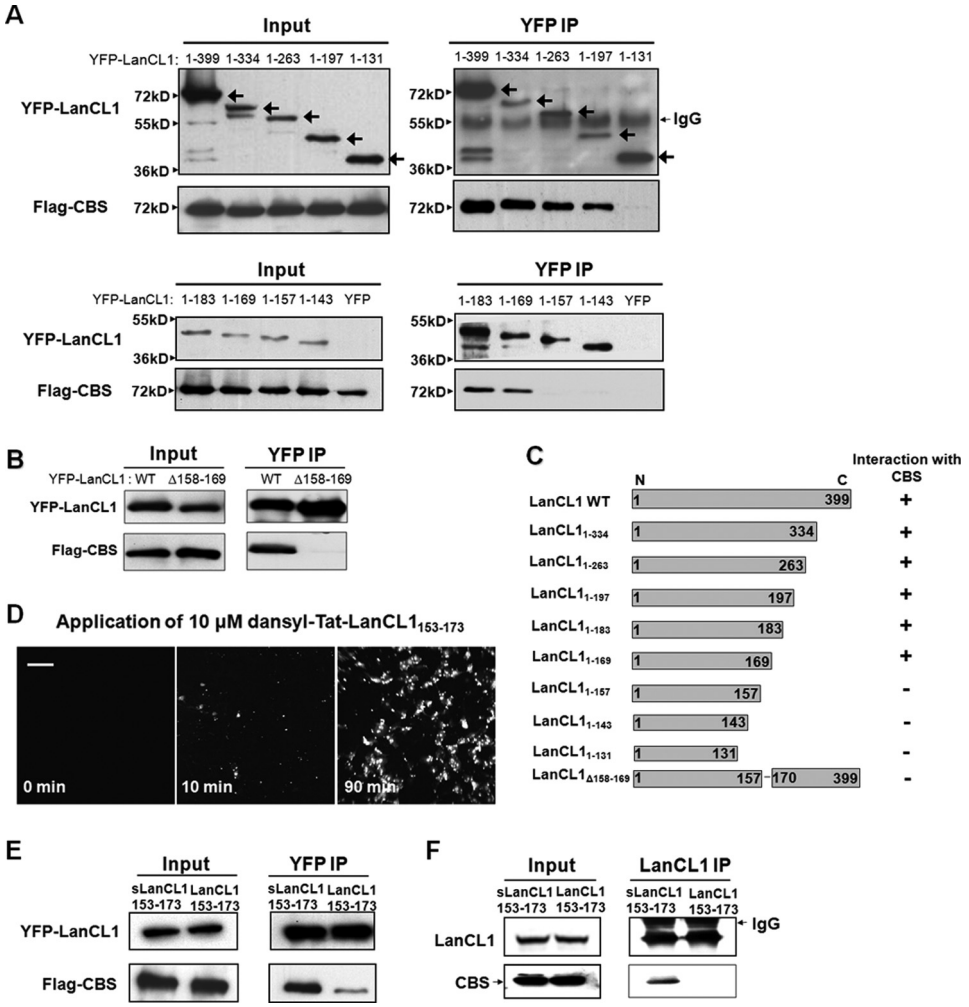

FIGURE 3. **Amino acids 158–169 of LanCL1 are necessary for CBS binding.** *A* and *B*, HEK293 cells co-transfected with FLAG-CBS and truncated LanCL1 mutants were used for co-IP by anti-YFP antibody. YFP-LanCL1$_{WT}$, YFP-LanCL1$_{1–334}$, YFP-LanCL1$_{1–263}$, YFP-LanCL1$_{1–197}$, YFP-LanCL1$_{1–183}$, or YFP-LanCL1$_{1–169}$ interacted with FLAG-CBS but not YFP-LanCL1$_{1–157}$, YFP-LanCL1$_{1–143}$, YFP-LanCL1$_{1–131}$, or YFP-LanCL1$_{\Delta158–169}$ (*B*). *C*, shown is a schematic illustration of truncated LanCL1 mutants used in *panels A* and *B*. + and − indicate the positive and negative interactions with CBS. *D*, time-course of intracellular dansyl-Tat-LanCL1$_{153–173}$ (10 μM) fluorescence after application to HEK293 cells is shown. *Scale bar*, 100 μm. *E*, bath application of the peptide Tat-LanCL1$_{153–173}$ (5 μM) for 1 h in the culture medium of HEK293 cells disrupted the co-IP of FLAG-CBS with YFP-LanCL1. Tat-sLanCL1$_{153–173}$ did not affect the co-IP. *F*, in cultured cortical neurons, bath application of TAT-LanCL1$_{153–173}$ (2.5 μM) for 1 h disrupted the endogenous co-IP of LanCL1 with CBS.

by centrifugation at 13,000 rpm for 60 min at 4 °C. Protein concentration was measured with the BCA method. The enzymatic reaction was carried out in a mixture containing 100 mM Tris-HCl (pH 8.5), 0.1 M serine, and 12.5 mM pyridoxal-5′-phosphate. The reaction was initiated by adding 0.2 M DL-homocysteine and protein extracts to the mixture. After a 1-h incubation at 37 °C, ice-cold 50% trichloroacetic acid was added to stop the reaction. Ninhydrin reagent was added to the supernatants of all tubes, and they were heated in a boiling water bath for 10 min. Tubes were cooled, and the absorbance was measured at 580 nm. Ethanol (75%) was used as the blank. The activity was calculated using 0.5 mM L-cystathionine as standard. CBS activity was described as nmol of cystathionine formed/mg of total protein during 1 h. All chemicals were from Sigma.

GSH and GSSG Assay—The intracellular GSH and GSSG levels were determined with GSH and GSSG assay kit (Beyotime Biotech Inc., Nantong, China). Co-transfected HEK293 cells or primary cultured cortical neurons were washed twice with PBS and harvested by scraping. Cellular pellets (10 μl) were mixed with 30 μl of 5% metaphosphoric acid, then frozen and thawed twice using liquid nitrogen and 37 °C water. The samples were centrifuged, and the supernatant was subjected to GSH and GSSG assay. The total glutathione level was measured by the 5,5′-dithiobis(2-nitrobenzoic acid)-GSSG recycling assay. The absorbance was measured at 412 nm. The amounts of total glutathione in samples were calculated according to the standard curve. After samples and standards were treated with 2-vinylpyridine to block GSH, the GSSG level was quantified similarly to the total glutathione. The amount of GSH was obtained by subtracting the amount of GSSG from the total glutathione. Protein concentration was assayed with a BCA kit using an identical volume of cellular pellets. Values were normalized to protein concentration and expressed as nmol of GSH or GSSG per mg of protein.

LanCL1-GSH Binding Assay—Lysates of HEK293 cells transfected with full-length YFP-LanCL1 or YFP-LanCL1$_{1–334}$ were incubated with GSH-Sepharose^{TM} 4B beads (GE Healthcare, 17-0756-01) for 1 h at room temperature, washed with PBS, and

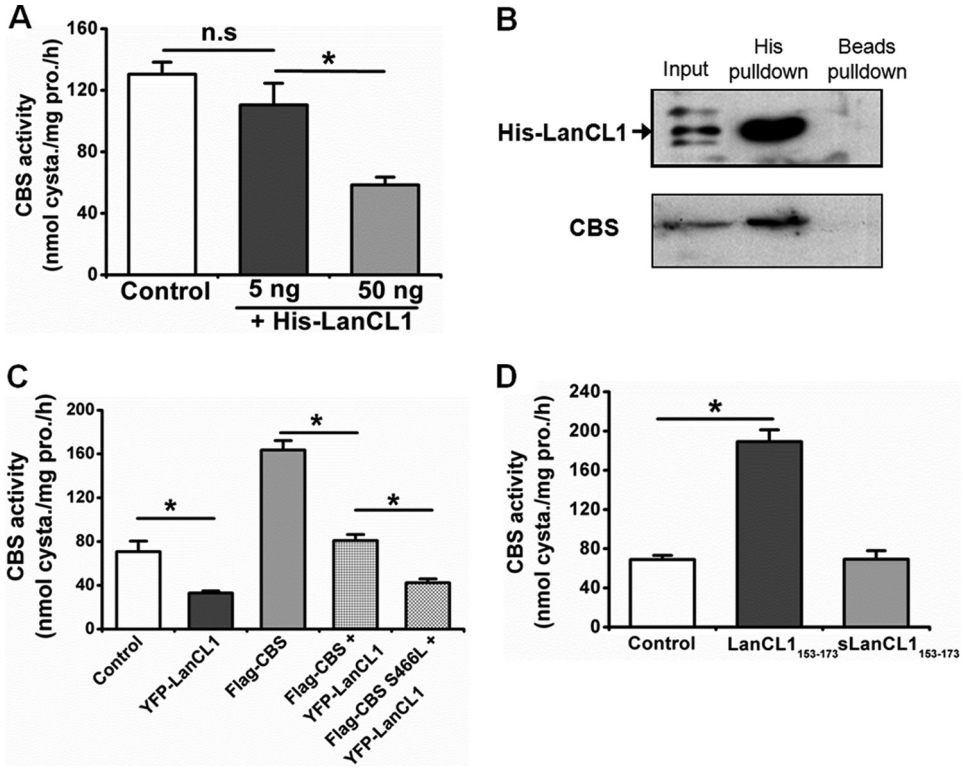

FIGURE 4. **LanCL1 inhibits CBS activity by binding to CBS.** *A*, application of purified His-LanCL1 to mouse cortex homogenates inhibited endogenous CBS activity. Units are nmol of cystathionine formed/mg of protein extract/h (*nmol cysta./mg pro./h*). CBS activity did not change when 5 ng of His-LanCL1/500 μg of protein extract was added and markedly decreased when 50 ng of His-LanCL1 was added (*n.s.*, no significant difference; *, $p < 0.05$; unpaired *t* test; $n = 4$). *B*, purified His-LanCL1 bound to endogenous CBS in mouse cortex homogenates. His-LanCL1 and endogenous CBS were pulled down by Ni-NTA beads from cortex homogenates. His-LanCL1 was detected by anti-His-tag antibody. *Bead pulldown*, as the negative control, represented the pulldown by Ni-NTA beads from the cortex lysates without His-LanCL1. *C*, shown are the inhibitory effects of heterogeneous LanCL1 on CBS activity. HEK293 cells transfected with YFP-LanCL1 had lower CBS activity than non-transfected cells. Co-transfection of YFP-LanCL1 and FLAG-CBS led to lower CBS activity than FLAG-CBS transfection alone. Co-transfection of FLAG-CBS_{S466L} and YFP-LanCL1 led to a lower level of CBS activity than co-transfection of wild-type FLAG-CBS and YFP-LanCL1 (*, $p < 0.05$; unpaired *t* test; $n = 4$). *D*, treatment with Tat-$LanCL1_{153-173}$ (5 μM) for 1 h enhanced CBS activity in HEK293 cells co-transfected with YFP-LanCL1 and FLAG-CBS (*, $p < 0.05$; unpaired *t* test; $n = 4$). The scrambled control had no impact on CBS activity. Data are the mean ± S.E.

eluted by 20 mM GSH in 50 mM Tris-HCl (pH 8.0). Samples were analyzed by SDS-PAGE and probed with anti-YFP antibody.

Cell Viability Assay—Twenty-four hours after H_2O_2 or glutamate exposure, neurons cultured on 96-well plates were incubated with 5 mg/ml methylthiazolyldiphenyl-tetrazolium bromide (MTT) (Sigma) for 4 h. After solubilization in DMSO, the absorbance was measured at 570 nm.

Apoptosis Assay—Hoechst staining was performed according to the instructions with the Hoechst 33258 kit (Beyotime Biotech). After fixation in 4% paraformaldehyde, neurons were washed with PBS and stained with Hoechst 33258. After washing, cells were mounted as above. The images were acquired and analyzed with an FV10-ASW 2.0 Viewer. 300–500 Hoechst-stained neurons were counted for each group.

Statistical Analysis—Data are given as the mean ± S.E. In some cases the results are expressed as percentages of the control value. Statistical differences were determined using the unpaired *t* test. $p < 0.05$ was considered statistically significant.

RESULTS

LanCL1 Is a Novel CBS-interacting Protein—Immunoblot analyses of tissues from adult mice showed that both CBS and LanCL1 were highly expressed in the brain (data not shown). Specifically, both proteins were widely expressed in cortex, hippocampus, cerebellum, and brainstem (data not shown).

LanCL1 antibody immunoprecipitated LanCL1 protein efficiently and co-precipitated CBS in mouse cortex. Control non-immune IgG did not immunoprecipitate LanCL1 or CBS (Fig. 1*A*). In HEK293 cells, endogenous LanCL1 also co-precipitated CBS (Fig. 1*B*). To confirm the interaction of LanCL1 and CBS, HEK293 cells were co-transfected with full-length LanCL1 (399 amino acids) tagged with YFP and CBS (551 amino acids) tagged with FLAG. Cellular extracts were immunoprecipitated by either anti-YFP antibody or anti-FLAG antibody. FLAG-CBS and YFP-LanCL1 co-precipitated each other in both cases (Fig. 1, *C* and *D*). Consistent with the co-IP results, immunocytochemistry showed that LanCL1 co-localized with CBS in cultured cortical neurons and HEK293 cells (Fig. 1*E*). Both LanCL1 and CBS displayed a cytoplasmic distribution (Fig. 1*E*).

N- and C-terminal Regulatory Domains of CBS Are Required for LanCL1-CBS Binding—Human CBS is a tetrameric protein, and each subunit consists of 551 amino acids. CBS contains an N-terminal regulatory domain (amino acids 1–69), a predominant catalytic core (amino acids 70–413), and a C-terminal autoinhibitory domain (amino acids 414–551) (19, 20). To better understand the LanCL1-CBS interaction, the binding region in CBS was investigated by co-IP of FLAG-tagged truncated CBS with full-length YFP-LanCL1 in HEK293 cells. Immunoblots of transfected cells demonstrated that all CBS constructs

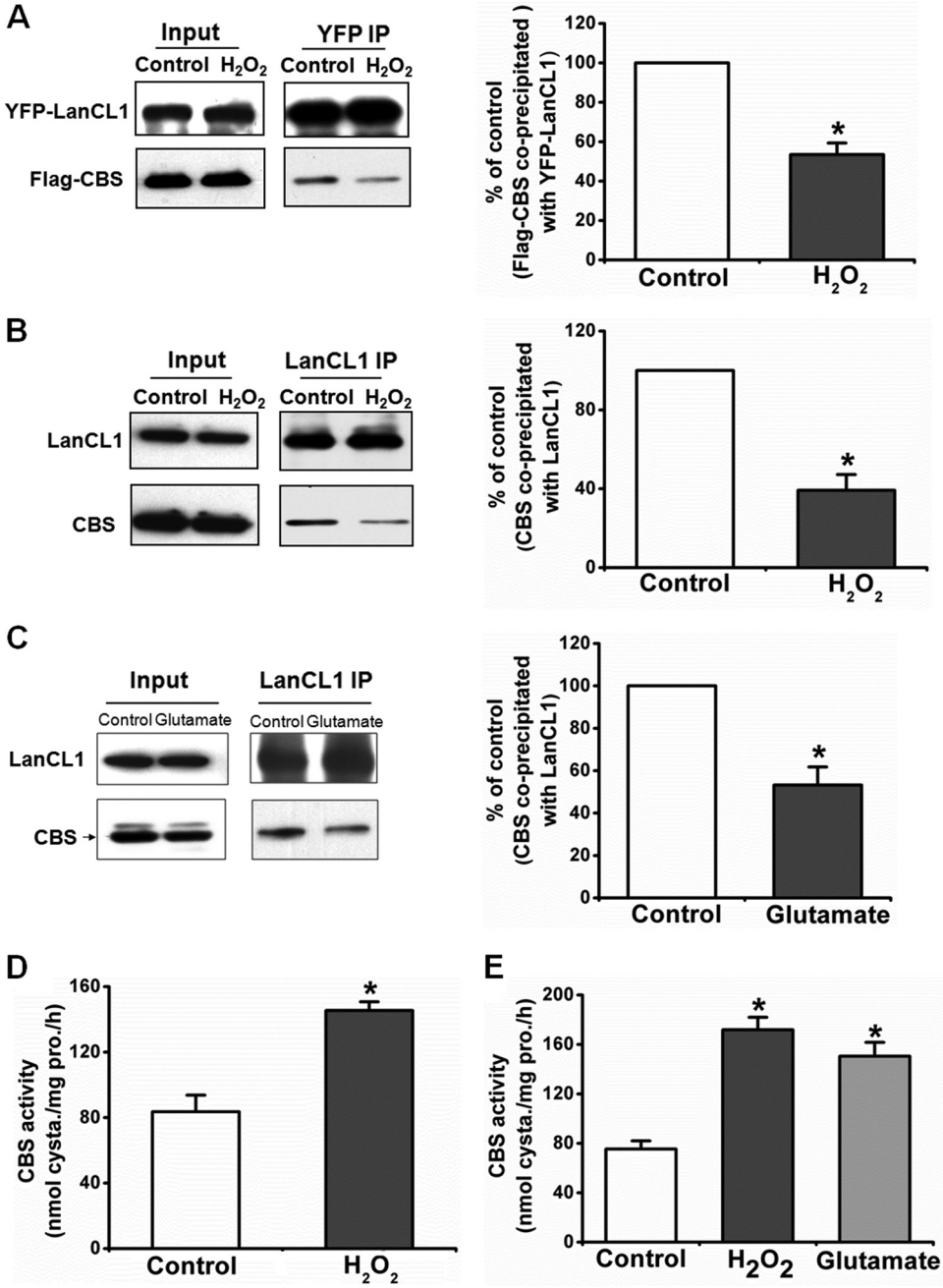

FIGURE 5. **Oxidative stress down-regulates the LanCL1-CBS interaction and elevates CBS activity.** *A*, co-transfected HEK293 cells were exposed to H_2O_2 (100 μM) for 2 h. *Left*, representative co-IP showed that LanCL1-CBS interaction in the H_2O_2-treated group was markedly weaker than that in the untreated group. *Right*, quantification of three independent co-IP tests is expressed as percentages of control (*, $p < 0.05$; unpaired *t* test; $n = 3$). *B* and *C*, primary cultured cortical neurons were exposed for 2 h to H_2O_2 (*B*) or glutamate (*C*) (100 μM for both) followed by co-IP. Co-IP showed that the endogenous LanCL1-CBS interaction was decreased by H_2O_2 or glutamate (*, $p < 0.05$; unpaired *t* test; $n = 3$ for both). *D*, higher CBS activity in co-transfected HEK293 cells was found after H_2O_2 exposure (*, $p < 0.05$; unpaired *t* test; $n = 4$). *E*, H_2O_2 or glutamate exposure promoted CBS activity in cortical neurons (*, $p < 0.05$; unpaired *t* test; $n = 4$). Data are the mean ± S.E.

were expressed correctly (Fig. 2*A*). Co-IP showed that deletion of either the N-terminal regulatory domain (FLAG-CBS_{70-551}) or the C-terminal regulatory domain (FLAG-CBS_{1-413}) alone impaired the binding to YFP-LanCL1, whereas deletion of both domains (FLAG-CBS_{70-413}) completely eliminated this binding (Fig. 2, *A* and *B*).

Amino Acids 158–169 of LanCL1 Are Necessary for LanCL1-CBS Binding—To identify the binding region on LanCL1, we generated a series of C-terminal deletion mutants (Fig. 3*C*) and found that YFP-$LanCL1_{1-169}$ and other longer fragments bound to FLAG-CBS, whereas YFP-$LanCL1_{1-131}$, YFP-$LanCL1_{1-143}$, and YFP-$LanCL1_{1-157}$ did not (Fig. 3*A*). These data suggest that the region from 158 to 169 in LanCL1 is required for its interaction with CBS. This identified binding region was confirmed by the LanCL1 mutant with only residues 158–169 deleted (YFP-$LanCL1_{\Delta 158-169}$), which failed to bind full-length FLAG-CBS (Fig. 3*B*). Interestingly, this region forms an α-helix in the inner helix barrel of the three-dimensional structure of LanCL1 (see Fig. 10) (PDB ID 3E73) (4).

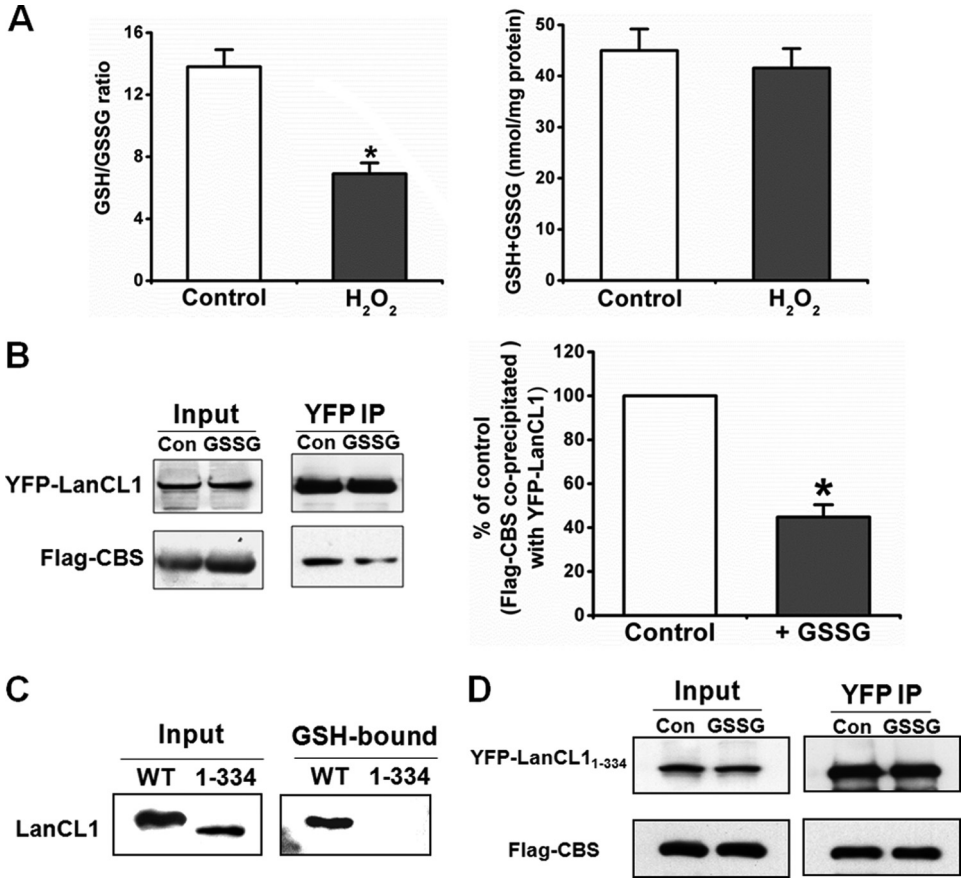

FIGURE 6. **Relationship between the GSH/GSSG redox status and LanCL1-CBS interaction.** *A*, GSH and GSSG levels were determined by the DTNB-glutathione reductase recycling assay and normalized to protein concentration. Transfected HEK293 cells were treated with H_2O_2 (100 μM) for 2 h. The ratio of GSH/GSSG (*left*) and the total glutathione (*right*) was determined (*, $p < 0.05$ *versus* control; unpaired *t* test; $n = 4$). Data are mean ± S.E. *B*, *left*, adding GSSG (3 nmol/mg) to the cellular protein extracts of transfected HEK293 cells decreased the co-IP of YFP-LanCL1 with FLAG-CBS. *Right*, quantified data of three independent co-IP tests (*, $p < 0.05$; unpaired *t* test; $n = 3$). *C*, after overexpression in HEK293 cells, binding of full-length YFP-LanCL1 (*WT*) and its C-terminal-truncated (1–334) variant with GSH cross-linked on Sepharose 4B beads was assayed *in vitro*. The blots were probed with anti-YFP antibody. *Input* denotes lysates of HEK293 cells, and *GSH-bound* indicates YFP-LanCL1 protein that bound to the GSH-Sepharose 4B beads. YFP-LanCL1$_{1-334}$ had no GSH binding ability. *D*, adding GSSG (3 nmol/mg) to cellular extracts of HEK293 cells co-transfected with FLAG-CBS and YFP-LanCL1$_{1-334}$ did not affect the co-IP. *Con*, control.

We subsequently generated the specific peptide Tat-LanCL1$_{153-173}$ and the scrambled control peptide Tat-sLanCL1$_{153-173}$. To determine whether the bath-applied Tat-LanCL1$_{153-173}$ was delivered into cells, the fluorophore dansyl chloride was conjugated to Tat-LanCL1$_{153-173}$ outside the transduction domain to obtain dansyl-Tat-LanCL1$_{153-173}$ (21). HEK293 cells exhibited fluorescence in the cytoplasm after treatment with dansyl-Tat-LanCL1$_{153-173}$ (10 μM) for 10 min, and the fluorescence intensified after 90 min, indicating intracellular uptake of the peptide (Fig. 3*D*).

Next, we determined whether Tat-LanCL1$_{153-173}$ perturbed the LanCL1-CBS interaction by examining its effects on the co-IP of CBS with LanCL1. After bath incubation of transfected HEK293 cells with 5 μM Tat-LanCL1$_{153-173}$ peptide for 1 h, cells were washed twice with PBS, harvested, and subjected to co-IP. The results showed that Tat-LanCL1$_{153-173}$ did not affect the immunoprecipitation efficiency but disrupted the co-IP between YFP-LanCL1 and FLAG-CBS (Fig. 3*E*) compared with the results of a parallel experiment using the scrambled peptide Tat-sLanCL1$_{153-173}$. Furthermore, application of 2.5 μM Tat-LanCL1$_{153-173}$ to cultured rat cortical neurons completely uncoupled endogenous CBS from LanCL1 (Fig. 3*F*), further confirming that LanCL1 interacts with CBS through residues 158–169.

LanCL1 Negatively Regulates CBS Enzyme Activity—To investigate whether LanCL1 regulates CBS activity, we expressed and purified the His-tagged recombinant protein, His-LanCL1. After incubating His-LanCL1 with mouse cortex lysates for 2 h, the catalytic activity of endogenous CBS in protein extracts was decreased in a dose-dependent manner (Fig. 4*A*). Next, we asked whether His-LanCL1 interacts directly with endogenous CBS to modulate its activity using pulldown with Ni-NTA beads. CBS was pulled down along with His-LanCL1, demonstrating the direct interaction between LanCL1 and CBS. As the negative control, Ni-NTA beads did not pull-down CBS from brain lysates without His-LanCL1 (Fig. 4*B*).

Next we confirmed the inhibitory effect of LanCL1 on CBS activity in HEK293 cells. The CBS activity of YFP-LanCL1-transfected cells was significantly lower than untransfected cells (Fig. 4*C*). Furthermore, CBS activity in HEK293 cells co-transfected with FLAG-CBS and YFP-LanCL1 was significantly lower than that in cells transfected with FLAG-CBS alone (Fig. 4*C*). These data suggested that overexpression of LanCL1 inhibits CBS activity. Consistent with the report that the

reduced CBS activity by S466L mutation causes hyperhomocysteinemia *in vivo* (22), we found that HEK293 cells co-transfected with FLAG-CBS_{S466L} and YFP-LanCL1 showed lower CBS activity than those co-transfected with FLAG-CBS and YFP-LanCL1 (Fig. 4*C*). In parallel with the lower CBS activity, we found a stronger interaction between FLAG-CBS_{S466L} and YFP-LanCL1 than that between FLAG-CBS and YFP-LanCL1 (Fig. 2*C*).

The results showed Tat-$LanCL1_{153–173}$ blocked the interaction between YFP-LanCL1 and FLAG-CBS in HEK293 cells (Fig. 3*E*), so we attempted to determine whether blockade of the interaction elevates CBS enzyme activity. Treatment of co-transfected HEK293 cells with 5 μM Tat-$LanCL1_{153–173}$ for 1 h significantly increased the CBS activity compared with that in untreated cells (Fig. 4*D*). As for the negative control, treatment with Tat-s$LanCL1_{153–173}$ had no such effect (Fig. 4*D*). Next, we assessed the role of endogenous LanCL1 on CBS activity in cultured cortical neurons. Consistent with the results in HEK293 cells, treatment of cortical neurons with 2.5 μM Tat-$LanCL1_{153–173}$ for 1 h significantly increased CBS activity compared with that in untreated neurons (as shown in the basal conditions in Fig. 8*A*).

Oxidative Stress Down-regulates LanCL1-CBS Interaction and Promotes CBS Activity in HEK293 Cells and Rat Cortical Neurons—CBS is activated in response to oxidative stress, such as H_2O_2treatment, and then contributes to redox homeostasis via the trans-sulfuration pathway (7). H_2O_2 can be reduced to H_2O by the reaction of glutathione peroxidase with GSH (14). Oxidative stress induced by 100 μM H_2O_2 in transfected HEK293 cells partially dissociated YFP-LanCL1 from FLAG-CBS (Fig. 5*A*), and increased CBS activity (Fig. 5*D*).

H_2O_2-induced oxidative stress (100 μM H_2O_2 for 2 h) partially uncoupled endogenous LanCL1 from CBS in cultured cortical neurons (Fig. 5*B*), consistent with the result from HEK293 cells. On the other hand, glutamate toxicity also contributes to intracellular oxidative stress in neurons (23, 24). Therefore, we also determined whether the LanCL1-CBS interaction and CBS activity in cortical neurons were affected by glutamate toxicity. Glutamate exposure (100 μM) for 2 h partially dissociated endogenous LanCL1 from CBS (Fig. 5*C*). After exposure to H_2O_2 or glutamate, elevation of CBS activity occurred in parallel with LanCL1-CBS dissociation in cultured cortical neurons (Fig. 5*E*).

LanCL1 Senses the GSH/GSSG Ratio to Regulate Its Binding to CBS—Considering that LanCL1 binds to both GSH and GSSG (3), we investigated whether LanCL1 directly senses the cellular GSH/GSSG status and subsequently regulates its binding to CBS. To explore the effects of H_2O_2-induced oxidative stress on cellular redox homeostasis, the intracellular levels of GSH and GSSG were measured. The basal GSH/GSSG ratio in transfected HEK293 cells was 13.8 ± 1.1 (mean ± S.E.), which is not significantly different from previous reports (25, 26). Compared with control, treatment with 100 μM H_2O_2 for 2 h significantly lowered the GSH/GSSG ratio to 6.9 ± 0.7 but did not affect the total glutathione (GSH+GSSG) level (Fig. 6*A*).

To address the direct connection between GSH/GSSG status and LanCL1-CBS interaction, we next altered the GSH/GSSG ratio by directly adding GSSG or GSH to the lysates of transfected HEK293 cells and then determined whether the interaction between YFP-LanCL1 and FLAG-CBS was affected. We added GSSG at 3 nmol/mg of cellular protein extract to decrease the GSH/GSSG ratio to 7, a level similar that induced by H_2O_2 treatment for 2 h. Co-IP results showed that the addition of GSSG dissociated YFP-LanCL1 from FLAG-CBS (Fig. 6*B*). These results indicated that a decrease in the GSH/GSSG ratio is sufficient to dissociate LanCL1 from CBS.

Furthermore, we found that LanCL1 overexpressed in HEK293 cells binds to glutathione cross-linked on Sepharose 4B beads and that the bound LanCL1 can be eluted by 20 mM free GSH (Fig. 6*C*), confirming the GSH binding property of LanCL1. It was reported that a point mutation of R364A in LanCL1 completely abolishes the GSH binding ability of LanCL1 (4). Our results showed that YFP-$LanCL1_{1–334}$, which lacks the 35 C-terminal residues including Arg-364, did not

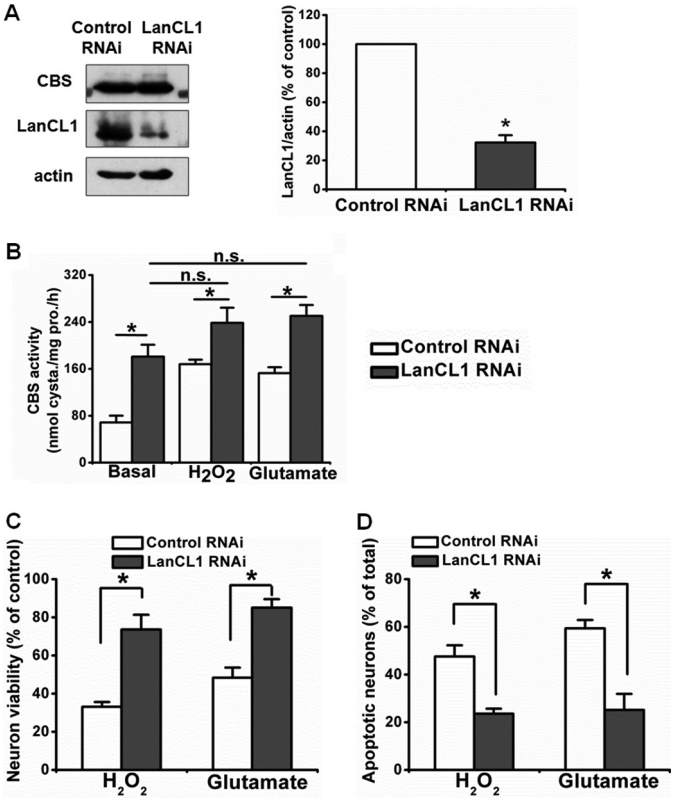

FIGURE 7. **LanCL1 knockdown increases CBS activity and protects neurons against H_2O_2 or glutamate.** *A*, *left*, shown are representative immunoblots of LanCL1 and CBS in neurons infected with LanCL1 RNAi or control RNAi lentivirus. LanCL1 RNAi decreased the expression of LanCL1. *Right*, shown is quantitative analysis of three independent experiments. The ratio of the band intensity of LanCL1 to actin in control neurons (*Control RNAi*) was normalized to 100% (*, $p < 0.05$; unpaired *t* test; $n = 3$). *B*, lentiviral knockdown of LanCL1 increased the CBS activity in neurons under basal conditions or with H_2O_2 or glutamate exposure. LanCL1 RNAi occluded the H_2O_2 or glutamate-induced increase of CBS activity (*, $p < 0.05$ *versus* control RNAi; *n.s.*, no significant difference *versus* basal; unpaired *t* test; $n = 5$). *C*, for cell viability measured by methylthiazolyldiphenyl-tetrazolium bromide assay, the values were normalized to the respective controls (defined as 100%). LanCL1 knockdown increased neuronal viability after H_2O_2 or glutamate exposure for 2 h and another 24 h in culture (*, $p < 0.05$; unpaired *t* test; $n = 3$). *D*, for apoptosis analyzed by Hoechst staining, the ratio of the number of apoptotic neurons to the total number was normalized to the respective control (defined as 100%). LanCL1 knockdown decreased the proportion of apoptotic neurons after H_2O_2 or glutamate challenge (*, $p < 0.05$; unpaired *t* test; $n = 3$). Data are mean ± S.E.

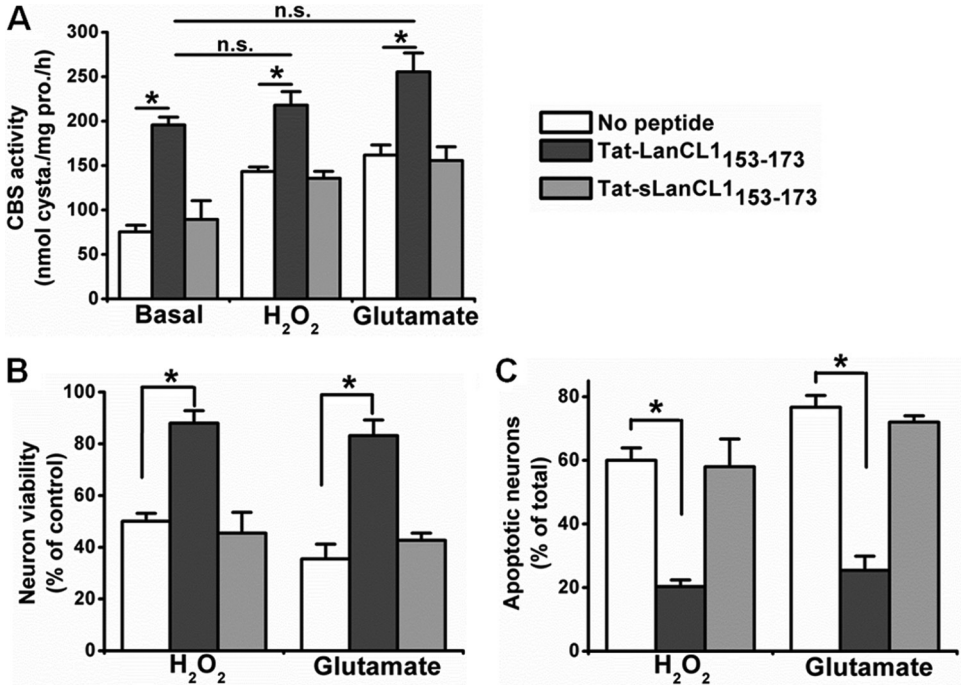

FIGURE 8. **Treatment with Tat-LanCL1$_{153–173}$ increases CBS activity and protects neurons against H_2O_2 or glutamate.** *A*, pretreatment with the specific blocking peptide Tat-LanCL1$_{153–173}$ (2.5 μM) for 1 h increased the CBS activity of neurons under basal conditions or with H_2O_2 or glutamate exposure but not the scrambled peptide Tat-sLanCL1$_{153–173}$. Tat-LanCL1$_{153–173}$ occluded the H_2O_2 or glutamate-induced increase of CBS activity (*, $p < 0.05$, *versus* untreated neurons; *n.s.*, no significant difference *versus* basal; unpaired *t* test; $n = 5$). *B*, treatment with Tat-LanCL1$_{153–173}$ (2.5 μM) for 1 h before H_2O_2 or glutamate insult rescued neuronal viability. Pretreatment with Tat-sLanCL1$_{153–173}$ under the same conditions did not affect viability (*, $p < 0.05$; unpaired *t* test; $n = 4$). *C*, analysis of neuronal apoptosis rates showed the neuroprotective effect of Tat-LanCL1$_{153–173}$ against H_2O_2 or glutamate insult but not the scrambled control (*, $p < 0.05$; unpaired *t* test; $n = 4$). Data are mean ± S.E.

bind to glutathione (Fig. 6*C*). The interaction between YFP-LanCL1$_{1–334}$ and FLAG-CBS was no longer affected by adding 3 nmol/mg GSSG to cellular extracts (Fig. 6*D*). Together, these results implied that LanCL1 functions as a sensor of oxidative stress through a GSH/GSSG-sensitive or a redox glutathione-dependent mechanism to regulate CBS. This process is dependent on the glutathione binding property of LanCL1.

LanCL1 Knockdown Elevates CBS Activity and Protects Neurons from Oxidative Stress—To verify the role of LanCL1 in CBS activity in neurons, we then generated LanCL1 RNAi lentivirus. Six days after RNAi infection, the expression level of LanCL1 decreased ~70% (Fig. 7*A*). Oxidative stress did not completely dissociate LanCL1 from CBS (Fig. 5), so it is possible that the inhibition of CBS activity by LanCL1 still occurred during oxidative stress. We then determined whether LanCL1 knockdown by lentivirus-mediated RNAi could release CBS activity and protect neurons from oxidative stress. Knockdown of LanCL1 with RNAi effectively enhanced CBS activity under basal conditions or under H_2O_2 or glutamate exposure (Fig. 7*B*). It should be noted that CBS activity in LanCL1 RNAi neurons was not significantly increased by H_2O_2 or glutamate insult (*n.s.* in Fig. 7*B*), demonstrating that CBS activation by LanCL1 knockdown occludes the CBS activation induced by oxidative stress.

We next determined whether LanCL1 knockdown is neuroprotective against oxidative stress. Neurons subjected to H_2O_2 or glutamate treatment for 2 h followed by a 24-h incubation in culture medium suffered injury. LanCL1 RNAi significantly restored neuronal viability (Fig. 7*C*) and decreased neuronal apoptosis (Fig. 7*D*) induced by H_2O_2 or glutamate. Therefore, LanCL1 knockdown increased CBS activity and then reduced neuronal damage under oxidative stress.

Disruption of LanCL1-CBS Interaction Elevates CBS Activity and Protects Neurons against Oxidative Stress—Next, we assessed the potential neuroprotective effect of the cell-permeable Tat-LanCL1$_{153–173}$ peptide. We pretreated cultured neurons with 2.5 μM Tat-LanCL1$_{153–173}$ or Tat-sLanCL1$_{153–173}$ for 1 h followed by H_2O_2 or glutamate exposure. Measurement of CBS activity revealed that Tat-LanCL1$_{153–173}$ markedly increased CBS activity under basal conditions or under H_2O_2 or glutamate exposure, whereas the scrambled peptide had no effect (Fig. 8*A*). It was similarly noted that H_2O_2 or glutamate stimulation did not significantly increase CBS activity in Tat-LanCL1$_{153–173}$-treated neurons (*n.s.* in Fig. 8*A*), implying that CBS activation by Tat-LanCL1$_{153–173}$ occludes the CBS activation induced by oxidative stress.

Measurements of neuronal viability and the apoptosis rate showed that Tat-LanCL1$_{153–173}$ prevented viability from decreasing (Fig. 8*B*) and inhibited apoptosis (Fig. 8*C*) in neurons subjected to H_2O_2 or glutamate exposure. In contrast, the scrambled peptide was not neuroprotective (Fig. 8, *B* and *C*). These results indicated that disruption of the LanCL1-CBS interaction increases CBS activity and then contributes to neuronal antioxidant defense.

The CBS Activator AdoMet Impairs LanCL1-CBS Binding and Protects Neurons against Oxidative Stress—The CBS activator *S*-adenosylmethionine (AdoMet) is reported to bind to the C-terminal regulatory domain of CBS (19, 27). AdoMet

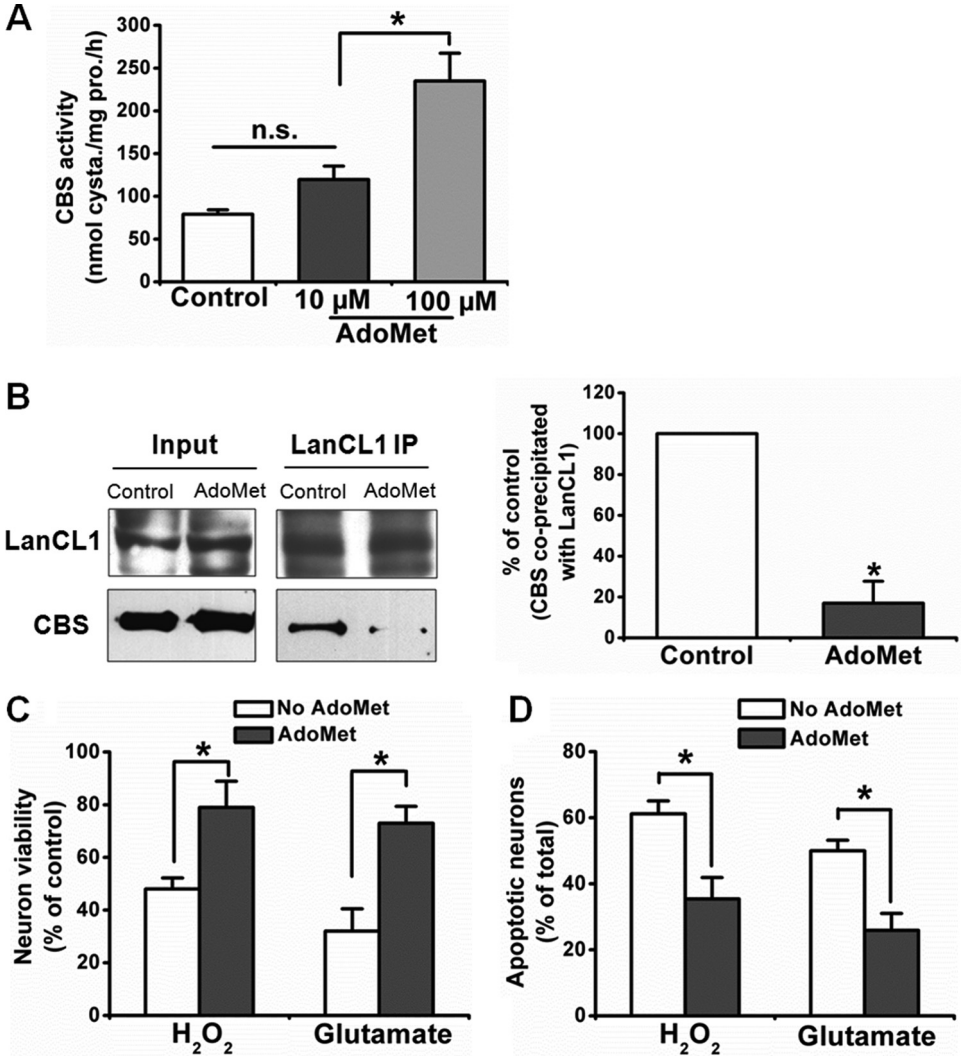

FIGURE 9. **The CBS activator, AdoMet, down-regulates the LanCL1-CBS interaction and exerts neuroprotection against H_2O_2 or glutamate.** *A*, application of AdoMet to neurons for 2 h activated CBS in a dose-dependent manner (*, $p < 0.05$; *n.s.*, no significant difference; unpaired *t* test; $n = 4$). *B*, *left*, treatment of cortical neurons with AdoMet for 2 h perturbed the co-IP of endogenous LanCL1 and CBS. *Right*, shown is quantification of three independent co-IP tests (*, $p < 0.05$; unpaired *t* test; $n = 3$). *C*, pretreatment with AdoMet (100 μM) for 2 h before H_2O_2 or glutamate insults in rescued neuronal viability (*, $p < 0.05$; unpaired *t* test; $n = 3$). *D*, apoptosis rates were also determined to evaluate the protective effect of AdoMet on neurons challenged by H_2O_2 or glutamate (*, $p < 0.05$; unpaired *t* test; $n = 3$). Data are mean ± S.E.

treatment for 2 h increased the CBS activity in neurons in a dose-dependent manner (Fig. 9*A*). We then assessed the effect of AdoMet on the LanCL1-CBS interaction. After neurons were treated with AdoMet (100 μM), LanCL1-CBS binding was tested by co-IP, and the results showed that AdoMet greatly suppressed this binding (Fig. 9*B*). Furthermore, treatment with 100 μM AdoMet for 2 h before H_2O_2 or glutamate exposure significantly blocked the reduction of neuronal viability (Fig. 9*C*) and diminished the apoptosis rate (Fig. 9*D*) 24 h after exposure. Uncoupling LanCL1 from CBS induced by AdoMet may be the mechanism by which AdoMet exerts neuroprotection via activating CBS.

DISCUSSION

Here, we found a novel physical and functional relationship between the GSH-binding protein LanCL1 and the enzyme CBS. Our findings suggest that LanCL1 has a natural tendency to form a complex with CBS. Previous studies have reported that CBS is essential for cell survival (18, 28). Considering its critical role in the sulfur metabolism system, it is not surprising that its activity under physiological conditions should be balanced at an appropriate level. Therefore, the physiological control of CBS activity by LanCL1 is important.

Major Contribution of LanCL1 to CBS Regulation—We identified the LanCL1 binding regions of CBS in the non-catalytic N and C termini. The N-terminal heme domain of CBS, composed of 70 amino acids, functions as a redox sensor and contributes to activating the CBS enzyme under oxidizing conditions (29). Therefore, the LanCL1-CBS association and dissociation may explain the effects of modifying the N- and C-terminal regions of CBS on its activity. CBS activity control by LanCL1 binding is adjustable according to cellular needs. In this study, under oxidative stress induced by H_2O_2 or glutamate, dissociation of LanCL1 from CBS released CBS activity. This is presumably a mechanism for the increased CBS activity, as the N- and C-terminal regions of CBS regulate its activity in response to oxidative stress. Considering how to evaluate the

contribution of LanCL1, we found that CBS activation in neurons induced by H_2O_2 or glutamate became insignificant after LanCL1 knockdown or disrupting the LanCL1-CBS interaction, suggesting a major place for LanCL1-CBS binding in CBS activity regulation under oxidative stress. This regulation constitutes an auto-corrective response to oxidative stress, allowing for rapid regulation of CBS activity in response to changes in cellular redox conditions.

Furthermore, the C-terminal 414–551 residues in CBS form a negative regulatory region that is responsible for the allosteric activation of CBS induced by AdoMet (19). Dissociation of LanCL1 from CBS induced by AdoMet may be the mechanism underlying the actions of AdoMet. It has been reported that S466L is one of the hyperhomocysteinemia-relevant mutations in CBS; however, the purified recombinant CBS_{S466L} shows enzyme activity similar to wild-type CBS *in vitro* (22). Our findings that the CBS_{S466L} mutant facilitates its binding with LanCL1 and reduces the CBS activity may be the mechanism underlying S466L mutation-induced hyperhomocysteinemia *in vivo*.

The Neuroprotection Induced by Blocking LanCL1-CBS Binding Is More Specific Than AdoMet—This study is the first to identify the role of LanCL1 in CBS-related neuroprotection. The trans-sulfuration pathway involving CBS is an important source of GSH, and diminished GSH levels increase cellular vulnerability to oxidative stress (30). We confirmed that maintenance of CBS activity at a higher level by LanCL1 knockdown or pretreatment with the blocking peptide Tat-$LanCL1_{153–173}$ targeting the LanCL1-CBS interaction in neurons achieves neuroprotection against oxidative stress.

Studies have reported that supplementation with AdoMet effectively protects neurons against oxidative damage and offers a useful approach for the treatment of neurodegenerative diseases (31, 32). AdoMet is also involved in the methylation cycle and acts as a methyl donor to membrane phospholipids, myelin, and other molecules important for normal brain function (33). Excess methylation reaction can cause specific Parkinson disease-like neuronal degeneration (34). It has also been reported that AdoMet can trigger transmethylation processes to induce apoptosis and necrosis in PC12 cells (35). The peptide Tat-$LanCL1_{153–173}$ used in the present study specifically blocked LanCL1 binding to CBS and showed significant neuroprotective effects. Considering its specificity, the blocking peptide targeting the LanCL1-CBS interaction may have advantages over AdoMet.

Oxidative stress is common during the brain damage, and antioxidants as well as free radical scavengers are effective for intervention and treatment (36, 37). We provide current evidence for the beneficial effects of Tat-$LanCL1_{153–173}$ in intervention against neuronal damage via activating CBS involved in neuroprotection such as up-regulation of endogenous antioxidant systems. Although animal studies are needed, the blocking peptide used here may have effective and safe therapeutic potential in treating oxidative stress-related neurodegenerative diseases.

Glutathione Binding of LanCL1 Decides the Oxidative Stress-sensitive Regulation of CBS by LanCL1—Oxidative stressors induce a cellular redox shift, including a decrease in the intracellular GSH/GSSG ratio (38). In this study oxidative stress caused by H_2O_2 or glutamate exposure decreased the intracellular GSH/GSSG ratio, dissociated the LanCL1-CBS interaction, and consequently increased CBS activity. To confirm that decreasing the GSH/GSSG ratio alone is sufficient to regulate the LanCL1-CBS interaction, exogenous GSSG was added to cell lysates to directly decrease the GSH/GSSG ratio to a level similar to that under oxidative stress. Under this condition, LanCL1-CBS interaction was also dissociated, suggesting the direct regulation of LanCL1 by the GSH/GSSG ratio.

How could the GSH/GSSG ratio regulate the LanCL1-CBS interaction? Our results suggested that this depends on the glutathione binding property of LanCL1, as the interaction of CBS and $LanCL1_{1–334,}$ the LanCL1 mutation that loses glutathione binding, was no longer regulated by the GSH/GSSG ratio. It is reported that GSH and GSSG have the same binding efficiency for LanCL1, and this binding is competitive (3), suggesting that they share the same binding site on LanCL1. Besides, there is evidence that the function of glutathione-binding proteins can be differentially regulated by GSH or GSSG binding, possibly through a conformational change (39). As for LanCL1, the CBS-binding site is located at the inner helix barrel, and the GSH binding site is located at the bottom of the barrel in the three-dimensional structure of LanCL1 (Fig. 10). So far, the three-dimensional structure of LanCL1 co-crystallized with GSSG has not been reported. However, based on our results, it may be that GSSG binding causes a conformational change of the LanCL1 barrel structure, leading to closure of the barrel and/or masking of the CBS binding sites and consequently dissociation of CBS from LanCL1.

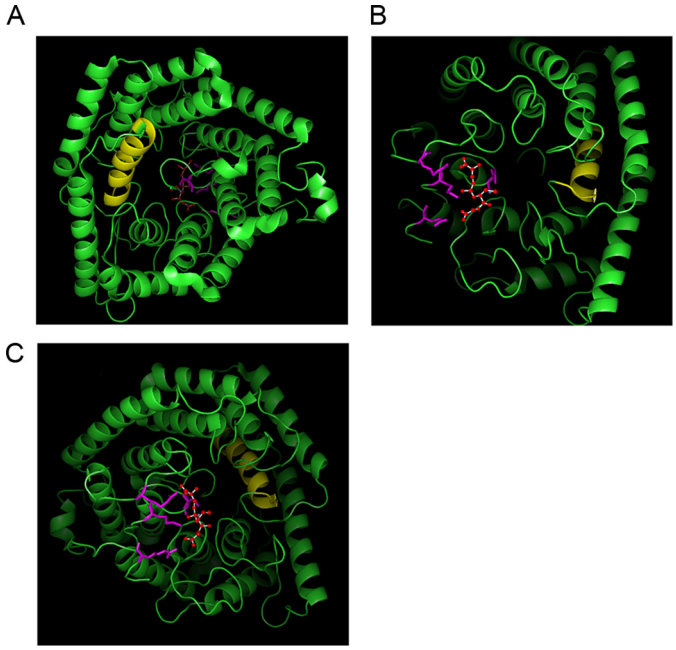

FIGURE 10. **CBS binding site and GSH binding site on the three-dimensional structure of human LanCL1 co-crystallized with GSH.** *A–C*, shown is a three-dimensional structure of human LanCL1 (PDB ID 3E73) from three points of view. The region including residues 158–169 of LanCL1, which is the CBS binding site, is colored *yellow*. Arg-4, Lys-317, Cys-322, and Arg-364, which directly interact with GSH, are shown as *purple sticks*. GSH is shown as *white sticks* and *red spheres*. The figures were drawn by PyMOL software.

Because LanCL1 is a sensor of the cellular GSH/GSSG ratio and meanwhile a regulator of CBS activity, LanCL1 contributes to cellular redox homeostasis. LanCL1 remains bound to CBS under basal conditions to prevent overactivation of CBS and is released from CBS once the redox balance becomes more oxidizing, enhancing CBS activity to compensate for the decreased GSH/GSSG ratio.

In summary, we have revealed a novel function of the glutathione-binding protein LanCL1 in regulating CBS activity in response to oxidative stress, demonstrating the physiological and pathological significance of the LanCL1-CBS interaction. Further investigation of the LanCL1-dependent regulation of CBS in animal models of human diseases may lead to potential intervention strategies for the treatment of oxidative stress-related diseases.

Acknowledgments—We thank Warren D. Kruger (Temple University) for providing human CBS cDNA and Dr. Bo Xiao (Sichuan University) for human LanCL1 cDNA and communication regarding this project. We also thank Dr. Iain C. Bruce (Zhejiang University) for assistance with correcting this manuscript.

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