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RESEARCH ARTICLE

Genetic and Pharmacological Modulation of Cellular Proteostasis Leads to Partial Functional Rescue of Homocystinuria-Causing Cystathionine-Beta Synthase Variants

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ABSTRACT Homocystinuria (HCU), an inherited metabolic disorder caused by lack of cystathionine beta-synthase (CBS) activity, is chiefly caused by misfolding of single amino acid residue missense pathogenic variants. Previous studies showed that chemical, pharmacological chaperones or proteasome inhibitors could rescue function of multiple pathogenic CBS variants; however, the underlying mechanisms remain poorly understood. Using Chinese hamster DON fibroblasts devoid of CBS and stably overexpressing human WT or mutant CBS, we showed that expression of pathogenic CBS variant mostly dysregulates gene expression of small heat shock proteins HSPB3 and HSPB8 and members of HSP40 family. Endoplasmic reticulum stress sensor BiP was found upregulated with CBS I278T variant associated with proteasomes suggesting proteotoxic stress and degradation of misfolded CBS. Co-expression of the main effector HSP70 or master regulator HSF1 rescued steady-state levels of CBS I278T and R125Q variants with partial functional rescue of the latter. Pharmacological proteostasis modulators partially rescued expression and activity of CBS R125Q likely due to reduced proteotoxic stress as indicated by decreased BiP levels and promotion of refolding as indicated by induction of HSP70. In conclusion, targeted manipulation of cellular proteostasis may represent a viable therapeutic approach for the permissive pathogenic CBS variants causing HCU.

KEYWORDS cystathionine beta-synthase, homocystinuria, misfolding, proteostasis, pharmacological, chaperone

INTRODUCTION

Classical homocystinuria (HCU; OMIM# 236200) is an autosomal recessive inborn error of sulfur amino acid metabolism caused by the lack of cystathionine beta--synthase (CBS) activity.[1,2] Deficient CBS activity results in accumulation of homocysteine, a toxic metabolic intermediate of methionine conversion to cysteine via transsulfuration pathway. Homocysteine buildup is generally accepted as a biochemical hallmark and the main culprit responsible for development of HCU clinical symptoms, such as connective tissue defects, osteoporosis, dislocated optic lenses, cognitive impairment, thromboembolism and often fatal strokes.[3] HCU is mostly caused by the presence of a missense point mutation in the CBS gene,[4] which alters a single amino acid residue in the CBS protein. Such pathogenic CBS variants have difficulty to fold properly into their native active conformations.[5] Pey et al. showed that several heterologously expressed and purified pathogenic CBS variants manifested instability of the regulatory domain, which is involved in oligomerization and binding of allosteric activator S-adenosylmethionine (SAM),[6] while having normal or higher catalytic activity compared to WT.[7] Thus, the data suggest that the majority of

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pathogenic missense mutations does not target key catalytic residues, but rather impairs stability, folding, regulation or interactions of CBS. Indeed, fibroblasts from HCU patients showed either no native CBS formation (degradation) or its accumulation into high molecular weight products (aggregation).[8] Therefore, HCU is considered a conformational disorder, which may be amenable to correction by modulation of cellular proteostasis network.[2]

Proteostasis (or protein homeostasis) network represents a set of interacting activities that maintain the health of cellular proteome including protein synthesis, folding, formation of supramolecular complexes, trafficking, disaggregation and degradation.[9] The main effectors involved in cellular proteostasis are molecular chaperones or chaperonins, also known as heat shock proteins (HSPs),[10] and ubiquitin/proteasome system (UPS).[11] HSPs are involved in proper protein folding and trafficking, while UPS is responsible for rapid degradation of damaged or misfolded proteins. Thus, in order to rescue activity and stability of a misfolded protein, one needs either to stimulate processes leading to refolding and increased conformational stability or to reduce activity of cellular protein degradation pathways. It has been shown that chemical chaperones, such as ethanol, dimethyl sulfoxide (DMSO) or trimethylamine-N-oxide (TMAO), rescued folding and activity of several pathogenic CBS variants either by their nonspecific mass effect on folding environment (osmolarity, water interactions) or induction of molecular chaperones.[12–16] More protein-specific small molecules, known as pharmacological chaperones,[17] typically comprise cofactors, substrates, competitive inhibitors or other ligands and offer an attractive avenue to target conformational disorders. Pyridoxine, a precursor of catalytically active CBS cofactor pyridoxal-5′-phosphate, has great clinical success in a milder (also called pyridoxine-responsive) form of HCU[18,19] Heme, a noncatalytic CBS cofactor of unclear function, was found essential for proper CBS folding and rescued expression and activity of CBS R125Q variant[20,21] SAM or its analogs showed highly variable effect among selection of pathogenic CBS variants,[22] but as a specific allosteric binder, SAM represents a great opportunity for development of a specific CBS stabilizer or activator.[7,23] In contrast, proteasome inhibitors, such as MG132 or bortezomib, prevent rapid degradation of misfolded proteins, which, in turn, increases their cellular steady-state levels and provides more time for refolding of unstable proteins into native-like active conformation.[24] The Kruger lab showed that bortezomib treatment of either yeast cells and human fibroblasts or transgenic CBS-deficient mice expressing several different pathogenic CBS variants increased their steady-state levels and, more importantly, rescued catalytic activity, which in mice resulted in a substantial decrease of plasma total homocysteine levels.[15,25–27] However, longer exposure or chronic treatment of HCU with proteasome inhibitors would come with serious toxicity and side-effects. Furthermore, a recent report showed that chronic treatment of transgenic HCU mice with a lower dose of proteasome inhibitors is better tolerated, but also less effective or completely ineffective in rescuing CBS activity and correcting homocysteine levels.[28] The authors argued that lack of efficacy could have been caused by inefficient upregulation of molecular chaperones, such as HSP90, HSP70 or HSP27, involved in refolding of CBS variants. Taken together, modulation of cellular proteostasis network by either promoting refolding or decreasing degradation of CBS variants represents a viable approach toward treatment of HCU.

Here, we studied how genetic or pharmacological stimulation of refolding processes, rather than slowing down protein degradation, affected rescue of pathogenic CBS variants in a mammalian cell model of HCU.

RESULTS

CBS misfolding perturbs proteostasis network and causes ER stress. To gain insight how expression of pathogenic CBS variant disturbs proteostasis network, we performed PCR array expression analysis targeting 84 HSPs and chaperones. Comparison between cells expressing CBS WT as a control and pathogenic CBS I278T variant as a test sample showed only five differentially expressed genes encoding for HSPs or chaperones (Fig. 1A). Specifically, small HSP family member HSPB3 was downregulated by 77%

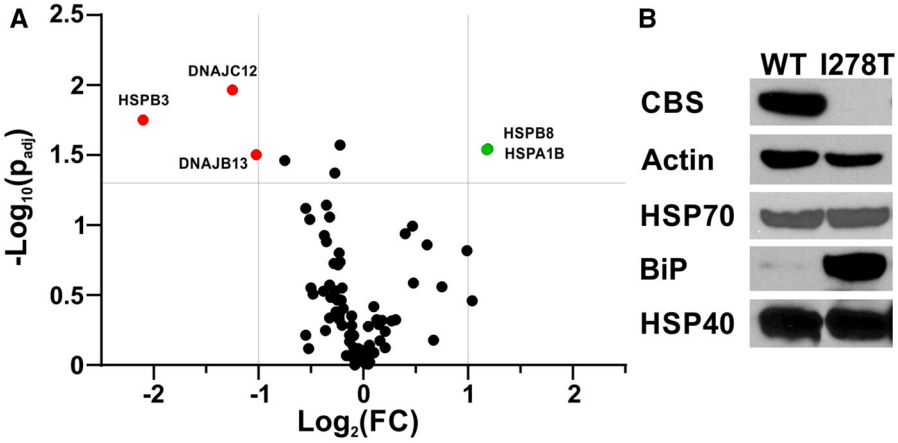

FIG 1 Expression profile of molecular chaperones in HCU. (A) Differential expression of genes encoding HSPs and chaperones in A23 CBS I278T compared to A23 CBS WT control ($n = 3$ independent biological replicates for both test and control samples). Significantly up- and downregulated genes are named and shown as green and red points, respectively. (B) Representative Western blot analysis of soluble protein extracts from A23 CBS WT and I278T (50 μg/lane) evaluating expression of CBS and selected molecular chaperones, such as HSP70, BiP and HSP40.

($P = 0.017$) in I278T compared to WT, while, on the contrary, another member of the same family HSPB8 was upregulated 2.28-fold ($P = 0.028$). Two HSP40 family members DNAJB13 and DNAJC12 were downregulated by 51% ($P = 0.031$) and 58% ($P = 0.011$), respectively, in I278T compared to WT. Lastly, a single HSP70 family member HSPA1B was upregulated 2.26-fold ($P = 0.029$) in I278T versus WT control. The results suggest that gene expression of a handful of HSPs is disturbed by overexpression of a pathogenic CBS I278T variant compared to WT as a control for correct CBS folding.

Validation of differential gene expression changes on a protein level using Western blot analysis was complicated due to lack of antibodies specific to gene products of the identified differentially expressed genes. Nevertheless, Western blot analysis confirmed no detectable steady-state levels of CBS I278T compared to WT control (Fig. 1B). In contrast to gene expression analysis, HSP70 and HSP40 did not show differential protein levels in the soluble extracts of WT and I278T variants. However, expression of endoplasmic reticulum (ER) stress sensor BiP (GRP78) was markedly upregulated in I278T variant compared to barely detectable levels in WT control. Expression of the selected effectors of proteostasis network has been further confirmed and visualized by confocal microscopy (Fig. 2). As anticipated, expression of CBS I278T was decreased by 85% on average compared to CBS WT as a control. BiP expression was upregulated 2.46-fold and showed both cytoplasmic and nuclear localization in I278T variant compared to just basal levels and nuclear localization in WT control (Fig. 2A and D). HSP70 showed both petechial and pan-cellular localization for both variants with a slightly higher signal and colocalization for I278T versus WT cells (Fig. 2B and D). Lastly, 20S proteasome subunit was upregulated 2.86-fold in I278T compared to WT and presented substantial colocalization with the misfolded CBS I278T variant (Fig. 2C and D). Together, the data suggest that a marked decrease in steady-state levels of CBS I278T variant is due to an increased cellular/ER stress and upregulation of proteasomal activity resulting in lower refolding and amplified degradation of this misfolded CBS variant.

Impact of HSPs' coexpression on CBS refolding. To stimulate cellular refolding activity, we transiently transfected cells overexpressing CBS WT as a control and CBS R125Q or I278T pathogenic variants as test samples with plasmids overexpressing HSPA1A or heat shock factor 1 (HSF1). HSPA1A encodes for a major molecular chaperone of HSP70 family involved in folding process of various cellular proteins, while HSF1 is a master regulator of heat shock response pathway ensuring proper protein folding and their cellular trafficking.[10] Co-expression of either HSPA1A or HSF1 resulted in a substantial recovery of CBS in soluble fractions of cell lines expressing CBS R125Q and

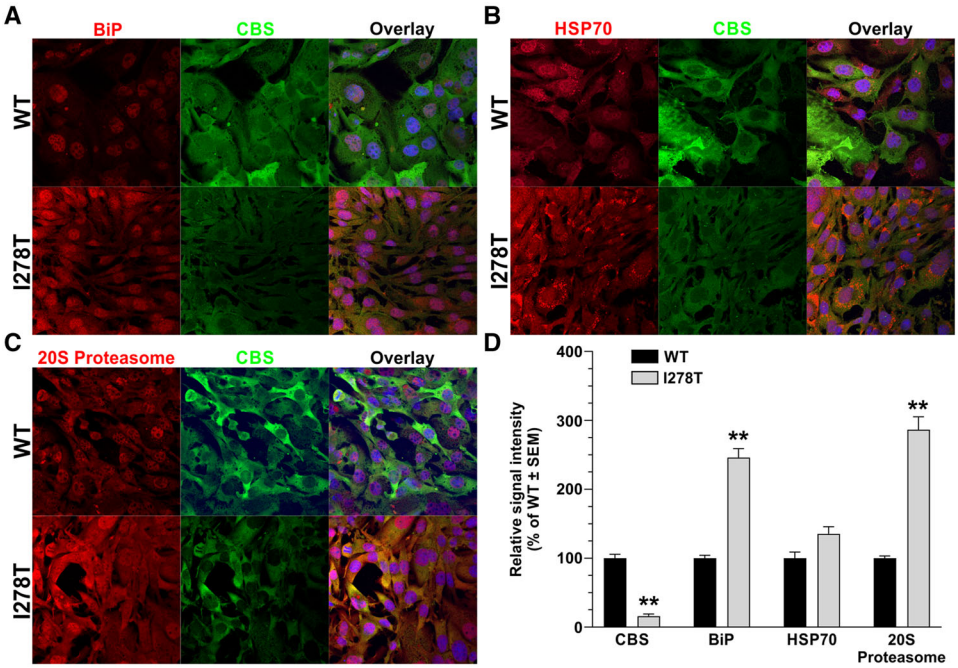

FIG 2 Disturbed proteostasis in HCU. The A23 CBS WT (top) and I278T cells (bottom) were grown on cell culture slides, fixed, permeabilized and probed for the indicated combination of targets as described in Materials and Methods section. Different effectors of proteostasis network are shown in red channel (A – BiP, B – HSP70 and C – 20S Proteasome), CBS in a green channel and nuclei in a blue channel (shown only in a composite image). (D) Fluorescent intensities for CBS, BiP, HSP70 and 20S Proteasome were normalized to background fluorescence, quantified using ImageJ package and analyzed using GraphPad Prism software (statistical significance: $^{**}P < 0.01$).

I278T variants, while steady-state levels of CBS WT were not affected (Fig. 3A). Although detection of a V5 tag attached to the introduced HSPA1A and HSF1 confirmed their overexpression, Western blot with HSP70 antibody failed to observe an increase in steady-state levels of HSP70, while HSF1 was clearly upregulated. More importantly, co-expression of HSPA1A and HSF1 resulted in a significant rescue of CBS R125Q activity: 5–7-fold increase compared to a nontransfected control along with recovery of CBS response to SAM stimulation (Fig. 3B). Specific activity of a rescued CBS R125Q variant corresponded to cca 10% of CBS WT levels. In case of CBS I278T, there was no rescue of enzymatic activity with co-expression of HSPA1A or HSF1. Taken together, our results suggest that stimulation of cellular refolding capacity may rescue expression, proper folding and, most importantly, activity of certain CBS pathogenic variants.

Impact of pharmacological modulation of proteostasis network on CBS rescue. As co-expression of HSPA1A or HSF1 did not rescue activity of CBS I278T (Fig. 3B), we continued only with a permissive CBS R125Q variant and evaluated pharmacological approach to rescue folding and activity of this variant. Specifically, we used eight compounds modulating several effectors and regulators of unfolded protein response (UPR) and protein homeostasis (Fig. 4). Generic experimental inducer of ER stress tunicamycin and inhibitor of cellular integrated stress response trans-ISRIB rescued expression of CBS R125Q variant the most (Fig. 4A). Celastrol, natural antioxidant, anti-inflammatory agent, inhibitor of TNFα-induced NF-κB activation and inducer of HSPs, as well as Dbeq, a potent, reversible inhibitor of p97 ATPase, which regulates both UPS and autophagic clearance system, resulted in a smaller rescue of CBS expression than tunicamycin or trans-ISRIB. The least effective were TRC051384, an HSF-1 activator and HSP70 expression inducer, and KIRA6, a potent IRE1alpha kinase inhibitor (Fig. 4A). Regardless of CBS protein recovery in a soluble fraction, all compounds rescued activity and SAM responsiveness of CBS R125Q variant to a similar extent: 3–12-fold and 12–15-fold in the absence and presence of SAM, respectively, compared to a

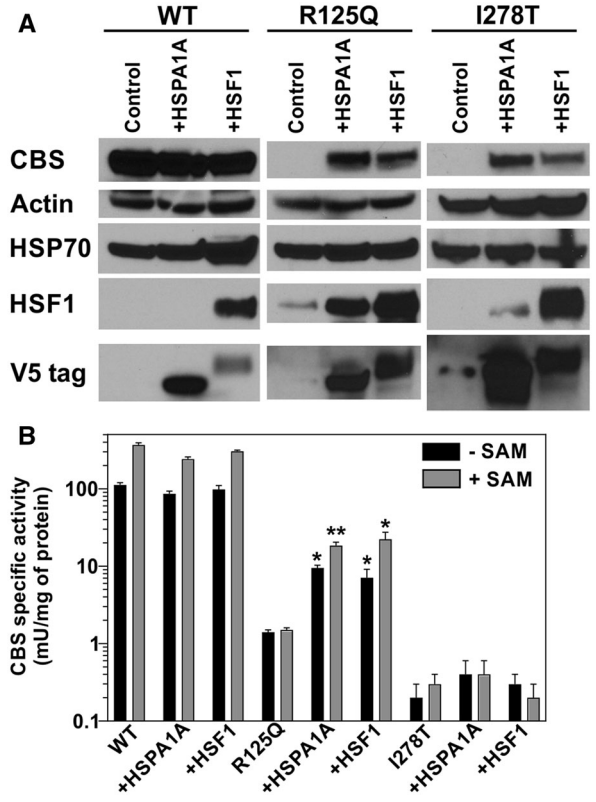

FIG 3 Partial rescue of CBS by co-expression of molecular chaperones. Western blot analysis (A) and CBS activity (B) in soluble fractions of A23 CBS WT, R125Q and I278T cells co-expressing either HSPA1A or HSF1 compared to mock-transfected controls. (A) Cell lysates (50 μg/lane) were resolved in 10% SDS-PAGE, transferred to PVDF membrane and probed with antibodies against CBS, actin, HSP70, HSF1 and V5 tag. (B) Cell lysates (150–500 μg) were analyzed by radiometric CBS activity assay in the absence and presence of 500 μM SAM. The results were compared to the respective mock-transfected controls and statistically analyzed. $^{*}P < 0.05$, $^{**}P < 0.01$.

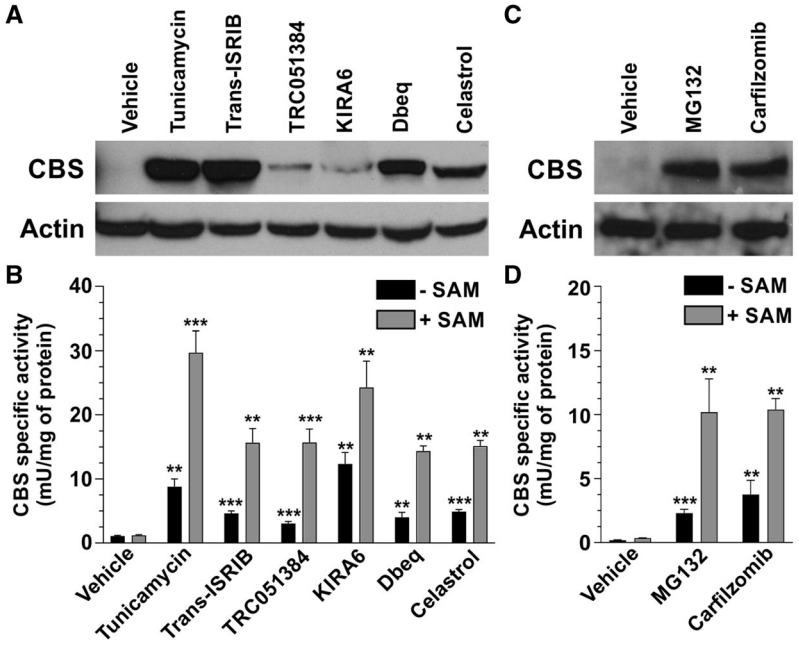

FIG 4 Rescue of CBS by pharmacological modulation of proteostasis network. Western blot analysis (A, C) and CBS activity (B, D) in soluble fractions of A23 CBS R125Q treated overnight with 0.5 μg/mL tunicamycin, 200 nM trans-ISRIB, 10 μM TRC051384, 5 μM KIRA6, 5 μM Dbeq or 5 μM celastrol (A, B) or after 8 h treatment with 20 μM MG132 or 20 μM carfilzomib (C, D). (A, C) Cell lysates (50 μg/lane) were resolved in 10% SDS-PAGE, transferred to PVDF membrane and probed with antibodies against CBS and actin used as a loading control. (B, D) Cell lysates (500 μg) were analyzed by radiometric CBS activity assay in the absence and presence of 500 μM SAM. The results were compared to vehicle-transfected controls and statistically analyzed. $^{**}P < 0.01$, $^{***}P < 0.001$.

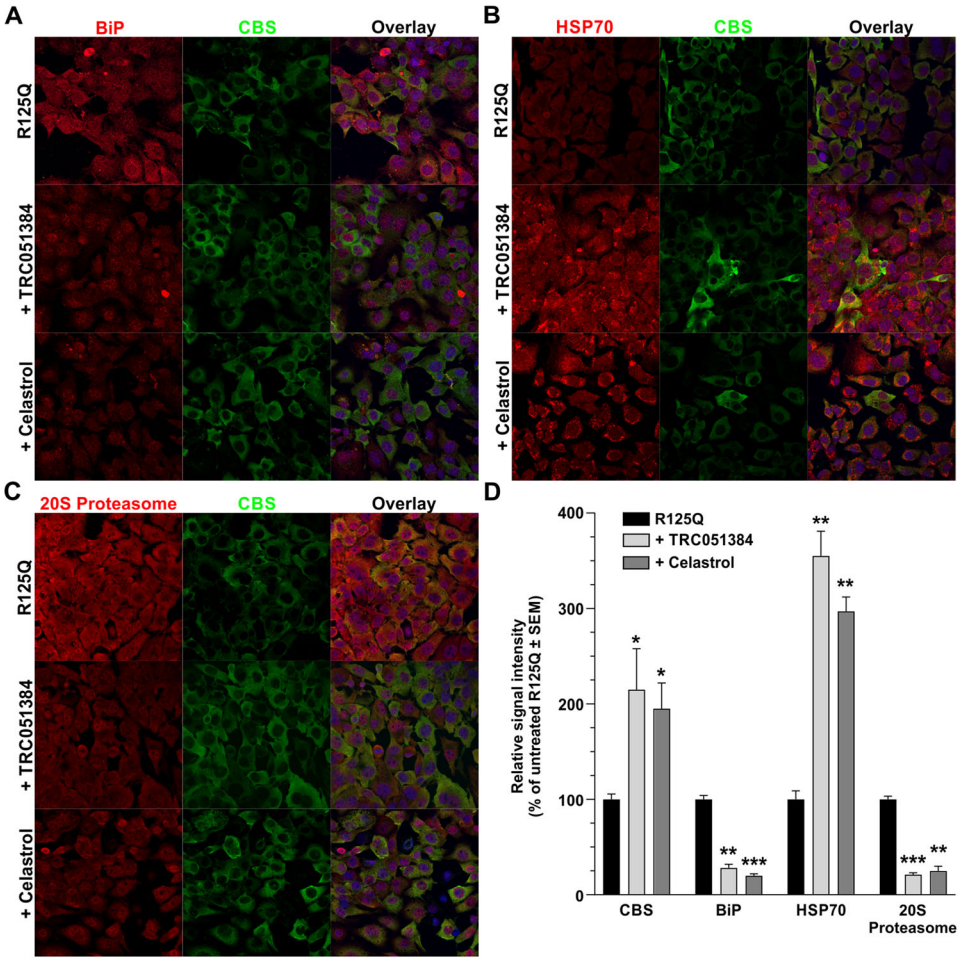

FIG 5 Pharmacological modulation of CBS refolding. The A23 CBS R125Q were grown on cell culture slides in the presence of vehicle only (top), 10 μM TRC051384 (middle) or 5 μM celastrol (bottom), fixed, permeabilized and probed for the indicated combination of targets as described in Materials and Methods section. Different effectors of proteostasis network are shown in red channel (A – BiP, B – HSP70 and C – 20S Proteasome), CBS in a green channel and nuclei in a blue channel (shown only in a composite image). (D) Fluorescent intensities for CBS, BiP, HSP70 and 20S Proteasome were normalized to background fluorescence, quantified using ImageJ package and analyzed using GraphPad Prism software (statistical significance: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

vehicle-treated control ($P < 0.01$; Fig. 4B). We also evaluated two proteasomal inhibitors MG132 and carfilzomib as inhibition of proteasome was found effective in transgenic HCU mouse models expressing different pathogenic human CBS alleles.[28] Treatment with both MG132 and carfilzomib rescued CBS R125Q expression as well as activity to a similar extent as previously mentioned compounds (Fig. 4C and D). Thus, our results suggest that pharmacological modulation of cellular proteostasis network by either promoting refolding or inhibiting degradation could lead to a reasonable rescue of CBS expression and activity.

Furthermore, we used confocal microscopy to visualize cellular response to pharmacological modulation of proteostasis network (Fig. 5). Specifically, we focused on promotion of refolding activities and thus used TRC051384 and celastrol as inducers of HSP70 response. Both compounds performed similarly and rescued expression of CBS R125Q variant by ~2-fold ($P < 0.05$; Fig. 5). Expression of BiP, which was strongly upregulated in control vehicle-treated CBS R125Q cells, decreased significantly by 72–80% ($P < 0.01$; Fig. 5A and D) after the treatment. As anticipated, TRC051384 and celastrol induced expression of HSP70 by ~3-fold ($P < 0.01$) compared to the controls with TRC051384 leading to a pan-cellular distribution, while celastrol resulted in more of a petechial HSP70 localization around nuclei (Fig. 5B and D). Promotion of CBS R125Q refolding by pharmacological treatment resulted in a downregulation of proteasomal

activity by 75–79% compared to vehicle-treated controls and lack of colocalization of CBS with proteasome ($P < 0.01$; Fig. 5C and D). Together, confocal microscopy analysis confirmed increased steady-state levels of CBS R125Q variant, decreased ER stress, increased cellular refolding capacity and decreased proteasomal localization of CBS R125Q in response to pharmacological modulation of proteostasis.

DISCUSSION

In the present study, we showed how expression of pathogenic CBS variants in a mammalian cell model of HCU disrupts cellular proteostasis network. Interestingly, our HSPs and chaperone gene expression analysis showed only a handful of genes differentially expressed in case of the most common pathogenic CBS I278T variant compared to CBS WT (Fig. 1). Two significantly upregulated genes were one of the isoforms of the main molecular chaperone HSP70 (HSPA1B) and a small HSP family member HSPB8. HSPB8 plays an important role in protein degradation, although it can also directly promote refolding of certain substrates.[29] HSPB8 recognizes misfolded proteins or aberrant peptides, recruits cochaperone BAG3 and directs its cargo towards autophagy degradation.[29,30] In this complex, HSPB8 is responsible for binding to misfolded proteins, whereas BAG3 likely activates autophagy machinery in proximity of the chaperone-loaded substrates. Although our expression analysis did not find upregulated BAG3, upregulation of HSPB8 in HCU may suggest that autophagy is also involved in rapid degradation of misfolded pathogenic CBS variants when the cellular refolding capacity is insufficient or UPS is being saturated by UPR. In contrast, the consequence of HSPB3 downregulation in HCU is less clear. HSPB3 is typically found upregulated upon proteotoxic stress and its oligomerization with another small chaperone HSPB2 negatively regulates its interaction with BAG3.[31] Thus, it is plausible that downregulation of HSPB3 makes BAG3 more available for complexing with HSPB8 and promotes chaperone-assisted selected autophagy of pathogenic CBS variants in HCU. Two members of the HSP40 family, DNAJC12 and DNAJB13, were downregulated in HCU model compared to WT control. HSP40 chaperones are essential in folding and refolding of misfolded proteins by stimulating the ATPase activity of HSP70, thus actually determining activity of HSP70s by stabilizing their interaction with substrate protein.[32,33] Downregulation of gene expression of these two HSP40 family members may suggest lower efficiency of refolding process in our cellular model of HCU. However, we did not observe any difference in total amount of HSP40 proteins in the HCU model compared to WT control. Lack of correlation between gene expression and protein levels for targets like HSP40 or HSP70 (Fig. 1) may be because of (i) differential protein vs. RNA turnover, (ii) no rate-limitation of mRNA levels with regard to protein synthesis, (iii) these proteins are part of multigene family, while antibody recognizes shared epitopes, or (iv) species differences as antibodies were raised against and validated for human, not Chinese hamster antigens. Interestingly, we found substantial upregulation of BiP, the ER UPR stress sensor,[34] which showed nuclear and cytoplasmic localization in A23 CBS I278T or R125Q cells compared to weak nuclear localization in A23 CBS WT cells (Fig. 2). BiP upregulation may actually represent cellular compensatory action to prevent UPR, because BiP acts as a suppressor of double-stranded RNA-dependent protein kinase-like ER (PERK), activating transcription factor 6 (ATF6) and inositol-requiring enzyme 1 (IRE1) signaling pathways.[34]

To increase cellular refolding capacity and thus to rescue pathogenic CBS variants, we either co-expressed HSP70 or HSF1 or pharmacologically modulated proteostasis and UPR (Fig. 3 to Fig. 5). Co-expression of the selected HSPs increased steady-state levels of CBS R125Q and CBS I278T, but enzymatic activity was rescued only in CBS R125Q to ~10% of CBS WT control. This is in contrast to published results showing that particularly CBS I278T allele is amenable to refolding and rescue. Specifically, CBS I278T was functionally rescued by proteasome inhibitor treatment when heterologously expressed in yeast, in human patient fibroblasts and transgenic HCU mice.[15,25,28] Blocking proteasomal degradation of all cellular proteins by itself was insufficient to restore activity of CBS I278T

and induction of UPR and specifically HSP70 expression was found required for bortezomib-induced rescue of function.[15] In our cellular model, we clearly expressed substantial amounts of HSPA1A, the main isoform of HSP70, as indicated by V5 tag detection; however, anti-HSP70 antibody did not show increased steady-state levels of cellular HSP70. This could be an issue of antibody specificity or this particular HSP70 isoform is not involved in CBS folding. Having said that, another pathogenic CBS R125Q variant was functionally rescued by both co-expression of HSPs and pharmacological treatment including proteasome inhibitors (Fig. 4). Pharmacological treatment using HSP70 inducers TRC051384 and celastrol indeed resulted in upregulation of HSP70 and substantial downregulation of BiP and 20S proteasome as shown by confocal microscopy (Fig. 5). Previous confocal analysis of two pathogenic CBS variants showed petechial localization for CBS T87N and homogeneous cytoplasmic distribution for CBS D234N.[35] Interestingly, distribution of CBS variants correlated with their residual activity: 4% and 42% for CBS T87N and D234N, respectively, suggesting that CBS misfolding affects cellular distribution/localization in addition to activity. We did not observe such difference in localization and rather saw only increased signal, i.e. amount of CBS antigen with the treatment.

If HCU is a conformational disorder and the treatment rescues folding of pathogenic CBS variants, why did we observe no and relatively low functional rescue of CBS I278T and R125Q variants, respectively, compared to CBS WT controls? From previous studies, we knew that both variants were responsive to refolding-promoting interventions: proteasome treatment for CBS I278T or pharmacological chaperoning effect of supplemental heme arginate in the case of CBS R125Q.[15,21,25,28] The reason may be a substantial activation of ER stress in our cellular HCU model due to proteotoxic effect of pathogenic CBS variant expression characterized by marked upregulation of BiP (Fig. 1, Fig. 2 and Fig. 5). Poor response to chemotherapy and proteasome inhibitor treatment in patients with diffuse large B-cell lymphoma was associated with upregulation of BiP and activation of ER stress (UPR).[36] Interestingly, BiP expression was decreased with treatment using pharmacological proteostasis modulators while increasing steady-state levels of CBS suggesting that there is indeed a negative correlation between CBS and BiP (Fig. 5). In addition, increased intracellular levels of homocysteine, a biochemical hallmark of CBS-deficient HCU, have been shown to cause ER stress and activation of UPR.[37,38] Furthermore, upregulation of BiP and ER stress may be specific to our cellular model, while other cells like yeast or HEK293 perhaps respond differently to expression of pathogenic CBS variants.[15,35] Lastly, other factors than HSP70 play a crucial role in efficacy of pathogenic CBS variant refolding. Although HSP70 is the main molecular chaperone responsible for CBS refolding, its activity is determined and regulated by other factors. For example, HSP40 determines activity of HSP70 by stimulating its ATPase activity.[32,33] As we found two members of the HSP40 family downregulated during expression of pathogenic CBS variant, upregulation of HSP40 could improve cellular CBS refolding capacity. Similarly, CBS refolding in yeast seems to be regulated by the balance between HSP70 and HSP26 steady-state levels.[13] HSP26 was found depleted during expression of CBS I278T variant compared to CBS WT control due to its association with misfolded CBS polypeptide with both polypeptides found ubiquitinated and thus destined for rapid proteasomal degradation.

In conclusion, here we demonstrated that expression and activity of pathogenic CBS variants can be rescued by genetic or pharmacological modulation of cellular proteostasis network toward promotion of refolding activities rather than by preventing protein degradation by proteasome inhibitors. Although effective, short-term high-dose proteasome inhibitor treatment is accompanied with substantial toxicity, while long-term low-dose treatment was found ineffective.[28] Therefore, pharmacological promotion of refolding by induction of HSPs could represent a feasible avenue for development of novel therapeutic approaches for HCU despite apparently complex interactions between various molecular effectors, whose role in CBS folding and HCU is still poorly understood.

MATERIALS AND METHODS

Cell culture. Chinese hamster DON lung fibroblast A23 cells naturally devoid of CBS activity were used as a mammalian host for overexpression of human CBS variants as described previously.[39] Stable cell lines for expression of CBS WT, R125Q and I278T variants were produced by transient transfection of A23 cells with the corresponding pcDNA3.1-based CBS constructs using Lipofectamine 2000 reagent (Invitrogen) and stable clones were bulk selected by passaging the cells using 1 mg/mL geneticin (Invitrogen) according to manufacturer's protocol. All cells were grown in a humidified incubator with 5% CO_2 atmosphere at 37°C using Minimum Essential Medium supplemented with 15% Fetal clone III serum, 100 μM nonessential amino acids, 100 μg/mL penicillin and 100 μg/mL streptomycin (all from Cytiva).

Mammalian plasmids overexpressing human HSPA1A and HSF1 were obtained from CCSB-Broad Lentiviral Expression Library,[40] propagated and isolated using EndoFree Plasmid Maxi Kit (Qiagen). A 30 μg of plasmid was transiently transfected into the corresponding A23 cells (100 mm dish, 70% confluency) using Lipofectamine 2000 reagent (Invitrogen) according to manufacturer's recommendations. After 48 h incubation, the cells were washed with PBS, harvested and either processed immediately or wet pellets were stored at −80°C.

For pharmacological modulation of proteostasis network, the A23 CBS R125Q cells were seeded into 100 mm dishes and cultured overnight to achieve ∼80% confluency. In the morning, medium was replaced for a fresh medium containing 0.5 μg/mL tunicamycin, 200 nM trans-ISRIB, 10 μM TRC051384, 5 μM KIRA6, 5 μM Dbeq or 5 μM celastrol and the cells were grown overnight. Treatment with 20 μM MG132 or 20 μM carfilzomib was carried out with ∼90% confluent cells and only for 8 h. At the end, cells were washed with PBS, harvested and cell pellets were stored at −80°C until further processing.

Expression analysis. Custom-designed RT2 PCR array (Qiagen) was used to evaluate expression of genes encoding selected HSPs and chaperones according to manufacturer's recommendations. The A23 cells stably expressing CBS WT or I278T variant at ∼70% confluency in T-75 flasks were harvested by trypsinization and total RNA was isolated using RNeasy mini kit following the standard protocol (Qiagen). The RT2 First Strand Kit (Qiagen) was used to obtain cDNA, which served as a template for real-time PCR using the array and SYBR green mastermix on Applied Biosystem's 7500 Fast real-time thermocycler. Differential expression from three independent biological replicates of control and test samples was calculated, compared and analyzed using manufacturer-provided Excel template (see Supplemental Data).

Protein expression was determined by Western blot analysis as described elsewhere.[14] Briefly, cell pellet was resuspended in cold 50 mM sodium phosphate pH7.0 containing 1% Triton X-100 and mammalian protease inhibitor cocktail (Sigma) in ratio 1:2 (w/v). Homogenous suspension was briefly (1–3 s) sonicated, incubated on ice for 20 min and spun down at 21,000*g* for 15 min at 4°C. Protein concentrations of the resulting supernatants were determined using Bradford assay (ThermoScientific). Typically, 50 μg of such cell lysates were separated on 10% or 4–12% TRIS-glycine gels (Biorad) and transferred onto the PVDF membrane (Biorad) using semi-dry apparatus. Antibodies (dilution used; manufacturer) used to probe the membranes are as follows: rabbit anti-CBS polyclonal (5,000×, lab-made), mouse anti-actin monoclonal (1,000×, Sigma cat#A4700), rabbit anti-HSP70 polyclonal (1,000×, Cell Signaling cat#4872), rabbit anti-HSP40 monoclonal (1,000×, Cell Signaling cat#4871), rabbit anti-HSF1 polyclonal (1,000×, Cell Signaling cat#4356), rabbit anti-BiP monoclonal (1,000×, CellSignaling cat#3177), mouse anti-20S proteasome subunit α type-1-6 monoclonal (1,000×, Enzo Life Sciences cat#BML-PW8195), mouse anti-V5 tag monoclonal (1,000×, Biorad cat#MCA1360GA). Anti-mouse or rabbit IgG secondary HRP conjugated antibodies (Jackson Immunoresearch cat# 111-035-003 and 115-035-003, respectively) and SuperSignal West Pico Plus chemiluminescent substrate (ThermoScientific) facilitated visualization of proteins on CL-Xposure films (ThermoScientific).

CBS activity assay. CBS activities were determined by radiometric assay as described elsewhere.[14] with the following modifications. Cell lysate (150–500 μg of total protein) was assayed in 100 mM TRIS.HCl pH8.6, 0.5 mg/mL BSA, 1 mM pyridoxal-5′-phosphate in the absence or presence of 500 μM SAM. After 10 min equilibration of the reaction mixture at 37°C, the reaction was initiated by addition of 7.5 mM L-serine and 10 mM L-homocysteine substrate mix containing C^{14}-labeled L-serine as a radiometric label and carried out for 4 h. Reactions were stopped by cooling down the assays on ice and cystathionine was separated from the substrates using paper chromatography (Whatman). Radioactivity was determined using automated counter (Beckman) and specific activity was calculated. One unit of CBS specific activity corresponds to 1 μmol of cystathionine produced by 1 mg of enzyme in 1 h at 37°C. CBS activity data were analyzed and plotted using GraphPad Prism. Statistical significance was designated by asterisks as follows: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

Confocal microscopy. The A23 CBS WT, I278T or R125Q cells were grown in a complete MEM media on eight-chamber cell culture slides (Falcon) to 70% confluency. Cells were then fixed with 4% paraformaldehyde for 10 min and permeabilized by methanol. For visualization and localization of desired targets, we used the following antibodies (dilution, manufacturer): mouse anti-CBS monoclonal (1,000×, Abnova) or rabbit anti-CBS polyclonal (2,500×, lab-made), rabbit anti-BiP monoclonal (200×, Cell Signaling), rabbit anti-HSP70 polyclonal (200×, Cell Signaling) and mouse anti-20S proteosome monoclonal (200×, Enzo Life Sciences). Appropriate anti-mouse and rabbit IgG secondary antibodies conjugated to either Atto 488 (Sigma) or Alexa Fluor 647 fluorophores (Molecular Probes) were used for immunostaining, while DAPI Fluoromont chemical stain (SouthernBiotech) was used to visualize nuclei. Slides were analyzed using Olympus FV-1000 microscope with Fluoview software for image acquisition. Images were normalized to background fluorescence, quantified using ImageJ package and analyzed

using GraphPad Prism software. Statistical significance was designated by asterisks as follows: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

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DATA AVAILABILITY STATEMENT

The authors confirm that the data supporting the findings of this study are available within the article. Supplemental data that support the reported findings of this study are openly available in Figshare (DOI: 10.6084/m9.figshare.24146337).

DISCLOSURE STATEMENT

No potential conflict of interest was reported by the author(s).

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