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Cystathionine Beta-Synthase Deficiency Causes Fat Loss in Mice

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Abstract

Cystathionine beta synthase (CBS) is the rate-limiting enzyme responsible for the *de novo* synthesis of cysteine. Patients with CBS deficiency have greatly elevated plasma total homocysteine (tHcy), decreased levels of plasma total cysteine (tCys), and often a marfanoid appearance characterized by thinness and low body-mass index (BMI). Here, we characterize the growth and body mass characteristics of CBS deficient *Tgl278T Cbs*$^{-/-}$ mice and show that these animals have significantly decreased fat mass and tCys compared to heterozygous sibling mice. The decrease in fat mass is accompanied by a 34% decrease in liver glutathione (GSH) along with a significant decrease in liver mRNA and protein for the critical fat biosynthesizing enzyme Stearoyl CoA desaturase-1 (*Scd-1*). Because plasma tCys has been positively associated with fat mass in humans, we tested the hypothesis that decreased tCys in *Tgl278T Cbs*$^{-/-}$ mice was the cause of the lean phenotype by placing the animals on water supplemented with N-acetyl cysteine (NAC) from birth to 240 days of age. Although NAC treatment in *Tgl278T Cbs*$^{-/-}$ mice caused significant increase in serum tCys and liver GSH, there was no increase in body fat content or in liver *Scd-1* levels. Our results show that lack of CBS activity causes loss of fat mass, and that this effect appears to be independent of low serum tCys.

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Introduction

Cystathionine beta synthase (CBS) is the key regulatory enzyme for the transsulfuration pathway, which is responsible for the conversion of methionine to cysteine. The enzyme catalyzes the condensation of homocysteine with serine to create cystathionine, the precursor of cysteine. Cysteine is the rate-limiting amino acid in the biosynthesis of the major intracellular anti-oxidant thiol glutathione (GSH). In serum cysteine exists mostly in an oxidized state, cross-linked either to cysteine in proteins or other thiol containing amino acids. The sum total of all cysteine forms is known as total cysteine (tCys). CBS deficiency (also known as classical homocystinuria) is the most common inborn error of sulfur metabolism and is characterized by extreme elevations of plasma total homocysteine (tHcy) and methionine along with low levels of plasma tCys [1]. Patients with CBS deficiency suffer from a variety of pathologies including thrombosis, osteoporosis, fatty liver, mental retardation, and dislocated lenses. Interestingly, the appearance of CBS deficient patients often resembles that of individuals with Marfan syndrome, characterized by arachnodactyly, scoliosis, low BMI, and reduced fat [2,3].

The relation between CBS deficiency and reduced fat has not been explored. One possible connection comes from studies relating tCys and BMI. Epidemiologic data from several thousand individuals show that tCys is positively associated both BMI and fat mass [4,5]. Rats on a low methionine diet show significant increases in both total weight and fat mass when the diets are supplemented with cysteine [6]. Mice homozygous for mutations in the glutathione catabolic enzyme gamma-Glutamyl transpeptidase have low plasma free cysteine levels (20% of control) and decreased body mass. Importantly, addition of the cysteine analogue N-acetylcysteine (NAC) to the drinking water of these animals results in dramatic increase in overall body weight [7]. Based on these observations, it has been hypothesized that low serum tCys levels may be the cause of leanness in CBS deficiency [4].

Our lab has previously developed a mouse model for CBS deficiency, *Tg-I278T Cbs*$^{-/-}$ [8]. These mice contain a homozygous deletion of the endogenous mouse *Cbs* gene, and also have a mutant human *CBS* gene containing the I278T point mutation under control of the zinc inducible metallothionein promoter. The mutant I278T CBS protein is enzymatically crippled, but can rescue the neonatal lethality associated with the *Cbs*$^{-/-}$ genotype. *Tg-I278T Cbs*$^{-/-}$ animals have extremely elevated tHcy levels (~250 μM), low tCys, and survive into adulthood at >90% efficiency. These animals have several phenotypes including small size, facial alopecia, osteoporosis, and a 20% shortening of median and maximal life-span [9].

In the present study, we have performed detailed growth and body mass composition studies on *TgI278T Cbs*$^{-/-}$ mice and show that these animals have significantly reduced body fat compared to sibling controls. We also have investigated the expression of central lipogenic gene, Stearoyl CoA desaturase-1 (*Scd-1*) in livers of CBS deficient *TgI278T Cbs*$^{-/-}$ mice. Finally, to test our hypothesis that serum tCys is the cause of fat loss, we raised the serum tCys in the *TgI278T Cbs*$^{-/-}$ mice by addition of N-acetyl cysteine (NAC) to the animals drinking water.

Materials and Methods

Mouse model

Mice used in the study are from C57BL6 strain background. *TgI278T Cbs*$^{-/-}$ mice were originally created as described previously [8]. *TgI278T Cbs*$^{-/-}$ mice were generated mating either transgene positive *Cbs*$^{-/-}$ or *Cbs*$^{+/-}$ male with a transgene-positive *Cbs*$^{+/-}$ female, in a cage with drinking water containing 25 mM of $ZnCl_2$. Control animals for all experiments were sibling transgene positive *Cbs*$^{+/-}$ animals. Pups were genotyped on 10th day for both the transgene and the Cbs knockout allele, as described previously [10]. Pups were weaned at 30th day and then placed in cages with non-$ZnCl_2$-supplemented water for long-term weight and photography studies. The cohorts of animals were weighed and photographed every 30th day starting from day 30 to day 420 of their age. Animals were fed standard rodent chow (Teklad 2018SX; Harlan Teklad, Madison, WI, USA) *ad libitum*. All studies were approved by the Fox Chase Cancer Center IACUC (#99-26).

N-acetyl cysteine (NAC) intervention

Treatment groups had N-acetyl L-cysteine (NAC) dissolved in their drinking water to a final concentration of 40 mM and were treated from birth through 240 days. Parents of treated as well as untreated group were on zinc water during mating and pregnancy. NAC water was prepared fresh from the chemical (Catalog No# A9165; Sigma, St. Louis, MO, USA) and was replaced once a week. Quantitation of NAC stability using a Biochrom 30 amino acid analyzer indicated that after one week at room temperature, 64% of the NAC was still present. Mice were weighed every 30th day starting from day 60 to day 240, at which time the mice were sacrificed. The liver, kidney and serum were then extracted, and the tissues and mouse corpses were then stored at -80°C for further analysis and DEXA studies.

We measured the effect of NAC on food and water intake as follows. Three *Tg-I278T Cbs*$^{+/-}$ mice (2–3 months old) were kept per cage (2 cages per sex) with sufficient pre-weighed rodent chow and either pre-measured regular water or NAC water and followed for 6 weeks. Mice were allowed to acclimatize with their respective waters for one week before measurements were taken. Food and water consumption was calculated weekly by subtracting the remaining water and food from the starting material after adjusting for spilled food and water leakage. Spilled food was collected by using a manual sieve that could separate food particles from cage bed and mouse excreta. Water leakage was measured by putting the water bottle in the cages without mice and measuring the remainder every week.

Dual energy X-ray absorptiometry (DEXA) studies

DEXA analysis was performed using a Lunar PIXimus II Densitometer (GE, software version 2.0, Lunar Corp., Madison WI) instrument located in the lab of Dr. Michael Tordoff at Monell Chemical Senses Center, Philadelphia, PA, USA. The mice were thawed prior to doing DEXA and results were obtained in terms of fat mass (FM; g), lean mass (LM; g), total tissue weight (FM+LM), % Fat [(FM/FM+LM)*100], bone mineral content (BMC; g) and bone mineral density (BMD; g/cm^2).

Amino acid analysis

Serum tHcy, tCys and other amino acids were measured using the Biochrom 30 amino acid analyzer (Cambridge, UK) as previously described [11]. In brief, 50 µl of serum was treated with 5 µl of 12% dithiothreitol (Room temperature; 5 min) followed by the addition of 55 µl of 10% sulfosalicylic acid. After incubation of 1 h at 4°C the centrifugation was done at 13, 000 rpm for 15 minutes at 4°C. The clear supernatant was used for amino acid measurement. tCys refers to the sum total of all forms of cysteine, including protein-bound, free-reduced, and free-oxidized including mixed disulfides. Similarly tHcy refers to sum total of all forms of homocysteine (protein bound, free-reduced and free-oxidized including mixed disulfides).

Glutathione analysis

A 10% homogenate of liver tissue was made in 2.5% sulfosalicylic acid at 4°C by employing a manual glass dounce homogenizer. The homogenate was kept at 4°C for 30 minutes followed by centrifugation at 10,000 rpm for 15 minutes. The clear supernatant was used for the quantification of free reduced form of glutathione (GSH) by employing Biochrom 30 amino acid analyzer.

Taqman quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)

RNA was extracted from the livers using the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) followed by cleaning and removal of contaminating DNA using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Quality of RNA was determined using an Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and quantification was done by NanoDrop. One-step qRT-PCR was done employing Taq Man Gene expression assays (Applied Biosystems, Foster City, CA, USA) for mouse *Scd-1* (Mm00772290_m1). Mouse beta actin (Mm01205647_g1) was used as endogenous control. Quantification of signal was achieved by using Applied Biosystems 7900 HT version 2.2.2 sequence detection system. Each sample was assayed in singlet (100 ng/well) for each gene, and relative signal strength was calculated using the $\Delta\Delta C_t$ method.

Western Blotting

Homogenates (20% w/v) of liver, kidney, spleen, visceral white adipose (from gonadal region) and subcutaneous white adipose tissues were prepared at 4°C in RIPA buffer (Thermo Scientific, USA) containing protease inhibitor cocktail tablet (Complete mini, Roche, Germany). Homogenates were then put on ice for one hour before centrifugation at 13, 000 rpm (4°C) for 15 minutes and supernatant was used for further analysis. Protein concentration was determined by using BCA protein assay kit (Thermo Scientific). For Western blotting, 30 µg of total protein extract was electrophoresed on a 10% Bis tris SDS Polyacrylamide gel (Invitrogen, USA) using MOPS running buffer (Invitrogen) followed by transfer on to a PVDF membrane (Bio Rad, USA). Scd-1 was detected by using a goat polyclonal antibody (1:200; sc-14719) and secondary anti-goat antibody (1:5,000; sc-2020; Horseradish peroxide conjugated) from Santa Cruz Biotechnology. Signal was visualized by using Supersignal west pico chemiluminescent kit (Thermo Scientific) and Alpha Innotech image analyser.

Statistical Analysis

For comparison of two samples, a two-sided unpaired students t-test was used with $P<0.05$ being considered significant. For multiple group comparison one-way ANOVA was done followed by Tukey's multiple comparison test using GraphPad Prism 4.0 software (Graphpad Software, La Jolla CA).

Results

TgI278T Cbs$^{-/-}$ mice weigh less and have age-related hair loss

A cohort of CBS deficient *TgI278T Cbs*$^{-/-}$ mice and *TgI278T Cbs*$^{+/-}$ sibling controls were weighed and photographed at 30 day intervals from 30 to 420 days of age. Both male and female *TgI278T Cbs*$^{-/-}$ mice appeared smaller and weighed significantly less as compared to age and gender matched control mice at all the times-tested ages (Fig. 1 and Fig. 2). Interestingly, the difference in size between control and *Tg-I278T Cbs*$^{-/-}$ mice increased over time, primarily because *Tg-I278T Cbs*$^{-/-}$ mice stopped putting on weight after 150 days, while the control animals kept growing throughout the entire period. We also found that the onset of the facial alopecia phenotype was between 105 and 120 days of age, and often this alopecia would spread to the backs of the animals as they continued to age.

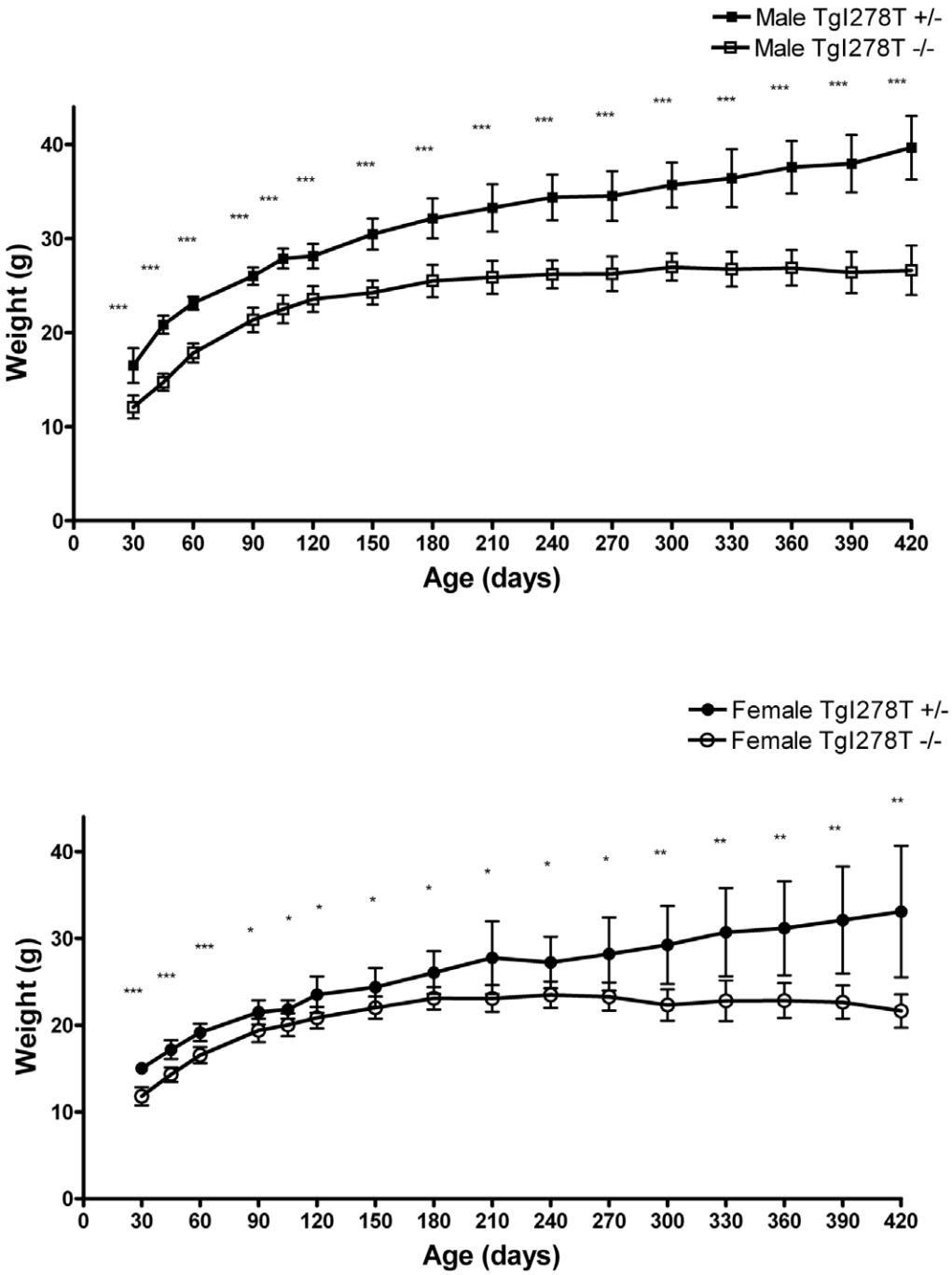

Figure 1. Comparison of the weight of CBS deficient *TgI278T Cbs*$^{-/-}$ mice with age and gender matched control *TgI278T Cb*$^{+/-}$ mice. Between eight and twenty mice were weighed at each time point. Graphs show the mean ± 95% confidence interval (CI). Significance between each age is shown by unpaired t test (*p<0.05; **p<0.01; ***p<0.001).
doi:10.1371/journal.pone.0027598.g001

Tgl278T Cbs$^{-/-}$ mice have reduced fat mass compared to control mice

Dual energy X-ray absorptiometry (DEXA) analysis was performed on a subset of *Tg-I278T Cbs*$^{-/-}$ and control animals when they reached 240 days of age (8 months old). DEXA allows the quantitation of overall fat mass, lean mass, and bone density. In both male and female *Tg-I278T Cbs*$^{-/-}$ mice there was an appreciable decrease in fat mass (Fig. 3A, regular water) and percentage body fat (Fig. 3C; regular water) as compared to control animals. In male mice the fat mass was decreased from 12.28±3.78 g to 4.68±0.41 g (P<0.001), while in female mice it showed the same trend decreasing from 7.96±5.25 g to 4.26±0.36 g, although the difference here was not statistically significant (P = 0.152). In contrast, the amount of lean mass in *Tg-I278T Cbs*$^{-/-}$ animals was only slightly affected (9% and 14% less in females and males respectively, see Fig. 3B; regular water). These findings indicate that most of the size difference between *Tg-I278T Cbs*$^{-/-}$ and control animals was due to difference in the animals fat mass.

In addition, the DEXA analysis confirmed our earlier observation using micro CT [9] that *Tg-I278T Cbs*$^{-/-}$ mice had osteoporosis as evidenced by decreased bone mineral density. We found that the mean bone mineral density was reduced significantly in both male and female *Tg-I278T Cbs*$^{-/-}$ mice compared to controls (Fig. 3D; regular water).

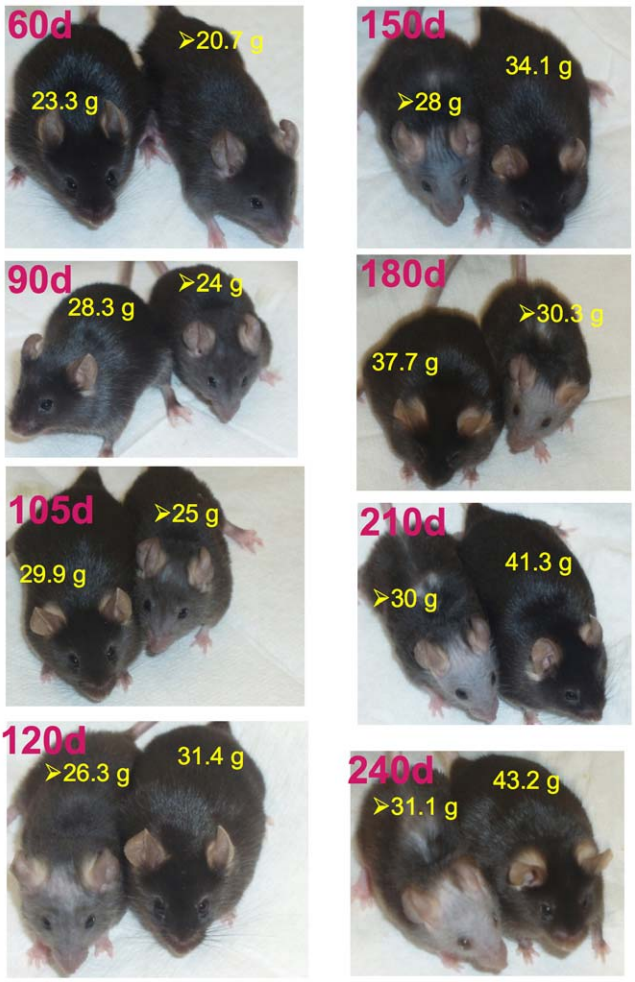

Figure 2. Photographic and weight comparison of a pair of sibling male *Tgl278T Cbs*$^{-/-}$ and *Tgl278T Cbs*$^{+/-}$ mice ranging from 60 to 240 days of age. Mice photographed from 60d-240d are the exact same pair. The *Tgl278T Cbs* $^{-/-}$ animal in each pair is indicated by the arrow.
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Decreased levels of serum cysteine and liver glutathione

Since human studies have suggested that serum tCys may play a role in regulating fat mass, we measured serum tCys in *Tg-I278T Cbs*$^{-/-}$ and control mice. Serum tCys was found to be 64% decreased in *Tg-I278T Cbs*$^{-/-}$ mice as compared to controls (P<0.001; Fig. 4A; regular water). Consistent with this observation, we found that liver glutathione levels were 34% decreased in *Tg-I278T Cbs*$^{-/-}$ mice (P<0.01; Fig. 4B; regular water). These findings show that lack of CBS activity lowers *in vivo* cysteine and glutathione levels despite the presence of cysteine in the diet.

Reduced expression of Scd-1 in *Tg-I278T Cbs*$^{-/-}$ mice

Previously we had performed microarray analysis on liver mRNA from *Tg-I278T Cbs*$^{-/-}$ and control animals [11]. We reexamined this data to look specifically at genes known to be involved in lipogenesis and fatty acid biosynthesis. Table S1 show the relative expression of these genes in *Tg-I278T Cbs*$^{-/-}$ in comparison to control mice. The transcript in this group with the greatest magnitude of change was *Scd-1*, with a seven-fold reduction in *Tg-I278T Cbs*$^{-/-}$ animals. *Scd-1* encodes the enzyme Stearoyl-coenzyme A desaturase, key lipogenic enzyme catalyzing the synthesis of monounsaturated fatty acids, mainly oleate and palmitoleate [12]. To confirm that *Scd-1* was indeed down-regulated in *Tg-I278T Cbs*$^{-/-}$, we performed quantitative real time PCR on *Tg-I278T Cbs*$^{-/-}$ mice and age and sex matched controls (Fig. 5A; regular water). Our results showed that expression of liver *Scd-1* message was only 6% of that observed in control animals (P<0.001). We also observed a 54% reduction in liver SCD-1 protein levels (Fig. 5B; regular water). We did not see any reduction SCD-1 levels in either of two white adipose tissues examined (Fig. 5C).

NAC treatment does not reverse phenotypes in *Tg-I278T Cbs*$^{-/-}$ mice

We next tested the hypothesis that the low levels of serum tCys and tissue glutathione levels might be responsible for the loss of fat mass and other phenotypes exhibited by *Tg-I278T Cbs*$^{-/-}$ mice. N-acetyl-L-cysteine (NAC), a cysteine analog, was added to the drinking water of both *Tg-I278T Cbs*$^{-/-}$ and control mice at a concentration 40 mM at the time of birth. We then measured weight at 60, 90, 105, 120, 150, 180, 210, and 240 days of age. We found that at all of the time points the average weight of NAC treated *Tg-I278T Cbs*$^{-/-}$ mice was less than NAC treated control animals (see Fig. 6 A,B). However, the relative difference in weight between the two genotypes was less than when the mice were on regular water (Fig. 1). The cause of this decrease in the difference became clear when we compared weights of mice on regular water vs. NAC water. We found that both control and *Tg-I278T Cbs*$^{-/-}$ animals exhibited reduced weights at each time point when on NAC water, but this difference was greater in control animals compared to *Tg-I278T Cbs*$^{-/-}$, especially as the animals aged (Fig. 6C). We also failed to observe any effect of NAC treatment on the reversing or delaying the alopecia phenotype in *Tg-I278T Cbs*$^{-/-}$ animals (Fig.S1A,B).

When the animals were 240 days old, DEXA analysis was performed on the NAC treated cohort. We observed large statistically significant decreases in fat mass, lean mass, percentage body fat, and bone mineral density in control animals on regular

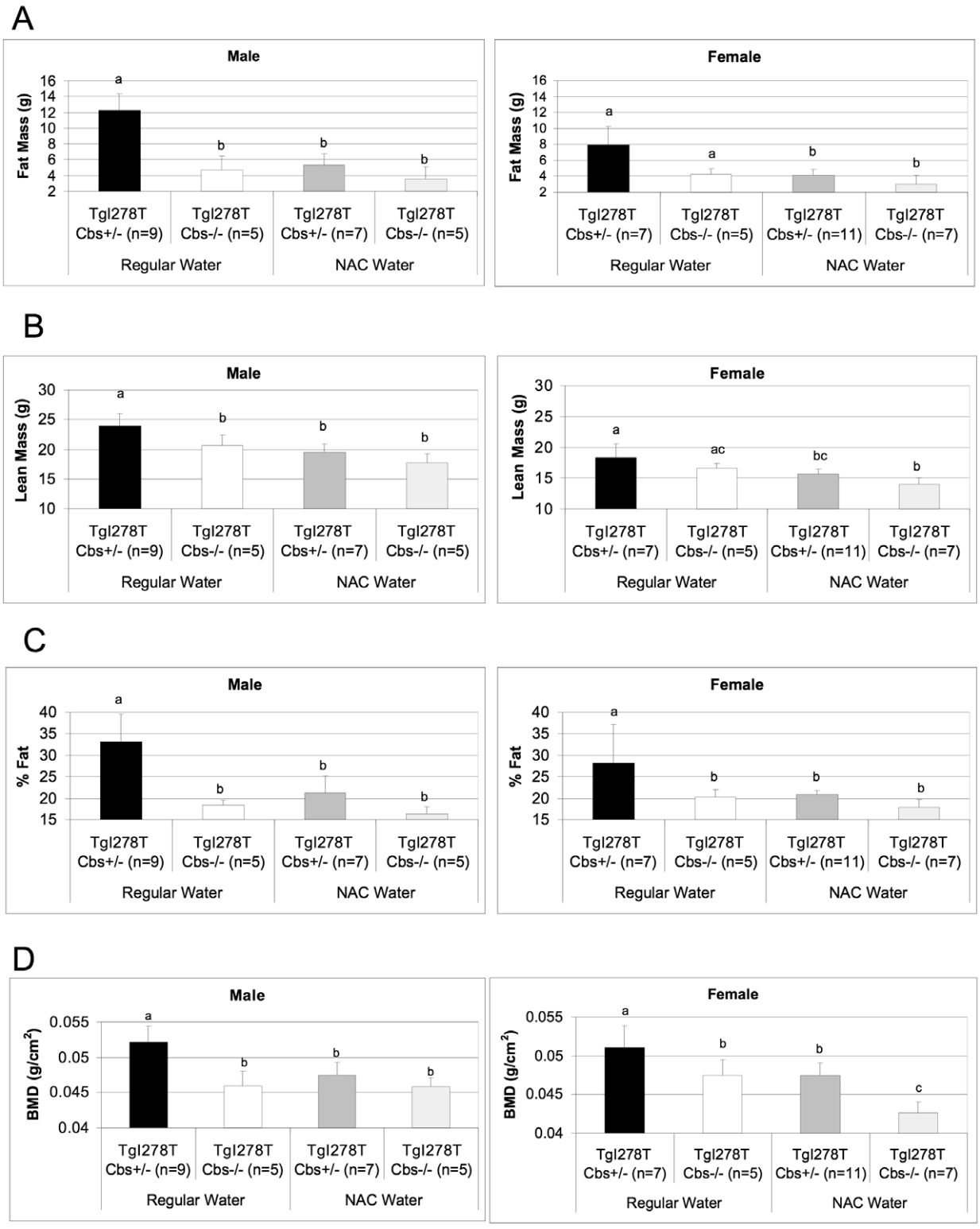
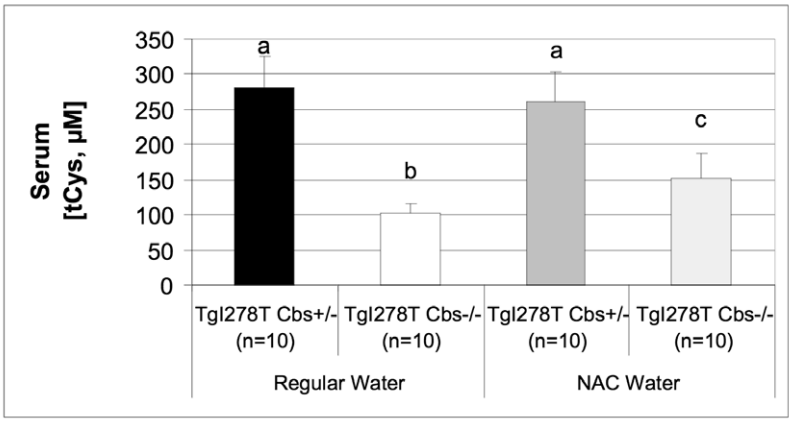


Figure 3. DEXA body composition studies on 240 day old *Tgl278T Cbs*$^{-/-}$ and *Tgl278T Cbs*$^{+/-}$ mice on regular and NAC supplemented water. All panels show effects on male and female animals separately. (**A**) Effect of genotype and NAC treatment on fat mass. (**B**) Effect of genotype and NAC treatment on lean mass. (**C**) Effect of genotype and NAC treatment on percentage of fat. (**D**) Effect of genotype and NAC on bone mineral density. Significance testing was performed using ANOVA followed by Tukey's multiple comparison test. All graphs show mean ± SD. Significant difference between columns are indicated by having a different letter at the top of the error bar. In case of more than two letters, even a single common letter at the top of the bar indicates non-significance.
doi:10.1371/journal.pone.0027598.g003

A



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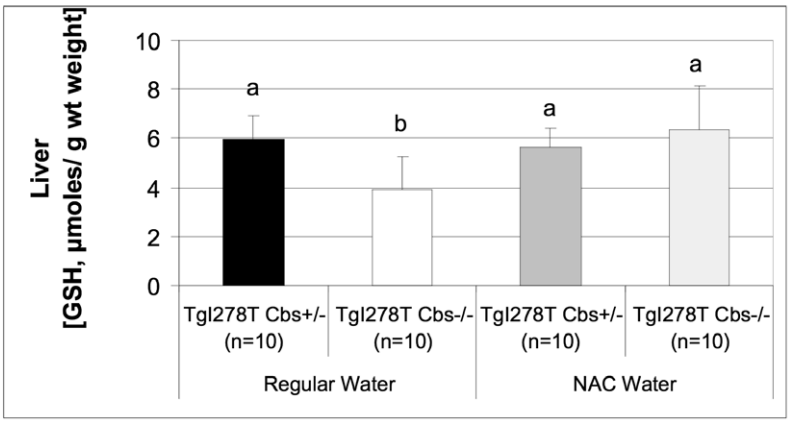

Figure 4. Effect of CBS genotype and NAC treatment on serum total cysteine and liver GSH. (**A**) Serum tCys. (**B**) Liver GSH. All four groups include 5 male and 5 female animals that were measured at 240 days of age. Graphs show the mean ± SD. Having different letters at the top of the error bar indicates significant differences between columns.
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water vs. NAC water (Fig. 3A-D). In contrast, *Tg-I278T Cbs*$^{-/-}$ animals were much less severely affected, with significant differences observed in lean, fat mass, and bone mineral density only in female animals. Finally, we examined *Scd-1* expression in NAC treated animals by both quantitative RT-PCR and Western analysis. We did not observe significant changes in the levels of *Scd-1* in either *Tg-I278T Cbs*$^{-/-}$ or control animals. (Fig. 5A,B; NAC water).

In summary these studies, in contrast to our *a priori* expectation, show that addition of NAC to drinking water did not restore fat mass or bone density to *Tg-I278T Cbs*$^{-/-}$ animals.

NAC treatment elevates tCys and liver glutathione in *Tg-I278T Cbs*$^{-/-}$ mice

Because NAC did not improve the phenotypes of *Tg-I278T Cbs*$^{-/-}$ mice, we examined if oral NAC was effective in raising serum tCys and liver glutathione. We found that in *Tg-I278T Cbs*$^{-/-}$ mice, tCys raised by 50%, from 101.7 to 152.6 µM ($p<0.001$, Fig. 4A). In contrast, we failed to observe any significant change in between NAC treated and untreated control mice (281 vs 261 µM, P = ns). Interestingly serum tHcy levels were slightly lower in NAC treated *Tg-I278T Cbs*$^{-/-}$ mice compared to untreated mice (383.6 vs. 334.5 µM, $P<0.05$). Amino acid profiling of twenty-seven additional amino acids and amine containing compounds did not reveal any other statistically significant changes (Table S2).

Since cysteine is the rate limiting amino acid in glutathione biosynthesis and in liver approximately 50% of the cysteine in glutathione is derived from methionine via transsulfuration pathway [13], we determined if liver GSH levels were modulated by NAC treatment (Fig. 4B). Like tCys, we found that NAC treatment raised liver GSH levels in *Tg-I278T Cbs*$^{-/-}$ animals, but had no effect on control animals. These experiments show that NAC treatment did have the desired physiological effects of raising tCys and liver glutathione in *Tg-I278T Cbs*$^{-/-}$ mice, but did not cause an increase in control animals.

Effect of NAC treatment on Food and water consumption

Because of the large decrease in weight and fat mass in control (*Tg-I278T Cbs*$^{+/-}$) on NAC water, we performed a 6 week study to

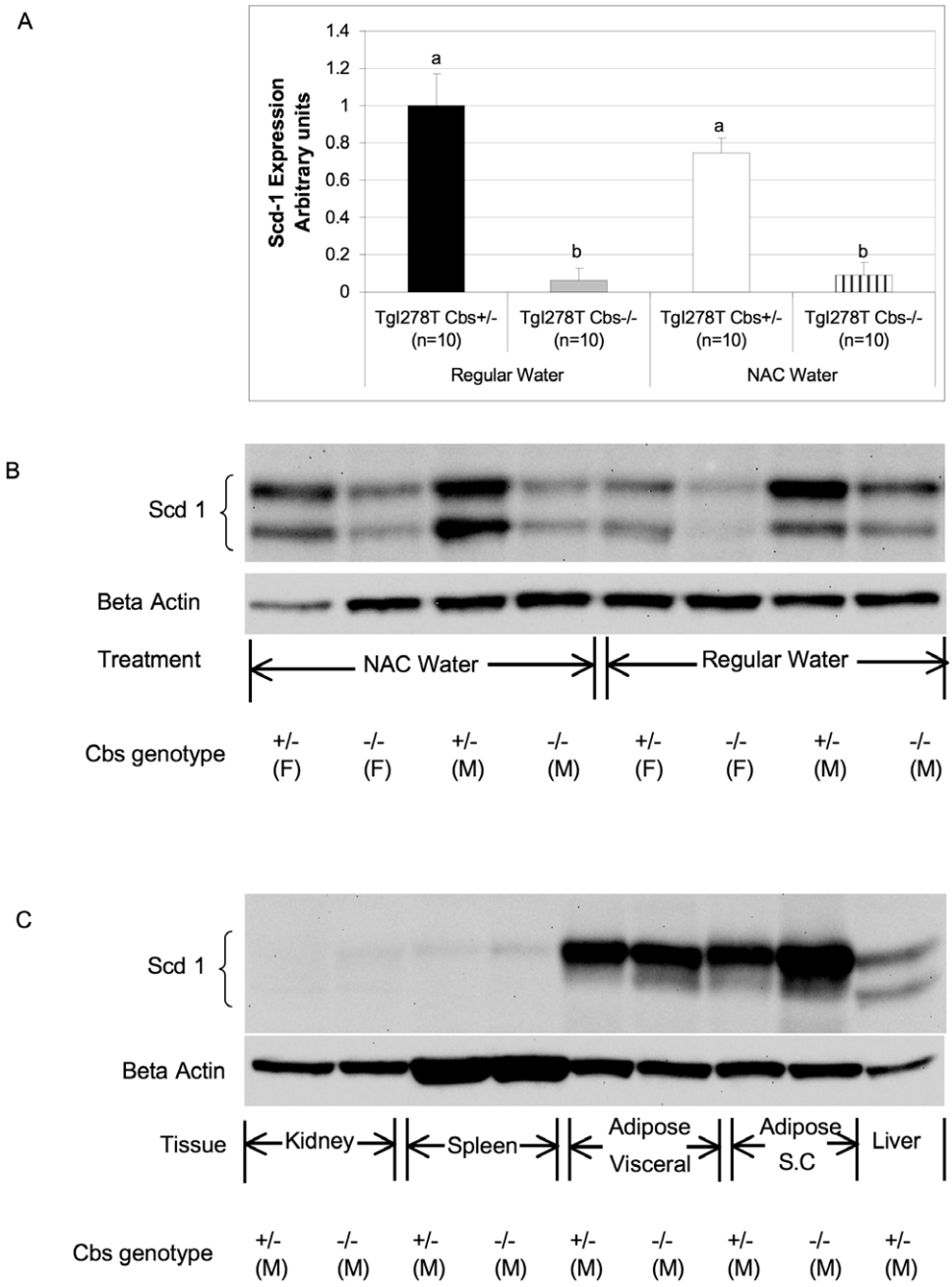

Figure 5. Effect of CBS genotype and NAC treatment on SCD-1 levels. (**A**) Liver *Scd-1* mRNA levels assessed by qRT-PCR. All four groups include 5 males and 5 females that are exactly 240 days of age. Graphs show the mean ± SD. Having different letters at the top of the error bar indicates significant differences between columns; (**B**) Liver SCD-1 protein levels as assessed by Western blotting. SCD1 runs as a doublet centered at 37 kDa. (**C**) SCD-1 expression in kidney, spleen, visceral, and subcutaneous fat from (*Tg-I278T Cbs*$^{+/-}$) and Cbs deficient (*Tg-I278T Cbs*$^{-/-}$) mice.
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see how NAC affected food and water consumption in eight to twelve week old mice. We found that although NAC added to the water decreased the water intake by around 40%, the food intake was only 10% less compared to mice on regular water (Fig. S2A,B). Similar percent reduction was estimated when results were calculated in terms of consumption/kg body weight/day (data not shown). However, we also observed that NAC treatment resulted in a 45% reduction in weight gain (Fig. S2C). These findings suggest that NAC may be affecting weight gain by means other than simply decreasing food intake.

Discussion

In this study we have shown for the first time that the decreased weight of the *Tg-I278T Cbs*$^{-/-}$ mouse model of CBS deficiency is driven primarily by decreased fat mass. Body composition study by DEXA reveals that 240 day old *TgI278T Cbs*$^{-/-}$ mice have a 46% (in females) to 62% (in males) reduction in fat mass than age and sex matched control animals. On the other hand, lean mass was only slightly affected (9% and 14% less in females and males respectively). Our findings integrate well with the observation that

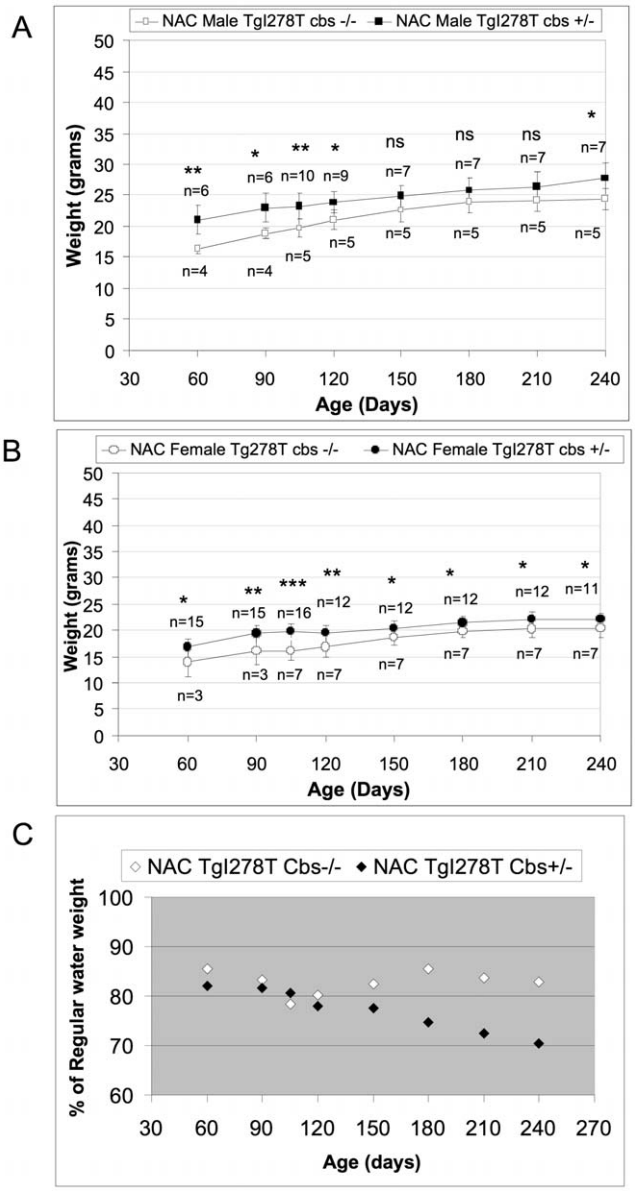

Figure 6. Comparison of weight of NAC treated *Tgl278T Cbs*$^{-/-}$ and *Tgl278T Cbs*$^{+/-}$ mice over time. (**A**) Weights of male mice. (**B**) Weights of female mice. (**C**) Shows the % of regular water weight for each genotype at each time point. Significance was done by unpaired t test: *p<0.05; **p<0.01; ***p<0.001; ns (non significant). Graphs show the mean ± SD.
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human CBS deficient patients generally have low BMI and decreased subcutaneous fat [2,3]. This large decrease in fat mass is associated with a reduction in mRNA expression and protein levels of *Scd-1*, a key enzyme involved in lipogenesis. *Scd-1* catalyzes the biosynthesis of unsaturated fatty acids which promotes obesity [12]. Mice with targeted disruption of *Scd-1* have reduced body adiposity, increased insulin sensitivity, and resistance to diet induced weight gain [14]. Moreover, *ob/ob* mice with mutations in *Scd-1* are significantly less obese than *ob/ob* controls [15]. Interestingly, both *Scd-1*$^{-/-}$ mice and *Cbs*$^{-/-}$ mice have been noted to have cutaneous abnormalities [16,17,18]. Recently, rats on a methionine restricted diet have been shown to have both low hepatic *Scd-1* levels, and low body fat [6]. Given these relationships it seems likely that the reduced level of hepatic *Scd-1* expression observed in *Tg-I278T Cbs*$^{-/-}$ mice is at least in part responsible for the reduction in fat mass.

In this study we have also explored the hypothesis that low plasma cysteine and tissue GSH resulting from loss of CBS activity might mediate both the observed fat loss and the down regulation of *Scd-1*. The motivation for this hypothesis comes from both epidemiological studies in humans, as well as dietary manipulation studies in rodents [4,6,7]. To test this hypothesis, we performed an intervention study in which we restored tCys by addition of NAC to the animals' drinking water. Chemically NAC is similar to cysteine, but the presence of acetyl moiety make it less susceptible to oxidation and more soluble in water, thus making it a better source of cysteine [19]. NAC is widely available as a human supplement and has been used in a variety of clinical trials to treat conditions such as bronchitis, cystic fibrosis, and HIV infection [20,21,22]. Measurements of serum tCys and liver GSH in NAC treated *Tg-I278T Cbs*$^{-/-}$ mice indicated that the treatment was successful in raising tCys and liver GSH. Liver GSH levels were entirely corrected, while serum tCys increased by 50%, although this was still somewhat lower than in control mice. Based on the hypothesis that low tCys and liver GSH are the driving force for the accumulation of fat mass, we expected mice on NAC intervention to weigh more and have increased fat mass. However, contrary to our expectation, we observed the opposite effect, i.e. NAC treated mice showed decreased total weight and fat mass. In addition, NAC treatment had no significant effect on *Scd-1* mRNA or protein levels and failed to reverse either the osteoporosis or alopecia phenotypes. In total, our data indicate that low serum tCys and tissue GSH are not the direct cause of fat mass, hair-loss, or osteoporosis phenotypes in *Tg-I278T Cbs*$^{-/-}$ mice.

Besides low cysteine, *Tg-I278T Cbs*$^{-/-}$ mice also have greatly elevated plasma homocysteine levels and liver S-adenosylhomocysteine (SAH levels) [9]. SAH is strong inhibitor of a variety of S-adenosylmethionine dependent methyltransferase enzymes, including DNA and histone methyltransferases [23,24,25,26,27]. Methylation of DNA and histones are critical events in the regulation of gene expression [28–29]. It is possible that the low levels of Scd-1 mRNA observed in the livers of *Tg-I278T Cbs*$^{-/-}$ animals may be related to inhibition of specific methyltransferase enzymes that normally are involved in regulating transcription from the *Scd-1* promoter. Studies examining the chromatin state of the Scd-1 promoter could confirm if this was in fact the case.

An unexpected finding of this study was that control animals exhibited a 48–56% (females-males) decrease in fat mass as a result of being given NAC water. Interestingly, we only observed a slight decrease in food intake in NAC treated animals, suggesting that the decrease in fat mass may be related to altered metabolism. It should be noted that there are several reports linking NAC in drinking water and weight loss. In one study it was found that NAC addition to drinking water resulted in decreased weight gain and BMI in rats on a high sucrose diet [30]. In another study, rats treated with NAC in drinking water showed significant decreases in relative body weight and a reduction of visceral fat [31]. The same study found that NAC inhibited lipid droplet formation in 3T3L1 adipocytes *in vitro*. Although reactive oxygen species (ROS) play an important role in adipocyte differentiation of 3T3L1 cells [32], and mediate insulin dependent signal transduction [33], other antioxidants including GSH, ascorbic acid and retinoic acid show no effect *in vitro* [31]. These results leave open the possibility that the reason we failed to see increased fat mass accumulation in *Tg-I278T Cbs*$^{-/-}$ mice treated with NAC is that the pro-lipogenic effects of elevation in serum cysteine was somehow counteracted by the anti-oxidant effect of NAC. One key difference between

NAC and cysteine, as that NAC is a potent anti-oxidant while cysteine is not. In the future the difference between NAC and cysteine could be addressed by altering the cysteine content of the mouse diet directly.

In summary, we have shown that *Tg-I278T Cbs*$^{-/-}$ mice lacking CBS activity have decreased serum tCys, decreased liver GSH, decreased fat mass, facial alopecia, reduced bone mineral density and more than 90% reduction in liver *Scd-1* mRNA compared to sibling control animals. However, we failed to see any improvement in these phenotypes when serum tCys and liver GSH were elevated by feeding the animals NAC for a period of 240 days. Our results suggest that the phenotypes observed in CBS deficient patients are probably not related to low tCys levels and reinforce the view that it is the elevated tHcy that is the pathologic agent in CBS deficiency.

Supporting Information

Figure S1 Effect of NAC treatment on hair loss phenotype. Representative pairs of sibling of *Tg-I278T Cbs*$^{-/-}$ and *Tg-I278T Cbs*$^{+/-}$ mice are shown over the course of 60 to 240 days of age. Panels on left show pair on regular water, while panels on right shows a different pair on NAC water.
(PPT)

Figure S2 Effect of NAC treatment on water and food consumption in *Tg-I278T Cbs*$^{+/-}$ control mice. (**A**) Water consuminption is shown for a six week period with the mean ± SD from each cage shown. (**B**) Food consumption is shown. (**C**) Total weight gained and total food consumed by NAC treated control mice compared to regular water control mice in 7 weeks (including one week of acclimatization). Paired t-test was done for all the panels.
(PPT)

Table S1 Expression levels of selected lipogenesis genes. Previously published microarray data from the livers of *Tg-I278T Cbs*$^{-/-}$ and control mice were analyzed for genes specifically involved in lipogenesis (see reference [9]).
(DOC)

Table S2 Comparison of serum amino acids in *Tg-I278T Cbs*$^{-/-}$ on normal and NAC containing water.
(DOC)

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Author Contributions

Conceived and designed the experiments: WDK SG. Performed the experiments: SG. Analyzed the data: WDK SG. Contributed reagents/materials/analysis tools: SG WDK. Wrote the paper: SG WDK.

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