COMPREHENSIVE INVITED REVIEW

**MAMMALIAN SULFUR AMINO ACID METABOLISM: A NEXUS BETWEEN
REDOX REGULATION, NUTRITION, EPIGENETICS AND
DETOXIFICATION**

María A. Pajares^{1,2,*} and Dolores Pérez-Sala¹

¹Department of Chemical and Physical Biology, Centro de Investigaciones Biológicas (CSIC), Ramiro de Maeztu 9, 28040 Madrid, Spain.

²Molecular Hepatology Group, Instituto de Investigación Sanitaria La Paz (IdiPAZ), Paseo de la Castellana 261, 28046 Madrid, Spain.

*To whom correspondence should be addressed at: Dept. of Chemical and Physical Biology, Centro de Investigaciones Biológicas (CSIC), Ramiro de Maeztu 9, 28040 Madrid, Spain. Phone: +34-918373112. Email: mapajares@cib.csic.es or ma.pajares@csic.es

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ABSTRACT

Significance. Transsulfuration allows conversion of methionine into cysteine using homocysteine as an intermediate. This pathway produces S-adenosylmethionine, a key metabolite for cell function, and provides 50% of the cysteine needed for hepatic glutathione synthesis. The route requires the intake of essential nutrients (e.g. methionine, vitamins) and is regulated by their availability. Transsulfuration presents multiple interconnections with epigenetics, ATP and glutathione synthesis, polyol and pentose phosphate pathways and detoxification that rely mostly in the exchange of substrates or products. Major hepatic diseases, rare diseases and sensorineural disorders, among others that concur with oxidative stress, present impaired transsulfuration.

Recent advances. In contrast to the classical view, a nuclear branch of the pathway, potentiated under oxidative stress, is emerging. Several transsulfuration proteins regulate gene expression, suggesting moonlighting activities. Additionally, abnormalities in homocysteine metabolism link nutrition and hearing loss.

Critical issues. Knowledge about the cross-regulation between pathways is mostly limited to the hepatic availability/removal of substrates and inhibitors. However, advances regarding protein-protein interactions involving oncogenes, identification of several post-translational modifications and putative moonlighting activities expand the potential impact of transsulfuration beyond methylations and homocysteine.

Future directions. Increasing the knowledge on transsulfuration outside the liver, understanding the protein-protein interaction networks involving these enzymes, the functional role of their post-translational modifications or the mechanisms controlling their nucleocytoplasmic shuttling may provide further insights into the pathophysiological implications of this pathway, allowing design of new therapeutic interventions.

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1. INTRODUCTION

In the mid-20th century intermediary metabolism was a subject of intense research. Elucidation of most steps in these routes and their essential regulatory mechanisms lead us to think that nothing much was left to discover, hence becoming a “classic” topic. At that time the dependence of tumor cells on glutamine and methionine was described, and initial attempts to understand this phenomenon were made (88,244,352). The existence of global DNA hypomethylation in tumor cells was later reported and shown to involve a decrease in the levels of a methionine derivative, S-adenosylmethionine (AdoMet, classically named SAM), the main cellular methyl donor (78,193). Afterwards, the concomitant existence of hypermethylation at specific DNA segments, frequently corresponding to promoters of tumor suppressors, was demonstrated (285) and additional connections between metabolism and cancer identified (284). Lately, research carried out in fields such as autophagy, inflammation or cardiovascular disease have contributed also to recall on the importance of sulfur amino acid metabolism renewing the interest in this field.

Identification of hyperhomocysteinemia (HHcy) as an independent risk factor for cardiovascular disease spurred research towards homocysteine (Hcy) metabolism (372). However, the number of pathologies associated with impaired sulfur amino acid metabolism and that concur with oxidative stress extends far beyond including, among others, several types of cancer, hearing loss, psoriasis, cirrhosis, Wilson or Alzheimer’s diseases (72,120,141,175,242,316). As will be described, this is not only due to the large number of AdoMet-dependent processes, but also due to connections of transsulfuration within one carbon metabolism and with epigenetics, detoxification and overall nutrition. As for other important metabolic pathways, most of the accumulated knowledge in this field was obtained from studies using the liver and hepatocytes.

Hence, the role of sulfur amino acid metabolism in other tissues or cells and their associated pathologies has been probably overlooked or just assumed to be similar in every context. Nevertheless, analyses of gene expression patterns in human and rodent tissues demonstrate the existence of the pathway in most organs/cells, the differences relying mainly in the methionine adenosyltransferase (MAT) and methyltransferase genes expressed.

Mammalian sulfur amino acid metabolism is centered around the methionine cycle and the pathway allowing conversion of Hcy into cysteine (Fig. 1). This last route is usually named transsulfuration in mammals, but reverse transsulfuration in other organisms where the term transsulfuration is reserved for the opposite reactions converting cysteine to Hcy (393). In some instances, transsulfuration is considered to comprise all the steps that allow the synthesis of cysteine from methionine and, for simplicity, we will follow this rule. The pathway is fed by methionine, the amino acid discovered in 1923 by John H. Mueller, and whose ingestion proved to be essential for adequate rat development (395). In contrast, cysteine, discovered in 1884 (18), becomes semi-essential due to transsulfuration. For this purpose, the methylene and the methyl groups bound to the sulfur atom of the methionine lateral chain need to be removed. Steps towards this end involve the synthesis and hydrolysis of several metabolites, AdoMet, S-adenosylhomocysteine (AdoHcy), Hcy and cystathionine. Among them, AdoMet synthesis is essential for life, and hence the *MAT* gene appears in the genome of the first synthetic organism (147).

Hcy, the third mammalian sulfur amino acid, is known since 1932 as a by-product of the incubation of methionine with sulfuric acid (32), and emerges here as a transsulfuration intermediate. Methionine and cysteine have a proteinogenic role and can be targets for modifications (25,386). In contrast, Hcy is a non-proteinogenic amino

acid that can modify proteins post-translationally (152). Therefore, its concentration must be controlled to preclude not only lysine N-homocysteinylolation and protein aggregation, but also activation by phosphorylation of protein kinase RNA-like endoplasmic reticulum (ER) kinase and ER stress and, in turn, phosphorylation and nuclear accumulation of Nrf2, a major redox regulator of gene expression (310). This control is achieved by Hcy export to the plasma and its use for remethylation, cystathionine and cysteine synthesis.

As will be discussed, several amino acids and metabolites of transsulfuration are key for epigenetic modifications, glutathione synthesis and detoxification reactions. Importantly, the synthesis and levels of these metabolites are intrinsically linked to nutritional intakes and the cellular redox status. Additionally, redox-sensitive transcription factors (i.e. NF κ B, AP-1) and stress kinases (i.e. JNK) directly or indirectly regulate transsulfuration during redox stress, together with the changes in redox pairs (i.e. GSH/GSSG, NADH/NAD⁺). Detailed knowledge of the crossregulation linking all these processes is therefore needed to understand the impact that impaired transsulfuration exerts on many essential aspects of Cell Biology.

2. OVERVIEW OF THE MAMMALIAN TRANSSULFURATION PATHWAY

Elucidation of the reactions that allow conversion of methionine into cysteine involve Cantoni's discoveries of MAT and AdoMet (39,40). There are three mammalian MATs that catalyze AdoMet synthesis from methionine and ATP, which is the first and rate-limiting step of transsulfuration (Fig. 1). Identification of AdoMet and deciphering its structure proved to be essential in Biology, where dozens of processes depend on this metabolite as donor of a variety of groups (41,194,248,351). Therefore, AdoMet becomes the first branch point in transsulfuration.

After decarboxylation, AdoMet serves as the aminopropyl donor for the synthesis of the polyamines spermidine and spermine (248). This process also generates 5'-methylthioadenosine (MTA), which inhibits a number of methyltransferases and is recycled back into methionine through the methionine salvage pathway (6). Furthermore, AdoMet is subjected to reductive cleavage through Fe-S centers to provide the 5'-deoxyadenosyl group needed by the eight human SAM radical proteins, as reviewed elsewhere (194) (Fig. 1). These reactions also produce methionine, which can reenter transsulfuration. Quantitatively, polyamine synthesis and SAM radical proteins consume <10% of hepatic AdoMet, whereas most of the 6-8 g of AdoMet synthesized daily in humans are used in methylation reactions (41,213,258,260). Nitrogen, sulfur, carbon or oxygen atoms of a large variety of substrates are the recipients of the methyl group donated by AdoMet in transmethylation reactions (41), which constitute the second step of transsulfuration (Fig. 1). Transmethylations produce AdoHcy, the demethylated form of AdoMet, which is a potent inhibitor of many methyltransferases (57). Hence, the importance of the AdoMet/AdoHcy ratio, also named the "methylation index". Adequate values of this index are maintained by AdoHcy elimination, which is the role of the next step catalyzed by AdoHcy hydrolase

or adenosylhomocysteinase (AHCY) (163). However, the reversibility of this reaction emphasizes the need to eliminate the Hcy and adenosine produced to preclude accumulation of the transmethylation inhibitor AdoHcy.

Transsulfuration reaches then a double branch point, represented by adenosine and Hcy (Fig. 1). Adenosine is used by adenosine deaminase (ADA) or adenosine kinase (ADK), leading to inosine and AMP, respectively. Conversely, Hcy is recycled back into methionine by remethylation, a process for which three methyl donors can be utilized, and that closes the methionine cycle. Hcy remethylation by methionine synthase (MTR) depends on methylcobalamin (vitamin B₁₂) and requires 5-methyltetrahydrofolate (MTHF) as methyl donor, thus linking transsulfuration with the folate cycle (361). The other two possibilities for Hcy remethylation involve highly similar homotetrameric enzymes, betaine homocysteine S-methyltransferase (BHMT) and BHMT2, that obtain the methyl group from betaine and S-methylmethionine, respectively (90,356). The source of these methyl donors is the diet (66,185), although mitochondrial choline oxidation also produces betaine (318). Hcy catabolism through transsulfuration involves its conjugation with serine or cysteine to synthesize cystathionine in a reaction catalyzed by cystathionine β -synthase (CBS) (89,360). Hydrolysis of cystathionine into cysteine constitutes the last reaction of transsulfuration that is driven by cystathionine γ -lyase or cystathionase (CTH) (347,353). Both CBS and CTH require pyridoxal phosphate (vitamin B₆) as cofactor (30,121) and, when using cysteine as substrate, synthesize hydrogen sulfide (H₂S), an important mediator in cellular signaling and cytoprotection (15,393). Thus, both enzymes provide additional connections with nutrition, redox regulation and signaling. Nonetheless, a reduced Hcy carrier can export the Hcy excess to the plasma (27), if required.

Intracellular levels of cysteine may reach 90-100 μM as a consequence of both dietary intake and transsulfuration (382). Cysteine is then used in several processes, including protein, glutathione and taurine synthesis. Hepatic synthesis of glutathione, the main low molecular weight antioxidant and free radical scavenger, utilizes ~50% of the cysteine produced through transsulfuration (256,382). The process involves two ATP consuming steps (247). In the rate-limiting reaction, cysteine is bound to the γ -carboxyl group of glutamic acid by heterodimeric γ -glutamylcysteine synthetase or glutamate cysteine ligase (GCL). Later on, glycine is added to γ -glutamylcysteine by homodimeric glutathione synthetase (GSS), rendering the reduced form of glutathione (GSH). GSH feedback regulates its own synthesis by inhibiting GCL, whereas expression of GCL subunits is regulated by Nrf2 (178,310).

Intracellular GSH levels are in the millimolar range, reaching 5-10 mM in the liver (5,424). During detoxification and free radical scavenging GSH is conjugated and oxidized into GSSG, respectively. GSSG is reduced back into GSH by glutathione reductase (GSR) using NADPH. According to different methodologies, the GSH/GSSG ratio is normally kept between 50-300 (5,71,424), exerting a preeminent role in the redox control of proteins, including some transsulfuration enzymes.

2.1. S-adenosylmethionine synthesis

According to protein function, the first group of genes in transsulfuration includes the three genes involved in AdoMet synthesis, named *MAT1A*, *MAT2A* and *MAT2B* (Fig. 2). The highly similar MAT α 1 and MAT α 2 catalytic subunits (84% identity) are encoded by *MAT1A* and *MAT2A*, respectively (125,183,277), whereas *MAT2B* codifies for the unrelated regulatory MAT β subunit (204). Basic information concerning these genes, including their chromosomal localization or the presence of SNPs is shown in Table 1. In hepatoma cells, several *MAT2B* splicing forms (V1, V2,

V2a and V2b) have been reported, which differ mainly in their N-terminal, although V2a and V2b also present internal deletions of the sequence (398,404). Recently, two alternative splicing forms have been reported for *MAT2A* that differ in intron 8 retention/elimination (287).

Expression of *MAT* genes differs among tissues and during development, *MAT2A* being preferentially expressed in extrahepatic tissues, fetal liver and tumor cells (111,366). *MAT2B* is expressed in most tissues following a similar pattern (232,404). In contrast, expression of *MAT1A* is maximal in human and rodent adult liver, whereas intermediate levels are detected in pancreas and lung and low levels occur in other rodent tissues (216,311,366). In normal liver, this expression pattern correlates with *MAT1A* promoter hypomethylation and hyperacetylation of its associated histones, whereas *MAT2A* promoter hypermethylation and histone hypoacetylation is observed (366,367). Conversely, in extrahepatic tissues *MAT1A* and *MAT2A* promoters present the opposite pattern of modification, but no such information is available for *MAT2B*.

Several studies have analyzed the promoters of the *MAT* genes (Fig. 2). Human and rodent *MAT1A* promoters include a variety of cis-acting elements whose functionality has been partially determined and to which redox-sensitive transcription factors bind (Fig. 2A). Among them, the role of glucocorticoid responsive elements was inferred from the induction of hepatic *Mat1a* mRNA by triamcinolone and dexamethasone (112). Positive regulation of the rat promoter activity was exerted by a member of the NF-1 protein family, whereas negative regulation was ascribed to differential binding of HNF factors in liver and hepatoma (10). In the human *MAT1A* promoter, upregulation by triiodothyronine was shown to involve binding of thyroid receptor TR β to C/EBP sites (396). C/EBP β binding and promoter hypomethylation were also responsible for increased expression in the mouse from fetal day 14 onward

(148), as expected from the crucial role of these factors in liver development and regeneration. The *MAT1A* promoter also contains an E-box to which MAT α 1/Mnt bind in normal liver. However, a switch towards cMyc/MafG/cMaf binding for *MAT1A* repression occurs upon induction of cholestasis and the associated oxidative stress (408). MAT α 1 and prohibitin 1 (PHB1), the mitochondrial chaperone that folds respiratory chain proteins, positively regulate each other expression in normal liver. Nevertheless, the change in occupancy of their E-boxes as described above downregulates both genes (96).

Human *MAT2A* promoter presents a variety of elements for binding of several factors that are redox-sensitive (Fig. 2B). Enhanced *MAT2A* expression in hepatocellular carcinoma (HCC) and HepG2 cells occurs upon binding of Sp1, cMyb, NF κ B and AP-1, (406,409), whereas in human colon RKO cells putrescine induces cJun and cFos binding to AP-1 sites (364). In quiescent liver, when *Mat1a* is highly expressed, E2F1, E2F3, E2F4 and pocket protein p130 bind to the rat *Mat2a* promoter, whereas during regeneration Sp1 binds when the gene is already being transcribed and p130 is displaced at the G1-S transition (317). The lncRNA PARTICLE shares the same genomic localization than *MAT2A* and a CpG island, whose methylation level changes after irradiation (266). PARTICLE levels increase in irradiated tumor and non-cancerous cell lines and, in this context, recruits G9a histone methyltransferase, Myb and SUZ12 (a subunit of Polycomb repressive complex PRC2) to silence the *MAT2A* promoter.

Studies on the *MAT2B* promoter are more limited, but upregulation upon binding of Sp1 and Sp3 factors to one Sp1 site have been described (Fig. 2C)(204). Later, when the existence of several *MAT2B* splicing forms was reported, it was found that NF κ B

and AP-1 binding only induces V1 expression (404). All these elements/factors offer additional options for regulation by oxidative stress.

Expression of *MAT1A* and *MAT2A* is post-transcriptionally regulated in fetal liver development and HCC (378) (Fig. 2A). During hepatocyte dedifferentiation, levels of AU-rich RNA binding factor 1 (AUF1) increase, favoring binding to the 3'-UTR of *MAT1A* mRNA and promoting its degradation (378). Conversely, stabilization of *MAT2A* mRNA occurs upon Hu antigen R (HuR) binding to AU-rich elements in its 3'-UTR. However, during liver development or AdoMet treatment of hepatoma cells the methylated-HuR levels increase and their binding to *MAT2A* mRNA induces its destabilization. Downregulation of expression by microRNA binding to the 3'-UTR of target mRNAs often occurs in carcinogenesis. Among them, miR-22 and miR-29b reduce rat *Mat1a* expression (184), and miR-664, miR-485-3p and miR-495 that of human *MAT1A* during hepatocarcinogenesis (405).

The three mammalian MAT isoenzymes were named consecutively, according to their order of elution from phenyl Sepharose columns. Their oligomeric composition corresponds to homotetramers (MAT I) and homodimers (MAT III) of MAT α 1 catalytic subunits or hetero-trimers (MAT II) including a MAT α 2 catalytic dimer and one MAT β regulatory subunit (115,261) (Fig. 3). MAT isoenzymes are paradigmatic cases of redox regulation, due to the differential effects exerted by the GSH/GSSG and NADPH/NADP⁺ ratios. The former modulates MAT I/III inhibition by GSSG (234,276), whereas a decrease of the latter favors NADP⁺ binding to MAT β subunits, subsequently increasing their affinity for MAT α 2 dimers (115). Thus, a connection between transsulfuration, glutathione synthesis, the polyol and pentose phosphate pathways and NAD⁺ synthesis is provided.

Both MAT α 1 and MAT α 2 subunits are organized in three domains that contribute to a large flat hydrophobic surface through which two monomers interact to constitute the homodimer (116,334) (Fig. 4). Two non-consecutive segments of the main chain make each domain and two active sites, opposite one to another, exist per dimer at the interface between MAT α subunits. Each monomer contributes part of the residues for each active site, and hence dimers are the minimum catalytic unit. The MAT α 1 central domain contains 5 of the 10 cysteines of this subunit and is involved in the interaction between dimers in MAT I (116,325). In contrast, MAT α 2 only contains 6 cysteines, 3 of them in the central domain, and MAT β includes two domains with short-chain dehydrogenase/reductase (SDR) homology that bind NADP⁺ and nucleotide-sugar substrates, respectively (334) (Fig. 5).

AdoMet synthesis requires methionine, ATP, K⁺ and Mg²⁺, and hence production of the methyl donor depends on the ingestion of methionine and ATP availability. Intracellular ATP (millimolar) and methionine (micromolar) concentrations differ notably, the availability of the latter becoming the limiting factor. The reaction catalyzed by MATs follows a S_N2 mechanism and involves two steps (reviewed in (228,277)). First, AdoMet synthesis by addition of the adenosyl moiety of ATP to the sulfur on the methionine lateral chain and, second, triphosphate hydrolysis to obtain the energy required for AdoMet to leave the active site. MAT isoenzymes differ in their affinities for methionine, which are 3.3-20 μ M for MAT II, 80 μ M for MAT α 2 homooligomers, 120 μ M for MAT I and $\sim$ 1 mM for MAT III; their V_{max} follow the opposite pattern (reviewed in (277)). Thus, intracellular AdoMet levels depend on the MAT isoenzyme expressed, a fact that becomes crucial for the liver, where $\sim$ 50% of the ingested methionine is metabolized (3,416). Estimations of the rodent hepatic methionine content under standard diets range between 120-220 nmol/g of tissue (88-

120 μ M) (71,72,100), conditions under which MAT III would not achieve its maximal capacity for AdoMet production.

Classically MATs were considered cytosolic proteins, and hence AdoMet synthesis a cytosolic process (Fig. 6). This view was further supported by the discovery of a transporter that imports AdoMet to cover mitochondrial needs (~30% of hepatic AdoMet) (2,140). Additionally, the small size of AdoMet was assumed to allow its transport through the nuclear pores to support nuclear methylations. However, recent reports demonstrated the existence of small nuclear pools of MAT α 1, MAT α 2 and MAT β (168,311,398), suggesting that at least part of the nuclear AdoMet required may be generated inside this compartment. Interestingly, rat MAT α 1 displays nuclear localization in those tissues/cell types with low *Mat1a* expression (311). This same study identified two overlapping areas in the rat MAT α 1 C-terminus involved in its cytoplasmic retention and nuclear localization. Basic residues delimiting these areas are mostly conserved in human MAT α 1, suggesting preservation of their role. Moreover, hepatic nuclear fractions contain MAT α 1 monomers and active MAT I tetramers and, among the epigenetic methylations analyzed, increased histone 3 K27 trimethylation (H3K27me3) correlates with nuclear accumulation (311). These data suggest that the nuclear enzyme sustains specific modifications, a fact later confirmed when the ability of nuclear MAT II to synthesize AdoMet for histone methylation was also demonstrated (168).

2.2. Selected examples of genes and enzymes of transmethylation

There are dozens of AdoMet-dependent methyltransferases that, among others, synthesize small molecules such as neurotransmitters or phospholipids, catalyze detoxification reactions (section 6) or introduce epigenetic modifications (section 4). Therefore, a complete overview of all these reactions is not feasible, and hence this

section will deal with three small molecule methyltransferases that are the main consumers of hepatic AdoMet. These are glycine (GNMT), phosphatidylethanolamine (PEMT) and guanidinoacetate (GAMT) N-methyltransferases.

Main characteristics of *GNMT*, *PEMT* and *GAMT* genes are shown in Table 1. Splicing forms have been reported for these genes according to the NCBI-SNP database and a specific report on *PEMT* (337). Moreover, human chromosomes 2 and 13 hold *GAMT* pseudogenes. Expression of *GAMT* is ubiquitous, whereas that of *GNMT* and *PEMT* is mainly hepatic. *GNMT* expression is induced soon after birth, but is abolished in many cell types and HCC (131,144). In several pathologies, reduced expression of these genes associates with their hypermethylation (12,146,206).

The *GNMT* promoter contains a CCAAT box, Sp1 and NF-Y binding sites and xenobiotic response elements (XRE) (200) (Fig. 7). Several androgen response elements have been described and an androgen agonist upregulates this promoter only in androgen receptor-positive prostate cancer cells (201). Known cis-acting elements in the *PEMT* promoter include estrogen response elements, which upregulate hepatic expression upon estrogen binding (309), whereas Sp1 binding downregulates *PEMT* during adipocyte differentiation (62). Information regarding regulation of *GAMT* expression is limited to the identification of regulatory elements in the promoter (TRANSFAC database) and its downregulation in liver cells upon atorvastatin induction of miR-124a (296). Again, the elements/factors identified in these promoters allow redox regulation of expression.

GNMT and GAMT are mainly cytoplasmic enzymes, whereas PEMT is a transmembrane protein of the ER (Fig. 7). GNMT reaches 1-3% of the total cytosolic protein in hepatocytes. Nevertheless, GNMT and GAMT also localize to the nucleus, where their role remains poorly explored (158,187). These three methyltransferases use

the (*S,S*)-AdoMet enantiomer produced by MATs to methylate their substrates, glycine, phosphatidylethanolamine and its mono- and di-methylated forms, and guanidinoacetate. Intracellular AdoMet levels are 10-90 μM depending on the cell type (54,86), and hence those methyltransferases with lower affinities are more sensitive to pathological reductions in methyl donor availability. Among them, GNMT with a K_m^{AdoMet} between 30-200 μM , whereas those of GAMT and PEMT are <20 μM . AdoHcy inhibits many methyltransferases, including PEMT (K_i^{AdoHcy} 3.8-68 μM), whereas GNMT is almost insensitive due to its high K_i^{AdoHcy} (138,171). An additional mechanism of control for GNMT is provided by MTHF inhibition (384), allowing preservation of AdoMet when its levels are low and remethylation through MTR is needed. Retinoic acid, glucocorticoids and glucagon increase GNMT protein and activity levels (264,319), but the mechanisms involved remain poorly explored.

Crystal structures show association of globular GNMT monomers into homotetramers (Fig 8). AdoMet binding to GNMT is cooperative and depends on the positive charge at the protein N-terminal (182,268) (Fig. 8A). MTHF also interacts with the N-terminal, precluding AdoMet binding and access to the active site (384) (Fig. 8B). Additionally, GNMT dimers bind 4S polycyclic aromatic hydrocarbon (PAH) at a different site.

Synthesis of phosphatidylcholine (PC) by PEMT requires trimethylation of the amino group of phosphatidylethanolamine, a process that includes phosphatidyl mono- and dimethyl-ethanolamine as intermediates (Fig. 7). This activity is very low in fetal liver, rises after birth (286), and in isolated hepatocytes is induced by several hormones (9,47). Regarding PEMT organization, only topological information exists and both monomeric or dimeric association states have been proposed (273,278). The model shows two AdoMet binding motifs close to the cytoplasmic side of the membrane (338).

Monomeric GAMT has a single domain structure, where the guanidinoacetate and AdoMet binding sites are located, and a Rossman fold for putative NAD⁺/NADP⁺ binding (180) (Fig. 8C). GAMT activity depends on three sulfhydryl groups, two of which cannot be modified after GSSG inhibition (269). This effect, together with NAD⁺/NADP⁺ binding, adds to the redox regulation of transsulfuration.

2.3. Homocysteine synthesis and catabolism

Genes involved in Hcy synthesis and utilization, *AHCY*, *CBS* and *CTH* constitute the third group of genes whose main characteristics are displayed in Table 1. Splicing forms have been reported for all these genes, as well as the *AHCY* paralogs *AHCYL1* and *AHCYL2* (77). *AHCY*, *CBS* and *CTH* display ubiquitous expression, both in human and rodents (54). Promoters of these genes also present elements for binding of redox-sensitive factors. The *AHCY* promoter contains POU2F1 and AP-1 binding sites that during genotoxic stress bind Oct-1 and cJun/ATF2, respectively (129,167) (Fig. 9).

The *CBS* 5'-UTR presents no TATA box, but a CAAT sequence, a variety of cis-acting elements and two promoters (Fig. 9). The use of alternative non-coding exons generates mRNAs that differ in their 5'-end (16,52). The most abundant forms are named *CBS-1a* and *CBS-1b* and are detected in adult and fetal tissues (16), whereas in hepatoma cells the main transcribed form uses the *-1b* promoter (108). Functional binding for Sp1/Sp3, NF-Y and NF-1 like factors to the CAAT-box in the *-1b* promoter was demonstrated, as well as USF-1 binding to the E-box (107-109,226). Sp1 and Sp3 activate both *-1a* and *-1b* promoters, but only the *-1b* promoter is synergistically upregulated (226). Tissue specific regulation of *CBS* may involve binding of Kruppel-like factors to both promoters (226). Livers of diabetic animals and glucocorticoid-treated hepatoma cells show increased *CBS* mRNA, protein and activity levels; insulin

reverts these effects reducing the *-Ib* promoter activity (306). Conversely, glucocorticoids upregulate Sp3 expression in hepatocytes, in turn, downregulating *CBS* (421).

The *CTH* promoter presents functional Sp1, NFκB and TCF/LEF binding sites and ATF4 binding to a cis-intronic site (95,254,392,403) (Fig. 9). Upregulation of *CTH* expression by bile acids in the liver, 17,β-estradiol in the myocardium and the PI3K/AKT pathway in hepatoma cells involve a farnesoid responsive element (FXR) and Sp1 binding (308,391,414). In contrast, *CTH* downregulation in Huntington's disease and HHcy involves Sp1 binding and promoter hypermethylation, respectively (208,282). Post-transcriptional downregulation of *CTH* expression in macrophages and cardiomyocytes implicates miR-216a and the miR-30 family, respectively (114,336).

Homotetrameric AHCY catalyzes the reversible hydrolysis of AdoHcy producing Hcy and adenosine (70); *in vitro*, the synthesis of AdoHcy is thermodynamically favored (Fig. 10A). AHCY is mainly a cytoplasmic enzyme, although its nuclear localization has been linked to AdoHcy elimination during cap methylation (304). This protein contains a motif for the tight binding of one NAD⁺/NADH molecule per subunit (370). During AdoHcy hydrolysis the NAD⁺ cofactor is initially reduced, but later oxidized to produce adenosine (279). AHCY has high affinity for copper (K_i 14 nM), its binding inducing NAD⁺ dissociation and enzyme inhibition (209). Adenosine inhibits AHCY, whereas cAMP competes with the nucleoside and counteracts its effects on AdoHcy hydrolysis (177). Each AHCY monomer includes two domains connected by helical segments that are involved in intersubunit interactions (Fig. 10A). The main chain extends from the catalytic domain, through the helical segments, into the cofactor's domain and returns to complete the catalytic domain (371). Structural data also show high similarity between the folding

patterns of AHCY and GNMT catalytic domains. The NAD^+ cofactor binds at a cleft between domains, through residues belonging to different monomers. This organization places the four cofactor domains at the center of the tetramer's structure and the catalytic domains at the periphery (370).

Homotetrameric CBS synthesizes cystathionine by condensation of Hcy with serine, although substitution of the latter by cysteine produces H_2S (15,160) (Fig. 10B). Additionally, CBS can use cysteine and H_2O or two cysteine molecules to synthesize H_2S , together with serine and lanthionine, respectively (160). The enzyme is mainly cytoplasmic, but nuclear CBS has also been detected. Its affinity for sulfur substrates is low, requires pyridoxal phosphate as cofactor (121), is allosterically activated by AdoMet and binds a heme group (101,360). The presence of this group connects with iron metabolism and allows redox sensing, but heme loss makes CBS insensitive to redox stress (360). CO and NO binding to the ferrous state of the heme group inhibit CBS (379). However, enzyme activity is increased under oxidative conditions by C346 glutathionylation, a process that putatively involves initial oxidation to sulfenic acid and enzymes such as glutaredoxin (265). Nevertheless, no additional redox sensing is provided by the presence of a CXXC oxidoreductase motif (360). The crystal structure shows a two-domain organization (Fig. 10B). The catalytic N-terminal domain holds the vitamin B_6 and heme binding sites, whereas AdoMet binding occurs at the regulatory C-terminal domain, whose structure has been identified in a large variety of proteins allowing their AdoMet-dependent regulation (174).

Homotetrameric CTH hydrolyzes cystathionine into cysteine in a reaction that requires vitamin B_6 as cofactor (30), but it also uses cysteine and/or Hcy as substrates in reactions where H_2S is produced (15,160,174) (Fig. 10C). CTH is a cytoplasmic enzyme whose activity is enhanced at birth, correlating with increased glutathione

needs, and that exhibits low affinity for its sulfur substrates (347). Crystal structures show a two-domain structure for the monomer, where vitamin B₆ binds to the larger domain through residues contributed by two subunits (353). Two loops at either side of the B₆ binding cleft approach each other in the presence of the vitamin leading to the closed conformation, where the N-terminal domain moves towards the contiguous monomer to become part of the adjacent B₆ binding site. This movement increases the hydrophobic interactions among monomers in the CTH tetramer (353).

3. REGULATION OF TRANSSULFURATION

This section is intended to summarize the main regulatory mechanisms of transsulfuration that involve, among others, the diet and protein-protein interactions.

3.1. Regulation of transsulfuration by the diet

The transsulfuration pathway requires several essential nutrients for its adequate function and regulation, among them, methionine, vitamins and cations. Therefore, the route is highly dependent on the dietary intake of these nutrients that may show a high variability depending on cultural, geographic and economic reasons. The protein source used determines the amount of methionine ingested, since its content tends to be lower in vegetables and digestion of plant proteins is less efficient (145). Protein rich diets activate FXR, a nutrient sensing nuclear receptor, upregulating hepatic amino acid catabolism and *Mat1a*, *Ahcy* and *Cth* expression (241) (Fig. 11). In contrast, methionine restriction extends lifespan in several organisms including rodents (271), an effect ascribed to reduced mitochondrial production of reactive oxygen species (ROS) due to inhibition of respiratory complexes I and III, rather than to increased antioxidant capacity (326,327). In fact, rats fed methionine-restricted diets show no alteration of GSH levels in most tissues, but reduced hepatic and renal concentrations (312); increased GSH plasmatic levels seem to rely on its hepatic efflux. Conversely, hepatic GSH increases in mice fed methionine-supplemented diets, whereas both deficiency and supplementation enhance GSSG levels (4). Overall, both nutritional regimes reduce the GSH/GSSG ratio. Longevity induced by nutritional restriction regimes in several experimental models also concurs with increased H₂S production through transsulfuration that seems tissue/cell specific (136). Moreover, in dwarf mice models of longevity reduced levels of growth and thyroid hormones upregulate hepatic *Cth* expression, CTH and CBS protein levels and H₂S synthesis (137). Lifespan can also be

extended by tissue-specific downregulation of AHCY through elimination of two AHCY-like proteins in flies (280), whereas its shortening is detected in mice lacking methionine sulfoxide reductase A (MsrA), which reverts oxidation at methionine residues that occurs during aging (257).

Some data suggest that methionine availability may affect its distribution between protein and AdoMet synthesis differentially. For instance, methionine-supplemented cultures of vascular endothelial and hepatoma cells show induction of elongation factors (EF-1 α , EF-1 β and EF-1 δ) and *MAT2B* expression, respectively (50,118). In contrast, restriction increases expression of the three *MAT* genes in hepatoma, whereas only *MAT2A* and *MAT2B* are upregulated in kidney Cos7 cells (118,233). Nevertheless, more work is required in this area to fully understand the putative connections.

In rodents, methionine restriction downregulates hepatic expression of *Ahcy*, *Cbs* and *Gnmt* (4) (Fig. 11). In the long-term, the liver and inguinal white adipose tissues are the main targets for expression changes induced by this diet (110). Hepatic alterations include upregulation of glutathione and xenobiotic metabolism by cytochrome P450, as well as up- (e.g. Myc) or down-regulation (e.g. NF κ B, Jun) of several redox-regulated transcription factors. Feeding of either methionine deficient or supplemented diets also increases hepatic SREBP1, CEBP β and CEBP α protein levels, whereas supplementation reduces PPAR α content (4). Whether such effects occur in other organs or if dietary restrictions in other amino acids could elicit these changes was not studied. Maternal methionine ingestion during pregnancy also affects expression in the offspring, supplementation upregulating transsulfuration genes, *GCLC* and *GSR* (151,375). Methionine deficiency and excess contribute to genomic instability and metabolic alterations that are related to cancer prognosis (38,294). Mice fed either type

of diet show decreased hepatic methionine concentrations, together with increased content of AdoMet, AdoHcy and Hcy (4). In addition, levels of 4-hydroxynonenal (HNE) protein adducts are increased, indicating enhanced lipid peroxidation and oxidative stress.

Methionine restriction also alters ATP synthesis through its effects on global transcriptional and translational programs (426). In this setting, oxidative phosphorylation is favored, allowing the best energy yield. AMPK, the main energy sensor, prevents significant changes in ATP concentrations (43), a task achieved in part by recycling adenosine derived from transsulfuration. Furthermore, the connection with ATP synthesis is also supported by AMPK activation through allosteric binding of AMP to a CBS domain in its γ -subunit (399). High AdoMet levels preclude AMPK activation by phosphorylation of its α -subunit, in turn, preventing HuR transport to the cytoplasm for mRNA stabilization, including that of *MAT2A* (237).

In many cancer cells the switch towards glycolysis (Warburg effect) reduces the yield of ATP produced and, if methionine is restricted, initial steps of transsulfuration become dependent on the contribution of serine to *de novo* purine synthesis and, subsequently to ATP pools (227). Crossregulation between pathways is further highlighted by the depletion of ATP and NAD^+ pools caused by MAT α 1 overexpression in CHO cells (324) and the regulation of autophagy by methylation and H_2S (355,357). Methionine controls yeast autophagy through the methylation status of the protein phosphatase 2A (PP2A) catalytic subunit; increased AdoMet availability favors PP2A methylation and then Npr2p dephosphorylation and inhibition of non-nitrogen-starvation-induced autophagy. Moreover, addition of a H_2S precursor to cultured Werner syndrome's fibroblasts also inhibits mTOR activation (357).

Micronutrients required by several steps of transsulfuration and some reactions refueling the pathway are obtained from the diet (Fig. 11). Marginal vitamin B₆ deficiencies seem quite common worldwide and lead to increased plasma cystathionine and serine levels (69). Effects of B₆ deficient diets vary from increased levels of plasma Hcy, hepatic Hcy and cysteine, together with decreased AdoMet content and impaired folate metabolism to small effects in the flux through transsulfuration and one carbon metabolism (231,314). In hepatoma cells, this deficiency increases cystathionine, cysteine and GSH levels (68), whereas production of H₂S remains unchanged (76). Vitamin B₁₂ and folate also influence transsulfuration, mainly through their roles in Hcy remethylation, where maintenance of MTR activity requires the reductive regeneration of B₁₂ catalyzed by methionine synthase reductase (MTRR). Additionally, low folate levels increase MATα2 acetylation, reducing its stability (411). Rats fed nicotinamide supplements show increased hepatic MAT and CBS activities (102), together with *Nnmt* and *Bhmt* promoter hypomethylation and *Cbs* promoter hypermethylation (207). Moreover, nicotinamide and 1-methylnicotinamide, the product of nicotinamide N-methyltransferase (NNMT), are neuroprotective against Hcy toxicity in cell culture (340).

Transsulfuration is also altered in obesity, where increased plasma cysteine levels are detected, putatively reflecting anomalous H₂S production that subsequently modifies glucose and lipid homeostasis (45). Leptin-mediated weight loss of ob/ob mice upregulates hepatic *Mat2a*, but downregulates *Ahcy*, *Gamt* and *Bhmt* (335). Experiments with *Pemt*^{-/-} mice fed high fat diets demonstrated the need of sufficient choline, rather than PC, to avoid obesity (150). Effects on transsulfuration also expand to dietary components/additives. The antioxidant resveratrol induces *MAT2B*-V1 and V2 expression, favoring exchange of MATβ interaction partners towards HuR and

SIRT1 and increasing AdoMet synthesis (410). Chronic ingestion of the sweetener aspartame impairs hepatic transsulfuration and depletes glutathione (99).

3.2. Regulation by metabolites of transsulfuration and related pathways

Initial studies focused on AdoMet, which controls its own synthesis inhibiting MAT I and MAT II, but stimulating MAT III (34,202). High AdoMet levels activate CBS and GNMT and inhibit methylenetetrahydrofolate reductase (MTHFR) and MTR activities (101,153), favoring Hcy catabolism versus remethylation, an effect to which the different K_m^{Hcy} of each enzyme further contribute. High AdoMet concentrations also preclude upregulation of *MAT2A* expression in hepatoma cells (233) and downregulate *BHMT* expression (272), effects that are mimicked by MTA produced during polyamine synthesis. Recent evidences also demonstrate that AdoMet levels regulate *MAT2A* splicing (287). In HEK293 cells, methionine starvation reduces AdoMet levels increasing the occupancy of a conserved hairpin (hp1) at the *MAT2A* 3'-UTR by the U6 snRNA m⁶A methyltransferase METTL16. The authors hypothesized that a longer dwell-time of METTL16 on hp1, not its methylation, dictates removal of the retained intron 8, enhancing *MAT2A* expression. These data establish a new link for transsulfuration, this time with the splicing machinery.

Methyltransferases are inhibited by AdoHcy and MTA (60), but AdoHcy is also able to counteract AdoMet inhibition of MTHFR. Hence, MTHF production increases, inhibiting GNMT to boost AdoMet availability (412). Adenosine and its derivatives, such as 3-deazaadenosine, inhibit AHCY leading to increased AdoHcy levels and a reduced methylation index (57), a property that prompted an extensive exploration of their potential use as antivirals. Hcy controls transsulfuration and protein synthesis, inducing *CTH* promoter hypermethylation and EF-1 δ expression in macrophages and vascular endothelial cells, respectively (50,208). Altogether a crossregulation between

the folate cycle, polyamine and protein synthesis and transsulfuration is established and further complicated by effects of transsulfuration metabolites on their own pathway. Additional effects will be analyzed in section 5.

3.3. New players identified through protein-protein interactions

Protein-protein interactions in transsulfuration are starting to be explored and, in fact, few studies have dealt with the effects of the known interactions. Databases such as IntAct or BioGRID contain results from high-throughput analysis of interest for this review, although many of the reported interactions have not been verified by additional procedures nor their functional role elucidated. Therefore, we will concentrate on a few protein targets with a known impact on transsulfuration.

Among MATs, the available information includes the already mentioned interaction of MAT α 2 with MAT β , leading to the MAT II heterotrimer, for which different interacting surfaces have been reported (115,203,261). Some studies also mentioned the interaction of MAT β with MAT α 1, but no further investigation has been carried out in this line. In colon RKO and hepatic HepG2 cancer cells increased levels of both MAT β -V1 and V2 splicing forms are detected, besides their interaction with HuR (Fig 12). This interaction enhances HuR cytoplasmic localization, subsequently increasing cyclin D1 and cyclin A expression (398). SIRT1 also binds to MAT β in this context, the MAT β /HuR/SIRT1 interaction enhancing the stability of the three proteins (410). The antioxidant resveratrol favors this tripartite interaction by inducing MAT β -V1 and V2 expression and weakening MAT α 2₂/MAT β binding (410). MAT β -V1 and V2 variants also interact with GIT1, MEK1 and ERK2 and the MAT β /GIT1 interaction is required to activate MEK1/ERK to favor cell growth in several types of cancer (288). Both MAT β forms joined GIT1 to interact with B-Raf and cRaf, but only MAT β interacts with Ras and contributes to protein stabilization (289). This array of

interactions further contributes to cell growth through the Ras/Raf/MEK/ERK signaling cascade in transformed cells.

Using tandem affinity purification followed by mass spectrometry and immunoprecipitation MAT α 2 was found as a nuclear interaction target for the MafK oncoprotein in mouse X63/0 plasmacytoma cells (168). This protein is a small Maf of the AP-1 family that forms either homodimers to repress antioxidant response elements (AREs) or heterodimers that act as activators or repressors of these elements. MAT α 2 behaves as MafK corepressor to reduce heme oxygenase-1 (*HO-1*) gene transcription in hepatoma Hepa-1 cells and immortalized mouse embryonic fibroblasts (iMEF) (168,170), a process in which the decrease of H3K4me2 and H3K9me2 marks on the gene enhancers is involved. Additionally, this same study identified more than a hundred nuclear MAT α 2 interaction targets in MEL cells and, among them, MAT β , histone methyltransferases (G9a, Ehmt1 and ALL1/KMT2A), several subunits of the Polycomb repressive complex 1 (PRC1), members of the chromatin remodeling complex Swi/Snf and the PARP1 complex. Nuclear MAT α 2 also interacts with methyltransferases SETDB1 and Suv39h1 in iMEF cells, where the MAT α 2/SETDB1 oligomer is recruited to a Maf recognition element on the *COX-2* promoter for its repression (170).

The oncogene PDRG1 interacts with MAT α 1 and MAT α 2 homo-oligomers and MAT II, reducing their AdoMet synthesizing activity and excluding MAT β from the heterotrimer (291). Interestingly, the PDRG1/MAT α 1 interaction seems restricted to the nucleus, where both proteins accumulate in acute liver injury and HCC (71,291,405). These are two pathologies in which expression of *PDRG1* and *MAT2A* increase, while the *MAT1A/MAT2A* switch is produced together with a reduction in global AdoMet levels. In normal liver, nuclear MAT α 1 also interacts with the Max network

transcriptional repressor Mnt (408). This MAT α 1 interaction changes towards cMyc, cMaf and MafG in cholestatic and cholangiocarcinoma tissues, where the levels of these three proteins increase. These complexes bind to the E-boxes in *MAT1A*, *c-MYC*, *c-MAF* and *MAFG* promoters to regulate their expression. Max is also an interaction target for MAT α 1, but this complex remains unaffected in cholestasis and cholangiocarcinoma (408).

The nucleocytoplasmic transport of MATs has not been specifically studied and neither PredictNLS or NetNES identified consensus localization or export signals in their sequences. Leptomycin inhibition of the CRM1 exportin did not affect nucleocytoplasmic distribution of rat MAT α 1 (311), despite the reported CRM1/XPO1 interaction with MAT in ancient metazoan (387). This result correlates with the low scores given by NetNES to the V360-L367 sequence in MAT α 1 and I305 in MAT β , and the negative scores for MAT α 2.

GNMT levels decrease drastically in HCC, thus impeding its protein-protein interactions. Among them, abolition of the GNMT/PREX2 interaction precludes ubiquitination-mediated proteasomal degradation of this PTEN inhibitor, leading to AKT activation and anomalous proliferation (205). Elimination of this interaction is probably a main contributor to HCC in *Gnmt*^{-/-} null mice. GNMT, as the PAH receptor, interacts with the aryl hydrocarbon receptor nuclear translocator (ARNT/HIF-1 β) in CHO cells (349). However, upon binding of benzo(a)pyrene, GNMT dissociates from this oligomer and translocates to the nucleus for the activation of an XRE in the *CYP1A1* promoter.

Hepatic CTH and BHMT interact with cytoplasmic high-mobility group box 1 (HMGB1) in ischemia/reperfusion (418). This protein contributes to nucleosome stability, facilitates transcription and, in the extracellular space, interacts with advanced

glycation end products and toll-like receptors stimulating the release of proinflammatory cytokines and activating the redox-regulated JNK and NF κ B pathways in response to organ damage (21,373). Moreover, HMGB1 binding to beclin1 promotes autophagy (358), whereas CTH and BHMT synthesize sulfide and methionine, respectively, that are inhibitory (355,357). Altogether, exchange of HMGB1 protein-protein interactions suggests a role in the control of autophagy.

3.4. Regulatory mechanisms through post-translational modifications

Human transsulfuration enzymes have been identified in high-throughput analysis of post-translational modifications (PTMs). Most of them present different degrees of phosphorylation, ubiquitination, acetylation and methylation, although some cases of succinylation, sumoylation or O-linked N-acetylglucosamine incorporation (OGlcNAc) are also reported. The initiator methionine is lost in MAT β (203), GAMT (23), PEMT, GNMT, AHCY (123) and CBS. Nevertheless, few are the cases in which the functional role of these modifications is known.

The three MAT subunits present several PTMs that for MAT α 1 are mostly related to its redox regulation. MAT I and MAT III isolated from rat liver are stable, whereas recombinant isoenzymes interconvert according to the protein concentration (251). Mutation of cysteines between positions 35 and 105 of the sequence alters the MAT I/III ratio (252), suggesting a role for these residues in isoenzyme interconversion. Rat hepatic isoenzymes contain the C35-C61 disulfide bond and *in vitro* studies showed that this modification precludes tetramer/dimer exchange (235,325). Inhibition by GSSG and its modulation according to the GSH/GSSG ratio also indicated production of an intrasubunit disulfide (276). Chemical modification and site-directed mutagenesis showed that cysteines, and especially C121, are very important for MAT I/III activity (252,274). Nitrosylation or hydroxylation at C121 erase activity. Structural information

allowed identification of residues (D355, R357 and R363) involved in the acid-base-catalyzed mechanism of nitrosylation (292). Crystal structures indicate that C35 and C61 are positioned to form the intrasubunit disulfide, whereas C121 locates at a flexible loop controlling access to the active site (116,117). These modifications may block the central domain β -sheet and the loop conformations to preclude dimer-dimer association and admittance of substrates into the catalytic site, respectively. These cysteines are preserved in human MAT α 1, allowing the same regulatory features, a fact that is precluded for MAT α 2, which lacks residues equivalent to C61 and C121.

PKC phosphorylation of rat MAT α 1 occurs on T342 and high-throughput studies found the equivalent modification in its human counterpart. This phosphorylation alters MAT I/III activity and weakens the monomer-monomer interactions (275). Location of this residue close to the areas involved in subcellular localization suggests a role for this phosphorylation in MAT α 1 distribution that deserves investigation. MAT α 2 and MAT β phosphorylation in hepatic stellate cells enhances their stability. However, while MEK modifies both subunits, ERK1/2 or B-Raf only phosphorylate MAT β (305).

P300 acetylates MAT α 2 on K81, favoring ubiquitination by the ubiquitin ligase UBR4 and its targeting for proteasomal degradation (411). MAT α 2 acetylation levels decrease in HCC, where deacetylation is performed by its interaction target HDAC3 (168). Nutrition influences acetylation levels, growth in folate-deficient media increasing MAT α 2 modification and degradation (411). Ubc9 sumoylates MAT α 2 on K340, K372 and K394 inducing its stabilization and, subsequently favoring MAT α 2/Bcl-2 interaction that also stabilizes Bcl-2 (363). Hence, the enhanced expression of these three proteins shown in tumor cells results in inhibition of apoptosis (365). MAT α 2 further contributes in an AdoMet-independent manner through its

binding to the *BCL2* promoter, upregulating its transcription. Conversely, apoptosis increases by addition of AdoMet due to reduced *UBC9* and *MAT2A* expression and the decreased MAT α 2 availability to induce *BCL2* transcription.

AMPK or PKA activation promotes GNMT phosphorylation (220,385), whereas N-terminal acetylation increases its inhibition by MTHF (221). These modifications connect ATP/ADP sensing and folate availability with the regulation of AdoMet utilization. PKA and PKC phosphorylation activate hepatic PEMT *in vitro* (47,380), whereas N-terminal N-glycosylation with high mannose oligosaccharides occurs in HEK293 cells (255). This last modification alters substrate specificity so that PEMT-2 hardly uses phosphatidylethanolamine, but produces PC and ether-linked PC, preferentially with polyunsaturated fatty acids (PUFAs) shorter than those synthesized by non-glycosylated PEMT-1. Regarding GAMT, only N-terminal acetylation has been reported (23).

CBS and CTH are also targets for PTMs. Carbachol stimulation of the cGMP/PKG pathway phosphorylates CBS on S227, stimulating H₂S production to control bladder tone (67). CBS glutathionylation on C346 enhances enzyme activity (265) and its sumoylation on K211 changes its localization towards the nuclear scaffold (162). For this purpose, the C-terminal domain has to interact with the sumoylation machinery, including SUMO-1. CTH phosphorylation by PKG or AKT1 on S377 regulates its function (307,415). In the carotid body, the major O₂ sensing organ, increased O₂ levels reduce H₂S synthesis through PKG phosphorylation of CTH. This involves kinase stimulation by the enhanced CO production of HO-2, whereas NO assumes PKG regulation in hypoxia.

4. EPIGENETIC MODIFICATIONS DEPENDENT ON TRANSSULFURATION

Epigenetic modifications that depend on transsulfuration are mainly DNA and histone methylations, although the recent identification of histone homocysteinylation provides a new link (400). There are three DNA methyltransferases (DNMTs) encoded by the *DNMT1*, *DNMT3A* and *DNMT3B* genes, but more histone methyltransferases are known. Thus, we will focus mainly on *EZH2*, encoding the Enhancer of Zeste Homologue 2 histone methyltransferase catalyzing H3K27me3.

4.1. Expression of epigenetic methyltransferases

Epigenetic methyltransferases present ubiquitous expression, although *DNMT3B* and *EZH2* exhibit higher levels in testis (53,315). All of them have splicing forms (Table 1) and *DNMT1* and *DNMT3B* genes locate close to *GAMT* and *AHCY* on human chromosomes 19 and 20, respectively. *DNMT* genes lack canonical TATA boxes and present several promoter sequences (24,156). A number of AP-1, E2F, TCF, Sp1, Sp3 and STAT3 SIE/GAS binding sites, some of which allow redox regulation, have been recognized in *DNMT1* and their functionality analyzed (Fig. 13). In HepG2 cells, ursolic acid decreases Sp1 expression, subsequently reducing *DNMT1* expression and phosphorylation (413). Moreover, *DNMT3A* and *DNMT3B* expression is upregulated by binding of Sp1 and Sp3 factors to Sp1 binding sites on their 5'-UTR sequences (156). *DNMTs* expression is increased by ROS, but inhibited by Nrf2 activators that also reduce their activity (143,310). The diet is also a factor influencing DNA methylation, thus nicotinamide supplements reduce this modification, while increasing MAT activity (207). Importantly, the methyl donor composition of the maternal diet (e.g. methionine content) affects DNA methylation levels in the infants and, among them, those of metabolic genes (283). In fact, maternal methionine supplementation upregulates expression of *DNMTs* in the offspring (151).

The *EZH2* promoter presents a variety of regulatory elements and functional binding has been reported in several settings, unveiling a complex regulation. Among others, *EZH2* repression is regulated by NFIB and p53 (297,359), whereas its activation is induced by cRel, RelA and RelB (263) (Fig. 13). Additionally, *EZH2* transcript levels in HCC are reduced by binding of several microRNAs to its 3'-UTR, including miR-26a, miR-214 and miR-98 (397,417,425). Conversely, the lncRNA ANCR mediates *EZH2* degradation in breast cancer (211).

4.2. Main features of epigenetic methyltransferases

Epigenetic methyltransferases are nuclear proteins that consume a modest amount of AdoMet compared to PEMT, GNMT and GAMT. Their K_m^{AdoMet} are 1.5 μM for *EZH2* and 3-14 μM for DNMTs, therefore only severe pathological decreases in AdoMet content would alter their function. Nevertheless, their different affinities for AdoMet make DNMTs more susceptible to its changes than *EZH2*.

DNMTs transfer a methyl group from AdoMet to the C5 on the cytosine located at CpG dinucleotides, which are mostly methylated in the genome (Fig. 13). CpG islands occupy 60% of human promoter sequences and variability in their methylation status determines expression of the corresponding gene, as this modification interferes with binding of transcription factors. DNMT structures differ mainly in their N-terminal regulatory domain. Homodimeric DNMT1 is the most abundant and guarantees maintenance of DNA methylation patterns during replication, allowing their transfer to the next generation (22). In contrast, homodimeric DNMT3A and DNMT3B are involved in *de novo* methylation (270). Their activity is modulated by DNMT3L homodimers, a third member of this family lacking methyltransferase activity, and together constitute heterotetrameric enzymes (341). Increased levels of DNMT3L induce the release of DNMT3A from the heterochromatin (159). Additionally, DNMT1

forms hetero-oligomers with *de novo* methyltransferases DNMT3A and DNMT3B through their N-terminals (172). This fact, together with spreading of methylation over the genome upon coexpression of these DNMTs, suggests their cooperation to control gene expression.

DNMT1 is involved in a variety of protein-protein interactions which are modulated by its PTMs in a position-specific manner. Therefore, only some examples will be mentioned. CDK5 phosphorylation on S154, interaction with the ubiquitin ligase UHRF1, multiple sumoylation by Ubc9 or deacetylation at residues K1349 and K1415 by SIRT1 activate DNMT1 (105,199,290,300). Increased stability occurs by interaction with the Hsp90 chaperone, deubiquitination by USP7, or by reducing/precluding methylation through AKT1 phosphorylation on S143 and interaction with β -catenin (92,299,344,423). Conversely, DNMT1 proteasomal degradation is promoted by SET7 methylation on K142 or its ubiquitination as a consequence of the lack of interaction with hyperacetylated Hsp90 (298,423). Additionally, the presence of certain PTMs in DNMT1 may be required for protein-protein interactions. For example, 14-3-3 binds S143 phosphorylated DNMT1 after replication, reducing its activity, and the MBT domain of PHF20L1 interacts with K142 monomethylated DNMT1, precluding its ubiquitination and degradation (93,94). Furthermore, interaction with the nuclear import regulator BRAP2 impedes DNMT1 nuclear localization, whereas the mismatch repair MSH2/MSH6 heterodimer recruits the methyltransferase to DNA damaged sites to reduce transcription at these locations (81,97).

DNMT3A and DNMT3B are targets of several PTMs that modify their activity. Among them, phosphorylation by ERK1/ERK2 blocks recruitment of DNMT3A to the Sox9 promoter (189), S386 and S389 phosphorylation by CK2 reduces methyltransferase activity and binding to the heterochromatin preventing methylation of

Alu or SINE repeats (74). Citrullination by peptidylarginine deiminase 4 stabilizes DNMT3A and seems key for p21 promoter methylation (75). Sumoylation disrupts DNMT3A interaction with HDAC1 and HDAC2, repressing transcription (134). DNMT3B interacts with SUMO-1 for its sumoylation (165) and with the hPc2 polycomb protein to repress fibroblast growth factor receptor 3 (173). During differentiation of human embryonic stem cells, phosphorylation of DNMTs occurs, as well as their interaction with the polymerase associated factor 1 transcriptional elongation complex (313). This complex binds to promoters of *OCT4* and *NANOG* genes that are involved in pluripotency, putatively contributing to their silencing.

Histone methylation occurs on arginine or lysine residues, which incorporate a maximum of two or three methyl groups, respectively. EZH2 is a subunit of PRC2 that catalyzes incorporation of the H3K27me3 repression mark, but also other protein methylations (Fig.13). Moreover, EZH2 recruits DNMTs to PRC2 and PRC3 complexes to regulate DNA methylation at EZH2-targeted promoters, a role to which interaction with the lncRNA AS1DHRS4 also contributes (210,381). EZH2 interacts with EED, SUZ12 and PHF1 subunits within PRC2 (191,328,333). EED is involved in directing PRC2 to the chromatin and SUZ12 seems essential for EZH2 activity, since its absence eradicates H3K27me2 and H3K27me3 marks (281). In contrast, PHF1 only stimulates catalysis of the H3K27me3 mark (328). Other EZH2 interactors include: C10orf12 and C17orf96 that do not bind simultaneously within PRC2 (8); the chromodomain Y-like protein that facilitates introduction and propagation of the H3K27me3 modification by increasing PRC2 methylation activity (420); and XNP, a member of the SNF2-like family, that contributes to transcriptional regulation or DNA repair (42).

EZH2 is a target for several PTMs. Among them, K348 acetylation by P300/CBP associated factor and S75 O-GlcNAcylation by interaction with OGT stabilize EZH2 (59,388). Conversely, phosphorylation by JAK2, CDK5 or CDK1, the latter induced by ncRNAs ANCR and HOTAIR, promote interaction with the E3 ubiquitin ligases FBXW1 or FBW7 for EZH2 ubiquitination and degradation (155,164,211,320). In some cases, an interplay between PTMs occurs for a coordinated regulation of EZH2, e.g. K348 acetylation decreases T345 and T487 phosphorylation favoring stability (388). Additionally, the presence or absence of certain PTMs promotes dissociation of EZH2 from PRC2. For example, lack of O-GlcNAcylation or Y244 phosphorylation by JAK3 disrupts the PRC2 complex, the latter allowing EZH2 interaction with Pol II and changing its role towards transcriptional activator (59,401). EZH2 activity is also modulated by PTMs, e.g. S21 phosphorylation by AKT blocks histone 3 binding and T350 modification by CDK1 and CDK2 is key to maintain the repressive H3K27me3 mark at the targeted loci (48,55). However, T416 phosphorylation by CDKs allows binding of NIPP1, a regulator of PP1, inhibiting dephosphorylation of EZH2 and impairing binding to polycomb target genes involved in proliferation (253). In general, EZH2 is abundant in proliferating organs and PRC2 deregulation is involved in tumorigenesis. Hence, considerable effort has been devoted to design molecules targeting the complex to control its activity. Among them, astemizol destabilizes EZH2/EED interaction reducing PRC2 methylation activity, and thus proliferation of lymphomas (181).

5. DEREGLATION OF TRANSSULFURATION IN PATHOLOGY

Oxidative stress is commonly detected in disorders in which transsulfuration is impaired, ranging from major liver pathologies to rare diseases and sensorineural disorders. Pathological changes involve all the regulatory levels from gene expression to activity, but differences are detected among diseases (Fig. 14). Most research in this field has been carried out in liver or hepatoma cells, and hence this section will consider mainly hepatic diseases. It is necessary to remind here that hepatic cell lines (mostly hepatomas), primary hepatocytes and even iPS hepatocytes show impaired expression of those genes traditionally considered “liver specific”, thus the need for overexpression (e.g. MAT α 1 or GNMT) reported in most studies.

5.1. Cancer and hepatocellular carcinoma

Common features of cancer cells are their high consumption of methionine and global DNA hypomethylation. These effects can be explained by an enhanced need of the amino acid for protein synthesis in proliferating cells and alterations in AdoMet synthesis due to changes in *MAT* expression patterns. Impairments of transsulfuration in tumor cells have been mostly studied in HCC, therefore we will focus preferentially in this malignancy that represents 85% of all primary liver cancers.

The story about cancer and transsulfuration started with the finding that growth of tumor cells depends strictly on methionine (88,352). Almost 20 years later, decreased MAT activity was found in rodent models of hepatoma together with changes in the MAT isoenzyme pattern (368). It took another 20 years to find the *MAT1A/MAT2A* expression switch that occurs in HCC, which involves changes in the methylation status of the gene promoters, rendering reduced *MAT1A* together with enhanced *MAT2A* and *MAT2B* expression (218,367). Changes in transsulfuration are however wider, including downregulation of *GNMT* (12,131), *PEMT* (376), *AHCY* (198) and *CBS* (12), but also

of several branches of the route, including Hcy remethylation (Fig. 14). This last observation justifies why Hcy cannot substitute methionine to support tumor cell growth. Additionally, several hepatoma cells express splicing variants for *MAT2B* (404) and are defective in *Gnmt* (394) and *Pemt* expression (62).

Several null mice for genes in this pathway have been generated since 2000. Most of them develop steatohepatitis and HCC due to their low (*Mat1a*^{-/-}) or high (*Gnmt*^{-/-}) hepatic AdoMet levels (215,236). The *Pemt*^{-/-} mouse constitutes an exception, showing severe steatosis without increased tumor incidence (150). This difference may rely on the ability to synthesize enough PC for hepatocyte function through the *de novo* CDP-choline pathway, whereas e.g. MAT α 2 seems unable to produce sufficient AdoMet to reach adequate concentrations to support the wide array of AdoMet-dependent reactions. Reduced *MAT1A* expression in HCC correlates with hypomethylation of *LIN28B* and downregulation of the tumor suppressor let-7, favoring invasion and metastasis (405). Moreover, the positive crossregulation between *MAT1A* and *PHB1* expression is also lost in HCC, where changes in the occupancy of their promoter E-boxes towards cMyc/cMaf/MafG reduce MAT α 1 and PHB1 content (96). The role of transsulfuration in HCC is further highlighted by the impact of miRNAs levels on *MAT1A* downregulation (miR-664, miR-485-3p, miR-405) and increased *EZH2* expression (miR-124) (405,422).

Pathological deregulation of transsulfuration has been associated with NF κ B and AP-1 signaling cascades that are redox-regulated. Sites for these transcription factors exist in the promoters of *MAT2A*, *MAT2B*, *PEMT*, *GAMT* and *GNMT*, but also in *BHMT* and *MTRR* promoters, although their role remains poorly explored. Upregulation of *MAT2B*, *JMJD3* and *NOS2* occurs upon binding to these elements, enhancing MAT β -V1 content, H3K27me3 demethylation and NO production, respectively (404).

Increased Hcy concentrations trigger the proinflammatory response mediated by NFκB, leading to enhanced ROS levels (126). In HCC, increased MATα2 levels would favor its corepressor role of MafK binding to ARE elements in target genes such as *HO-1* (168), therefore altering its role in iron reutilization, anti-inflammation or cytoprotection. Additional contributors to this picture are HuR and AUF1, which favor stabilization of *MAT2A* and degradation of *MAT1A* mRNAs, respectively (377).

Changes in expression of “liver specific” genes are mimicked by their cytosolic protein levels, although for MATs this is accompanied by enhanced *MAT2A* expression, *MAT2B* splicing forms and generation of the MAT II isoenzyme (36,277,404). As a consequence, an increase in MAT oligomers with a low $V_{\max}$ for AdoMet synthesis occurs. However, the picture further complicates with heterotrimers containing MATβ splicing forms, whose impact on AdoMet synthesis remains unclear. Nevertheless, a N-terminal MATβ truncation similar to MATβ-V2 still associates with MATα2 dimers and the resulting heterotrimer exhibits a higher $V_{\max}$ than MAT II *in vitro* (115).

Low AdoMet concentrations have been measured in several models of cancer, correlating with decreased protein and activity levels of methyltransferases consuming large amounts of AdoMet (e.g. PEMT). In contrast, modest users of AdoMet (e.g. EZH2) increase their presence in HCC correlating with tumor progression (58), whereas overexpression of NNMT occurs in a variety of cancers consuming large amounts of AdoMet to support growth (374). Moreover, low AHCY activity and the homozygous R49H mutation in this protein associate with HCC (188,348), putatively modifying the methylation index. Furthermore, increased NO levels due to iNOS upregulation will inhibit MAT I/III and CBS activities, through C121 nitrosylation and NO· binding to the heme group, respectively (13,379), but may also impair remethylation via BHMT nitrosylation as shown in cholestasis (214). In contrast, NO stimulates *CTH*

transcription, and thus, H₂S production and sulfhydration of the NFκB subunit RelA, increasing DNA binding (332). Combined effects of NO on CBS and CTH are probably compensatory regarding H₂S production through transsulfuration, in turn, acting on tumor growth.

Importantly, *MAT2A* expression does not follow the same trend in all tumors, being increased in liver, colon and breast cancers (364,407). According to the Human Protein Atlas, reduced MATα2 staining is reported in glioma or thyroid tumors and disparities in staining for most transsulfuration proteins exist in different cancer types. The NCI60 human cancer cell panel shows reduced *CBS* expression as compared to HepG2 hepatoma cells (419) and *PDRG1* overexpression occurs in several tumors coinciding with downregulation of p53 and/or miR-214, as expected from their diminished binding to the *PDRG1* promoter and mRNA, respectively (154,291,390). This last evidence suggests that in tumors with increased PDRG1 and MATα2 levels modulation of AdoMet synthesis and DNA methylation can involve their interaction. In this line, aspects such as whether this interaction is restricted to a specific subcellular compartment and the existence of putative MATα2 localization changes remain unexplored.

5.2. Ethanol abuse

In 1947, it was reported that patients with alcohol liver cirrhosis exhibit a delayed clearance of plasma methionine after an overload of this amino acid (175). It is also long-known that the disease often concurs with deficiencies in B-vitamins required in transsulfuration. Later on, impaired transsulfuration, reduced hepatic MAT and PEMT activities and low AdoMet concentrations were described in these patients (84,141). The MAT I/III activity ratio was decreased by enhanced MAT III levels, concomitant with MAT I loss (33). These results were confirmed in animal models of

ethanol and CCl₄ intoxication (64,127), where parallel depletion of glutathione and alteration of the GSH/GSSG ratio were detected. Moreover, inhibition of GSH synthesis with buthionine sulfoximine (BSO), an inhibitor of GCL, also decreased MAT activity and AdoMet concentrations, effects that were prevented by glutathione monoethyl ester (EGSH) (65). Additionally, GSH transport into the mitochondria was reduced by the decreased fluidity of the inner mitochondrial membrane induced by ethanol, effects prevented by AdoMet (63). Ethanol fed micropigs showed increased plasma Hcy and hepatic AdoHcy levels, centrilobular microsomal CYP2E1 and acetaldehyde and malondialdehyde protein adducts (127), and ethanol intoxicated rats presented DNA hypomethylation, increased DNA strand breaks and *c-MYC* expression (217). The oxidative stress and proinflammatory response elicited by ethanol are linked, and reflected in the expression changes induced by NFκB activation. Expression in human cirrhosis resembles changes in HCC, the *MAT1A* promoter is hypermethylated, and hence, expression reduced together with that of *GNMT*, *CBS* and remethylating genes (12) (Fig. 14). Additionally, lncRNA HEIH levels increase in cirrhosis and HCC, enhancing its binding to EZH2 and recruitment to the target genes (402).

5.3 Acute liver injury

Acetaminophen (APAP) intoxication is the leading cause of acute liver injury in the USA and UK. Its metabolism involves the production of NAPQI, an intermediate able to modify and inactivate proteins, and requires GSH (301). Therefore, administration of the glutathione precursor N-acetylcysteine (NAC) is the treatment of choice to maintain adequate GSH concentrations. As expected from the link between GSH synthesis and transsulfuration, APAP intoxication in rodents reduces hepatic *Gamt* and *Bhmt* expression and increases that of *Mat2a* with the consequent reduction in AdoMet levels (330). Effects in other transsulfuration related genes include transient

increases in *Gnmt*, *Ahcy* and *Gclc* transcripts. Cytosolic MAT α 1 levels remain unaffected by APAP in rat liver, but homotetrameric association is preferred and MAT activity decreases (71). The net result is a reduction in AdoMet content, together with a stronger decrease in AdoHcy and GSH concentrations, leading to an increased methylation index and reduced GSH/GSSG ratio, respectively. Additionally, the decreased GSH/GSSG ratio induced by APAP correlates with nuclear accumulation of MAT α 1. NAC supplementation to H35 transfected cells prevented changes in the GSH/GSSG ratio and MAT α 1 localization (71,293). Nuclear MAT activity remains unaffected by APAP, despite the increase in MAT I content and H3K27me3 levels. The underlying cause for the lack of activity changes remains unknown, but may rely on the putative modification of MAT α 1 by NAPQI. Furthermore, the importance of transsulfuration and associated reactions in acute liver injury is also supported by the better response exhibited by mouse strains with high *Bhmt2* expression against APAP intoxication (212). This effect may depend on the use of S-methylmethionine as methyl donor, doubling the yield of methionine recovered by Hcy remethylation.

More data linking acute liver injury and transsulfuration have been obtained in models of D-galactosamine (Gal) intoxication, that cause depletion of uridine nucleotides and the subsequent impairment of RNA and protein synthesis, and GSH depletion by BSO. Gal-intoxication induces the hepatic *Mat1a/Mat2a* switch and reduction of *Gnmt* and *Bhmt* expression, while increasing *Mat2b*, *Ahcy*, *Mtr*, *Gclc*, *Gclm* and *Pdrgl* transcript levels (71,291) (Fig. 14). Generally, changes in mRNA levels correlate with abnormalities in their half-lives. Expression and cytoplasmic protein levels follow the same trend, and homotetrameric association is preserved in GNMT, AHCY and BHMT, whereas MAT α 1 is mainly homodimeric (33,71). Subsequently, the corresponding activities change, according to the protein levels and the association

state, and the hepatic metabolite concentrations are altered. Hence, AdoMet concentration decreases and Hcy, methionine and GSSG levels increase, reducing the methylation index and GSH/GSSG ratio (71).

Gal- and BSO intoxications reduce MAT α 1 and BHMT cytoplasmic content, while inducing their nuclear accumulation in H35 cells (71,293). Gal induces a similar nucleocytoplasmic redistribution of GNMT, whereas AHCY and PDRG1 only increase their nuclear levels (71,291). Both agents diminish the GSH/GSSG ratio and prevention of this change by NAC or EGSH administration precludes anomalous nucleocytoplasmic distribution of MAT α 1 and BHMT due to Gal or BSO, respectively (71,293). In contrast to APAP intoxication, nuclear MAT α 1 accumulation in Gal-intoxicated livers rises MAT I content and MAT activity (71). Moreover, Gal-treated hepatoma H35 cells also show increased H3K27me3 levels correlating with nuclear MAT α 1 accumulation and NAC administration prevents alterations in this repression mark. Lack of significant effects in DNA methylation may rely on the parallel Gal-induced nuclear accumulation of PDRG1 (291). Interaction between PDRG1 and MATs reduces their activity, putatively decreasing nuclear AdoMet concentrations, despite MAT I increase. In this context, nuclear methyltransferases with higher affinities for the methyl donor (e.g. EZH2) would be less affected by its limited availability. Altogether, these data suggest that redox changes induced in acute liver injury play an important role in subcellular distribution of transsulfuration enzymes and their nucleocytoplasmic activities. Therefore, it may be worth to increase research in this field towards new drugs that avoid/modulate these effects to expand the available therapeutic options.

5.4. Wilson disease

Wilson disease is characterized by hepatic copper accumulation and impairment of its transport into the bile, although some patients present neurological deficiencies.

These symptoms are a consequence of various mutations in the *ATP7B* copper transporter gene. D-penicillamine is widely used for the treatment of Wilson disease and, in 1975, its coadministration with AdoMet showed improvement of liver function (104).

Oxidative stress, as well as alterations in methionine metabolism have been identified early in life in two models of this disease, the Long Evans Cinnamon rat (LEC, with a 900 bp deletion in *Atp7b*) and the toxic milk mouse (tx-j, carrying the G712D mutation). Livers of 9-weeks old LEC rats already present the *Mat1a/Mat2a* expression switch, decreased *Mtr*, *Ahcy* and, especially, *Mat2b* transcript levels (72) (Fig. 14). In cells, BSO prevented downregulation of *Mat2b* by copper, whereas its chelator neocuproine was ineffective and increased cell death, as expected from the elevated production of the toxic neocuproine-Cu(I) complex. Once more, hepatic cytoplasmic protein levels in LEC rats matched expression, leading to decreased MAT activity and GSH/GSSG ratios, alongside increased MAT I and MAT II levels (72). Enhanced hepatic methionine concentrations were noticed, probably due to copper activation of MTR, whereas AHCY inhibition cannot explain the low AdoHcy levels found, which result in an increased methylation index. Furthermore, *in vitro* experiments proved that copper alters the folding/association of MAT α 1 and MAT α 2 and inhibits the isoenzymes (72). This effect may be the basis for the reduced hepatic AdoMet levels and MAT activity observed in older LEC rats (339), mimicking cirrhosis and HCC.

Evolution of hepatic expression changes in transsulfuration and related reactions with age have been studied using tx-j mice (197). This work revealed downregulation of *Ahcy* from fetal stages until adulthood, together with biphasic expression trends for other genes. Namely, fetal downregulation of *Dnmt* genes, *Mat2a* and *Mtr* and their

upregulation from 12-20-weeks of age onward occurs, whereas *Mat1a* expression boosts in mice 12-weeks old. Therefore, AHCY, the methylation index and global DNA methylation are reduced. Additionally, decreased expression of genes involved in fatty acid oxidation (*Ppara* and *Cpt1A*), lipid synthesis (*Srebp1c*) and ER stress (*Grp78*) are found, together with the corresponding changes in SREBP1c and PPAR α proteins. In tx-j mice, administration of D-penicillamine or betaine reduces copper levels and normalizes DNA methylation (246). However, these agents exert additional effects, D-penicillamine increases AHCY activity, whereas betaine reduces *Ahcy* expression, increases AdoMet levels, the methylation index and *Dnmt3b* expression. Choline supplementation of the maternal tx-j mice diet induces global DNA hypermethylation in the fetus, while preventing downregulation of *Mat2a*, *Dnmt1*, *Dnmt3a*, *Dnmt3b*, *Ahcy*, *Mtr*, *Srebp1c*, *Cpt1A* and *Ppara* expression (245). In general, DNA hypomethylation correlates with upregulation of *Dnmt* genes, whereas AdoHcy levels and the methylation index associate with changes in copper levels (197). Altogether, Wilson disease models show impairment of transsulfuration with alterations mimicking mainly those described for HCC and cirrhosis, but also with additional defects e.g. in folding.

5.5. Hearing loss

According to the 2013 report of the World Health Organization, there are 360 million people worldwide with moderate to profound hearing loss and the problem affects especially the aged population. Surprisingly, 50% of the factors involved (e.g. noise, ototoxic drugs) can be prevented, and association of hearing loss with deficits in micronutrient ingestion exists. Epidemiological studies also noticed a relationship between deafness and increased plasma Hcy levels, but ~10-20% of the human population shows such an increase due to several factors (29). Decreased serum folate levels, correlating with low vitamin B₁₂ or HHcy, are also detected in age-related and

sudden sensorineural hearing loss (35,195). Moreover, these studies provide evidences of the association between inner ear atherosclerosis and deafness (157). This connection with Hcy and folate metabolisms prompted the design of intervention studies that improved age-related hearing loss and reduced systemic Hcy levels (87).

Data obtained from animal models involve Hcy and folate metabolism in hearing loss. *Cbs*^{+/-} mice present high Hcy levels in the *stria vascularis*, the structure involved in the metabolic control of the inner ear, and the *spiral ligament* (190). Moreover, these mice exhibit increased hearing thresholds, oxidative stress and reduced density of the microvasculature, abnormal features that are prevented by folate administration. Reduced *Bhmt* and increased *Ahcy* expression are found in the *stria vascularis* of *Cx30*^{-/-} mice (61), which have impaired connections among ciliary cells in the organ of Corti that are essential for hearing. Nevertheless, the first study devoted to cochlear Hcy metabolism was performed in mouse quite recently (238). Genes of transsulfuration and related reactions show the expected expression pattern for a non-hepatic organ. Namely, high levels of *Mat2a*, *Mat2b*, *Ahcy* and *Adk* transcripts, intermediate levels for *Mtr* expression and low for *Mat1a*, *Ada*, *Cbs* and *Bhmt2*. As an exception, similar *Mtr* and *Bhmt* transcript levels are detected. Some proteins, BHMT and AHCY, present anomalous mobilities in SDS-PAGE gels that are compatible with the existence of PTMs or specific cochlear splicing forms that remain unknown.

The impact of the nutritional deficiency of folic acid was analyzed using two mouse models, C57BL/6J mice that are prone to hearing loss and CBA mice with delayed onset of the disability. In both cases, folic acid deficiency accelerates the onset of hearing loss (238,239), but CBA mice require a long-term exposure to the diet that also leads to anemia, therefore suggesting that the impact of the diet depends on the genetic background (Fig. 15). Nevertheless, the folate-deficient diet causes cochlear

expression and protein changes in C57BL/6J mice intended to decrease Hcy production (*Ahcy* and AHCY), moderate its remethylation (reduced *Bhmt*, *Mtr*, and BHMT, but increased MTR) and catabolism (decreased CBS), in an attempt to diminish protein N-homocysteinylation (238). However, changes in Hcy catabolism may have a dark side by decreasing H₂S production by CBS and subsequently contributing to microvasculature alterations. Several cochlear parameters indicate oxidative stress and an attempt for its control: i) p22phox and MnSOD levels increase; ii) *Cat*, *Gpx4* and *Gss* expression decreases; iii) GSH and GSSG concentrations rise maintaining the GSH/GSSG ratio; and iv) 3-nitrotyrosine levels increase in the *stria vascularis* and the cochlear ganglion.

Other epidemiological studies showed the putative protection offered by ω3-PUFA against hearing loss (85,119). This possibility was initially explored in short-term supplementation studies of animal models, rendering contradictory results. However, a long-term supplementation study of C57BL/6J mice shows modest protection against hearing loss, together with attenuated cochlear inflammation and oxidative stress (240). Regarding transsulfuration and associated reactions, decreased expression of *Ahcy*, *Cbs*, *Cth* and *Adk* was detected with age, as well as for *Mtr* and *Bhmt* remethylation genes, whereas *Ada* was upregulated. Protein levels remained mostly unmodified with age, except for a CBS increase that was prevented by ω3-supplementation, while boosting BHMT content. This effect could rely on choline recycling as a consequence of anti-inflammatory mediator production from PC-incorporated PUFAs. This possibility is supported by the increased hepatic *Pemt* and *Gnmt* expression detected in ω3-supplemented animals, although their cochlear expression remains unknown. Conversely, the increased CBS detected with age could reflect an enhanced H₂S need to regulate the synthesis of anti- and pro-oxidant enzymes

and inflammatory mediators (161). In fact, several synthetic drugs that provide this gasotransmitter have been used to treat deafness with diverse outcomes. Protection against noise-induced hearing loss is provided by nicotinamide riboside (31), a NAD^+ precursor. This treatment prevents noise effects in hearing and neurite degeneration in the *spiral ganglion* and in mitochondrial SIRT3 that requires this cofactor. SIRT3 malfunction induces ROS and reduction of GSH content (342). However, effects of this treatment on AHCY that also uses NAD^+ were not analyzed, despite the potential improvement of nicotinamide riboside administration in cochlear Hey metabolism.

6. DETOXIFICATION MECHANISMS LINKED TO TRANSSULFURATION

Detoxification of xenobiotics and endogenous electrophilic compounds that are generated in excess under pathophysiological conditions involves a series of processes some of which require metabolites derived from sulfur amino acid metabolism, such as AdoMet or GSH (26,249,267). Additionally, these reactions produce the oxidized forms of different cofactors (e.g. NADP^+) that regulate transsulfuration and related reactions (115,250). Drug metabolism is essential for processing towards inactive metabolites and for the transformation of prodrugs into their active metabolic forms. Enzymes involved in these processes catalyze conversion of the original compound into a more polar metabolite (phase I) or conjugation reactions for the transformation of endogenous compounds and xenobiotics into more hydrophilic products (phase II) for excretion or inactivation of pharmacological drugs. These enzymes present ubiquitous expression, but their highest levels exist in liver. In this review, we have focused in a few of such processes that exemplify the connections with transsulfuration.

6.1. Aldo-ketoreductases

The aldo-ketoreductase (AKR) family of enzymes plays an important role in the reduction of carbonyl-containing compounds, which requires adequate levels of the NADPH cofactor (250). This family comprises more than 150 members with high sequence conservation and a similar monomeric structure. AKR1B1 and AKR1B10 are probably the best studied due to their involvement in the development of diabetic complications and/or cancer. These roles are related to their ability to metabolize aldehyde-substrates such as glucose, endogenous reactive carbonyls such as HNE and acrolein that are cytotoxic, and several anti-cancer drugs (79,179). Under physiological conditions, these enzymes play a constitutive and/or inducible cell-protective role. Thus,

in certain cell lines, oxidants or electrophilic compounds induce *AKR* expression through factors such as Nrf2 (166,224).

AKR1B1 catalyzes removal of ~30% of glucose excess in diabetes. Hence, its pathological role derives from an increased flux through the polyol pathway resulting in elevated NADPH consumption and redox imbalance (295,345). Additionally, the sorbitol excess generated can induce osmotic stress that together with AKR1B1 role in inflammation can further contribute to diabetic complications. In fact, certain AKR1B1 polymorphisms have been associated with complications such as diabetic cataract, retinopathy, nephropathy, vasculopathy, etc. (73).

Several AKRs have been associated with cancer and, particularly, AKR1B1 and AKR1B10 are overexpressed in lung, liver and breast tumors (192). The proposed mechanisms include the AKR1B10 abilities to: detoxify endogenous carbonyls increasing survival (389); metabolize exogenous carbonyls leading to chemoresistance (132); associate with acyl-coenzyme A carboxylase promoting fatty acid synthesis and, subsequently, tumor proliferation (225); and competence to convert retinaldehyde into retinol hampering activation of retinoic acid receptor and cell differentiation (103). AKR1B10 also metabolizes PAH to quinones with higher carcinogenic potential (303), thus interfering with PAH binding to the transsulfuration enzyme GNMT. Nevertheless, the role of AKRs in tumor promotion appears more complex, since *AKR1B10* expression increases in well-and moderately-differentiated liver tumors, but decreases in advanced-poorly differentiated tumors (135). In HCC and preneoplastic liver, *AKR1B10* expression is increased, whereas siRNA-mediated knockdown inhibits HCC cell proliferation (329,369). However, high AKR1B10 levels also predict favorable prognosis in HCC, after curative hepatectomy (124). Altogether, it remains unclear

whether the enzyme exerts a defensive role for the host in early tumor stages that is lost in more advanced steps.

Importantly, AKRs can participate in prostaglandin synthesis and are redox regulated through various oxidative and electrophilic modifications on cysteines in their C-terminal segment, close to the active site. These modifications either increase (e.g. nitrosylation and acrolein) or decrease enzyme activity (e.g. cyclopentenone prostaglandins (cyPG), HNE, glutathionylation) (346). Moreover, the cofactor NADPH also protects AKRs from inactivation by 4-oxonon-2-enal (82,230), whereas GSH availability and glutathionylation impact AKR activity allowing recovery from cyPG-modified or nitrosylated states, respectively, protecting the enzyme against irreversible inactivation (14,80). These last effects provide additional connections between transsulfuration, GSH synthesis and detoxification.

6.2. Glutathione S-transferases

Glutathione S-transferases (GSTs) are multifunctional enzymes that play a key role in the maintenance of the redox status and cellular detoxification. Conjugation of multiple polar compounds with GSH is their classical function, but they also form channels in intracellular membranes, reduce the availability of a variety of compounds by covalent and non-covalent binding (267), catalyze protein glutathionylation, thus participating in the defense against oxidative damage (362), and perform reactions involved in prostaglandin and steroid hormone synthesis (28). This superfamily comprises cytosolic, mitochondrial and microsomal members, whose structural and functional features have been reviewed elsewhere (267). Cytosolic GSTs can also move to the nucleus and differences in their nucleocytoplasmic activities correlate with protein-adduct production in either compartment (350).

Increased GST activity occurs in tumors, correlating with chemoresistance. In fact, GST has been considered a marker of hepatic carcinogenesis (321). GST induction can act as defense mechanism against chemicals, but once overexpressed it may provide an advantage for transformed cells that become able to survive the toxic insult. However, in tumor cells the GSH required for GST activity may not be sustained due to impaired transsulfuration, making them dependent on a larger import of cysteine. GSTP1-1 is one of the most exhaustively studied members of this family. Nrf2 upregulates GSTP1 expression in HCC through its binding to ARE elements in the promoter (196). Upregulation is also detected at initial stages of chemical hepatic carcinogenesis, as expected from the ability of these agents to change its transcription (133,169). However, modification of GSTP1-1 in response to several electrophilic compounds and drugs (e.g. HNE, cyPG, oxidants) modulates its oligomerization and activity (322). Among others, agents inducing oxidative stress or cyPG inhibit GST, eliciting reversible dimerization or irreversible tetramerization through cysteine crosslinking, respectively (323). Many of these compounds are also GST substrates, thus a context-dependent balance between their detoxification and enzyme inhibition is established. In fact, GSH or non-hydrolyzable GSH analogs protect GST against these modifications and oligomerization (106,322). Protein-protein interactions involving monomeric GST can be impaired by oligomerization, subsequently leading to activation of stress cascades by the release of its putative interaction partners. In fact, this mechanism supports sustained JNK activation directing to apoptosis (1,20).

GST provides protection against injury by various agents, e.g. its induction protects against acute liver failure (113). Surprisingly, *Gstp*^{-/-} mice exhibit increased resistance against APAP-induced liver injury, without induction of other GST family members, but with compensatory rises in enzymes maintaining redox status (e.g.

antioxidant protein 2, thioredoxin peroxidases) (176). This reduced toxicity also correlates with increased global protein glutathionylation, including modification of GSH biosynthesis enzymes, and could represent an adaptive mechanism providing protection in the absence of GSTP (243). Altogether, the protective mechanisms involving GST are constituted by a complex network of regulatory proteins and compensatory systems to which e.g. the different BHMT2-dependent susceptibilities to APAP may contribute. Moreover, although human and mouse GSTPs are highly homologous, critical residues for modification by oxidants and electrophiles (C101) are not preserved in the mouse protein, and hence the response to these agents may differ between species.

6.3. Thiopurine methyltransferase

Thiopurines such as 6-mercaptopurine (6MP) and its precursor azathioprine are the treatments of choice for inflammatory bowel disease, Crohn's disease and ulcerative colitis, but 6MP is also prescribed in childhood acute lymphoblastic leukemia (ALL) and azathioprine as immunosuppressor (11,331). Azathioprine is converted mainly by GST into 6MP, a process dependent on the activity and polymorphisms of this protein family (229). Three competing routes further metabolize 6MP using thiopurine methyltransferase (TPMT), hypoxanthine phosphoribosyltransferase (HPRT) or xanthine oxidase. Thioxanthine, an intermediate of the xanthine oxidase pathway, inhibits TPMT (26), while enhancing HPRT activity (331). 6MP combined with methotrexate is administrated as maintenance treatment for ALL, but methotrexate, as folate analog, inhibits purine and pyrimidine synthesis and dihydrofolate reductase, impairing folate recycling and remethylation (49).

TPMT is a cytoplasmic phase II enzyme, whose physiological substrate remains unknown. Its expression is almost ubiquitous, although hepatic and renal levels are the

highest. *TPMT* polymorphisms reducing activity are frequent and, in these cases, toxic effects of thiopurine treatments can increase mortality (229), therefore the importance of pharmacogenetic *TPMT* tests in chemotherapy. Azathioprine and 6MP are hepatotoxic drugs, but this effect is also observed with 6-methylmercaptopurine the product of *TPMT* (83). Although the exact contribution of *TPMT* to AdoMet consumption is not well established, these therapies will increase AdoMet needs in tumor cells that have reduced MAT activity, thus favoring 6MP metabolism through competing pathways. The 6-thioguanine nucleotides generated can substitute adenine and guanine in the DNA, inhibiting replication and contributing to DNA damage and apoptosis (302). AdoMet addition to MOLT lymphoblasts showed *TPMT* stabilization and prevention of 6MP cytotoxicity (249). However, the decreased DNMT activity and global hypomethylation observed seem to depend on a metabolite not produced by *TPMT* (139). Altogether, the ATP required for AdoMet synthesis to meet *TPMT* and DNMT needs depends on *de novo* purine synthesis that is inhibited by methylated products of 6-thioinosine monophosphate also generated by *TPMT* (249), thus closing the loop and favoring cytotoxicity.

7. HUMAN MUTATIONS IN TRANSSULFURATION

An increasing number of patients with homocystinuria, hypermethioninemia, mild liver dysfunction and/or neurological defects due to genetic deficiencies in enzymes of transsulfuration and associated reactions have been identified along the years. The variety of symptoms and deficiencies associated with transsulfuration impairment may rely on the diversity of AdoMet-dependent processes, but may also obscure defects concerning other metabolites with a more restricted utilization. The impact of the mutations found to date can be inferred from the structural information that is slowly becoming available. Nevertheless, since oligomerization is in many cases essential to achieve the biological function, knowledge about the folding and association events is also crucial to interpret the role of certain mutations with an apparently minor impact.

7.1. Human mutations identified in transsulfuration genes

According to the NCBI-SNP database on February 2017, missense mutations, frameshifts and splicing forms have been identified in most transsulfuration genes (Table 1). Homozygous and compound heterozygous mutations are commonly found, but some heterozygous mutations have been also identified. In general, these mutations are inherited in an autosomal recessive trait, but some exceptions exist. Homocystinuria (MIM 236200), discovered in 1962 in patients with mental retardation, is the most studied disorder (44). It is characterized by increased urinary excretion of homocistine and methionine and the associated symptoms include mental retardation, skeletal abnormalities and myopia. Newborn screenings are carried out worldwide, allowing detection of an increasing number of *CBS* mutations. The most common mutation, I278T, associates with the risk of spina bifida and leads to reduced protein stability and minimal activity (56). Prevalence of CBS deficiency varies notably among countries,

but is estimated between 1:58000 and 1:1000000. Response to vitamin B₆ depends on the mutation, for example patients carrying the I278M or A114V mutations are pyridoxine responsive, whereas those with the G307S mutation are non-responsive.

Cystathionuria (MIM 219500) due to mutations in the *CTH* gene was described in a mental institution in 1959 (128). The estimated prevalence is 1:14000. These patients show increased plasma concentrations and urinary excretion of cystathionine. Again, different outcomes to vitamin B₆ treatment have been described. The T67I mutation of ancient origin is the most common and responsible for the loss of CTH activity (91). Recombinant T67I protein shows reduced affinity for the B₆ cofactor, as expected from its localization in the same chain than R62 that is involved in vitamin binding (186). R62H and Q240E mutations also affect CTH activity, the former by altering cofactor binding and increasing conformational flexibility near the active site and the latter by changing kinetic parameters (186). Additional mutations include deletion of residues G57-Q196, consequently eliminating a large portion of the catalytic site and putatively modifying tetramer association.

The number of cases of isolated persistent hypermethioninemia due to *MAT1A* (MIM 250850), *AHCY* (MIM 613752) and *GNMT* (MIM 606664) mutations is more limited, and hence their worldwide prevalence remains unknown. *MAT1A* deficiency presents with elevated plasma methionine levels and is usually asymptomatic, although some cases with neurological abnormalities have been described. Demyelination was initially observed in patients with indels leading to truncated MAT α 1 forms ~350 residues long, but later detected in individuals carrying shorter protein forms (185X) or single mutations (R292C/R356L) (259,262,354). AdoMet treatment improved myelination in some patients (262,354). R264H is the most common *MAT1A* mutation and the only autosomal dominant (51). This mutation reduces enzyme activity, an effect

ascribed to the involvement of the *wild type* arginine in a salt bridge essential for dimerization.

Patients carrying *GNMT* mutations exhibit high plasma methionine and AdoMet levels, together with increased serum transaminases and mild hepatomegaly. The few mutations identified to date are point mutations that reduce activity. L49P disturbs the second α -helix, H176N decreases stability and N140S nearly abolishes catalysis (219,222,223).

AHCY deficient patients present elevated plasma AdoHcy, AdoMet and methionine levels, their symptoms including mental and motor retardation, facial dysmorphism and myocardiopathy (17). The few point mutations identified to date affect the catalytic domain. R49C and Y143C include new cysteine residues putatively contributing to alter the cofactor's oxidative state, while others like D86G suppress negative charges in this domain (17,19,383). Some mutations also increase thermosensitivity or contribute to form inactive aggregates. Interestingly, a translocation involving *TAX1BP1* and *AHCY* genes has been described in breast cancer HCC1806 cells (142), although it remains unclear whether the fused gene is translated. Such anomaly may interrupt several processes, including TAX1BP1 interaction with the NF κ B signaling pathway and its coactivation of nuclear receptors, as well as AdoHcy hydrolysis with the corresponding reduction of the methylation index and methyltransferase inhibition.

Cerebral creatine deficiency syndrome 2 (MIM 612736) due to mutations in the *GAMT* gene leads to developmental delay, mental retardation and muscular hypotonia, among other symptoms. Patients have low plasma and reduced excretion of creatine. Point mutations W20S and C169Y reduce GAMT activity (37), whereas T136M alters the protein core (7). Indels are also detected in the *GAMT* gene causing changes in the

sequence and early stop codons (7,149). Some truncated proteins lack D134, an essential amino acid for AdoMet binding, and consequently their activity is abolished.

Data about the impact of mutations in the remaining transsulfuration genes are scarce. Nevertheless, the V175M mutation in PEMT causes activity loss and associates e.g. with the susceptibility to non-alcoholic fatty liver disease (343), whereas the C-terminal E344A and R356H mutations in MAT α 2 seem to predispose to thoracic aortic disease (122). These MAT α 2 mutations alter association and/or activity due to their respective locations at the end of an α -helix required for electrostatic interactions with the other monomer or MAT β (E344A) and near the AdoMet binding site (R356H). Regarding *DNMTs* and *EZH2*, mutations have been reported as stated in Table 1, but no specific disease has been related to changes limited to these genes.

7.2. Impact of mutations in association and subcellular localization

Most transsulfuration proteins are homo or hetero-oligomers, and association is a requirement to attain activity. Subunit assembly requires not only preservation of the overall structure, but also of the charge or hydrophobic characteristics of the interacting interfaces. Some of these features may be altered in the variant proteins obtained by alternative splicing that are detected in pathologies that concur with oxidative stress. In fact, those reported to date result in: i) change (MAT β , PEMT) or elimination (AHCY) of part of the N-terminal sequence; ii) deletion or alteration of other parts of the sequence (MAT β , PEMT, GAMT); iii) insertions (CBS); and iv) the combination of these factors (e.g. MAT β -V2a). Additionally, mutations leading to early stop codons or frameshifts may have a similar impact.

Examples of special interest to explain these effects are MAT α 1 truncated forms. The anomalous association of MAT α 1 350X or 351X with *wild type* MAT α 2 subunits in an inactive oligomer was initially proposed to explain demyelination in

these patients (130). Later on, when shorter forms (185X) were found in patients with these symptoms and the MAT I/III structure became available (116), it was evident that association requires three β -sheets, provided each by a different domain, and that 185X cannot meet this constraint. In contrast, the 350X truncation eliminates ~50% of the C-terminal domain preserving the sequence for the β -sheet, whereas in 351X the remaining sequence of this domain changes (228). Therefore, only the 350X subunit may achieve an intermediate folding state resembling that allowing association with MAT α 2. Provided this is the case, two scenarios can take place: i) monomer association in an inverted fashion to render a MAT α 1/MAT α 2 heterodimer with a single active site completed; and ii) the 350X intermediates could trap MAT α 2 intermediates and preclude the last folding steps. A putative hetero-oligomeric association of 351X and MAT α 2 intermediates seems even more complicated, as only two “*wild type*” domains could be involved, in turn, increasing the instability of the association or generating an unproductive intermediate due to the alternative C-terminal sequence.

Subcellular localization can be also modified by mutations. Cytoplasmic retention and nuclear localization of MAT α 1 involves C-terminal basic residues, their elimination putatively altering subcellular distribution. In fact, nuclear accumulation and 70% reduction of MAT activity is detected in the rat N351X protein (311). T87N and D234N mutations in CBS also affect localization (46), despite their situation out of the protein's C-terminal end required for the interaction with the sumoylation machinery (162). Effects were stronger for the T87N mutant that abolishes activity and tends to aggregate (46). This highly conserved residue locates in a loop within the N-terminal domain, its side chain putatively stabilizing the hydrophobic dimer interface, as well as establishing interactions with additional residues. Substitution of threonine by

asparagine may disrupt the array of interactions established by the former, in turn, destabilizing the oligomer.

8. INTEGRATED VIEW OF TRANSSULFURATION

The conversion of methionine into cysteine through transsulfuration involves the synthesis of key metabolites that allow the interplay of this pathway with many others. AdoMet-dependent methylations are key for this exchange transferring the methyl group to multiple substrates for the synthesis of a variety of compounds or the regulation of processes through protein, RNA and DNA modification (218). Here, we have highlighted AdoMet connections including the synthesis of sarcosine (219), creatine (158) and PC (273), drug detoxification (249) and epigenetic modifications (285,400), but we cannot forget that this network expands far beyond. Besides one carbon metabolism, Hcy connections are evolving to regulatory actions on ER stress and epigenetics, mainly involving protein homocysteinylation (152). Its conversion into cysteine supports GSH synthesis (256,382), key for drug detoxification, but also provides H₂S for additional regulatory mechanisms (393). Detection of transsulfuration enzymes in new subcellular localizations (71,162,187,304,311), together with their involvement in unexpected functions (e.g. GNMT as PAH receptor) are changing the classical view.

Nowadays we can no longer speak about transsulfuration as a cytoplasmic process, excepting some transmethylation events, but rather about a pathway with cytoplasmic and nuclear branches as shown in liver (Fig. 16). The hepatic composition of each branch includes as core components MATs, AHCY and CBS (71,162,304,311), whereas differences may rely on the associated methyltransferases. Nevertheless, some are found in both compartments, namely GNMT and GAMT (158,187), as well as the remethylating enzyme BHMT (293). In normal liver, the nuclear content of these enzymes is rather small, but active oligomers exist (e.g. MAT I) together with additional protein states putatively responsible of their moonlighting activities. This is

the case of GNMT dimers, acting as PAH receptors for the activation of XREs (349), and could be that of MAT α 1 monomers as activators of the *MAT1A* E-box (408), although this last option remains unexplored.

Nuclear accumulation of transsulfuration proteins is observed in APAP- (MAT α 1) and Gal-induced (MAT α 1, AHCY, GNMT, BHMT) acute liver injury, concomitantly with their cytoplasmic decrease (71,293) (Fig. 16). This altered nucleocytoplasmic distribution correlates with a lower GSH/GSSG ratio and, in some instances, can be prevented with glutathione precursors, mainly NAC. Reductions in the GSH/GSSG ratio and the cytoplasmic content of transsulfuration enzymes are common to other hepatic pathologies, suggesting that similar changes in subcellular distribution could take place in those diseases. In fact, nuclear MAT α 1 accumulation has been also reported in HCC (405). Nuclear protein accumulation correlates with increases in processes requiring enzyme activity (e.g. MAT α 1 and H3K27me3 or AHCY and cap methylation), indicating that transsulfuration occurs in this compartment. Moreover, it seems plausible that protein accumulation results from an attempt to increase the flux through the nuclear pathway in order to respond to different types of insults, but whether this effect is aided by the nuclear import/export of metabolites such as AdoMet or AdoHcy remains unknown.

Most of the data presented in this review suggest a preeminent role for redox regulation in expression of transsulfuration genes that can be exerted through E-boxes, AP-1 sites or AREs on several gene promoters, which may bind redox regulated transcription factors (e.g NF κ B). In normal liver, binding of MAT α 1/Mnt or PHB1, an ARE coactivator (96), to the *MAT1A* E-box regulate its high expression levels, whereas USF-1 binding on the *CBS* E-box is responsible for basal activity (108,408). Changes in the factors bound to the E-box and/or an interplay with additional elements alters

expression of the corresponding gene (e.g. cMyc/MafG/cMaf binding reduces *MAT1A* expression). Binding of NFκB, AP-1 or other factors to AP-1 sites increase expression of *MAT2A*, *MAT2B*, *EZH2*, *GSTP1-1* in cancer (98,169,404,409) and of *CTH* upon lipopolysaccharide administration (392). The presence of AREs in *GSTP1-1* and *AKR* promoters allows their regulation through AP-1 or Nrf2 binding as part of the antioxidant response (133,166). These effects are also complemented by alterations in the methylation status of the promoters and their associated proteins due to redox effects on methyltransferases/demethylases or the availability of AdoMet, but also through the interplay of DNA methylation with additional epigenetic modifications dependent on other metabolic pathways (e.g. deacetylation requires NAD^+). Thus, the roles of transsulfuration as provider of AdoMet, hydrolyser of AdoHcy and as a “receptor” of epigenetic methylations reciprocally connect this pathway and Epigenetics (Fig. 17). Some of these aspects may also influence post-transcriptional mechanisms, as is the case of miRNA expression that is upregulated in diseases concurring with oxidative stress (e.g. miR-664 in HCC) or the methylation status of HuR (378,405).

Oligomerization, a prerequisite for the function of many transsulfuration enzymes, can be redox regulated as shown for MAT isoenzymes. Moderate decreases in the GSH/GSSG ratio lead to dimeric MAT III accumulation, putatively favoring production of the C35-C61 disulfide bond during folding (235,325), whereas its severe reduction may impair folding and generate monomeric proteins (234,276). In contrast, decreased NADPH/NADP⁺ ratios favor MAT II generation by enhancing NADP⁺ binding to MATβ-V1 and, subsequently its affinity for MATα2 dimers (115). It remains unclear whether this mechanism favoring MAT II assembly is also feasible for other MATβ splicing forms detected in HCC. Altogether, oxidative stress diminishes AdoMet production by supporting the low $V_{\max}$ isoenzyme MAT II vs. MAT I/III and connects

transsulfuration with the synthesis/levels of redox couples (NADPH/NADP⁺, GSH/GSSG), making it susceptible to their imbalance during detoxification. Again, reciprocal links between transsulfuration and detoxification are established, the former providing AdoMet and cysteine for TPMT activity and GSH synthesis, respectively, while the latter generates oxidized species (i.e. NADP⁺) that impair transsulfuration.

Protein-protein interactions provide additional regulatory options. Among them, interactions of EZH2 within the PRC2 sustain its methyltransferase activity for the introduction of the H3K27me3 repression mark on target genes, whereas interaction of PDRG1 with MAT α subunits reduces AdoMet synthesis (291). This aspect is of special importance in the nucleus, where the PDRG1/MAT α 1 interaction occurs, but also during acute liver injury when nuclear accumulation of both proteins arises. Here, again redox regulation may have a role, as suggested by the increased tripartite MAT β /HuR/SIRT1 stabilizing interaction induced by the antioxidant resveratrol (410), which also connects members of transsulfuration with epigenetic processes. Moreover, GNMT and CTH also establish protein-protein interactions that are involved in xenobiotic response (e.g. GNMT/ARNT/HIF-1 β) or in the putative control of autophagy (e.g. CTH/HMGB1) (349,358).

Another level of regulation includes PTMs and their effects on activity or subcellular localization. Here, redox regulation (e.g. MAT α 1 nitrosylation or CBS glutathionylation) adds to a large variety of additional mechanisms that connect transsulfuration with acetyl-CoA (e.g. MAT α 2 and EZH2 acetylation) or ATP levels (e.g. PEMT, DNMTs and EZH2 phosphorylation) to regulate activity of the different transsulfuration enzymes. In general, oxidative stress reduces initial steps of transsulfuration (e.g. AdoMet synthesis), but enhances the last steps towards cysteine production (e.g. CBS activity). In some instances, an interplay between different PTMs

is established. This is the case of acetylation that interferes with certain phosphorylation events favoring EZH2 stability (388). CBS sumoylation drives the enzyme to the nucleus (162), but no information exists about putative PTMs involved in the nuclear accumulation of transsulfuration enzymes observed in acute liver injury. Collectively, information concerning PTMs (e.g. protein methylations) has been mostly obtained in high-throughput studies, and hence their role remains mostly unknown.

Dietary effects are exerted mainly through the availability of essential substrates or cofactors (e.g. methionine and B-vitamins) for transsulfuration. However, additional aspects include its coordination with other pathways, such as protein synthesis (e.g. EF-1 α induction) and autophagy (e.g. PP2A methylation) by methionine excess (50,355,357), or the regulation of expression of transsulfuration genes through nutrient sensing elements (e.g. FXR in *Cth*) (241). Furthermore, methionine restriction changes global transcriptional and translational programs, increasing for example GSH and xenobiotic metabolism by cytochrome P450 (110) and ATP synthesis by oxidative phosphorylation (426). Thus, the diet further contributes to reciprocal connections between transsulfuration, additional metabolic pathways and expression events.

9. CONCLUSIONS AND FUTURE DIRECTIONS

Many diseases, but also nutritional changes and detoxification events, concur with alterations in transsulfuration that extend from gene expression towards enzyme activity, modifying the availability of essential metabolites for the synthesis of cellular components and regulatory mechanisms. The redox status is involved in a variety of such alterations, through differential binding of transcription factors, introduction of PMTs, changes in subcellular localization and putatively by promoting pathological protein-protein interactions. However, most of these aspects need additional examination. This picture is further complicated by the presence of key core proteins of transsulfuration in the nucleus, where they show activity, thus indicating the existence of cytoplasmic and nuclear branches of the pathway. Aspects as whether nuclear transsulfuration is enough to support e.g. epigenetic modifications, the actual nuclear metabolite concentrations (e.g. AdoMet) or if the cytoplasm still contributes for this purpose remain unknown. Accumulation of core proteins during oxidative stress points towards an effort to improve the flux through nuclear transsulfuration, but there is insufficient information concerning putative PTMs or protein-protein interactions involved in nucleocytoplasmic shuttling. Moreover, differences in the oligomeric state of some enzymes between subcellular compartments suggest changes in their structures, which may potentially result in moonlighting activities and/or new interaction surfaces that deserve further investigation. To date, these moonlighting functions link transsulfuration with detoxification and control of gene expression, but the set of regulated genes and transsulfuration proteins involved is limited. Furthermore, the impact of human mutations in these aspects is largely unknown, and hence deserves some attention. The neurological symptoms associated with human mutations and the large variety of disease that concur with impairment of transsulfuration also highlights

the need to increase the knowledge on this pathway in non-hepatic tissues. Altogether, transsulfuration presents an interesting interplay between Nutrition, a variety of metabolic pathways, redox regulatory processes, epigenetic and detoxification events that are far from being completely understood and that constitute a continuous source of surprises.

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ABBREVIATIONS

ADA, adenosine deaminase

ADK, adenosine kinase

AdoMet or SAM, S-adenosylmethionine

AdoHcy, S-adenosylhomocysteine

AHCY, S-adenosylhomocysteine hydrolase or adenosylhomocysteinase

AKR, aldo-keto reductase

APAP, acetaminophen or paracetamol

ARE, antioxidant response element

AUF1, AU-rich RNA binding factor

BHMT and BHMT2, betaine homocysteine S-methyltransferases

BSO, buthionine sulfoximine

CBS, cystathionine β -synthase

CTH, cystathionase or cystathionine γ -lyase

cyPG, cyclopentenone prostaglandin

DNMT, DNA methyltransferase

EGSH, glutathione monoethyl ester

ER, endoplasmic reticulum

EZH2, Enhancer of Zeste Homologue 2

FXR, farnesoid responsive element

Gal, D-galactosamine

GAMT, guanidinoacetate N-methyltransferase

GCL, γ -glutamylcysteine synthetase or glutamate cysteine ligase

GNMT, glycine N-methyltransferase

GSH, glutathione reduced form

GSR, glutathione reductase

GSS, glutathione synthetase

GSSG, glutathione oxidized form

GST, glutathione S-transferase

H₂S, hydrogen sulfide

H3K27me₃, H3K4me₂ and H3K9me₂, histone 3 trimethylation or demethylation in lysines 27, 4 or 9

HCC, hepatocellular carcinoma

HDAC, histone deacetylase

HO-1, heme oxygenase

Hcy, homocysteine

HHcy, hyperhomocysteinemia

HMGB1, high-mobility group box 1

HNE, 4-hydroxynonenal

HPRT, hypoxanthine phosphoribosyltransferase

HuR, Hu antigen R or ELAVL1

LEC, Long Evans Cinnamon

MAT, methionine adenosyltransferase

MAT α 1, methionine adenosyltransferase catalytic subunit α 1

MAT α 2, methionine adenosyltransferase catalytic subunit α 2

MAT β , methionine adenosyltransferase regulatory subunit

6-MP, 6-mercaptopurine

MTA, methylthioadenosine

MTR, methionine synthase

MTHF, 5-methyltetrahydrofolate

MTHFR, methylenetetrahydrofolate reductase

NAC, N-acetylcysteine

NNMT, nicotinamide N-methyltransferase

NO, nitric oxide

OGlcNAc, O-linked N-acetylglucosamine incorporation

PAH, 4S polycyclic aromatic hydrocarbon

PC, phosphatidylcholine

PDRG1, p53 and DNA damage-regulated gene 1

PEMT, phosphatidylethanolamine N-methyltransferase

PHB1, prohibitin 1

PKA, PKC and PKG, protein kinases A, C and G

PP1 and PP2A, protein phosphatases 1 and 2A

PRC1, PRC2 and PRC3, polycomb repressive complexes 1, 2 and 3

PTM, posttranslational modification

PUFA, polyunsaturated fatty acid

ROS, reactive oxygen species

SDR, short-chain dehydrogenase/reductase

SNP, single nucleotide polymorphism

TPMT, thiopurine methyltransferase

Tx-j, toxic-milk

XRE, xenobiotic responsive element

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CLASSICAL VIEW

CURRENT VIEW

A **B** **C**

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CYTOPLASM