Mutations in the cystathionine β-synthase (CBS) gene are the cause of classical homocystinuria, the most common inborn error in sulfur metabolism. The p.G307S mutation is the most frequent cause of CBS deficiency in Ireland, which has the highest prevalence of CBS deficiency in Europe. Individuals homozygous for this mutation tend to be severely affected and are pyridoxine nonresponsive, but the molecular basis for the strong effects of this mutation is unclear. Here, we characterized a transgenic mouse model lacking endogenous Cbs and expressing human p.G307S CBS protein from a zinc-inducible metallothionein promoter (Tg-G307S Cbs−/−). Unlike mice expressing other mutant CBS alleles, the Tg-G307S transgene could not efficiently rescue neonatal lethality of Cbs−/− in a C57BL/6J background. In a C3H/HeJ background, zinc-induced Tg-G307S Cbs−/− mice expressed high levels of p.G307S CBS in the liver, and this protein variant forms multimers, similarly to mice expressing WT human CBS. However, the p.G307S enzyme had no detectable residual activity. Moreover, treating mice with proteasome inhibitors failed to significantly increase CBS-specific activity. These findings indicated that the G307S substitution likely affects catalytic function as opposed to causing a folding defect. Using molecular dynamics simulation techniques, we found that the G307S substitution likely impairs catalytic function by limiting the ability of the tyrosine at position 308 to assume the pyridoxal−cystathionine intermediate. These results indicate that the p.G307S CBS is stable but enzymatically inert and therefore unlikely to respond to chaperone-based therapy.

Cystathionine β-synthase (CBS) deficiency (MIM# 613381) is the most common inborn error of sulfur metabolism and is the cause of classical homocystinuria (1). It is a recessive disorder typically caused by missense mutations. The overall frequency of CBS deficiency in Western populations based on a meta-analysis of screening is about 0.5–1 per 100,000, but this can vary widely between countries due to the presence of specific founder mutations (2). CBS converts homocysteine to cystathionine employing serine as co-substrate and pyridoxal phosphate (PLP) as prosthetic group. CBS-deficient patients are characterized by extreme elevations in plasma total homocysteine (tHcy) and suffer a variety of pathologies, including thrombosis, osteoporosis, mental retardation, and dislocated lenses. Whereas over 164 different CBS mutations have been observed in patients, some alleles are more frequently seen in patients from specific populations. Ireland has a relatively high rate of CBS deficiency (1/65,000) primarily due to the expansion of one particular founder allele, c.919G>A, which results in a glycine to serine substitution at position 307 of the CBS protein (p.G307S) (3). p.G307S accounts for 71% of CBS mutations in Ireland, 21% in the United Kingdom, 18% in Australia, and about 7% of patients in the United States (4–6). It is rarely observed in other countries. No patients containing this allele have been reported to respond to pyridoxine (6–9).

Mammalian CBS is a 63-kDa protein that forms homotetramers and is expressed at high levels in liver and kidney. It has three functional domains: an N-terminal heme-binding domain, a middle catalytic domain, and a C-terminal regulatory domain (10). The role of heme in the enzyme appears to be primarily structural, although there is some evidence that it may be involved in redox regulation (11–13). The catalytic domain binds the co-factor PLP, which plays a key catalytic role in enzyme reaction (14), and the C-terminal domain binds S-adenosylmethionine (SAM), which acts as an allosteric activator of the enzyme (15). Deletion of the C-terminal domain results in a constitutively active enzyme that no longer responds to SAM (16). Significantly, a small deletion of eight amino acids in the regulatory domain results in a dimeric form of the enzyme that still responds to SAM activation. Importantly, dimeric CBS (but not tetrameric CBS) has been crystallized both in the presence and absence of SAM, and high resolution protein structures have been created (17–19).
addition, CBS structures from C-terminally deleted human CBS and Drosophila melanogaster CBS have also been determined (20).

Cell-based heterologous expression systems have been useful in understanding the effects of missense mutations on CBS enzymatic function. In these systems, many mutant CBS proteins have been shown to be responsive to various treatments that alter the protein-folding environment, including the addition of chemical chaperones and the manipulation of cellular chaperone proteins (21–24). However, cell-based systems do not always reflect the true in vivo situation. For example, the p.R266K and p.S466L mutations show high levels of enzymatic activity in cell-based heterologous expression systems but cause massive protein instability when expressed in vivo in mouse liver (25, 26). Recombinant human p.G307S CBS has been produced in several different heterologous expression systems, including Escherichia coli, Saccharomyces cerevisiae, and Chinese hamster ovary CHO-K1 cells (21, 23, 24). In almost all cases, the heterologous protein lacked enzyme activity. The one exception was a report from our laboratory showing that p.G307S CBS expressed in S. cerevisiae lacking the Hsp26 gene was enzymatically active (28). However, the behavior of p.G307S in a mouse model has not been explored.

To fully understand the in vivo consequences of p.G307S CBS, we have created a mouse (Tg-G307S CBS+/−) that lacks endogenous mouse CBS and expresses p.G307S under the control of the zinc-inducible mouse metallothionein (MT-I) promoter. In this paper, we describe and characterize these animals and use structural modeling and molecular dynamics simulation techniques to understand the effects of the mutation on enzyme structure and dynamics.

Results

Tg-G307S does not rescue neonatal lethality associated with CBS−/−

CBS knockout mice on a C57BL6 strain background show a high frequency of neonatal death, with >90% of the homozygous animals dead by 4 weeks of age due to liver damage (29). Previously, our laboratory has shown that this neonatal lethality can be rescued by the introduction of a transgene expressing either WT or certain mutant human CBS alleles. For this study, we constructed a vector that contains a hemagglutinin-tagged p.G307S CBS encoding cDNA downstream of the mouse MT-I promoter flanked by two “locus control regions” that help alleviate position effect differences in expression. This construct is identical (except for the mutation) to our earlier constructs (30). The construct was injected into C57BL6/C3H F2 embryos. From 44 offspring, we obtained eight transgene-positive founders. All of these founders (3, 4, 13, 14, 23, 26, 29, and 36) were then crossed to WT C57BL6 to determine whether the transgene was germline-transmissible. Transgene-positive offspring were obtained from all but one of the lines, and the progeny of four lines (4, 13, 26, and 36) was then assessed for zinc-inducible transgene expression. Immunoblot analysis of the liver indicated that three lines expressed the transgene (13, 26, and 36), whereas one line did not (Fig. 1A). Of the expressing lines, line 36 had the highest level of expression, whereas line 26 had very low expression.

Mice from the three expressing lines were then tested to see whether they could suppress the neonatal lethality phenotype. Suppression of neonatal lethality was observed previously in CBS−/− mice that express that p.I278T or p.S466L alleles (25, 31). Mice were first crossed with CBS+/− mice (C57BL6) to obtain Tg-G307S CBS+/− mice, and these mice were then intercrossed to generate Tg-G307S CBS−/− mice. For all three lines, the total distribution of CBS+/+, CBS+/−, and CBS−/− offspring observed at 10 days of age was not significantly different from the expected number (Table 1). However, in all three lines we observed that the vast majority of Tg-G307S CBS−/− offspring died either before weaning (around 30 days of age) or slightly after weaning (Table 2). This was true whether or not the offspring contained the transgene, implying that the transgene was not able to rescue the neonatal lethality associated with loss of CBS.

The line (36) that showed the highest rates of Tg-G307S CBS−/− survivors (37.5%) also had a high percentage of brown offspring, suggesting that this line had more C3H genetic material than the other two lines. This is possible because the injected embryos are F2, C3H/C57BL6 hybrids and thus may vary in their percentage of C3H and C57BL6 content. Because it has been previously shown that CBS−/− on a C3H background has a higher survival (32), we decided to backcross this line three more times either to C3H or C57BL6 and compared neonatal survival rates. After three rounds of backcrossing, we found significantly higher survival on the C3H background, with 60% (39/60) Tg-G307S CBS−/− animals surviving, compared with only 23% (6/26) on the BL6 background (p = 0.0008). These studies confirm that neonatal lethality associated with CBS−/− is significantly reduced on the C3H background. We used animals from the 36 line for the experiments described below.

p.G307S expression and activity

We first measured human CBS mRNA levels in livers of zinc-treated Tg-G307S CBS−/− mice and Tg-hCBS CBS−/− mice and found that levels of induction were similar between the two lines (Fig. 1B). With regard to liver CBS protein levels, female zinc-treated Tg-G307S CBS−/− mice had CBS protein levels that were very similar to the amount observed in female Tg-hCBS CBS−/− mice (Fig. 1C). Male Tg-G307S mice showed more variable expression with some mice having good induction and others having poor induction. On native gels, p.G307S efficiently form multimers, although they run a bit slower than observed for WT hCBS. Enzyme activity, however, was undetectable, <0.1% of that observed for WT hCBS.

Consistent with the lack of enzyme activity, we observed that Tg-G307S CBS−/− mice had mean serum tHcy of 279 ± 54 μM (n = 8), which is about a 100-fold increase from that observed in Tg-G307S CBS+/+ or Tg-G307S CBS+/− animals (2.9 μM, n = 4, p < 0.0001). Mean serum methionine was 95 ± 36 μM (n = 8), not significantly different from that found in control animals (82 μM, n = 4, p = not significant). At 33–35 days of age (just after weaning), the surviving Tg-G307S CBS−/− mice are
In previous work, our laboratory has shown that it was possible to restore function of certain CBS point mutations in mice by treatment with the proteasome inhibitors bortezomib and oprozomib (34). The mechanism of rescue is thought to involve the induction of molecular chaperone proteins, which can promote the proper folding of mutant CBS protein (28). We performed two different experiments in which we examined the effect of these compounds on Tg-G307S CBS−/− mice. In the first experiment, six Tg-G307S CBS−/− mice were surgically implanted with an osmotic pump that delivered bortezomib at a dose of 0.49 mg/kg/day (Fig. 3A). After 7 days, the mice were euthanized, and blood and liver were collected for analysis.

Most of the treated mice showed increased levels of steady-state CBS protein but had either no or miniscule levels of liver CBS activity. Serum tHcy in bortezomib-treated mice was similar to that observed in vehicle-treated animals. As a positive control for these experiments, we also treated two Tg-R266K CBS−/− (26) and one Tg-I278T CBS−/− mouse (31), and all three mice showed high levels of liver CBS activity and low tHcy, similar to that observed in zinc-induced untreated Tg-hCBS CBS−/− mice that express WT hCBS.

In the second experiment, we treated three Tg-G307S CBS−/− mice with the oral proteasome inhibitor oprozomib at a dose of 40 mg/kg/day for 4 days (Fig. 3B). For this experiment, we included three oprozomib-treated Tg-R266K CBS−/− mice as positive controls, and three mock-treated Tg-R266K CBS−/− and Tg-G307S CBS−/− animals as negative controls. Again, we failed to see any evidence of rescue with the Tg-G307S CBS−/− mice, but we saw a good response in two of the three of the Tg-R266K CBS−/− animals. Taken together, these two experiments indicated that human G307S is not rescuable by proteasome inhibitors.

**G307S molecular modeling**

To gain some structural insight into the effect of the G307S alteration, we examined the available experimental structures of human and *D. melanogaster* CBS (18–20, 35–38). There are a total of nine PDB entries of human CBS (1JBQ, 1M54, 4COO,
Analysis of p.G307S CBS

Table 2

| Cbs\(^{-/}\) survival | Tg + Cbs\(^{-/}\) (survivors/total) | Tg + Cbs\(^{-/}\) (% survival) | Tg - Cbs\(^{-/}\) (survivors/total) | Tg - Cbs\(^{-/}\) (% survival) | p value (Tg+ vs. Tg-) |
|----------------------|------------------------------------|-------------------------------|------------------------------------|-------------------------------|-----------------------|
| Line no.             |                                    |                               |                                    |                               |                       |
| 13                   | 1/14                               | 7.1%                          | 1/3                                | 33%                           | 0.08                  |
| 26                   | 1/5                                | 20%                           | 1/7                                | 14.3%                         | 0.79                  |
| 36                   | 9/24                               | 37.5%                         | 1/7                                | 14.3%                         | 0.49                  |

Figure 2. Comparison of Tg-G307S Cbs\(^{-/}\) and Tg-G307S Cbs\(^{+/}\) sex-and age-matched littermates. CBS genotype, age, and weight of each sibling pair are shown.

4L0D, 4L27, 4L28, 4L3V, 4PCU, and 5MMS) and three PDB entries of Drosophila CBS (3PC2, 3PC3, and 3PC4). Each structure contains the common dimer found in this family of PLP-dependent enzymes (39). The dimers found in the human structures are shown in Fig. 4A. We compared the conformations of the segment around Gly-307 in these structures and observed that the most significant variations among them are the backbone conformations of residues 307 and 308 and the rotamer of the tyrosine side chain at position 308 (Tyr-308). Chain B of the 4PCU structure has a different backbone conformation for residues 307 and 308 in which the peptide group connecting these two residues is flipped over (gray in Fig. 4B) relative to other structures of CBS. In addition, Tyr-308 occurs in the gauche-minus state (\(\chi_1\) dihedral angles \(\sim -60^\circ\), bright yellow in Fig. 4B) in most of the structures but in the trans rotamer (\(\chi_1\) dihedral angle \(\sim 180^\circ\), cyan in Fig. 4B) in 1JBQ and 1M54. The Drosophila structure 3PC4 contains PLP (magenta in Fig. 4B) with a covalently attached serine substrate (PLP-Ser), which is an intermediate in the enzyme reaction. In this structure, the Tyr-308 side chain in the gauche-minus conformation is within hydrogen-bonding distance of the serine side chain of PLP-Ser (distance 2.8 Å), suggesting an important role for this residue in the activation of the PLP-Ser hydroxyl (OG) for a dehydratase step and an attack of homocysteine (dashed line in Fig. 4B). The flipped backbone conformation in 4PCU chain B does not allow an interaction between residue Tyr-308 and the serine substrate (distance 6.1 Å), whereas Tyr-308 in the trans position also cannot make this interaction (distance 7.4 Å).

We modeled the next step in the reaction pathway by adding homocysteine to the PLP-serine intermediate found in PDB structure 3PC3, as described under “Materials and methods.” With the resulting external aldimine of cystathionine, we created an ensemble of 2,000 conformations generated by the Omega2 program (40). Using the covalently bound PLP of CBS as a molecule for alignment, we replaced it with the various conformers in the same location. We then screened the 2,000 aldimine conformers for their binding energy by calculating the energy of the bound complex and subtracting the energy of unbound CBS. We scored these structures without minimization. A feasible placement for the external aldimine of cystathionine is a cavity created by the movement of the Tyr-308 side chain from gauche-minus (Fig. 5A) to trans (Fig. 5B). Otherwise, there is a severe clash with Tyr-308 and surrounding protein atoms. We hypothesize that the position of Tyr-308 and its ability to flip between the two positions, gauche-minus and trans, is required for the enzyme reaction to proceed.

Based on these observations, we hypothesized two possible mechanisms by which G307S could eliminate CBS catalytic function. First, G307S could cause the protein backbone to “flip” (like the 4PCU-B structure), resulting in Tyr-308 that is entirely out of position to interact with the Ser residue of the external aldimine. Second, the G307S mutation might hinder the ability of the Tyr-308 to move from the gauche-minus to the trans rotamer. To explore these possibilities, we performed molecular dynamics simulations of both the WT human CBS and G307S CBS by modeling the serine side chain at position 307 in both the A and B chains of PDB entry 4COO (see “Materials and methods”). It should be noted that there is a slight asymmetry between the two chains, which means they will not behave identically in the simulations. We performed 220 ns of simulation each for the WT and mutant homodimers: ns of equilibration of the system followed by 200 ns of production simulation. After the equilibration phase, the simulations were relatively stable as demonstrated by RMSD changes from the starting structure (Fig. S1). Fluctuations of the structure from a mean value (RMSF) for each chain were calculated with the program Theseus and are shown in Fig. 6. Besides the C terminus and a long loop around position 60, the region around residue 307 is the most dynamic in both the WT and mutant simulations.

We analyzed the simulations for differences in conformation in the vicinity of residue 307. Such conformational changes can be identified by monitoring the dihedral angles of these residues. In Fig. 7A, the backbone dihedral angles \(\phi\) and \(\psi\) are plotted against each other in the standard Ramachandran plot for residues 305–310. Each point is a different snapshot in the A and B chains in the wildtype (WT) and G307S homodimer simulations. In chain A, there is a noticeable shift in the dihedral angles of both residues 307 and 308 when comparing the WT and G307S simulations. In chain B, the changes are localized to residues 306 and 307. In both cases, the \(\psi\) dihedral of the first residue in each pair is altered as is the \(\phi\) of the next residue. This is typical of backbone flips in protein structures (39). In the simulation of the mutant dimer, chain A has flipped during the equilibration phase from the conformation seen in the WT protein (Gly-307: \(\phi,\psi = 85^\circ,147^\circ\); Tyr-308: \(\phi,\psi = -153^\circ,166^\circ\)) to another conformation that is more consistent with conformations typically seen for serine residues (Ser-307: \(\phi,\psi = 100^\circ,20^\circ\); Tyr-308: \(\phi,\psi = -80^\circ,150^\circ\)). This is close to the conformation
observed in chain B of the 4PCU structure (Gly-307: H9278/H9274/H11005, 106°, H11002 11°; Tyr-308: H9278/H9274/H11005/H11002 52°, 156°, see Fig. 4B). This flipped conformation of the mutant is relatively stable, although it occasionally moves back to the starting conformation. Similarly, the WT protein occasionally flips but is predominantly in the commonly observed conformation present in the starting structure. This is clear in plots of H9278 and H9274 as a function of time for these residues during the 220-ns simulation (Figs. S2 and S3). In the B chain, the change in conformation occurs in H9274 of residue 306 and H9278 of residue 307 (Fig. 7A).

We examined the behavior of the Tyr-308 side chain during the simulations. Kernel density estimates of φ,ψ during the WT and mutant simulations shows that the conformation of the side chain is relatively stable in both the A and B chains (Fig. 7B). The χ1 dihedral of Tyr-308 in the WT protein simulation at ∼−80° is distorted from the typical rotameric value (−60°) observed in the simulations of the mutant protein. This is due to the φ,ψ values of Tyr-308 in the WT protein, which disfavor and therefore distort the gauche-minus rotamer, as observed in analysis of the backbone-dependent preferences of side-chain conformations in proteins (41, 42).

Finally, in Fig. 8, we show superposed, representative structures of chain A of the WT and mutant simulations, demonstrating the position of Tyr-308 with respect to the active site PLP. As shown in Fig. 8, Tyr-308 in the mutant simulation has moved further away and is rotated relative to the PLP compared with WT. This would be consistent with the idea that Tyr-308 would no longer be in position to interact with the PLP-serine intermediate.

Discussion

In this report, we describe the creation and characterization of a mouse model for the p.G307S mutation and used computational modeling to explain its behavior. The mouse model expresses human p.G307S protein from a zinc-inducible promoter in a background devoid of endogenous mouse Cbs. Our key findings are as follows: 1) p.G307S expression is unable to rescue the neonatal lethality associated with being homozygous null for Cbs in the C57BL6 background; 2) p.G307S CBS appears to be stable and forms multimers but has minimal residual enzyme activity; 3) p.G307S cannot be rescued by treatment with proteasome inhibitors; and 4) a molecular dynamics simulation of p.G307S suggests that this mutation affects the position and orientation of a key tyrosine at position 308 thus hindering the CBS enzyme reaction. We will discuss each of these four points below.

Cbs−/− animals on a C57BL6/6J background were present in litters at the expected Mendelian ratios at 10–14 days, but >80% of these animals died by 30 days of age (29). Our laboratory previously showed that not only could WT human CBS rescue this neonatal lethality, but so could two patient-derived
missense alleles (p.I278T and p.S466L) (25, 31). However, more recently we found that another patient-derived mutant transgene, p.R266K, could not rescue the lethality (26), and here we report that p.G307S could not as well. Neonatal lethality does not seem to be associated with residual enzyme activity in the various mutants. Although p.G307S is the most severely impaired, the residual enzyme activity observed in p.R266K is in the same range as that observed for the p.I278T and p.S466L mutations. In addition, our laboratory has not observed any difference in tHcy levels in 18-day-old mice from rescued and nonrescuable strains.3 We have previously speculated that the difference in neonatal lethality may actually reflect an additional “moonlighting” function that is disrupted by some mutations but not others (31), but to date no such function has been found. Fortunately, we were able to circumvent the neonatal lethality issue by backcrossing into the C3H background, allowing us to generate sufficient numbers of adult Tg-G307S Cbs<sup>−/−</sup> mice to study.

In mouse liver lysates, p.G307S CBS appears to be quite stable, forms multimers similar to WT CBS, but is enzymatically dead. This contrasts with the three other patient-derived missense alleles that have been expressed in mice. Lysates made from p.I278T, p.S466L, and p.R266K mice all exhibited low levels of CBS protein compared with mice expressing WT hCBS and had detectable levels of residual CBS activity (25, 26, 31). Although unstable, two of these alleles (p.R266K and p.S466L) showed proper multimer formation on native gels, whereas one (p.I278T) exhibited aggregation and smearing. Interestingly, p.I278T and p.R266K are known to be associated with clinical response to pyridoxine in humans, but the p.G307S allele is not pyridoxine responsive.

Mice expressing p.S466L, p.I278T, and p.R266K CBS proteins all had significant tHcy lowering and restoration of enzyme function when treated with proteasome inhibitors (26, 34). Previous work from our laboratory and others suggests that most missense CBS mutations affect protein stability and that treatment of these proteins with agents that promote proper folding (i.e. chaperones) can in many cases increase residual enzyme activity (21–24). The process of protein folding may be thought of as an equilibrium between properly folded (func-

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Figure 4. A, structural superposition of structures of the human CBS homodimer. PLP is shown as magenta sticks; heme is shown as red sticks, and the backbones of residues 307 and 308 adjacent to the active site are shown in green and yellow, respectively. B, close-up of the active site of CBS. Residues 304–310 are shown as sticks. PLP and its analogues are shown in magenta. Atoms are colored by element type (oxygen = red, nitrogen = blue, phosphorus = orange, and carbon = different colors for each residue). The most common backbone conformation for this segment places Tyr-308 into two different rotamers: gauche-minus in yellow and trans in cyan. A hydrogen bond (black dashed line) exists between the hydroxyl of Tyr-308 and the hydroxyl attached to the γ-carbon (OG) of serine-PLP in PDB entry 3PC4. An alternative backbone conformation of these residues from PDB entry 4PCU chain B is shown as gray sticks; this conformation places Tyr-308 in a different position from the other structures.

Figure 5. Modeling of the external aldimine of cystathione in CBS enzyme. A, Tyr-308 in the gauche-minus conformation (yellow sticks) occupies volume required for the homocysteine moiety in the E-Cyst model. The empty volume in the absence of the external aldimine of cystathione is shown in a gray wire frame. The E-Cyst ligand does not fit into this volume when Tyr-308 is in the gauche-minus position. B, E-Cyst ligand fits into this volume when Tyr-308 is in the trans position (cyan sticks).

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3 S. Gupta and W. Kruger, unpublished data.
**Figure 6.** Fluctuations of the main chain in molecular dynamics simulations along the sequence of CBS. Multiple structural alignments of 2,000 frames of chains A and B from each simulation were performed separately with the program Theseus. The values of RMSF represent root-mean-square deviations of C-α atoms from the mean structure calculated by Theseus.

**Figure 7.** A, analysis of molecular dynamics simulations of the human CBS homodimer of the enzyme domain. Ramachandran plots for residues 305–310 for chains A and B of simulations of the wildtype (WT, magenta) and mutant (MUT, blue) homodimers. The A chain of mutant dimer exists predominantly in a flipped state at residues 307–308, whereas the WT protein is predominantly unflipped (similar to the starting conformation and most structures of human CBS). The B chain of mutant dimer exists predominantly in two flipped states at residues 306–307, whereas the WT protein is entirely in an unflipped conformation (similar to the starting conformation and most structures of human CBS). B, Kernel density estimates of the χ₁ dihedral of Tyr-308 from the WT and mutant simulations.
Analysis of p.G307S CBS

Unlike most other CBS mutations, it does not seem to affect protein folding or stability, and it is highly unlikely that G307S is resucliable by chaperone treatment strategy. These findings imply that other avenues of treatment such as enzyme replacement or gene therapy should be explored.

Materials and methods

DNA constructs and mouse generation

All nucleotide numbering is based on the GenBank™ RefSeq NM_000071.1. The DNA mutation numbering system is based on the cDNA with +1 corresponding to the A or the ATG translation initiation codon in the reference sequence. Codon 1 is the initiator in the protein sequence. Site-directed mutagenesis was used to introduce a c.919G>A (p.G307S) change into pLW2, which contains a hemagglutinin epitope-tagged version of the human CBS cDNA (30) using the Quick Change XL site-directed mutagenesis kit from Stratagene (La Jolla, CA). The entire ORF of the resulting clone, pLW2:G307S, was sequenced to verify no additional changes occurred due to the mutagenesis process. Plasmid pLW2:G307S was subsequently digested with MfeI and cloned into the EcoRI site of MT-LCR expression vector 2999 (45). The resulting plasmid was designated pLW3:G307S.

Transgenic mice containing pLW3:G307S were created as described previously (30). Approximately 70 injected C57BL/6/C3H F2 embryos were then transferred to pseudopregnant mice, which resulted in the birth of 44 pups. Tails from the resulting pups were then screened for the presence of the transgene by PCR as described. To create Tg-G307S Cbs+/− mice, transgene-positive offspring were bred to C57BL/6J Cbs−/− mice for at least three generations before again intercrossing. Mice were fed standard rodent chow (Teklad 2018SX) containing 0.6% methionine by weight. All animal protocols were approved by the Fox Chase Cancer Center IACUC.

RNA, protein, and metabolite analysis

RNA was extracted from livers using TRIzol (Life Technologies, Inc.) followed by cleanup using Qiagen RNeasy mini kit. Gene expression analysis was done using TaqMan probes for human CBS (Hs00163925_m1, Applied Biosystems) and mouse β-actin (Mm01205647_g1) as described previously (26). Each sample was assayed in duplicate for both probes, and averages were used for statistical analysis. Relative signal strength was calculated using the ΔΔCt method (46).

CBS Western blotting was performed as described previously (26, 31, 47). For native Western blotting, samples were run at 4 °C under nondenatured conditions employing 4–12% Tris-glycine gels (Novex, Life Technologies, Inc.). CBS activity was analyzed in the presence of SAM (250 μM) as described previously (30). Reactions contained 30 μg of dialyzed liver extracts, 5 mM L-serine, 10 mM DL-homocysteine, and 50 μM pyridoxal.
phosphate in a 50-μl volume. One unit of activity is defined as nanomoles of cystathionine formed per mg of protein/h.

Serum tHcy and methionine were measured using a Biochrom 30 amino acid analyzer as described previously (30). The sum total of all forms of homocysteine, including protein-bound, free-reduced, and free-oxidized, is referred to as tHcy.

**Proteasome inhibitor studies**

Bortezomib (Velcade, Millennium Pharmaceuticals, Cambridge, MA) was obtained from the pharmacy at Fox Chase Cancer Center. Adult Tg-G307S Cbs−/− mice were subcutaneously implanted with Alzet microosmotic pumps (model 2001; pumping rate 1 μl/h) containing bortezomib diluted in 0.9% NaCl to deliver a final dose of 0.49 mg/kg/day. Solutions were on zinc water 11 days before the pump implant and throughout the duration of drug studies. Mice were sacrificed after 2 days on pump to harvest liver and serum.

ONX0912 was obtained from Onyx Pharmaceuticals. ONX0912 was administered as a suspension in 1% (w/v) carboxymethylcellulose in water to deliver a final dose of 40 mg/kg/day. Solutions were made fresh each day. Each animal was dosed once per day by oral gavage. Animals were dosed for 4 days in a row and were on zinc water 11 days before the dosing and during the dosing to keep the transgenic protein expressed. After the final dose, animals were sacrificed, and tissues were harvested 6 h after last dose.

**Modeling CBS bound to the external aldimine of cystathionine**

The CBS enzyme containing the external aldimine of cystathionine (E-Cyst), as described previously (14), was modeled in the following manner. An L-homocysteine molecule from PDB entry 1JVI was joined covalently to the aminoacylate intermediate in the structure of Drosophila CBS from PDB entry 3PC3 (20), and by visual inspection, the homocysteine portion was placed where it made the fewest clashes with active-site residues. Clashes were assessed within the human CBS active site with the UCSF Chimera software (48), after superposition of the enzymatic domain of human CBS in PDB entry 4L3V (19) with the Drosophila structure in 3PC3. Most clashes of the homocysteine portion of the E-Cyst ligand were with either Tyr-308 or Tyr-223. Clash reduction was performed by energy minimization of only the ligand atoms while holding the protein atoms fixed, including side-chain rotamers. The minimization was done with the Antechamber setup within Chimera, after assigning partial charges with the Gasteiger method, and using the AMBER ff14SB force field. Subsequently, 2,000 other low energy conformers of the E-Cyst ligand were generated with Open Eye Omega software (40) for additional minimization of clashes.

To examine the effect of the G307S mutation on the position and conformation of Tyr-308, we performed molecular dynamics simulations of both the WT dimer of human CBS from PDB entry 4COO and the mutant dimer created by modeling the serine side chain at position 307 in both monomers of 4COO. The 4COO structure was chosen because it is the highest resolution structure of human CBS (2.0 Å) and contains PLP. The asymmetric unit of 4COO consists of a biological dimer of CBS with two monomers with slightly different structures. The simulations were performed on a homodimer consisting of residues 43–401, i.e. without the presence of the C-terminal regulatory domains. We included the lysine-linked PLP in the active site and the heme prosthetic group (see below). We computed molecular dynamics simulations in Gromacs version 5.1.2 (49) with the Charmm36 (50) all-atom force field and explicit solvent using the SPCE water model (51). We added 0.15 mM Na+ and Cl− to neutralize the system. We equilibrated the system with a 10-ns NVT simulation, followed by a 10-ns NPT simulation for a total of 20 ns of equilibration time. Production runs were carried out for 200 ns for both the mutant and the WT dimer structures after confirming convergence of the energy, temperature, and RMSD of the equilibrated structures.

To parameterize PLP covalently linked to Lys-119, we first built a model of PLP attached to lysine from the structure in PDB entry 4COO. This model included all side-chain carbons of the lysine and the ω-nitrogen of lysine, which was then linked to PLP. From this structure, we added hydrogens using PyMOL and adjusted the geometry of planar groups manually. With this model of the lysine-linked PLP, we generated parameters for PLP using the CHARMM utility CgenFF (https://cgenff.paramchem.org),4 version 4.0.1 (52, 53). After generating CgenFF parameters, we built hybrid lysine backbone-PLP parameters by selectively copying CgenFF parameters from the native CgenFF force field and converting them to CHARMM36 force field parameters by matching the atom types for the backbone atoms. We used the Python script cgenff_charmm2gmx.py provided by the CgenFF site to build the .itp and .prm files necessary for Gromacs input. We built a topology for a new amino acid type, named “LYP,” into Gromacs by adjusting the rtp.itp file in Gromacs version 5.1.2. We used the native support in CHARMM36 to include the heme. All of the files and parameters used in the described simulations are in the supporting information.

For calculations on simulated structures, we used a variety of scripts and software to measure dihedrals, distance from TYROH to SEROG, and the RMSD from the reference structure to all simulated frames. For dihedrals, we used an in-house script to calculate backbone and side-chain dihedral angles. To calculate the distance from the TYROH–SEROG, we aligned the two structures using the THESEUS program, which performs a maximum likelihood superposition (27). We used chain A of the reference 3PC4 structure to do the alignments to both the simulated chain A and chain B for all simulated frames, and then we calculated the OH–OG distances as the Euclidean distance. Similarly, for the RMSD calculations, we used THESEUS to align all simulated frames for the A and B chain to the reference structure 4COO.

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