Amino Acids (2014) 46:1471–1480
DOI 10.1007/s00726-014-1712-4

ORIGINAL ARTICLE

Bleomycin hydrolase and hyperhomocysteinemia modulate the expression of mouse proteins involved in liver homeostasis

Joanna Suszyńska-Zajczyk · Jacek Wróblewski · Olga Utyro · Magdalena Łuczak · Łukasz Marczak · Hieronim Jakubowski

Received: 23 December 2013 / Accepted: 21 February 2014 / Published online: 16 March 2014

Abstract The liver is the major contributor to homocysteine (Hcy) metabolism and fatty liver disease is associated with hyperhomocysteinemia. Bleomycin hydrolase (Blmh) is an aminohydrolase that also participates in Hcy metabolism by hydrolyzing Hcy-thiolactone. To gain insight into hepatic functions of Blmh, we analyzed the liver proteome of $Blmh^{-/-}$ and $Blmh^{+/+}$ mice in the absence and presence of diet-induced (high methionine) hyperhomocysteinemia using 2D IEF/SDS-PAGE gel electrophoresis and MALDI–TOF mass spectrometry. We identified eleven liver proteins whose expression was significantly altered as a result of the *Blmh* gene inactivation. The differential expression ($Blmh^{-/-}$ vs. $Blmh^{+/+}$) of four liver proteins was lower, of two proteins was higher, and was further modified in mice fed with a hyperhomocysteinemic high-Met diet. The down-regulated proteins are involved in lipoprotein metabolism (ApoA1, ApoE), antigen processing (Psme1), energy metabolism (Atp5h, Gamt), methylglyoxal detoxification (Glo1), oxidative stress response (Sod1), and inactivation of catecholamine neurotransmitters (Comt). The two up-regulated proteins are involved in nitric oxide generation (Ddah1) and xenobiotic detoxification (Sult1c1). We also found that livers of $Blmh^{-/-}$ mice expressed a novel variant of glyoxalase domain-containing protein 4 (Glod4) by a post-transcriptional mechanism. Our findings suggest that Blmh interacts with diverse cellular processes—from lipoprotein metabolism, nitric oxide regulation, antigen processing, and energy metabolism to detoxification and antioxidant defenses—that are essential for liver homeostasis and that modulation of these interactions by hyperhomocysteinemia underlies the involvement of Hcy in fatty liver disease.

Keywords Bleomycin hydrolase · High methionine diet · Hyperhomocysteinemia · Mouse liver proteome

Abbreviations

Hcy	Homocysteine
Blmh	Bleomycin hydrolase
Cbs	Cystathionine β-synthase
ApoA1	Apolipoprotein A1
ApoE	Apolipoprotein E
Psme1	Proteasome activator complex subunit 1
Atp5 h	ATPase subunit d
Gamt	Guanidinoacetate-*N*-methyltransferase
Glo1	Methylglyoxylase 1
Sod1	Superoxide dismutase
Comt	Catechol-*O*-methyltransferase
Ddah1	Dimethylarginine dimethylaminohydrolase 1
Sult1c1	Phenolsulphotransferase

J. Suszyńska-Zajczyk and J. Wróblewski contributed equally to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s00726-014-1712-4) contains supplementary material, which is available to authorized users.

J. Suszyńska-Zajczyk · J. Wróblewski · O. Utyro · M. Łuczak · Ł. Marczak · H. Jakubowski
Institute of Bioorganic Chemistry, Poznań, Poland

J. Suszyńska-Zajczyk · H. Jakubowski (✉)
Department of Microbiology and Molecular Genetics, Rutgers-New Jersey Medical School, International Center for Public Health, 225 Warren Street, Newark, NJ 07101-1709, USA
e-mail: jakubows@njms.rutgers.edu

H. Jakubowski
Department of Biochemistry and Biotechnology, University of Life Sciences, Poznań, Poland

IEF/SDS-PAGE	Isoelectric focusing/sodium dodecylsulphate polyacrylamide gel electrophoresis
MALDI–TOF	Matrix-assisted laser desorption ionization–time of flight

Introduction

Elevated homocysteine (Hcy)[1] levels have long been known to be linked to liver disease. For example, fatty liver is a common finding in genetic hyperhomocysteinemia due to cystathionine β-synthase (CBS) deficiency (Mudd et al. 2001) and in nutritional hyperhomocysteinemia due to excessive alcohol intake, folate deficiency, or methionine overload (Werstuck et al. 2001; Hirsch et al. 2005; Kaplowitz et al. 2007). Although there is evidence suggesting that Hcy-induced endoplasmic reticulum and oxidative stress mediate liver damage by promoting apoptotic cell death, inflammation, insulin resistance, and deregulated lipid metabolism (Kaplowitz et al. 2007), the exact mechanism underlying Hcy-induced liver damage is unclear. Analysis of mRNA expression shows that several ATP-binding cassette transporters and nuclear hormone receptors involved in liver homeostasis are differentially expressed in *Cbs*$^{-/-}$ mouse liver (Hamelet et al. 2007). A recent proteomic study shows that dietary hyperhomocysteinemia alters the expression of mouse liver proteins involved in Hcy metabolism, urea cycle, and antioxidant defenses (DiBello et al. 2010).

Bleomycin hydrolase (Blmh) is a thiol-dependent cytoplasmic aminopeptidase expressed in various organs, including the liver (Kamata et al. 2007). In addition to a proteolytic activity, Blmh exhibits a hydrolase activity toward Hcy-thiolactone (Zimny et al. 2006; Borowczyk et al. 2012), a chemically reactive metabolite implicated in the pathology of hyperhomocysteinemia (Jakubowski 1997, 2013; Chwatko et al. 2007). Metabolism of Hcy-thiolactone is impaired in *Blmh*-null mice, which also exhibit increased sensitivity to Hcy-thiolactone toxicity (Borowczyk et al. 2012). In addition, the *Blmh*-null mice have several other phenotypes, including neonatal mortality, tail dermatitis (Schwartz et al. 1999), brain pathology (Montoya et al. 2007), and impairments in the presentation of some antigens (Towne et al. 2007), suggesting important roles of Blmh in these organs.

In contrast, the function of Blmh in the liver has not been studied and how the alteration of Hcy metabolism in *Blmh*-null mice affects liver homeostasis is not known. To identify metabolic pathways regulated by Blmh and to gain insight into its physiological roles, we examined the liver proteome in *Blmh*-null mice in the absence and presence of diet-induced hyperhomocysteinemia.

Materials and methods

Mice and diets

Colonies of *Blmh*$^{-/-}$ mice on the C57BL/6J genetic background (Schwartz et al. 1999) and wild-type *Blmh*$^{+/+}$ littermates were bred and housed at the Rutgers-New Jersey Medical School Animal Facility. The mice were fed a normal rodent chow (LabDiet 5010, Purina Mills International, St. Louis, MO, USA) (Borowczyk et al. 2012). To induce hyperhomocysteinemia, 4-week-old mice were provided 1 % methionine in drinking water for 8 weeks (high-Met diet) (Zhou et al. 2001; Velez-Carrasco et al. 2008; Borowczyk et al. 2012). Four experimental groups of animals were studied (4 animals/group): (1) *Blmh*$^{-/-}$ mice, control diet; (2) *Blmh*$^{+/+}$ mice, control diet; (3) *Blmh*$^{-/-}$ mice, high-Met diet; (4) *Blmh*$^{+/+}$ mice, high-Met diet. Animal procedures were approved by the Institutional Animal Care and Use Committee at the Rutgers-New Jersey Medical School.

Genotyping

Genomic DNA isolation and genotyping to establish the status of the *Blmh* locus (Schwartz et al. 1999) were carried out as described in the Supplemental Experimental Procedures.

Hcy assays

Total Hcy and *N*-Hcy proteins were assayed by HPLC-based methods with post-column derivatization and fluorescence detection as described previously (Jakubowski 2008; Jakubowski et al. 2008, 2009).

Proteomic analyses

Protein sample preparation, 2D IEF/SDS-PAGE image analysis, and protein identification by MALDI–TOF mass spectrometry (Luczak et al. 2011) were carried out as described in the Supplemental Experimental Procedures.

Glod4 mRNA analysis

Total RNA extraction, reverse transcription, and PCR amplification using primers specific for Glod4 isoforms 1, 2 and 3 (http://www.uniprot.org/uniprot/Q9CPV4) were carried out as described in Supplemental Experimental Procedures.

Data treatment and statistical analysis

For each animal in the four experimental group (4 animals/group), the analyses were repeated 2–3 times. The relative

abundance of each protein spot (% volume) was calculated as its volume divided by the total volume of all spots. Data are expressed as mean ± SD. Data for each protein spot had a normal distribution. The differences between the groups were analyzed by ANOVA. Unpaired Student's *t* test was used to test differences between two groups. Statistical analyses were carried out using Statistica 8.0 software.

Results

Dietary hyperhomocysteinemia in *Blmh*$^{-/-}$ and *Blmh*$^{+/+}$ mice

We studied liver proteomes of *Blmh*$^{-/-}$ mice and their *Blmh*$^{+/+}$ littermates fed a standard chow diet. Because Blmh protein is involved in Hcy metabolism, we also studied effects of *Blmh* gene inactivation on the liver proteome in mice, in which hyperhomocysteinemia was induced by feeding with a high-Met diet for 8 weeks. Plasma tHcy levels increased to 39 ± 19 and 77 ± 45 μM in *Blmh*$^{-/-}$ and *Blmh*$^{+/+}$ mice fed with a high-Met diet, from a basal level of 6.8 ± 2.2 and 7.4 ± 2.2 μM, respectively, in mice fed the standard chow diet (Borowczyk et al. 2012). *N*-Hcy protein increased to 8.4 ± 2.8 and 5.4 ± 2.9 μM in hyperhomocysteinemic *Blmh*$^{-/-}$ and *Blmh*$^{+/+}$ mice, respectively, from the corresponding basal levels of 2.8 ± 0.8 and 1.2 ± 0.4 μM in non-homocysteinemic animals (Borowczyk et al. 2012).

Identification of proteins differentially expressed in *Blmh*$^{-/-}$ mouse liver

Mouse liver protein separation by IEF/SDS-PAGE yielded several hundred distinct protein spots (Supplementary Fig. S1), 100 of which have been identified by MALDI–TOF mass spectrometry (Supplementary Table S1). Ten of these proteins were found to have significantly changed expression in *Blmh*$^{-/-}$ mice relative to *Blmh*$^{+/+}$ littermates, while eleven had significantly changed expression in response to high-Met diet (Table 1). The expression levels of the other

Table 1 Differentially expressed liver proteins regulated by *Blmh*$^{-/-}$ genotype and/or high-Met diet

Protein description (spot #)	Gene name	Fold change *Blmh*$^{-/-}$ vs. *Blmh*$^{+/+}$		Fold change Std vs. 1 % Met diet	
		Control diet	1 %-Met diet	*Blmh*$^{+/+}$	*Blmh*$^{-/-}$
Lipoprotein metabolism					
Apolipoprotein A1 (#77)	*ApoA1*	−1.10	−1.89[a]	−1.47[a]	−2.56[a]
Apolipoprotein E (#39)	*ApoE*	−1.16[b]	−1.79[b]	−1.06	−1.61[a]
Antigen processing					
Proteasome activator complex subunit1 (#69)	*Psme1*	1.00	−4.00[a]	1.22[b]	−2.27[a]
Energy metabolism					
ATP synthase subunit d (#78)	*Atp5h*	−1.30[b]	−2.50[a]	1.35[b]	−1.16
Guanidinoacetate *N*-methyltransferase (#76)	*Gamt*	−1.04	−1.82[a]	1.62[a]	−1.08
Iron metabolism and homeostasis					
Ferritin light chain (#81)	*Ftl*	1.00	−1.02	1.68[a]	1.65[b]
Oxidative stress response					
Peroxiredoxin 2 (#73)	*Prdx2*	1.14	1.14	1.28[a]	1.28[a]
Superoxide dysmutase 1 (#91)	*Sod1*	−1.33[a]	1.08	1.10	1.40[a]
Catechol metabolism					
Catechol-*O*-methyl transferase (#75)	*Comt*	−1.06	−1.56[a]	1.36[a]	−1.06
Nitric oxide generation					
Dimethylarginine dimethylaminohydrolase 1 (#41)	*Ddah1*	1.45[a]	2.41[a]	−1.14[a]	1.45[a]
Methylglyoxal detoxification					
Methylglioxalase 1 (#71)	*Glo1*	−1.30[b]	−1.64[a]	−1.19	−1.14
Xenobiotic detoxification					
Phenol sulfotransferase (#40)	*Sult1c1*	1.60[a]	1.25[a]	−1.05	−1.25[a]

(Spot #) refers to the numbering on the IEF/SDS-PAGE gels in Fig. 1 and Supplementary Fig. S1

Significantly different: [a] $P < 0.001$, [b] $P < 0.01$, [c] $P < 0.05$

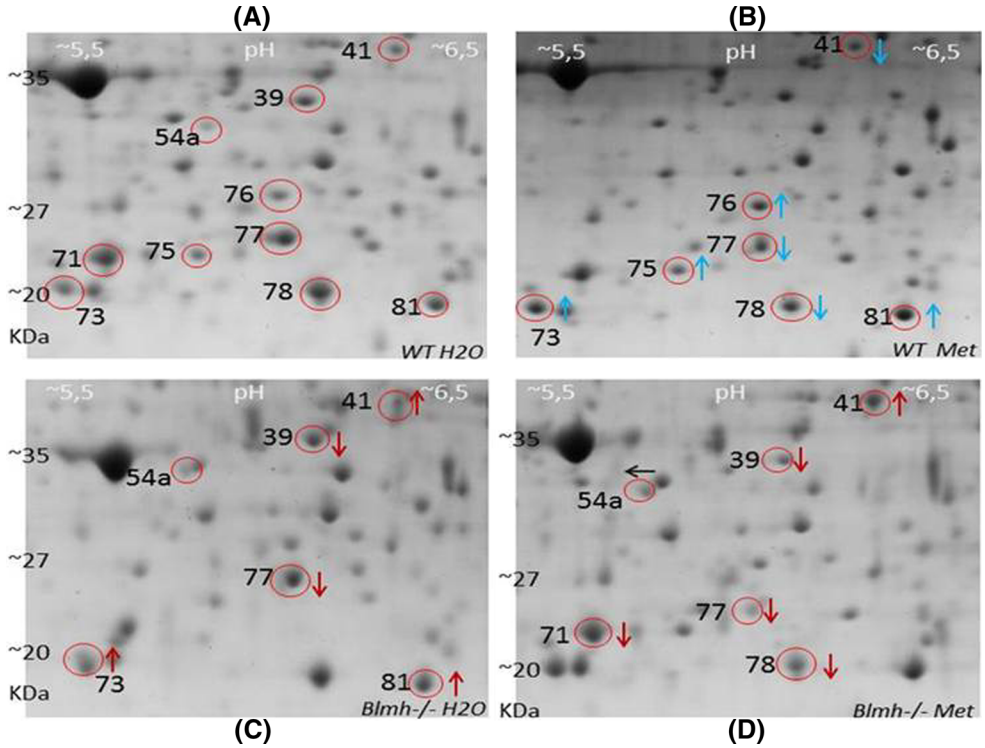

Fig. 1 Close-up views of representative IEF/SDS-PAGE gels showing mouse liver proteins whose expression was affected by *Blmh* genotype and/or high-Met diet. Analyses were carried out for the following groups of mice: **a** $Blmh^{+/+}$, control diet; **b** $Blmh^{+/+}$, high-Met diet; **c** $Blmh^{-/-}$, control diet; **d** $Blmh^{-/-}$, high-Met diet. *Up* and *down arrows* indicate the direction of the change dependent on *Blmh* genotype (**c**, **d**) and high-Met diet (**b**). Isoelectric point of one identified protein (Glod4, spot 54a) was changed to a more acidic value in $Blmh^{-/-}$ mice, indicated by a *black horizontal arrow* in **d**

88 identified proteins were not affected by the *Blmh* genotype or the high-Met diet, but one protein exhibited pI that was dependent on *Blmh* genotype. Close-up views of representative IEF/SDS-PAGE separations of differentially expressed proteins are shown in Fig. 1. Quantification of the levels (% volume) for each of the differentially expressed proteins is shown in Fig. 2.

Liver proteins regulated by *Blmh* genotype

In mice fed with a standard chow diet the differential expression ($Blmh^{-/-}$ vs. $Blmh^{+/+}$ mice) of four liver proteins was lower (−1.16 to −1.33-fold, $P < 0.01$), and of two proteins was higher (1.45–1.60-fold, $P < 0.001$) (Table 1). The proteins down-regulated in $Blmh^{-/-}$ mice include those involved in lipoprotein metabolism (apolipoprotein E, ApoE), energy metabolism (ATP synthase subunit d, Atp5h), methylglyoxal detoxification (methylglyoxalase 1, Glo1), and antioxidant defense (superoxide dismutase, Sod1). The two proteins that were up-regulated in $Blmh^{-/-}$ mice are involved in the regulation of nitric oxide generation (dimethylarginine dimethylaminohydrolase 1, Ddah1) and xenobiotic detoxification (phenol sulfotransferase, Sult1c1) (Table 1). Isoelectric point of one liver protein, glyoxylase domain-containing protein 4 (Glod4), was changed to a more acidic value in $Blmh^{-/-}$ mice (Fig. 1c, spot 54a).

Liver proteins regulated by hyperhomocysteinemia

In $Blmh^{+/+}$ mice, the high-Met diet significantly down-regulated two liver proteins (Ddah1 and apolipoprotein A1, ApoA1; −1.14 to −1.47-fold, $P < 0.001$) and up-regulated six (proteasome activator complex subunit 1—Psme1, Atp5 h, Gamt, ferritin light chain—Ftl, Prdx2, catechol-*O*-methyltransferase—Comt; 1.22 to 1.68-fold, $P < 0.01$) (Table 1). With the exception of Atp5q and Ddah1, the expression of these Hcy-affected proteins was not significantly affected the $Blmh^{-/-}$ genotype alone (Table 1).

Liver proteins regulated by *Blmh* genotype in the presence of hyperhomocysteinemia

The magnitude of changes in the differential expression of liver proteins ($Blmh^{-/-}$ vs. $Blmh^{+/+}$) was increased in mice fed with a hyperhomocysteinemic high-Met diet (Table 1). For example, the differential expression ($Blmh^{-/-}$ vs. $Blmh^{+/+}$) of ApoE, Atp5h, and Glo1 was −1.79 to −2.5-

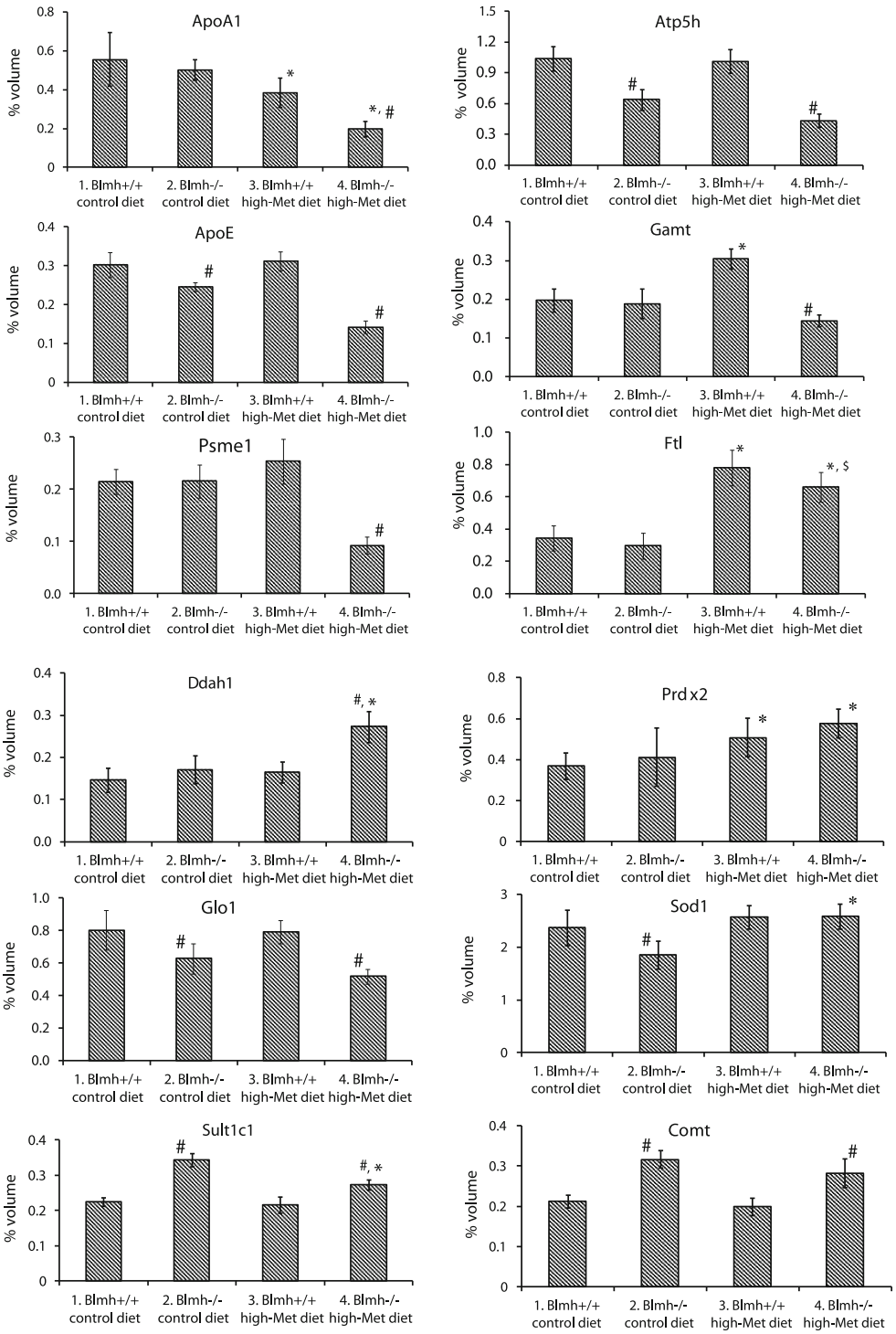

Fig. 2 Mouse liver protein expression levels as a function of *Blmh* genotype and/or high-Met diet. *Each panel* shows expression level (% volume) for the indicated liver protein determined for the following groups of mice: *1* $Blmh^{+/+}$, control diet; *2* $Blmh^{-/-}$, control diet; *3* $Blmh^{+/+}$, high-Met diet; *4* $Blmh^{-/-}$, high-Met diet. $^{\#}$, $^{\$}$, *Significant effects of the $Blmh^{-/-}$ genotype ($^{\#}P < 0.001$, $^{\$}P < 0.02$) and high-Met diet ($^{*}P < 0.0001$), respectively

fold lower ($P < 0.001$) while the differential expression of Sult1c1 and Ddah1 was 1.25–2.51-fold higher ($P < 0.001$) when the $Blmh^{-/-}$ and $Blmh^{+/+}$ mice were fed with high-Met diet. The expression of five proteins (ApoA1, Psme1, Psme1, Gamt, Comt) became dependent on *Blmh* genotype only in mice fed with high-Met diet and was decreased −1.56 to −4.00-fold in $Blmh^{-/-}$ animals (Table 1). The change in the isoelectric point of Glod4 to a more acidic value that occurred in $Blmh^{-/-}$ mice fed with a control diet (Fig. 1c, spot 54a) was also observed in $Blmh^{-/-}$ mice fed with a high-Met diet (Fig. 1d, spot 54a).

Western blot analysis was performed for ApoA1 and Ftl to validate the IEF/SDS-PAGE results. As shown in Fig. 3, ApoA1 was lowered by high-Met diet both in $Blmh^{-/-}$ and

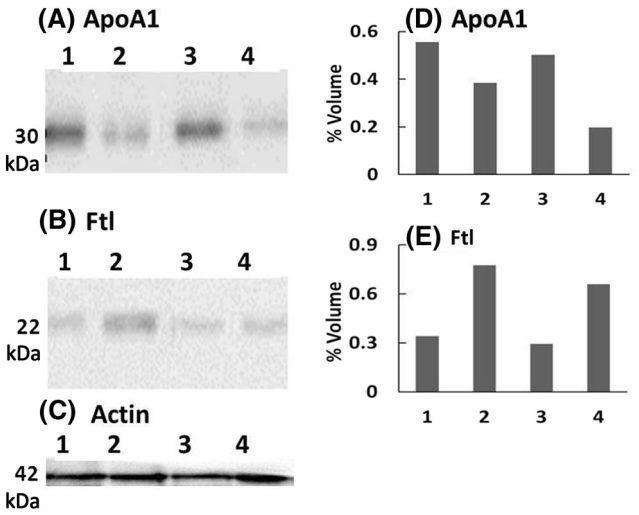

Fig. 3 Western blot validation of IEF/SDS-PAGE results. **a**, **b** Western blots for ApoA1 and Ftl, respectively. **c** Western blot for actin that indicates equal loading in the four *lanes*. **d**, **e** '% volume' for ApoA1 and Ftl, respectively, from IEF/SDS-PAGE analyses of the same samples. The following groups of mice were studies: *lane 1* $Blmh^{+/+}$, control diet; *lane 2* $Blmh^{+/+}$, high-Met diet; *lane 3* $Blmh^{-/-}$, control diet; *lane 4* $Blmh^{-/-}$, high-Met diet

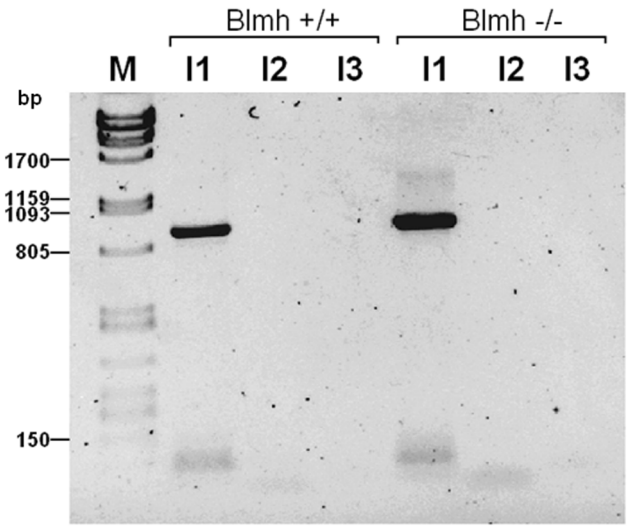

Fig. 4 RT-PCR analysis of liver Glod4 mRNA in $Blmh^{-/-}$ and $Blmh^{+/+}$ mice. Glod4 mRNA was amplified with primers specific for isoform 1, 2, or 3 and the products were analyzed by agarose gel electrophoresis. PCR product of expected size, 920 bp, formed only with isoform 1 primers and is shown in lanes labeled I1. No PCR product formed with isoform 2 and 3 primers, as shown in lanes labeled I2 and I3, respectively. *Lane M* shows λ/PstI standards. Identical results were obtained for six individual mice, three $Blmh^{-/-}$ and three $Blmh^{+/+}$

$Blmh^{+/+}$ mice (Fig. 3a), similar to the results obtained by the IEF/SDS-PAGE analysis (Fig. 3d). Western blot analysis also showed that Ftl, was elevated by high-Met diet in $Blmh^{-/-}$ mice and to a lesser extent in $Blmh^{+/+}$ mice (Fig. 3b), consistent with the results of the IEF/SDS-PAGE analyses (Fig. 3e).

Glod4 isoform 1 mRNA is expressed in livers of $Blmh^{-/-}$ and $Blmh^{+/+}$ mice

To determine whether a more acidic isoelectric point (pI) of the Glod4 protein expressed in $Blmh^{-/-}$ mice (relative to Glod4 expressed in $Blmh^{+/+}$ animals) is due to the expression of different isoforms, we analyzed Glod4 mRNA by PCR using primers specific for Glod4 isoforms 1, 2 and 3 (http://www.uniprot.org/uniprot/Q9CPV4). We found that Glod4 isoform 1 was expressed in livers of $Blmh^{-/-}$ and wild-type $Blmh^{+/+}$ mice while isoforms 2 and 3 were not expressed at a detectable level (Fig. 4). These findings suggest that that the low pI Glod4 variant observed on IEF/SDS-PAGE gels (Fig. 2c, d) is formed in livers of $Blmh^{-/-}$ mice by a post-transcriptional mechanism.

Discussion

In the present work, we used *Blmh*-null mice in a proteomic study to discover metabolic pathways regulated by the *Blmh* genotype in the liver and to determine how the regulation is affected by dietary hyperhomocysteinemia (high-Met diet). We found that: (1) proteins involved in lipid homeostasis (ApoE), energy metabolism (Atp5h), oxidative stress response (Sod1), nitric oxide generation (Ddah1), and metabolic detoxification (Glo1, Sult1c1) were differentially expressed in livers of *Blmh*-null mice; (2) dietary hyperhomocysteinemia interacts with the $Blmh^{-/-}$ genotype to amplify its effects on protein expression; (3) proteins involved in lipoprotein metabolism (ApoA1), antigen processing (Psme1), energy metabolism (Gamt), catechol metabolism (Comt) show differential expression in $Blmh^{-/-}$ mice only in animals fed with a hyperhomocysteinemic diet; (4) One protein (Glod4) showed a shift to a more acidic isoelectric point in $Blmh^{-/-}$ mice. These findings suggest that Blmh interacts with diverse cellular pathways that are essential for normal liver homeostasis and that these interactions are modulated by hyperhomocysteinemia.

The function of Blmh in the liver has not been explored previously. As shown in the present work, in *Blmh*-null mice fed with a control diet, four liver proteins were identified with decreased expression (ApoE, Atp5h, Sod1, and Glo1) and two liver proteins were identified with increased expression (Ddah1 and Sultc1). Thus, in the absence of hyperhomocysteinemia, Blmh interacts with proteins involved in lipid metabolism (ApoE), energy metabolism (Atp5h), oxidative stress response (Sod1), nitric oxide generation (Ddah1), glyoxal detoxification (Glo1), and xenobiotic detoxification (Sult1c1).

In livers of *Blmh*-null mice fed with a hyperhomocysteinemic diet, seven proteins were identified with decreased expression (ApoA1, ApoE, Psme1, Atp5h, Gamt, Comt, Glo1) and two were identified with increased expression (Ddah1, Sultc1). Effects of $Blmh^{-/-}$ genotype on protein expression were more pronounced and had a greater magnitude (25–400 %) in mice fed with hyperhomocysteinemic diet, compared with a control diet (16–60 %). Hyperhomocysteinemia alone changed the expression of one protein (Atp5h) that was also affected by the $Blmh^{-/-}$ genotype, as well as of five proteins (ApoA1, Gamt, Ftl, Prdx2, and Comt) that were not affected by the $Blmh^{-/-}$ genotype. Taken together, these findings indicate that hyperhomocysteinemia and *Blmh* genotype have distinct effects on protein expression and that there is an interaction between hyperhomocysteinemia and $Blmh^{-/-}$ genotype that modulates protein expression.

Down-regulation of liver ApoA1 was observed in other models of hyperhomocysteinemia. For example, plasma Hcy and ApoA1 levels are negatively correlated in human subjects (Liao et al. 2006; Mikael et al. 2006). Such correlations are consistent with findings in mildly hyperhomocysteinemic $Mthfr^{+/-}$ and $Cbs^{+/-}$ mice showing that Hcy inhibits hepatic ApoA1 synthesis, both at ApoA1 protein and mRNA level (Mikael et al. 2006). A similar decrease in ApoA1 levels was also found in $Cbs^{-/-}ApoE^{-/-}$ mice, although in this model Hcy appears to affect ApoA1 expression at translational, but not transcriptional level (Liao et al. 2006).

Down-regulation of ApoA1 and ApoE is associated with atherosclerosis. For example, in humans ApoA1 is negatively correlated with plasma Hcy and low ApoA1 level is a risk factor for cardiovascular disease (Gueant-Rodriguez et al. 2011; Xiao et al. 2011). *ApoE*-null mice spontaneously develop atherosclerosis and the process is accelerated by hyperhomocysteinemia (Hofmann et al. 2001). Our findings that ApoA1 and ApoE are down-regulated by the $Blmh^{-/-}$ genotype suggest that Blmh has an anti-atherogenic function.

Psme1 participates in immunoproteasome assembly as a component of the PA28 activator complex and is required for efficient antigen processing. The PA28 activator complex enhances the generation of MHC class I-binding peptides by altering the cleavage pattern of the proteasome. In humans, immunoproteasome components, including Psme1 (PA28α) are elevated in livers of patients with cirrhosis and chronic active hepatitis (Vasuri et al. 2010). Our finding that Psme1 is down-regulated in $Blmh^{-/-}$ mice fed with high-Met diet, but not a control diet, suggests that Blmh participates in antigen processing in hyperhomocysteinemic animals and thus has a pro-inflammatory function. This suggestion is consistent with the findings of other investigators showing that Blmh plays a role in generating MHC class I-presenting peptides and influences presentation of some antigens in mice by trimming them to the proper size of 9–10 residues, although this role is largely redundant with other aminopeptidases (Towne et al. 2007).

Atp5h is a mitochondrial ATP synthase that catalyzes ATP synthesis, utilizing an electrochemical gradient of protons across the inner membrane during oxidative phosphorylation. The ATP-synthase system is disturbed under pathophysiological conditions (Das 2003), including methylmalonic aciduria due to the dysfunction of methylmalonyl CoA mutase, a vitamin B12-dependent enzyme. In aciduria, toxic metabolites, such as Hcy and methylmalonic acid, accumulate in tissues and body fluids. These metabolites compromise ATP synthase activity, which limits ATP generation and may lead to 'slow onset' excitotoxicity and finally cell death (Das 2003). Our finding that Atp5h is down-regulated by the $Blmh^{-/-}$ genotype suggests that Blmh interacts with ATP synthase to maintain adequate energy generation in the liver. Because Blmh is not known to be present in mitochondria, the interaction is most likely indirect.

Glo1 catalyzes the first step in a two-step detoxification of methylglyoxal, a byproduct of glycolysis formed by the degradation of dihydroxyacetone phosphate and glyceraldehyde-3-phosphate (Thornalley 1993). Methylglyoxal is highly toxic due to its chemical reactions with cellular components that lead to protein modification (advanced glycation end-products), oxidative stress, and apoptosis (Distler and Palmer 2012). Human *GLO1* gene polymorphism is associated with a predisposition to the development of alcoholic liver cirrhosis (Spitsyn et al. 2001). Decreased GLO1 activity in situ results in an accumulation of methylglyoxal and increased formation of advanced glycation end products, which can accelerate aging and chronic degenerative diseases. In mice, aging-dependent reduction in Glo1 delays wound healing (Fleming et al. 2013). Our present finding that Glo1 is down-regulated by the $Blmh^{-/-}$ genotype suggests Blmh is required for efficient methylglyoxal detoxification.

We found that two methyltransferases, Gamt and Comt, respond similarly to the $Blmh^{-/-}$ genotype or high-Met diet: both are down-regulated by the $Blmh^{-/-}$ genotype only in hyperhomocysteinemic mice and up-regulated by the diet in $Blmh^{+/+}$ animals. Gamt catalyzes methylation of guanidinoacetate with S-adenosylmethionine (AdoMet) in the second step of creatine synthesis in the liver and kidney. Creatine is transported through the blood, and taken up by tissues with high energy demands, such as the brain and skeletal muscle. Creatine synthesis is the major user of methyl groups from AdoMet—accounting for ~75 % of Hcy formation (Mudd et al. 1980)—and is an important determinant of plasma Hcy in rats (Brosnan et al. 2004). In humans, this is reflected by a positive correlation between plasma Hcy and creatine (Rauh et al. 2001). Comt catalyzes the transfer of a methyl group from AdoMet to

catecholamines, one of the major degradative pathways of catecholamine transmitters. Comt is also important in the metabolism of catechol drugs used in the treatment of hypertension, asthma, and Parkinson's disease (PD). L-Dopa treatment is associated with the increase in plasma Hcy level in patients with PD (De Bonis et al. 2010). Concomitant treatment with a Comt inhibitor attenuates L-dopa-induced elevation of Hcy level (Hu et al. 2013). Our present findings that Gamt and Comt are up-regulated by hyperhomocysteinemia and down-regulated by the $Blmh^{-/-}$ genotype in the presence of hyperhomocysteinemia suggest that Blmh is required for creatine biosynthesis and catechol degradation, respectively, in hyperhomocysteinemic animals.

Of the liver proteins that were not affected by the $Blmh^{-/-}$ genotype alone, five were up-regulated (Psme1, Gamt, Ftl, Prdx2, and Comt) and one (ApoA1) was down-regulated in mice fed with a hyperhomocysteinemic high-Met diet. Up-regulation of three of these proteins has been observed previously in related models of hyperhomocysteinemia. For example, in mice fed with 0.5 % Met in drinking water for 2 weeks Ftl, Prdx2, and Psme1 are up-regulated in $Cbs^{+/+}$ animals, while Psme1 was up-regulated also in $Cbs^{+/-}$ mice (DiBello et al. 2010). ApoA1 was also found to be down-regulated in other models of hyperhomocysteinemia (discussed above).

We identified two proteins, Ddah1 and Sult1c1 that were up-regulated by the $Blmh^{-/-}$ genotype. Ddah1 regulates nitric oxide synthesis by removing an inhibitor, asymmetrical dimethylarginine (ADMA), generated by protein degradation (Ogawa et al. 1987). Hcy inhibits Ddah1 activity, causes ADMA accumulation, which in turn inhibits nitric oxide synthase activity, thereby contributing to endothelial dysfunction (Stuhlinger and Stanger 2005). Sult1c1 is a member of the sulfotransferase enzymes family that has significant roles in the regulation of hormones, neurotransmitters, and the detoxification of xenobiotics (Yalcin et al. 2013). In humans, the decrease in sulfotransferase expression and/or activity is linked to liver disease. For example, in both alcohol- and diabetes-induced cirrhosis, a reduction of major hepatic sulfotransferase isoforms (SULT1A1, SULT1E1, and SULT1A3) is observed, while the milder steatosis reduces only SULT1A1 and SULT1A3. This can lead to xenobiotic accumulation and toxicity due to diminished SULT1A1, alteration in androgen synthesis and metabolism due to modified SULT2A1, alteration in estrogen metabolism due to modified SULT1E1, or accumulation of catecholamines due to diminished SULT1A3 levels in diseased liver (Yalcin et al. 2013). Our findings that Ddah1 and Sult1c1 were up-regulated by the $Blmh^{-/-}$ genotype suggest that the absence of Blmh induces protective responses that enhance nitric oxide generation as well as xenobiotic or neurotransmitter inactivation.

We identified one liver protein, Glod4, whose isoelectric point (pI) was dependent on the *Blmh* genotype: it was shifted to a more acidic value in $Blmh^{-/-}$ mice. Glod4 is a mitochondrial protein that has three isoforms with molecular weights of 33,317 Da (isoform 1), 32,410 Da (isoform 2), and 30,771 Da (isoform 3). Isoforms 1, 2, and 3 having p*I* values of 5.28, 5.14, and 5.24, respectively, differ slightly in their primary amino acid sequences and are synthesized from different mRNAs (http://www.uniprot.org/uniprot/Q9CPV4). Thus, we predicted that the more acidic Glod4 isoform expressed in $Blmh^{-/-}$ mice could correspond to isoform 2 (pI 5.14), whereas $Blmh^{+/+}$ mice express isoform 1 (pI 5.28). To test these predictions, we amplified Glod4 mRNA using primers specific for each isoform. However, we found that the expression of mRNA for different Glod4 isoforms was not affected by *Blmh* genotype and that Glod4 isoform 1 mRNA was expressed both in $Blmh^{-/-}$ and $Blmh^{+/+}$ livers, while isoform 2 and 3 mRNAs were not expressed. These findings suggest that the appearance of a low pI Glod4 isoform in $Blmh^{-/-}$ liver is due to a post-translational modification of isoform 1, the nature of which remains to be established.

We have not defined how the absence of the Blmh protein/function could interact with specific proteins in the mouse liver to modulate their expression. One possible mechanism could involve changes in protein modification by Hcy-thiolactone. As hyperhomocysteinemia elevates essentially all Hcy metabolites while $Blmh^{-/-}$ genotype elevates only one metabolite—Hcy-thiolactone—our findings suggest that Hcy-thiolactone is likely to be responsible for the down-regulation of protein levels in $Blmh^{-/-}$ mice fed with high-Met diet. A possible mechanism of decreased expression of specific proteins in the liver of $Blmh^{-/-}$ mice could involve their modification by Hcy-thiolactone—protein *N*-homocysteinylation—followed by increased proteolytic turnover of *N*-Hcy protein (Glowacki et al. 2010; Zaabczyk et al. 2011). In this scenario, ApoA1, ApoE, Psme1, Atp5h, Gamt, Comt, and Glo1 would be targeted for *N*-homocysteinylation. Indeed, in humans ApoA1 is known to undergo *N*-homocysteinylation (Jakubowski 2002) that is positively correlated with Hcy levels (Ishimine et al. 2010). Consistent with this scenario are our findings showing that plasma total protein *N*-homocysteinylation increases in $Blmh^{-/-}$ mice (Borowczyk et al. 2012). However, another mechanism has to be responsible for the up-regulation of Ddah and Sult1c1. Thus, it would be interesting to determine whether *N*-homocysteinylation status of specific liver proteins is altered in $Blmh^{-/-}$ mice.

In conclusion, our findings suggest that Blmh interacts with diverse cellular processes—from lipoprotein metabolism, nitric oxide regulation, antigen processing, and energy metabolism to detoxification and antioxidant defenses—essential for normal liver homeostasis and that modulation of these interactions by hyperhomocysteinemia underlies

the involvement of Hcy in fatty liver disease. Taken together, our findings suggest that Blmh has a protective role in the liver, particularly in hyperhomocysteinemia.

Acknowledgments We thank John Lazo for kindly providing a breeding pair of *Blmh*-null mice. This work was supported in part by grants from the American Heart Association, the National Science Center, Poland (2011/01/B/NZ1/03417, 2011/02/A/NZ1/00010, 2012/07/B/NZ7/01178, and 2013/09/B/NZ5/02794) and MNiSW, Poland (N401 065321504, N N302 434439).

Conflict of interest The authors declare that they have no conflict of interest.

References

Borowczyk K, Tisonczyk J, Jakubowski H (2012) Metabolism and neurotoxicity of homocysteine thiolactone in mice: protective role of bleomycin hydrolase. Amino Acids 43:1339–1348

Brosnan JT, Jacobs RL, Stead LM, Brosnan ME (2004) Methylation demand: a key determinant of homocysteine metabolism. Acta Biochim Pol 51:405–413

Chwatko G, Boers GH, Strauss KA, Shih DM, Jakubowski H (2007) Mutations in methylenetetrahydrofolate reductase or cystathionine beta-synthase gene, or a high-methionine diet, increase homocysteine thiolactone levels in humans and mice. Faseb J 21:1707–1713

Das AM (2003) Regulation of the mitochondrial ATP-synthase in health and disease. Mol Genet Metab 79:71–82

De Bonis ML, Tessitore A, Pellecchia MT, Longo K, Salvatore A, Russo A, Ingrosso D, Zappia V, Barone P, Galletti P, Tedeschi G (2010) Impaired transmethylation potential in Parkinson's disease patients treated with L-Dopa. Neurosci Lett 468:287–291

DiBello PM, Dayal S, Kaveti S, Zhang D, Kinter M, Lentz SR, Jacobsen DW (2010) The nutrigenetics of hyperhomocysteinemia: quantitative proteomics reveals differences in the methionine cycle enzymes of gene-induced versus diet-induced hyperhomocysteinemia. Mol Cell Proteomics MCP 9:471–485

Distler MG, Palmer AA (2012) Role of Glyoxalase 1 (Glo1) and methylglyoxal (MG) in behavior: recent advances and mechanistic insights. Front Genet 3:250

Fleming TH, Theilen TM, Masania J, Wunderle M, Karimi J, Vittas S, Bernauer R, Bierhaus A, Rabbani N, Thornalley PJ, Kroll J, Tyedmers J, Nawrotzki R, Herzig S, Brownlee M, Nawroth PP (2013) Aging-dependent reduction in glyoxalase 1 delays wound healing. Gerontology 59:427–437

Glowacki R, Bald E, Jakubowski H (2010) Identification and origin of Nepsilon–homocysteinyl–lysine isopeptide in humans and mice. Amino Acids 39:1563–1569

Gueant-Rodriguez RM, Spada R, Moreno-Garcia M, Anello G, Bosco P, Lagrost L, Romano A, Elia M, Gueant JL (2011) Homocysteine is a determinant of ApoA-I and both are associated with ankle brachial index, in an ambulatory elderly population. Atherosclerosis 214:480–485

Hamelet J, Demuth K, Paul JL, Delabar JM, Janel N (2007) Hyperhomocysteinemia due to cystathionine beta synthase deficiency induces dysregulation of genes involved in hepatic lipid homeostasis in mice. J Hepatol 46:151–159

Hirsch S, Poniachick J, Avendano M, Csendes A, Burdiles P, Smok G, Diaz JC, de la Maza MP (2005) Serum folate and homocysteine levels in obese females with non-alcoholic fatty liver. Nutrition (Burbank, Los Angeles County, Calif) 21:137–141

Hofmann MA, Lalla E, Lu Y, Gleason MR, Wolf BM, Tanji N, Ferran LJ Jr, Kohl B, Rao V, Kisiel W, Stern DM, Schmidt AM (2001) Hyperhomocysteinemia enhances vascular inflammation and accelerates atherosclerosis in a murine model. J Clin Invest 107:675–683

Hu XW, Qin SM, Li D, Hu LF, Liu CF (2013) Elevated homocysteine levels in levodopa-treated idiopathic Parkinson's disease: a meta-analysis. Acta Neurol Scand 128:73–82

Ishimine N, Usami Y, Nogi S, Sumida T, Kurihara Y, Matsuda K, Nakamura K, Yamauchi K, Okumura N, Tozuka M (2010) Identification of *N*-homocysteinylated apolipoprotein AI in normal human serum. Ann Clin Biochem 47:453–459

Jakubowski H (1997) Metabolism of homocysteine thiolactone in human cell cultures. Possible mechanism for pathological consequences of elevated homocysteine levels. J Biol Chem 272:1935–1942

Jakubowski H (2002) Homocysteine is a protein amino acid in humans. Implications for homocysteine-linked disease. J Biol Chem 277:30425–30428

Jakubowski H (2008) New method for the determination of protein N-linked homocysteine. Anal Biochem 380:257–261

Jakubowski H (2013) Homocysteine in protein structure/function and human disease—chemical biology of homocysteine-containing proteins. Springer, Wien

Jakubowski H, Boers GH, Strauss KA (2008) Mutations in cystathionine {beta}-synthase or methylenetetrahydrofolate reductase gene increase *N*-homocysteinylated protein levels in humans. FASEB J 22:4071–4076

Jakubowski H, Perla-Kajan J, Finnell RH, Cabrera RM, Wang H, Gupta S, Kruger WD, Kraus JP, Shih DM (2009) Genetic or nutritional disorders in homocysteine or folate metabolism increase protein *N*-homocysteinylation in mice. Faseb J 23:1721–1727

Kamata Y, Itoh Y, Kajiya A, Karasawa S, Sakatani C, Takekoshi S, Osamura RY, Takeda A (2007) Quantification of neutral cysteine protease bleomycin hydrolase and its localization in rat tissues. J Biochem 141:69–76

Kaplowitz N, Than TA, Shinohara M, Ji C (2007) Endoplasmic reticulum stress and liver injury. Semin Liver Dis 27:367–377

Liao D, Tan H, Hui R, Li Z, Jiang X, Gaubatz J, Yang F, Durante W, Chan L, Schafer AI, Pownall HJ, Yang X, Wang H (2006) Hyperhomocysteinemia decreases circulating high-density lipoprotein by inhibiting apolipoprotein A–I protein synthesis and enhancing HDL cholesterol clearance. Circ Res 99:598–606

Luczak M, Formanowicz D, Pawliczak E, Wanic-Kossowska M, Wykretowicz A, Figlerowicz M (2011) Chronic kidney disease-related atherosclerosis—proteomic studies of blood plasma. Proteome Sci 9:25

Mikael LG, Genest J Jr, Rozen R (2006) Elevated homocysteine reduces apolipoprotein A-I expression in hyperhomocysteinemic mice and in males with coronary artery disease. Circ Res 98:564–571

Montoya SE, Thiels E, Card JP, Lazo JS (2007) Astrogliosis and behavioral changes in mice lacking the neutral cysteine protease bleomycin hydrolase. Neuroscience 146:890–900

Mudd SH, Ebert MH, Scriver CR (1980) Labile methyl group balances in the human: the role of sarcosine. Metabolism 29:707–720

Mudd SH, Levy HL, Kraus JP (2001) Disorders of transsulfuration. In: Scriver CR, Beaudet AL, Sly WS, et al (eds) The metabolic and molecular bases of inherited disease, vol 2. Mc Graw-Hill, New York, pp 2007–2056

Ogawa T, Kimoto M, Sasaoka K (1987) Occurrence of a new enzyme catalyzing the direct conversion of NG, *NG*-dimethyl-L-arginine to L-citrulline in rats. Biochem Biophys Res Commun 148:671–677

Rauh M, Verwied S, Knerr I, Dorr HG, Sonnichsen A, Koletzko B (2001) Homocysteine concentrations in a German cohort of 500 individuals: reference ranges and determinants of plasma levels in healthy children and their parents. Amino Acids 20:409–418

Schwartz DR, Homanics GE, Hoyt DG, Klein E, Abernethy J, Lazo JS (1999) The neutral cysteine protease bleomycin hydrolase is essential for epidermal integrity and bleomycin resistance. Proc Natl Acad Sci USA 96:4680–4685

Spitsyn VA, Nafikova A, Spitsyna N, Afanas'eva IS (2001) Genetic predisposition to development of toxic liver cirrhosis caused by alcohol. Genetika 37:698–707

Stuhlinger MC, Stanger O (2005) Asymmetric dimethyl-L-arginine (ADMA): a possible link between homocyst(e)ine and endothelial dysfunction. Curr Drug Metab 6:3–14

Thornalley PJ (1993) The glyoxalase system in health and disease. Mol Aspects Med 14:287–371

Towne CF, York IA, Watkin LB, Lazo JS, Rock KL (2007) Analysis of the role of bleomycin hydrolase in antigen presentation and the generation of CD8 T cell responses. J Immunol 178:6923–6930

Vasuri F, Capizzi E, Bellavista E, Mishto M, Santoro A, Fiorentino M, Capri M, Cescon M, Grazi GL, Grigioni WF, D'Errico-Grigioni A, Franceschi C (2010) Studies on immunoproteasome in human liver. Part I: absence in fetuses, presence in normal subjects, and increased levels in chronic active hepatitis and cirrhosis. Biochem Biophys Res Commun 397:301–306

Velez-Carrasco W, Merkel M, Twiss CO, Smith JD (2008) Dietary methionine effects on plasma homocysteine and HDL metabolism in mice. J Nutr Biochem 19:362–370

Werstuck GH, Lentz SR, Dayal S, Hossain GS, Sood SK, Shi YY, Zhou J, Maeda N, Krisans SK, Malinow MR, Austin RC (2001) Homocysteine-induced endoplasmic reticulum stress causes dysregulation of the cholesterol and triglyceride biosynthetic pathways. J Clin Invest 107:1263–1273

Xiao Y, Zhang Y, Lv X, Su D, Li D, Xia M, Qiu J, Ling W, Ma J (2011) Relationship between lipid profiles and plasma total homocysteine, cysteine and the risk of coronary artery disease in coronary angiographic subjects. Lipids Health Dis 10:137

Yalcin EB, More VR, Neira K, Lu ZJ, Cherrington NJ, Slitt AL, King RS (2013) Down-regulation of sulfotransferase expression and activity in diseased human livers. Drug Metab Dispos

Zaabczyk M, Glowacki R, Machnik A, Herod P, Kazek G, Jakubowski H, Undas A (2011) Elevated concentrations of Nvarepsilon–homocysteinyl–lysine isopeptide in acute myocardial infarction: links with ADMA formation. Clin Chem Lab Med CCLM/FESCC 49:729–735

Zhou J, Moller J, Danielsen CC, Bentzon J, Ravn HB, Austin RC, Falk E (2001) Dietary supplementation with methionine and homocysteine promotes early atherosclerosis but not plaque rupture in ApoE-deficient mice. Arterioscler Thromb Vasc Biol 21:1470–1476

Zimny J, Sikora M, Guranowski A, Jakubowski H (2006) Protective mechanisms against homocysteine toxicity: the role of bleomycin hydrolase. J Biol Chem 281:22485–22492