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Analysis of Differential Neonatal Lethality in Cystathionine β-Synthase Deficient Mouse Models using Metabolic Profiling

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Abstract

Cystathionine beta-synthase (CBS) is a key enzyme of the transsulfuration pathway that converts homocysteine to cystathionine. Loss of CBS activity due to mutation results in CBS deficiency, an inborn error of metabolism characterized by extreme elevation of plasma total homocysteine (tHcy). C57BL6 mice containing either a homozygous null mutation in the cystathionine β-synthase (*Cbs*$^{-/-}$) gene or express an inactive human CBS protein (*Tg-G307S Cbs*$^{-/-}$) are born in mendelian numbers, but the vast majority die between 18 and 21 days of age due to liver failure. However, adult *Cbs* null mice that express a hypomorphic allele of human CBS as a transgene (*Tg-I278T Cbs*$^{-/-}$) show almost no neonatal lethality despite having serum tHcy levels similar to mice with no CBS activity. Here, we characterize liver and serum metabolites in neonatal *Cbs*$^{+/-}$, *Tg-G307S Cbs*$^{-/-}$ and *Tg-I278T Cbs*$^{-/-}$ mice at 6, 10, and 17 days of age to understand this difference. In serum, we observe similar elevations in tHcy in both *Tg-G307S Cbs*$^{-/-}$ and *Tg-I278T Cbs*$^{-/-}$ compared to control animals, but methionine is much more severely elevated in *Tg-G307S Cbs*$^{-/-}$ mice. Large scale metabolomic analysis of liver tissue confirms that both methionine and methionine-sulfoxide are significantly more elevated in *Tg-G307S Cbs*$^{-/-}$ animals, along with significant differences in several other metabolites including hexoses, amino acids, other amines, lipids, and carboxylic acids. Our data is consistent with a model that the neonatal lethality observed in *CBS*-null mice is driven by excess methionine resulting in increased stress on a variety of related pathways including the urea cycle, TCA cycle, gluconeogenesis, and phosphatidylcholine biosynthesis.

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Author Contributions

SG, LW, KNM, KQT, and BW performed experiments; WDK, SG, and MJS analyzed the data; SG, WDK, TB designed experiments; WDK, SG, TB, and KNM contributed to writing the manuscript. WDK is the guarantor of the work and as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Conflict of Interest Statement

The authors declare there are no conflicts of interest in connection with this article.

Keywords

Inborn error; homocysteine; methionine; metabolomics; mouse models

Introduction

The sulfur containing amino acid methionine (Met) is an essential dietary amino acid that plays a key role in protein synthesis, biological methylation reactions, and polyamine biosynthesis. In mammals, Met is an essential dietary amino acid, but cysteine is not (1). This is because Met can be converted to cysteine via the action of the irreversible cystathionine β-synthase (CBS) enzyme, which produces cystathionine from serine and methionine-derived homocysteine. (Fig 1). In humans, biallelic loss of function mutations in the CBS gene result in clinical CBS deficiency (or classical homocystinuria), which is characterized by ten to twenty-fold increases in plasma total homocysteine (tHcy) and Met (2). Human CBS deficiency affects a variety of organ systems including the visual, skeletal, nervous, and vascular. Specific phenotypes include dislocated lenses, osteoporosis, behavioral abnormalities, and thrombosis of major and minor vessels. Treatment of human CBS deficiency is based on lowering plasma homocysteine. CBS deficient patients on tHcy lowering treatments do significantly better than those that are not (3–7). The earlier the treatment is instituted, the better the outcome. The vast majority of disease-causing human CBS mutations are missense mutations, which often result in proteins that are unstable and degraded (8). The most common human CBS allele in European populations is p.I278T, with an allele frequency as high as 1% (9). This allele is associated with pyridoxine responsiveness in humans, but does not appear to be pyridoxine responsive when expressed in either mice, yeast, or bacteria (10). Another common allele is p.G307S, which is associated with pyridoxine non-responsive disease and does not have residual activity in heterologous expression systems (11, 12).

Unlike humans, mice homozygous for a null allele of CBS (*Cbs*$^{-/-}$) tend to die very young. This neonatal lethality is most severe in the C57BL6 background, with >90% of the animals dying before six weeks of age with evidence of extensive liver damage (13, 14). This neonatal lethality can be rescued by expression of a zinc-inducible transgene encoding a mutant human CBS enzyme (*Tg-I278T*) that has only about 3–5% activity of wild-type CBS in mouse liver extracts (15), but is not rescued by an identical construct expressing the enzymatically dead G307S transgene (*Tg-G307S*) (12). Surprisingly, adult *Tg-I278T Cbs*$^{-/-}$ mice have serum tHcy that is similar to that found in the few adult non-transgenic *Cbs*$^{-/-}$ survivors, raising the question of whether the accumulation of homocysteine in blood or tissues is actually the cause of the neonatal lethality (15). It should also be noted that adult *Cbs*$^{-/-}$ and *Tg-I278T Cbs*$^{-/-}$ mice have only very modest elevations in serum Met compared to human CBS-deficient patients.

To further understand the cause of the differences in neonatal survival we have performed a series experiments comparing the pathology and metabolism of three different strains of mice with different CBS activity levels: *Tg-G307S Cbs*$^{-/-}$ (no liver CBS activity), *Tg-I278T Cbs*$^{-/-}$ (low activity), and control *Cbs*$^{+/-}$ mice (high activity). The metabolome of these mice

were analyzed at days 6, 10, and 17 of neonatal age. Our results show that there are important metabolic changes that occur in this period, and that the key differences in outcome between *Tg-G307S Cbs*$^{-/-}$ and *Tg-I278T Cbs*$^{-/-}$ mice are likely due to the accumulation and persistence of high concentrations of Met in the liver.

Materials and Methods

Mouse genetics and generation

Transgenic mice were created as previously described (12, 15). For the studies performed here, *Tg-G307S* founder 13 was used. The *Tg-I278T* founder is the same as used in (10). For matings, mice were given 25 mM $ZnSO_4$ from the start of the mating until the sacrifice/weaning of pups. Unless otherwise stated, matings consisted of a *Tg*$^{+}$ *Cbs*$^{-/-}$ male crossed to a *Tg*$^{+}$ *Cbs*$^{+/-}$ female. Because these crosses did not generate *Cbs*$^{+/+}$ offspring, we used a mixture of *Tg-I278T Cbs*$^{+/-}$ and *Tg-G307S Cbs*$^{+/-}$ mice as the control arm for the experiments. Mice were sacrificed at 6d, 10d, and 17d for cohort studies. Mice were fed standard rodent chow (Teklad 2018SX containing 6 g Met/kg) unless otherwise stated. For low methionine rescue experiments TD110591 (0.5 g Met/kg) and TD120114 (1.5 g Met/kg) diets were used. For cystathionine rescue experiments L-cystathionine and DL-propargylglycine were purchased from Sigma. All studies were approved by the FCCC IACUC.

Histology

Haematoxylin and eosin (H&E) and Caspase 3 staining was performed on tissue sections fixed in 10% Formalin, dehydrated and embedded in paraffin. H&E stained sections were used for morphological evaluation purposes and unstained sections for immunohistochemical (IHC) studies. IHC staining was carried out according to standard methods. Briefly, 5-μm formalin-fixed, paraffin embedded sections were deparaffinized and hydrated. Sections were then subjected to heat-induced epitope retrieval with 0.01 M citrate buffer (pH 6.0). Endogenous peroxidases were quenched by the immersion of slides in 3% hydrogen peroxide solution. The sections were incubated overnight with primary antibodies to cleaved caspase-3 (1:200,rabbit, polyclonal, Cat No. 9661 Cell Signaling, Danvers, MA) at 4 °C in a humidified slide chamber. Immunodetection was performed using the Dako Envision+ polymer system and immunostaining was visualized with the chromogen 3,3'-diaminobenzidine. The sections were then washed, counterstained with hematoxylin, dehydrated with ethanol series, cleared in xylene, and mounted. As a negative control, the primary antibody was replaced with normal rabbit IgG to confirm absence of non-specific staining.

Serum analysis

Serum total homocysteine (tHcy) and Met were measured under reducing conditions by Biochrom 30 amino acid analyzer as described (16). Serum ALT was assessed as described (14).

Liver Metabolite determination

Metabolites in the liver samples were measured by several different methods. Liver tHcy was measured by Biochrom 30 amino acid analyzer under reducing conditions as described (17). Other methionine recycling metabolites (Met, AdoMet, AdoHcy, and cystathionine) were measured by liquid chromatograpy tandem mass spectrometry (LC-MS/MS) on a 5500 QTrap (Sciex, Framingham, MA) as previously described (18, 19). For this analysis liver tissue was deproteinized with 4 volumes of perchloric acid (0.4M). Following centrifugation, internal isotope standards were added to the clear PCA extract and 10 μL injected into the LC-MS/MS system. Two levels of quality control samples were used to monitor assay performance that was <10% variance for all metabolites.

Targeted metabolomics in liver tissue was performed using the commercially available MxP® Quant 500 kit (Biocrates, Innsbruck, Austria), following the manufacturer's instructions provided. Liver tissue was prepared for analysis by deproteinization with 85% methanol in 15% phosphate buffered saline. Following centrifugation, 10 μL of clear extract, calibrators (7 levels) and quality controls (3 levels) were added to the 96 well kit plate. The MxP® Quant 500 kit can potentially identify 630 metabolites across 23 classes of compounds by LC-MS/MS and flow-injection analysis (FIA) MS/MS. Multiple reaction monitoring (MRM) of LC-MS/MS and FIA-MS/MS data was analyzed using Analyst 6.0 (Sciex, Framingham, MA) for peak identification. Data was further uploaded to Met*IDQ* (Biocrates, Innsbruck, Austria) for analyte quantitation. Additional software, Metabo*Indicator*™ (Biocrates, Innsbruck, Austria) was used to potentially calculate 232 metabolic indicators, based on sums of metabolites or ratios of metabolites.

Statistics and bioinformatics

Targeted metabolite data was analyzed using MetaboAnalyst 5.0 software (metaboanalyst.ca). Metabolites with >50% of the values below the detection limit (<LoD) values were eliminated, and for those with less than 50%, the <LoD values were replaced with 1/5 the minimum positive value for that variable. The data was processed/normalized by: (i) Sample normalization by sum (scale each sample so that the mean of metabolite concentrations is the same for all samples), (ii) log-normalization, and (iii) standardizing the values of each metabolite to have mean 0 and s.d. 1). Significantly different metabolites were identified using one-way ANOVA with an adjusted p-value cut-off of 0.05. Fisher's least squared difference was used in post-hoc analysis. For heat map generation, metabolites with a significant ANOVA were clustered using Pearson distance measure and Ward's linkage. For two-sample comparisons a 2-sided student's t-test was used.

Western Blotting and CBS Enzyme Activity

CBS western blots and enzyme activity were performed as previously described (20). Liver homogenates (10%) of 6, 10 and 17 day mice were prepared in 10 mM PH 8 Tris HCl buffer containing protease inhibitor (Complete mini tablet). Protein concentration was determined by Bradford assay. For western blotting 7% NuPAGE Tris acetate gel (Invitrogen, Thermo Fisher Scientific) was ran using 30 μg protein followed by transfer to PVDF membrane. CBS was detected by using polyclonal primary antibody (1:10,000) and secondary anti-rabbit antibody (1:30,000) conjugated with horse radish peroxidase. Signal was detected by

SuperSignal West Pico Chemiluminescent kit (Thermo Scientific) and visualized by Alpha Innotech imager.

CBS Activity was conducted on dialyzed liver extracts in an assay reaction containing 10 mM DL-homocysteine, 5 mM L-serine, 50 μM Pyridoxal phosphate and 250 μM AdoMet. Activity was calculated in terms of nmoles cystathionine formed/mg protein/hr.

Results

Tg-G307S Cbs$^{-/-}$ mice die around 20 days of age from massive liver failure

We initially compared the birth and neonatal lethality ratios in *Tg-I278T Cbs*$^{-/-}$, *Tg-G307S Cbs*$^{-/-}$ mice, and the original *Cbs*$^{-/-}$ mice all in a C57BL6 background (12, 13, 15). When genotyping is done at 10 days of age, no statistically significant deviation from the expected mendelian ratios of *Cbs*$^{-/-}$ mice in any of the three strains was observed (Table 1). However, by the time the animals were weaned at 30 days, the *Tg-G307S Cbs*$^{-/-}$ strain and the *Cbs*$^{-/-}$ strain had only about 5–9% of the *Cbs*$^{-/-}$ mice alive. In contrast, 92% of the mice were still alive in crosses generating *Tg-I278T Cbs*$^{-/-}$ animals. Since the *Tg-I278T* transgene is expressed from the zinc-inducible mouse *Mt-1* promoter, we also performed some crosses in which the mating cages were not given zinc-containing water, which would reduce transgene expression and residual CBS activity. In these crosses we found a significantly increased rate of neonatal lethality (8% vs. 48%, P<0.0001, Fishers exact). The bit of rescue that still occurs is most likely due to leaky expression of the transgene in the absence of zinc.

To more precisely determine when and how *Tg-G307S Cbs*$^{-/-}$ mice were dying, we monitored several litters daily from birth till the time of weaning (about d30). We observed almost no deaths through 17d, but starting at 18d we began to observe some of the *Tg-G307S Cbs*$^{-/-}$ pups becoming morbid and dying shortly thereafter. Almost all of the deaths occurred between 18d to 22d. Necropsies on moribund *Tg-G307S Cbs*$^{-/-}$ and age matched *Tg-I278T Cbs*$^{-/-}$ mice showed that *Tg-G307S Cbs*$^{-/-}$ animals exhibited a distinct discoloration of the liver and had blood in the small intestine, while *Tg-I278T Cbs*$^{-/-}$ animals were essentially indistinguishable *Cbs*$^{+/-}$ controls (Supplemental Figure 1A). Histopathological analysis of liver, stomach, thymus, spleen and bone marrow revealed significant amounts of hepatocyte necrosis, as well and mucosal atrophy in the stomach, apoptotic cells in the thymus, and atrophy in the spleen and bone marrow. These same tissues appeared normal in *Tg-I278T Cbs*$^{-/-}$ mice.

To understand the timing and the pathology in *Tg-G307S Cbs*$^{-/-}$ mice, we collected blood and tissue from cohorts of *Tg-G307S Cbs*$^{-/-}$, *Tg-I278T Cbs*$^{-/-}$, and sibling control *Tg*$^{+}$ *Cbs*$^{+/-}$ mice at either 6d, 10d, and 17d. The extent of liver damage in these mice was determined by measuring serum levels of alanine aminotransferase (ALT) and by histopathology. In control animals, ALT levels were quite low in 6d and 10d mice, but did show about a three-fold increase at 17d, suggesting there may be some developmental stress on the liver at this time (Figure 2A). In *Tg-I278T Cbs*$^{-/-}$ mice we see a similar pattern, although the increase at 17d is somewhat more pronounced (100 vs 370 units). Strikingly, in *Tg-G307S Cbs*$^{-/-}$ neonates, ALT levels rise from 17.3 units at 6d, to 176 at 10d, to 2311 units at 17d. In addition, we observed significant amounts of caspase 3 staining in 17d *Tg-G307S Cbs*$^{-/-}$

liver sections (Figure 2B). H and E stained liver sections from *Tg-G307S Cbs*$^{-/-}$ mice showed mild levels of steatosis (fat droplets) at 6d, that become more severe at 10d and 17d (Figure 2C, Supplemental Figure 1B). In contrast, *Tg-I278T Cbs*$^{-/-}$ mice had similar amounts of steatosis as *Tg-G307S Cbs*$^{-/-}$ at 6d, but this did not worsen over time. Liver and small intestine of 6d and 10d *Tg-G307S Cbs*$^{-/-}$ mice did not show any discoloration and blood. Taken together, the ALT and histopathology data confirm that *Tg-G307S Cbs*$^{-/-}$ mice suffer from significant liver damage just before they enter the period in which they become morbid.

Serum analysis

Both *Tg-G307S Cbs*$^{-/-}$ and *Tg-I278T Cbs*$^{-/-}$ mice showed large increases in serum tHcy relative to controls at all three time points, with tHcy increasing as the mice aged in all three genotypes (Figure 3A). In the 6d neonates, we observed a significant difference between *Tg-G307S Cbs*$^{-/-}$ and *Tg-I278T Cbs*$^{-/-}$ mice (29 vs 90 μM, P=0.008), however this difference disappeared as the mice became older. In fact, the mean tHcy in the *Tg-I278T Cbs*$^{-/-}$ strain was actually slightly higher than *Tg-G307S Cbs*$^{-/-}$ at 17d (344 μM vs. 284 μM), although this difference was not significant. It should be noted that the serum concentration of tHcy in 17d mice are similar to what is observed in adult mice of the same genotypes (17). These findings suggest that differences in serum tHcy are most likely not the direct cause for the differences in neonatal lethality outcomes.

Serum Met showed a different pattern (Figure 3B). *Tg-G307S Cbs*$^{-/-}$ mice had extremely high Met concentrations in neonates. Six day old neonates had a mean serum Met of 1967 μM, by far the highest amount we have ever observed in a *Cbs*$^{-/-}$ mouse. This high level of Met was maintained through 10d and then decreased slightly to 1225 μM by 17d. In contrast, the Met concentrations in *Tg-I278T Cbs*$^{-/-}$ neonates while elevated compared to controls, was significantly lower. In these mice Met peaked at 1020 μM at 10d, declined to 446 μM at 17d, and finally to 54 μM at the time of weaning (30d). Our findings show that while *Tg-I278T Cbs*$^{-/-}$ mice appear to able to resolve their serum hypermethioninemia during the neonatal period, *Tg-G307S Cbs*$^{-/-}$ mice cannot.

Liver Methionine Pathway Metabolites

In order to understand the relationship between metabolites in the blood and the liver, we initially measured the concentration of the all of the methionine recycling pathway metabolites and cystathionine in neonatal liver lysates using targeted LC-MS/MS assays (Figure 4). Similar to what we observed in serum, liver Met concentrations were significantly elevated in both *Tg-G307S Cbs*$^{-/-}$ and *Tg-I278T Cbs*$^{-/-}$ mice compared to controls at both 6d and 10d, but by 17d the *Tg-I278T Cbs*$^{-/-}$ Met concentration had lowered to near control levels, while in the *Tg-G307S Cbs*$^{-/-}$ livers it remained extremely elevated. In contrast, liver tHcy was elevated throughout the neonatal period in both *Tg-I278T Cbs*$^{-/-}$ and *Tg-G307S Cbs*$^{-/-}$. AdoMet levels appeared to parallel the Met values, while AdoHcy was more similar to the Hcy pattern. Interestingly, the AdoMet/AdoHcy ratio in all three strains decreased as the mice aged, but was consistently a bit higher in *Tg-G307 Cbs*$^{-/-}$ mice. The most unexpected finding was the large difference in cystathionine levels between *Tg-G307S Cbs*$^{-/-}$ and *Tg-I278T Cbs*$^{-/-}$ mice. While *Tg-G307S Cbs*$^{-/-}$ mice had barely

detectable cystathionine, *Tg-I278T Cbs*$^{-/-}$ mice had concentrations that were only slightly less than control animals.

The cystathionine finding was surprising given our previous findings that adult *Tg-I278T Cbs*$^{-/-}$ animals only had 3–5% of the CBS activity of *Cbs*$^{+/+}$ mice (20). To explore this further, we measured liver CBS activity and protein levels in 6, 10, and 17d neonatal liver lysates from the same two strains as well as control animals expressing wild-type human CBS (Supplemental Figure 2). Unexpectedly, we found that the lysates of younger age mice actual had quite a bit more CBS activity than adult *Tg-I278T Cbs*$^{-/-}$ mice, although this amount was still significantly less than age-matched controls. Western blotting indicated that CBS protein was more abundant in the younger animals, suggesting that the mutant protein might be more stable. No activity and very little CBS protein was produced in *Tg-G307S Cbs*$^{-/-}$ neonates.

Metabolomic Analysis in Liver Tissue

To look at other metabolic pathways, we performed a targeted metabolomic analysis of 6d, 10d, and 17d neonatal livers from the same three genotypes (n=6 per group) using the Biocrates MxP Quant 500 kit, which can quantitate up to 630 different biological metabolites. After filtering for compounds that were below detection, we were able to detect 142 to 153 different metabolites depending on age and genotype. These metabolites include a variety of different types of compounds including lipids, amines, nucleobases, steroids, carboxylic acids, vitamins, and sugars.

We first examined the metabolites for differences between genotypes at each time point using univariate ANOVA followed by unsupervised hierarchical clustering using the metabolites that showed significant ($p<0.05$) differences (Figure 5, Supplemental File 1). At the 10d and 17d time points, the algorithms correctly clustered the samples by genotype, however at the 6d time point, *Tg-I278T Cbs*$^{-/-}$ samples were intermingled with the *Tg-Cbs*$^{+/-}$ controls. The number of significant differences increased with age, i.e., at 6d there were 12 metabolites, 10d there were 35, and at 17d there were 67 metabolites. Overall, the heat maps indicate that differences observed between the genotypes increased with neonatal age. Principal Component Analysis (Supplemental Figure 3) shows that at the 6d time point there was significant intermingling of the samples of all three genotypes, but by 10d the genotypes each had their unique signatures. Interestingly, by 17d the *Tg-I278T Cbs*$^{-/-}$ and the control samples appeared to be much closer to each other than to the *Tg-G307S Cbs*$^{-/-}$ samples. Besides the individual metabolites, we also examined 182 metabolic indicators. These indicators are ratios or sums of metabolites that can reveal information about overall differences in metabolic flux and have been cited as being biomarkers in the literature. We analyzed the indicators in an identical fashion as the individual metabolites (Supplemental Figure 4, Supplemental File 1). Overall, these indicators showed a similar clustering pattern as observed by the individual metabolites.

We also noted that in all three genotypes, there were multiple metabolites whose concentration changed as a function of neonatal age. Therefore, we performed univariate ANOVA followed by unsupervised clustering on each genotype comparing metabolite levels as a function of age (Figure 6). With the exception of a single *Tg-I278T Cbs*$^{-/-}$ 10d sample,

the unsupervised clustering correctly grouped the mice by age for each genotype. One interesting difference between genotypes is whether the metabolites increase or decrease as they age. In control mice about half of the metabolites increase with age and half decrease. However, in both *Tg-I278T Cbs*$^{-/-}$ and *Tg-G307S Cbs*$^{-/-}$ mice the vast majority of the changed metabolites seem to decrease with age. Our results show that the liver metabolic profiles in these neonatal mice is not static, but in fact changes substantially as the mice age and this appears to be influenced by the CBS status of the mice.

To explore the genotype/time interaction a bit further, we used two-factor ANOVA to examine the influence of both time and genotype on metabolite levels. We identified a total of 61 metabolites that showed significant differences due to genotype, 95 metabolites that changed due to neonatal age, and 74 metabolites whose concentration had a significant interaction effect between age and genotype (Supplemental File 1).

Pathway Analysis

To try and understand the biological relationship between individual metabolites and CBS deficiency, we examined specific metabolic pathways in which multiple metabolites were altered. Pathways examined included the Met-breakdown pathway, purine metabolism, choline pathway, and downstream sulfur metabolite pathways.

In the absence of CBS, the only way to catabolize Met is to produce glutamate and 4-methylthio-2-oxobutanoic acid (MTOB) from Met and α-ketoglutarate by the action of tyrosine transaminase. The glutamate produced from this reaction then has its ammonia eliminated from the body by conversion to urea. It should also be pointed out that, extracellular MTOB causes apoptosis via its breakdown to methanethiol and malondialdehyde (21, 22). We found that 17d old *Tg-G307S Cbs*$^{-/-}$ mice had elevated levels of Met, glutamate, ornithine and citrulline, consistent with the idea that *Tg-G307S Cbs*$^{-/-}$ mice are trying to eliminate their excess Met via the urea cycle (Figure 7). In regard to purine biosynthesis, the low levels of xanthine and hypoxanthine found in *Tg-G307S Cbs*$^{-/-}$ mice could be explained if these mice were diverting their folates from formyl THF used in purine synthesis toward methyl-THF used for homocysteine remethylation (Supplemental Figure 5). Choline is important for both the formation of betaine used in homocysteine remethylation and for the production of phosphatidylcholine via the Kennedy pathway (Supplemental Figure 6). Analysis of this pathway suggests that both *Tg-I278T Cbs*$^{-/-}$ and *Tg-G307S Cbs*$^{-/-}$ mice are utilizing choline to make betaine for homocysteine remethylation, resulting in increased sarcosine production. The reduction of overall phosphatidylcholine derived lipids observed in *Tg-G307S Cbs*$^{-/-}$ could be explained by this increased choline usage for remethylation. The downstream sulfur amino acid taurine is decreased in *Tg-G307S Cbs*$^{-/-}$ livers, while the CBS co-substrate serine is increased (Supplemental Figure 7). Interestingly, cysteine levels, which are low at 6d and 10d in *Tg-G307S Cbs*$^{-/-}$ mice, are not different from controls at 17d. The large increase in the bile acids glycocholic acid (GCA) and cholic acid (CA) in *Tg-G307S Cbs*$^{-/-}$ mice are probably related to the reduced levels of their taurine conjugated cognates: Tauromurocholic acid (TMCA) and Taurochenodeoxycholic acid (TCDCA).

Some other specific metabolites that are interesting include Met sulfoxide (Met-SO), alpha amino butyrate (AABA), asymmetric and symmetric dimethylarginine (ADMA and SDMA), and hexoses. Met-SO is oxidized Met and the Met-SO/Met ratio is an indicator of the intracellular redox environment. We found that Met-SO paralleled Met, as expected. Despite the large differences in the total levels of Met and Met-SO, the ratio was surprisingly constant, with only a very slight increase in the Met-SO/Met ratio occurring a 10d (Supplemental Figure 8A). This suggests that the overall redox environment is not being significantly impacted by the loss of CBS. Alpha amino butyrate is reduced significantly in *Tg-G307S Cbs*$^{-/-}$ livers at 6d and 10d (Supplemental Figure 8B). This metabolite is produced from transamination of α-ketobutyrate. The reduced levels could be explained by the lack of cystathionine production, thereby eliminating the production α-ketobutyrate by CGL catalyzed cysteine formation. ADMA produced via SAM-dependent methylation reaction was significantly elevated in both 10d and 17d *Tg-G307S Cbs*$^{-/-}$ mice, while SDMA was elevated only at the 10d time point (Supplemental Figure 8C). The elevation in these molecules would be consistent with the hypothesis that elevated liver AdoMet in *Tg-G307S Cbs*$^{-/-}$ might increase arginine methyltransferase activity by increased substrate availability. Hexoses decreased relative to controls in both *Tg Cbs*$^{-/-}$ strains, with the decrease being more severe in *Tg-G307S Cbs*$^{-/-}$ mice (Supplemental Figure 8D). Because the liver is a center for gluconeogenesis, this finding suggests that gluconeogenesis may be impaired in these animals. A possible explanation is that methionine is normally converted to sugars via the alpha ketobutyrate produced by cleavage of cystathionine by the enzyme cystathionine gamma lyase. Thus, loss of CBS would inhibit this source of carbons for glucose production.

There were a few metabolites that were significantly changed in *Tg-I278T Cbs*$^{-/-}$ vs. controls, but not *Tg-G307S Cbs*$^{-/-}$ livers. At 10d, propionylcarnitine was significantly decreased, while at 17d carnitine and valerylcarnitine were decreased (See Supplemental File 1). Carnitines are important in fatty acid utilization for energy and are ultimately derived from Met and lysine. Another metabolite that had an unusual pattern was homoarginine, which appeared to be specifically decreased in 10d old *Tg-I278T Cbs*$^{-/-}$mice but increased in comparison to controls in 17d *Tg-G307S Cbs*$^{-/-}$ livers (Supplemental Figure 9E).

Failed Neonatal Lethality Rescue Experiments

Based on our metabolite analysis, we decided to try two different interventions to see if we could overcome the neonatal lethality in *Tg-G307S Cbs*$^{-/-}$ mice. Our first intervention was to shift the birthing cages to a low Met diet, as the Met in both serum and liver were much more elevated in *Tg-G307S Cbs*$^{-/-}$. In previous work from our lab, we found that a diet containing about 1/12 the normal Met content (0.5g/kg) was effective in lowering plasma tHcy and correcting the adult age-related phenotypes in *Tg-I278T Cbs*$^{-/-}$ mice (23). However, when nursing mothers were put on this diet, they and all the pups died within the next 10 days. We presumed this was because nursing mothers require large amounts of Met to support the protein synthesis required for lactation. Therefore, we repeated the experiment using a diet that was not quite a stringent, with about 1/4 the normal amount of Met (1.5 g/kg). At this level of Met the mothers survived and, therefore, pup survival could be

monitored. We found that 17 of 21 *Tg-G307S Cbs*$^{-/-}$ pups on this diet died between 14 and 19 days of age, and three more died before 42 days of age. To see if this intervention actually lowered serum Met in the pups, we sacrificed one litter (n=6) of pups, genotyped them, and measured serum tHcy and Met. Surprisingly, the one *Tg-G307S-Cbs*$^{-/-}$ animal in the litter had 160 μM tHcy and 2610 μM Met. Thus, giving low Met diet to mothers did not appear to lower Met in the *Tg-G307S Cbs*$^{-/-}$ pups.

A second intervention involved supplementing *Tg-G307S Cbs*$^{-/-}$ pups with cystathionine. This was motivated by our finding that *Tg-G307S Cbs*$^{-/-}$ mice had almost no detectable liver cystathionine, while *Tg-I278T Cbs*$^{-/-}$ mice had quite a bit (Figure 4). In addition, there was a report by Maclean et al. that inhibition of cystathionine γ-lyase (CGL) by the inhibitor propargylglycine (PPG) could enhance liver injury and steatosis by tunicamycin, suggesting potential beneficial functions for cystathionine (24). In our first experiment, we injected 8 *Tg-G307S Cbs*$^{-/-}$ baby mice subcutaneously (s.c.) with either 100 μl of saline (n=2) or 2 mM cystathionine (n=6) every two or three days (three times a week) starting from 6d. Starting on 17d, the first mouse died and by 20d a total of five of the mice had died and one more was moribund. The moribund (cystathionine treated) and two remaining animals (one saline and one cystathionine treated) were then euthanized, and serum and liver cystathionine were measured. In all three animals, cystathionine was below the level of detection, suggesting that the intervention was not able to restore liver cystathionine levels (Supplemental Figure 9A). Therefore, we performed an additional intervention in which we injected a combination of cystathionine and PPG into baby *Tg-G307S Cbs*$^{-/-}$ mice, reasoning that the PPG would help keep the cystathionine levels high. Three *Tg-G307S Cbs* $^{-/-}$ and four *Tg-G307S Cbs*$^{+/-}$ mice were injected with 100 μl combination of a 2 mM cystathionine and 17 mM PPG solution, every two or three days (three times a week) starting at 6d. One of the *Tg-G307S Cbs*$^{-/-}$ appeared moribund on 16d, while the other two died between 19–21d. Livers were harvested from the 16d *Tg-G307S Cbs*$^{-/-}$ moribund animal, as well as four of the control animals (26d), and cystathionine levels were determined (Supplemental Figure 9B). All five of these samples had cystathionine concentrations higher than those observed in untreated mice of the same genotype. This finding indicates that the s.c. injection of the combination was effective in raising liver and serum cystathionine. Also, it should be noted that the cystathionine concentration in the moribund *Tg-G307S Cbs*$^{-/-}$ mice was similar to that observed in 17d *Tg-I278T Cbs*$^{-/-}$ mice. Taken together, these findings do not support the idea that low cystathionine is the cause of the liver failure observed in *Tg-G307S Cbs*$^{-/-}$ mice.

Discussion

The purpose of the experiments described here was to attempt to understand the differences in the neonatal lethality phenotype between *Tg-G307S Cbs*$^{-/-}$ mice and *Tg-I278T Cbs*$^{-/-}$ mice. The most striking finding is that both serum and liver Met levels are much more elevated in *Tg-G307S Cbs*$^{-/-}$ mice, but tHcy is not much different. The finding of high concentrations of Met in *Tg-Cbs*$^{-/-}$ neonates was unexpected given that adult *Tg-Cbs*$^{-/-}$ mice tend to have only very modest elevation in serum Met. This is in stark contrast to human CBS deficient patients in which severe hypermethioninemia is observed in almost all untreated patients (25). In *Tg-Cbs*$^{-/-}$ mice of both genotypes, elevated serum Met is

observed as early as 6d and remains elevated through 17d. Met in the liver shows a similar trend, although in *Tg-I278T Cbs*$^{-/-}$ mice the Met elevation is largely resolved by 17d. Consistent with our data, Majtan et al. found that at 18d non-transgenic *Cbs*$^{-/-}$ mice also had extremely elevated plasma and tissue Met and that *Tg-I278T Cbs*$^{-/-}$ mice placed on a high methionine diet had fatal intestinal hemorrages (26, 27). At 17d, *Tg-G307S Cbs*$^{-/-}$ mice show a large increase in the liver damage marker ALT and have a significant percentage of apoptotic cells, while the difference in liver Met between the two strains is nearly 14-fold. Although there are reports of elevated methionine in patients and mini-pigs with severe liver dysfunction (28–30), the observation that methionine elevation occurs before there is any evidence of liver damage strongly suggests that the elevation in methionine is not a consequence of liver damage itself. Rather, the difference in liver Met levels is likely related to the fact that *Tg-I278T Cbs*$^{-/-}$ mice have significant levels of residual CBS activity, while *Tg-G307S Cbs*$^{-/-}$ mice are entirely devoid of activity. Without CBS activity, the major catabolic pathway for methionine recycling metabolites, i.e. transsulfuration, is eliminated.

Interestingly, the amount of residual activity in *Tg-I278T Cbs*$^{-/-}$ mice is higher in liver extracts from younger animals and seems to decrease over time. Based on previous work from our lab, the I278T mutation can be rescued in living cells by treatment with chemical chaperones, deletion of a small HSP, or treatment with proteasome inhibitors (8, 20, 31). These findings suggest that the mutant protein is sensitive to perturbations in the cellular proteostasis environment. We suspect that the change in residual activity observed in the extracts may reflect changes in the liver protein-folding environment as the liver develops. Consistent with this idea is our metabolic profiling data which clearly shows that the metabolome of both control and mutant CBS livers show significant differences between 6d and 17d.

Somewhat surprisingly, liver and serum tHcy were only marginally higher in *Tg-G307S Cbs*$^{-/-}$ compared to *Tg-I278T Cbs*$^{-/-}$. This finding in conjunction with the Met data suggests that once homocysteine reaches some threshold inside the cell, there is increased flux to Met to keep it low. Besides Met, we also observed that both strains had elevations in liver AdoMet and AdoHcy levels. However, the AdoMet/AdoHcy ratio in the liver was as high or higher than that observed in control animals at each time examined. Because AdoHcy is a competitive inhibitor of AdoMet-dependent methyltransferases in one carbon methyl-transfer reactions (32), these findings suggest that inhibition of methylation is not responsible for the neonatal lethality differences between the two strains. Finally, it should be noted that the levels of cystathionine in *Tg-I278T Cbs*$^{-/-}$ mice are surprisingly high in relation to the amount of CBS activity in the liver. For instance, at 17d *Tg-I278T Cbs*$^{-/-}$ livers have a 92% reduction of the enzyme activity compared to the 17d *Cbs*$^{+/-}$ controls, but only a 21% reduction in cystathionine. The finding of relatively high cystathionine levels relative to extremely low *in vitro* CBS activity was also observed in the HO mouse model of CBS deficiency (33). However, it should be noted that for both these studies *in vitro* enzyme assays were performed under V_{max} conditions, where the amount of homocysteine used in the assay is 10 mM (K_m for CBS is ~2 mM). The highest concentration of homocysteine we observed in any of our liver lysates was 0.5 mM (see Figure 4), indicating that the enzyme is working at sub-V_{max} conditions, where K_m becomes more important in determining

metabolite flux. Although I278T hCBS is impaired in V_{max}, its K_m is actually similar to the wild-type enzyme (34). Therefore, the elevated levels of Hcy in *Tg-I278T Cbs*$^{-/-}$ livers would partially compensate for the reduced V_{max} of the I278T enzyme with regards to pathway flux.

One possible mechanism by which elevated Met is driving liver damage is the degradation pathway of Met. Normally excess Met in the Met recycling pathway can be catabolized by its conversion to cystathionine by CBS. Since CBS is an irreversible enzyme, its action has the effect of removing Met and Met-recycling metabolites (SAM, SAH, and homocysteine) from the pathway. In the absence of CBS, the only way to remove Met recycling metabolites is via the enzyme tyrosine aminotransferase, which transfers the amine group from Met to α-ketogutarate to form 4-Methylthio-2-oxobutanoic acid (MTOB) and glutamate (Figure 7). The glutamate molecule is then acted on by glutamate dehydrogenase, releasing ammonia that is ultimately converted into urea by the urea cycle. Although MTOB was not measured directly, glutamate, ornithine and citrulline were all significantly elevated in 17d livers from *Tg-G307S Cbs*$^{-/-}$ mice, consistent with increased flux through this pathway. Importantly, it has been reported that extracellular MTOB specifically causes apoptosis most likely via a pathway that produces methional that is then broken down to form the toxic metabolites methanethiol and malondialdehyde (21, 22). Consistent with this idea, Yamada and colleagues found that mice lacking cystathionine gamma lyase put on high Met diets died of acute liver failure, with the accumulation of methional in the liver and blood (35). In addition, it was recently reported that *Tg-I278T Cbs*$^{-/-}$ mice put on a high methionine diet for several weeks also died with accumulation of blood in the intestine (27).

Metabolomic analysis on the livers of neonatal animals suggested some other possible pathways that could potentially be involved in *Tg-G307S Cbs*$^{-/-}$ neonatal deaths. At 17d, we observed significantly reduced levels of choline and choline derived phosphatidylcholines (PCs) and lysophosphatidylcholines (Lyso PCs) in the livers of Tg-G307S *Cbs*$^{-/-}$ compared to Tg-I278T *Cbs*$^{-/-}$ mice. We suspect this is due to the diversion of choline for homocysteine remethylation, leaving reduced amounts for synthesis of PCs and Lyso PCs. Phosphatidylcholine is an essential component of very low-density lipoprotein complex that facilitates the transport of triacylglycerols out of liver. Consistent with this idea, it has been reported that *Tg-I278T Cbs*$^{-/-}$ mice have reduced plasma triacylglycerol levels (36). Mice put on choline-deficient diets show significant levels of steatosis and can eventually develop hepatocellular carcinoma (37, 38). However, there are no reports in the literature of choline deficiency causing acute liver failure. Another major difference between the two strains was in taurine and its downstream metabolites. *Tg-G307S Cbs*$^{-/-}$ mice exhibited a 28% reduction in liver taurine, and a 47–82% reduction in taurine conjugated bile acids, and a whopping 18-fold and 450-fold increase in non-taurine conjugated precursors. Interestingly, mice with a homozygous null allele of cysteine dioxygenase (a key enzyme in the production of taurine) do show about a 20% increase in neonatal lethality compared to controls (39). However, this is a much smaller level of lethality than we observe, and it should also be noted that these mice have a much larger drop in liver taurine levels (>95% decrease). Thus it seems unlikely that either low choline or low taurine levels by themselves could be causing liver failure, although it is possible that there may be some sort of synergy between the two pathways. Consistent with our observations, adult HO mice have also been

reported to show reductions in liver phosphocholine and betaine concentrations as well (40, 41).

Despite considerable effort, we were unable to rescue the neonatal lethality in *Tg-G307S Cbs*$^{-/-}$ mice either with a Met restricted diet, or by supplementation with cystathionine. With regards to Met restriction, we suspect that the reason this approach failed is that the major source of nutrition for the pups is mothers milk, and despite the mothers taking in less dietary Met, they probably maintain normal levels of Met in casein and other proteins that make up milk. In fact, this idea may explain our observation that mothers could not tolerate the same low Met diet that non-nursing mice could tolerate for at least 210 days (23). Specifically, we hypothesize that the dames will catabolize their own Met stores in order to produce protein to feed their young. With regards to cystathionine, we injected the neonates repeatedly with cystathionine, and were able to confirm that this treatment could raise cystathionine levels. However, this treatment did not enhance survival implying that loss of cystathionine or its downstream metabolites is not the primary cause of the neonatal lethality observed in *Tg-G307S Cbs*$^{-/-}$ mice.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Non-Standard Abbreviations

CBS	Cystathionine β-synthase
tHcy	total (free and reduced) homocysteine
TCA	tricarboxylic acid cycle
Met	methionine
H&E	haematoxylin and eosin
IHC	immunohistochemical
ALT	alanine aminotransferase
AdoMet	S-adenosylmethionine
AdoHcy	S-adenosylhomocysteine
LC-MS/MS	liquid chromatograpy tandem mass spectrometry
<LoD	below level of detection

ANOVA	analysis of variance
d	day
THF	tetrahydrofolate
SEM	standard error of mean
MTOB	4-methylthio-2-oxobutanoic acid
GCA	glycocholic acid
CA	cholic acid
TMCA	tauromurocholic acid
TCDCA	Taurochenodeoxycholic acid
Met-SO	methionine sulfoxide
AABA	α-amino butyrate
ADMA	asymmetric dimethylarginine
SDMA	symmetric dimethylarginine.
CGL	cystathionine γ-lyase
PPG	propargylglycine
PCA	perchloric acid

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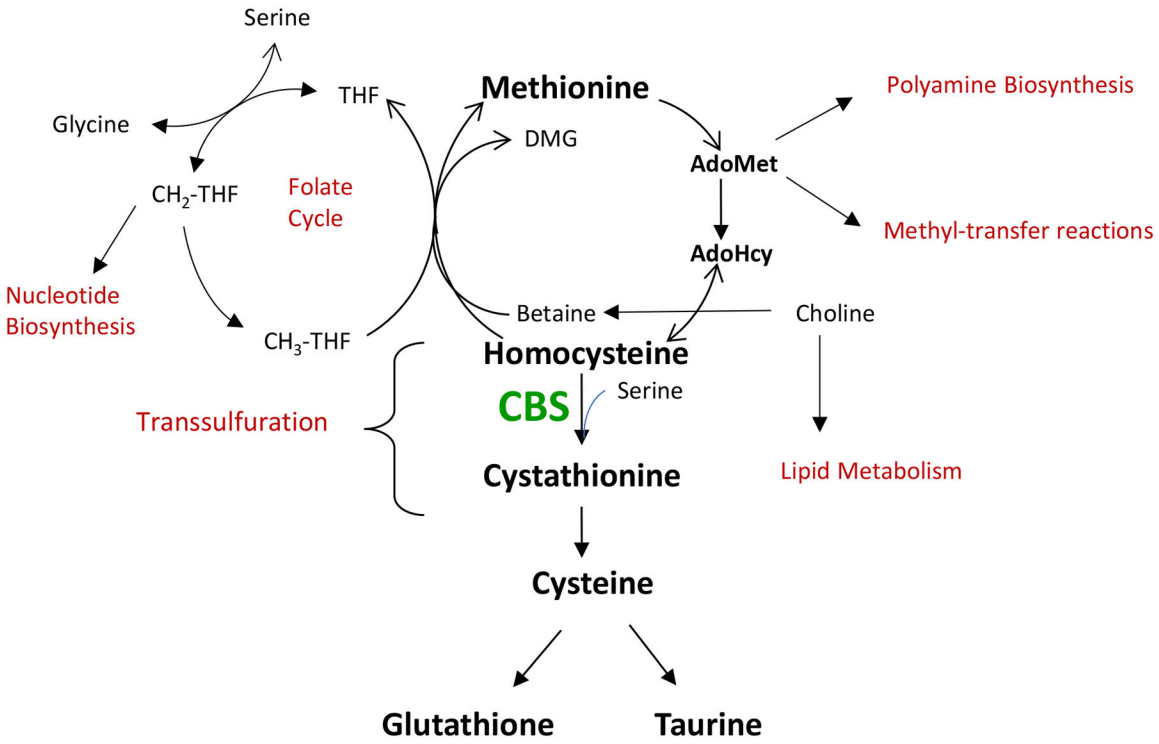

Figure 1. Overview of the role of CBS in metabolism.

Key metabolites are shown in black, while important related metabolic pathways are shown in red.

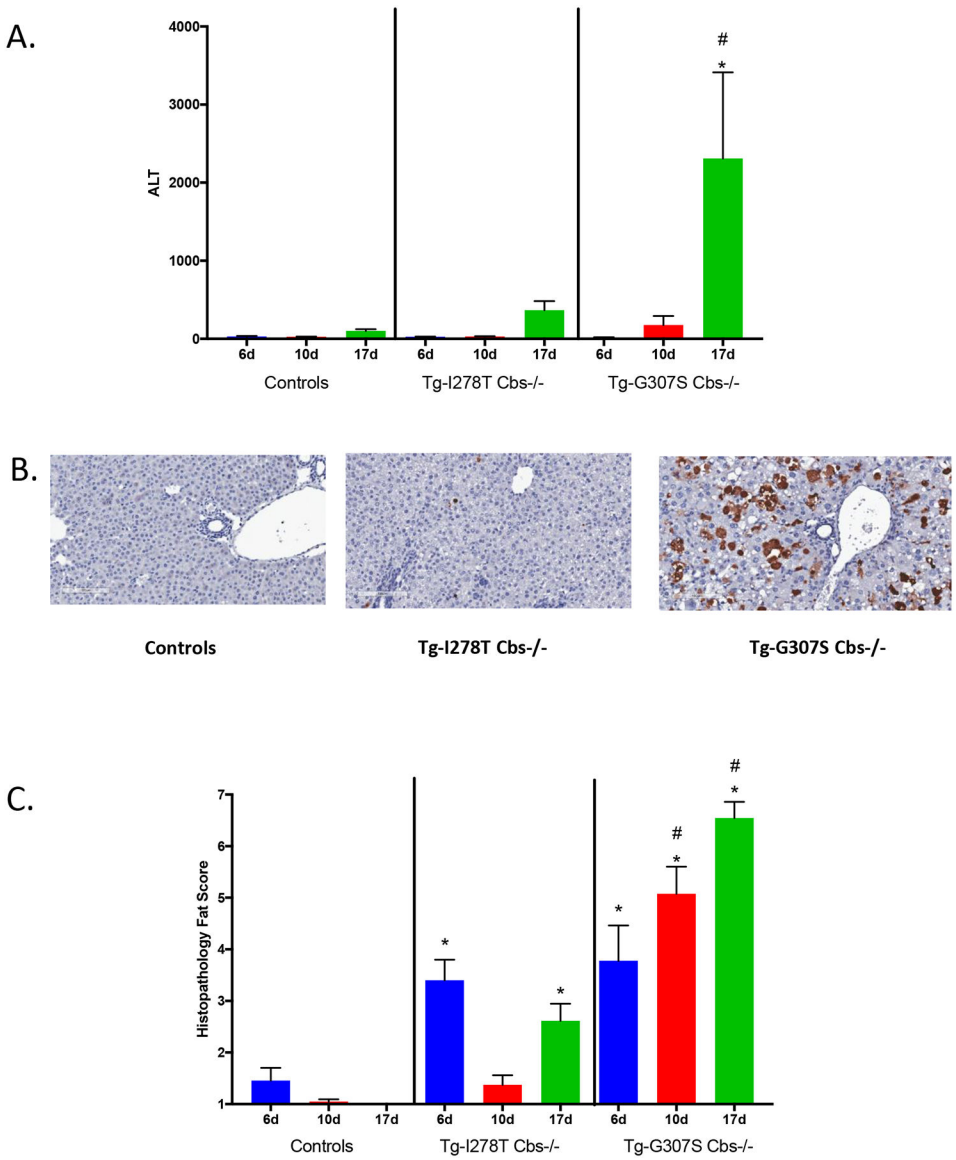

Figure 2. Comparison of liver damage and pathology in *Tg-G307S Cbs*$^{-/-}$, *Tg-I278T Cbs*$^{-/-}$, and *Tg+ Cbs*$^{+/-}$ controls.

A. Serum ALT levels at indicated times. N=4 for 6d and 10d; N=5 for 17d. Error bars show SEM. B. Histopathology score for relative level of steatosis in liver sections. All samples were assessed by a blinded observer (S.G.) on a seven point scale, with 7 being most severe. N=5 to 22 per bar. * indicates P<0.01 from same aged control, # indicates P<0.01 between same aged *Tg-I278T Cbs*$^{-/-}$ and *Tg-G307S Cbs*$^{-/-}$.

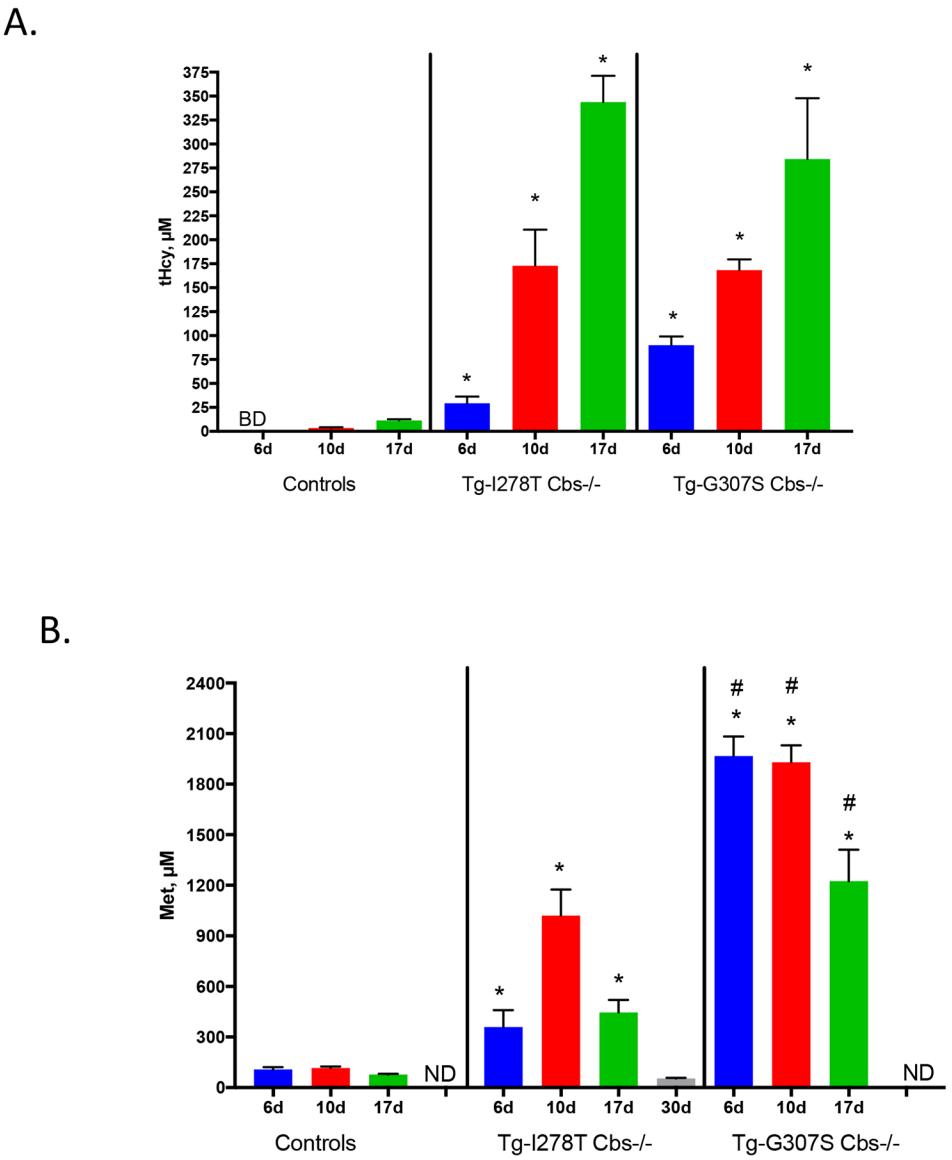

Figure 3.

Serum tHcy and Met in neonates. A. Serum tHcy. N≥5. Error bars show SEM. B. Serum Met. * indicates P<0.01 from same aged control, # indicates P<0.01 between same aged *Tg-I278T Cbs*$^{-/-}$ and *Tg-G307S Cbs*$^{-/-}$. ND = Not done. BD = Below detection.

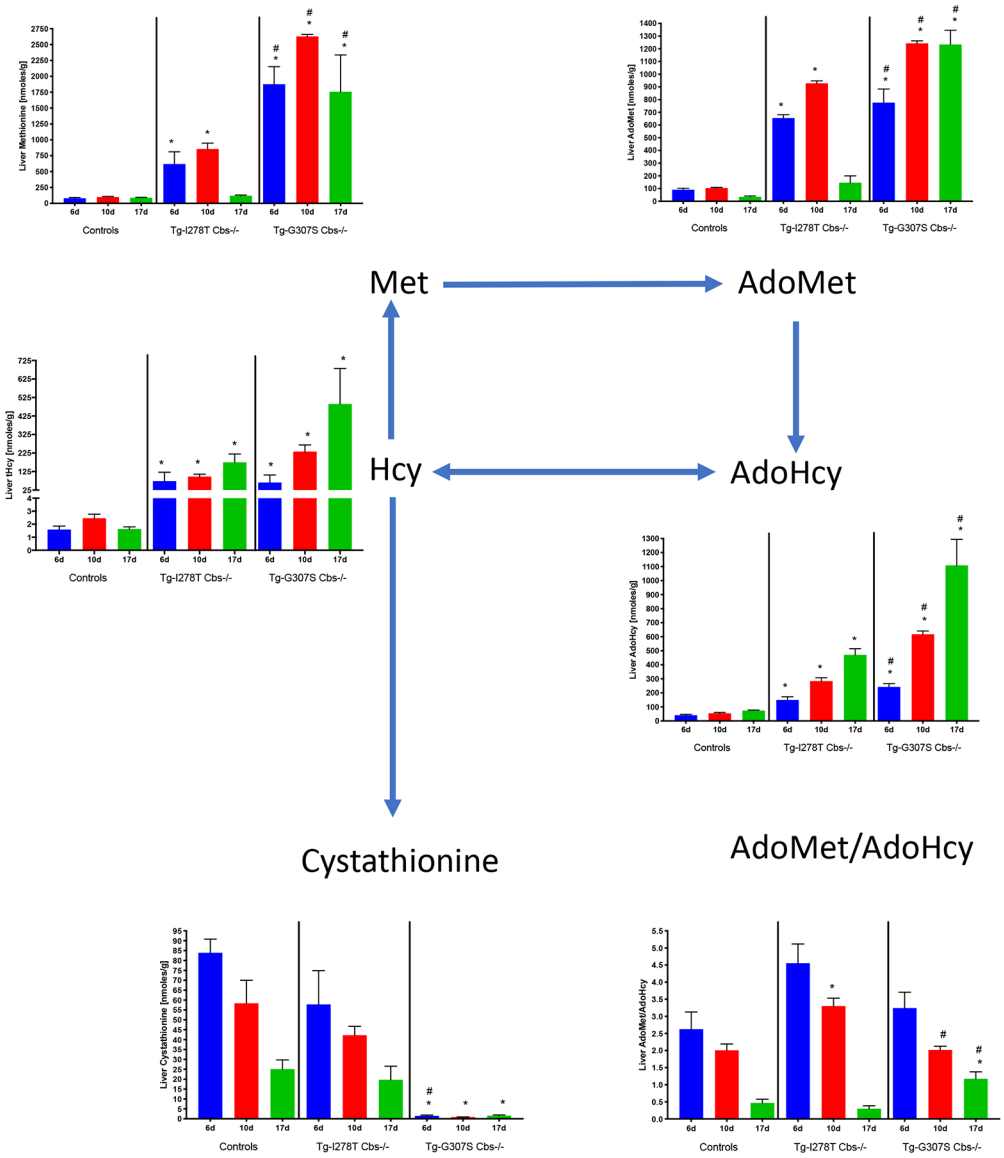

Figure 4.

Liver Met recycling metabolites. Graphs are placed adjacent to metabolites in pathway figure. Error bars show SEM (n=3 per sample except controls were n=6). * indicates P<0.01 from same aged control, # indicates P<0.01 between same aged *Tg-I278T Cbs*$^{-/-}$ and *Tg-G307S Cbs*$^{-/-}$. Total homocysteine (tHcy) is measured through Biochrom 30 amino acid analyzer. Rest were done by LC-MS/MS assay.

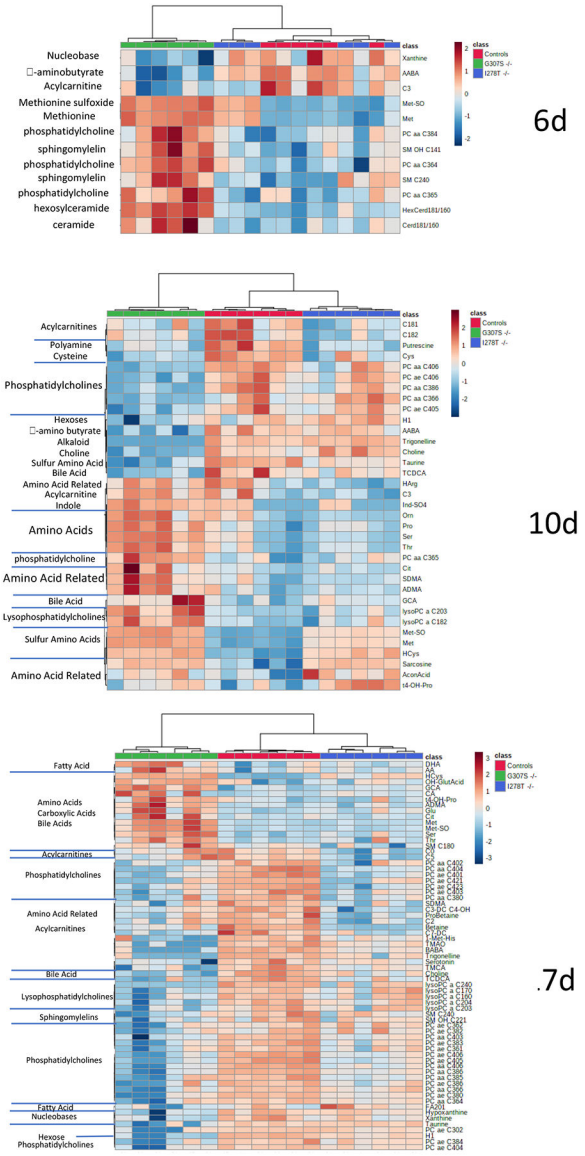

Figure 5.

Hierarchical clustering of metabolite concentrations by genotype at different ages. A. 6d cluster. B. 10d cluster. C 17d cluster. On the right of the clustering are the names of the metabolites using the Biocrates nomenclature. On the left, we show the general categories for the indicators. Note that due to space limitations we do not show all the categories for the 10d and 17d clusters. Red indicates higher than mean levels, while blue indicates lower than mean levels.

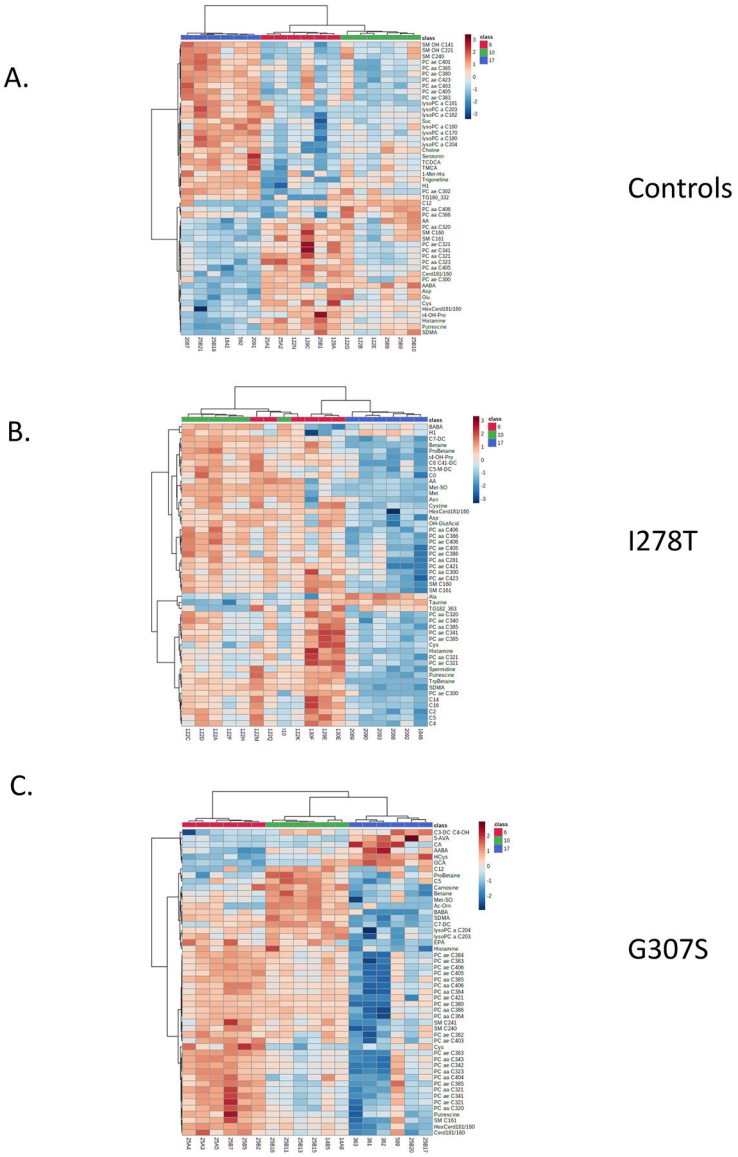

Figure 6.

Hierarchical clustering of metabolites by neonatal age in different genotypes. A. Control (*Cbs*$^{+/-}$) mice. B. *Tg-I278T Cbs*$^{-/-}$. C. *Tg-G307S Cbs*$^{-/-}$.

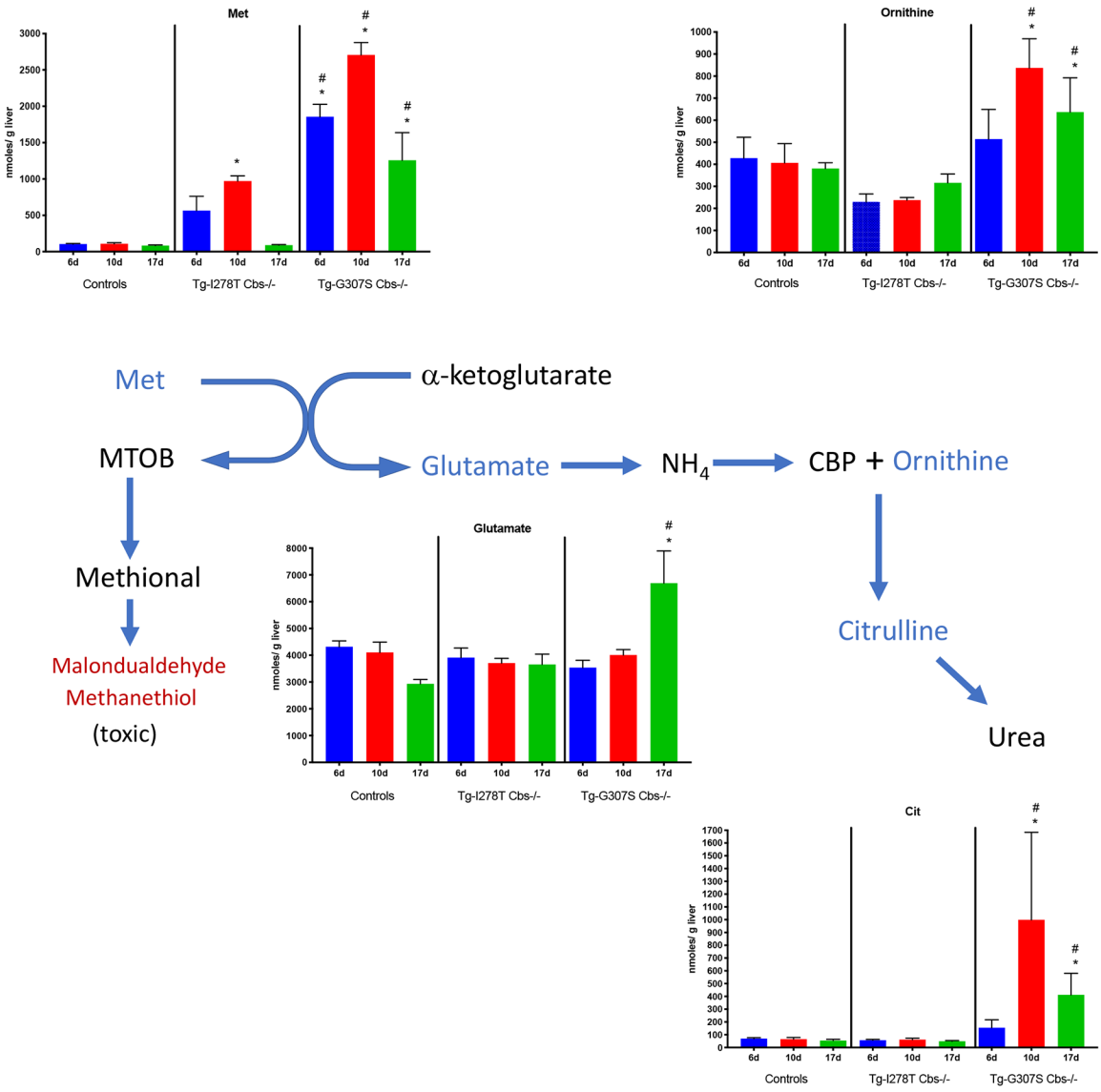

Figure 7. Met transamination catabolic pathway.

Flow chart shows pathway. Measured metabolites are shown in blue. Red metabolites are known to cause cellular toxicity. Graphs show metabolite levels in mice of indicated age and genotype. * indicates P<0.01 from same aged control, # indicates P<0.01 between same aged *Tg-I278T Cbs*$^{-/-}$ and *Tg-G307S Cbs*$^{-/-}$. CBP = Carbamoyl phosphate.

TABLE 1.

Neonatal lethality in Cbs-deficient mice

Transgene	Cross-type (*Cbs* $^{-}$status)	Total Progeny	Expected *Cbs* $^{-/-}$	Observed *Cbs* $^{-/-}$ (10d)	P-value (Chi^2)	*Cbs*$^{-/-}$ Alive at 30d	% lethal	Zn^{2+}
none	*Cbs*$^{+/-}$ *x Cbs*$^{+/-}$	276	69	58	NS	9	85	Yes
Tg-G307S	*Cbs*$^{+/-}$ *x Cbs*$^{+/-}$	274	68.5	56	NS	5	91	Yes
Tg-I278T	*Cbs*$^{+/-}$x *Cbs*$^{-/-}$	155	77.5	84	NS	79	8	Yes
Tg-I278T	*Cbs*$^{+/-}$x *Cbs*$^{-/-}$; *Cbs*$^{+/-}$ *x Cbs*$^{+/-}$ *	82	32	25	NS	13	48	No

* 46 progeny came from *Cbs*$^{+/-}$x *Cbs*$^{-/-}$, 36 from *Cbs*$^{+/-}$ *x Cbs*$^{+/-}$