

## Review Article

# Targeting the methionine addiction of cancer

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Received March 15, 2022; Accepted April 14, 2022; Epub May 15, 2022; Published May 30, 2022

**Abstract:** Methionine is the initiator amino acid for protein synthesis, the methyl source for most nucleotide, chromatin, and protein methylation, and the carbon backbone for various aspects of the cellular antioxidant response and nucleotide biosynthesis. Methionine is provided in the diet and serum methionine levels fluctuate based on dietary methionine content. Within the cell, methionine is recycled from homocysteine via the methionine cycle, which is linked to nutrient status via one-carbon metabolism. Unlike normal cells, many cancer cells, both *in vitro* and *in vivo*, show high methionine cycle activity and are dependent on exogenous methionine for continued growth. However, the molecular mechanisms underlying the methionine dependence of diverse malignancies are poorly understood. Methionine deprivation initiates widespread metabolic alterations in cancer cells that enable them to survive despite limited methionine availability, and these adaptive alterations can be specifically targeted to enhance the activity of methionine deprivation, a strategy we have termed “metabolic priming”. Chemotherapy-resistant cell populations such as cancer stem cells, which drive treatment-resistance, are also sensitive to methionine deprivation, suggesting dietary methionine restriction may inhibit metastasis and recurrence. Several clinical trials in cancer are investigating methionine restriction in combination with other agents. This review will explore new insights into the mechanisms of methionine dependence in cancer and therapeutic efforts to translate these insights into enhanced clinical activity of methionine restriction in cancer.

**Keywords:** Methionine, metabolism, one-carbon, epigenetics, oxidative stress, nutrition, cancer therapy

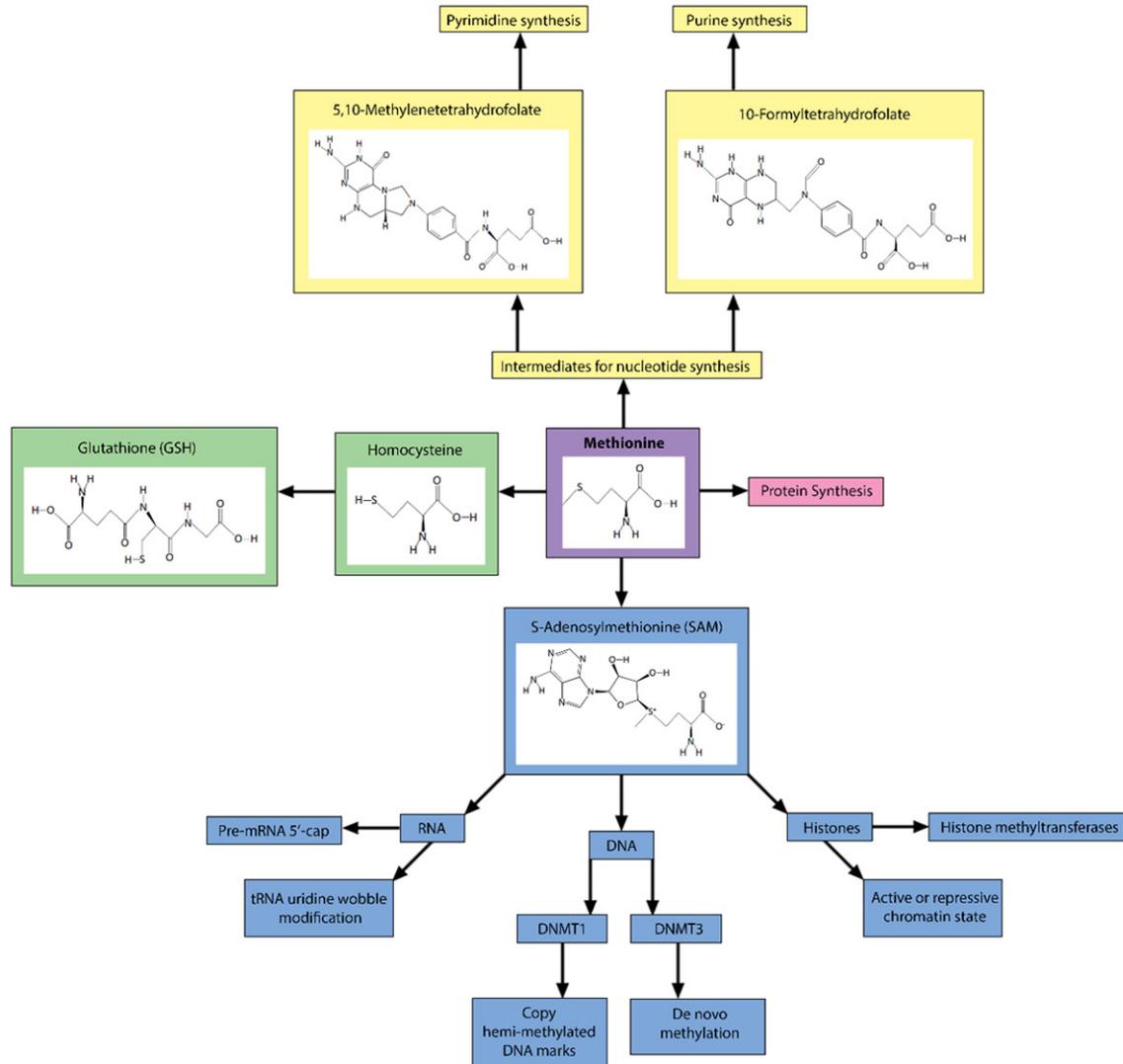
### Methionine cycle and one-carbon metabolism: linking carbon flux to nutrient status

Methionine is an essential amino acid for protein synthesis as well as many biochemical reactions required for cell viability and growth (**Figure 1**). S-adenosylmethionine (SAM) is the universal methyl donor for RNA, DNA, and chromatin methylation, and is synthesized from methionine via methionine adenosyltransferase (MAT) in the first step of the methionine cycle (**Figure 2A**) [1, 2]. MAT I (a tetramer) and MAT III (a dimer) are generally expressed in the liver, where high SAM synthesis occurs, and are encoded by *MAT1A*, while MAT II (a dimer) is expressed in most other cell types and is encoded by *MAT2A* [3-5]. SAM is then converted to S-adenosylhomocysteine (SAH) through various transmethylation reactions. SAH is hydrolyzed to homocysteine by SAH hydrolase (AHCY or SAHH), which can then be re-methylated to methionine by methionine synthase (5-methyltetrahydrofolate-homocysteine meth-

yltransferase; MTR or MS) or betaine-homocysteine methyltransferase (BHMT) to complete the methionine cycle [1, 2]. Alternatively, homocysteine can be diverted into the transsulfuration pathway by cystathionine- $\beta$ -synthase (CBS) to become cystathionine, which is then converted to cysteine by cystathionase (CTH) for use in glutathione production and maintenance of redox homeostasis [1, 3]. Additionally, SAM is utilized for polyamine synthesis, which produces S-methyl-5'-thioadenosine (MTA) as a byproduct that can then be recycled back to methionine in the methionine salvage pathway [1, 3]. The immediate precursor to methionine in the salvage pathway, 2-keto-4-methylthiobutyrate (MTOB), can also be converted to methionine, which is capable of activating the apoptotic cascade, linking methionine metabolism to cell fate decisions [1].

The methionine and folate cycles are directly linked via so-called one-carbon metabolism to stabilize metabolite levels, particularly during

## Cancer's methionine addiction

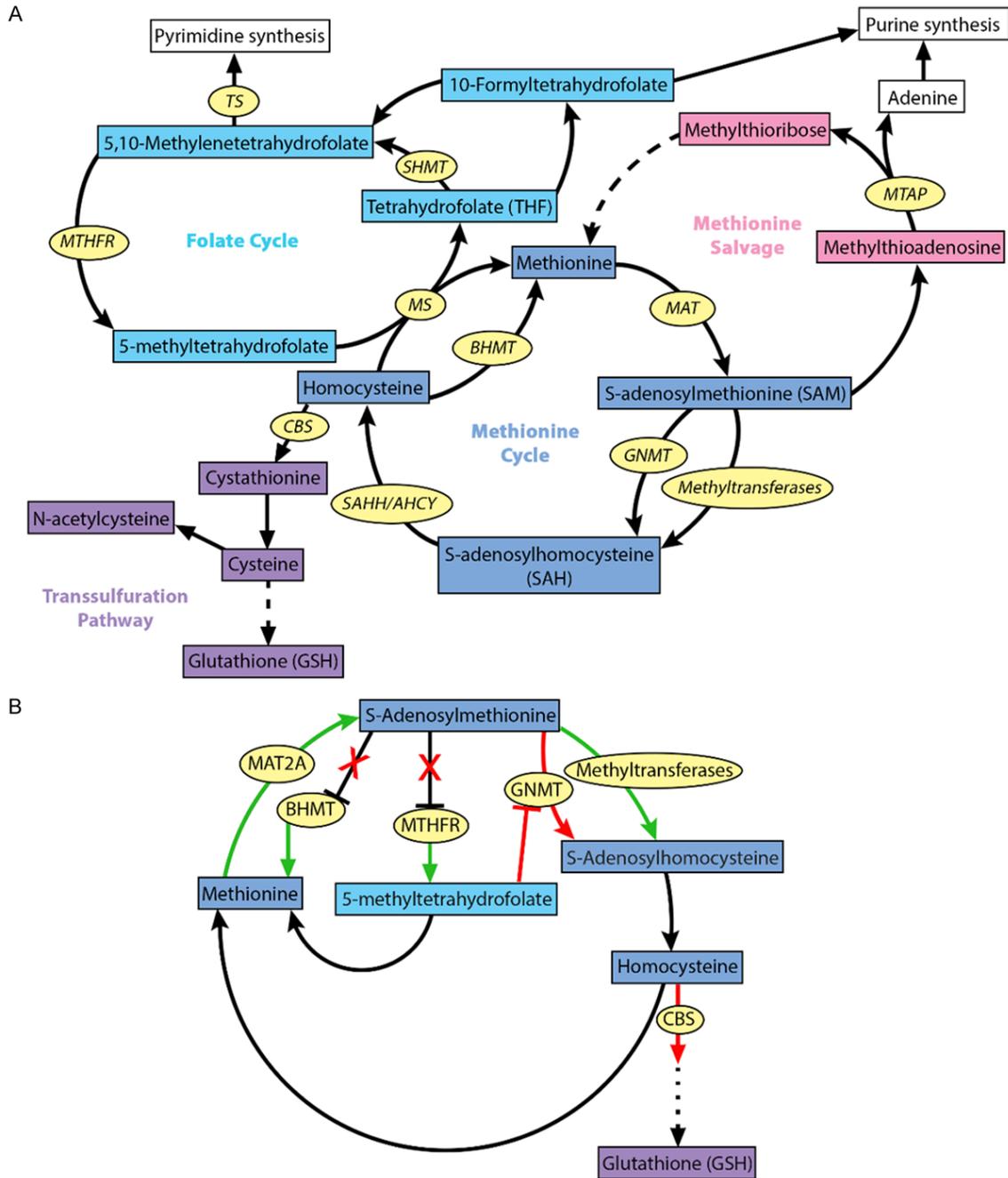


**Figure 1.** Diverse cellular functions of methionine in cell survival, growth and division. Methionine is an essential amino acid, meaning it must be provided through diet. The main cellular functions for methionine are protein synthesis, generation of intermediates and cofactors for nucleotide and glutathione synthesis, and generation of S-adenosylmethionine (SAM), the universal methyl donor for methylation of RNA, DNA, and histones.

times of low methionine availability to maintain flux through the methionine cycle instead of diverting intermediates towards other pathways [6] (**Figure 2B**). When SAM is abundant, indicative of high methionine levels, SAM inhibits the enzymes methylenetetrahydrofolate reductase (MTHFR) and BHMT to limit conversion of homocysteine to methionine, allowing homocysteine to be diverted for transsulfuration [5, 6]. SAM also activates CBS, further diverting homocysteine towards transsulfuration [5-7]. When methionine concentrations are low, SAM levels decline, releasing inhibition of MTHFR and BHMT, and suppressing activa-

tion of CBS to maintain flux through the methionine cycle. Low methionine synthesis leads to accumulation of 5-methyltetrahydrofolate (5-mTHF), which inhibits glycine N-methyltransferase (GNMT) to direct SAM usage towards DNA methyltransferases (DNMTs) [6]. Moreover, MAT2A expression is selectively increased when methionine levels are low [8-10], suggesting that SAM biosynthesis is regulated in an effort to maintain cellular SAM levels. These coordinated interactions allow a cell to buffer declining methionine and SAM levels in times of low methionine input to maintain protein and polyamine synthesis as well as meth-

## Cancer's methionine addiction



**Figure 2.** Methionine and Folate Cycles are Linked by One-Carbon Metabolism. A. The methionine and folate cycles functionally interact to generate intermediates and cofactors for various anabolic processes. Available methionine largely depends on dietary input. Methionine cycle intermediates can be diverted into the methionine salvage pathway to promote purine synthesis and into the transsulfuration pathway to aid in the production of reduce glutathione. B. The methionine and folate cycles interact to divert intermediates based on methionine availability. SAM normally inhibits MTHFR and BHMT and promotes CBS to divert intermediates towards nucleotide and glutathione synthesis when methionine and SAM are plentiful. During times of low SAM, this inhibition is released, increasing the synthesis of methionine from homocysteine as well as allowing 5-methyltetrahydrofolate to begin to accumulate. 5-methyltetrahydrofolate blocks GNMT to allow methyltransferases to utilize available SAM to maintain RNA, DNA, and histone methylation. MAT2A selectively increases during low methionine, further increasing SAM synthesis. Together, this regulation maintains intermediates around the folate and methionine cycle in times of low methionine input. Abbreviations: TS, tetrahydrofolate synthase; MTHFR, methylenetetrahydrofolate reductase; SHMT, serine hydroxymethyltransferase; MS, methionine synthase; MAT, methionine adenosyltransferase; MAT2A, methionine adenosyltransferase 2A; GNMT, glycine N, methyltransferase; SAHH/AHCY, adenosylhomocysteinase; BHMT, betaine-homocysteine S-methyltransferase; CBS, cystathionine- $\beta$ -synthase; MTAP, S-methyl-5'-thioadenosine phosphorylase.

ylation reactions, all of which are necessary for cell growth and survival.

### **Cancer metabolic vulnerabilities: an addiction to methionine**

Oncogenic alterations in cancer cells modify their nutrient intake and utilization, leading to altered metabolic states that fuel their enhanced proliferation and growth [11]. One such tumor-specific metabolic alteration is methionine dependence, namely, the requirement of exogenous methionine for tumor growth and the inability of transformed cells to grow when methionine is replaced by homocysteine in cell media or the diet [12-15]. This effect appears to be specific to transformed cells, as normal cells maintain growth and viability, even on prolonged methionine deprivation when supplemented with homocysteine [12-14]. When human sarcoma HOS-1A cells were co-cultured with fibroblast FS-3 cells, methionine deprivation selectively suppressed growth of the cancer cells while maintaining normal growth of the fibroblasts [16]. To date, many cancer cell lines evaluated, including prostate, breast, bladder, colon, glioma, kidney, melanoma, and leukemia cells, along with fresh patient tumors cultured *ex vivo*, have been found to exhibit some degree of methionine dependence [1, 13, 14, 17-19]. Methionine deprivation has also been found to decrease invasion and wound healing, suggesting it may be useful in targeting metastatic subclones [20, 21]. Viewed from this context, cancer cells are methionine auxotrophs "addicted" to exogenous methionine, a metabolic dependence intrinsic to the transformed phenotype that is not manifested by most normal cells [22-24].

One exception to the lack of methionine-dependence in normal cells was observed in healthy human lymphocytes cultured in methionine-free media. One study cultured both normal and malignant B and T lymphocytes in methionine-free media and all cell lines with the exception of one failed to grow and colony formation was abolished [25]. However, other studies conducted since have raised concern that the level of homocysteine used was not sufficient to support cell growth. Another study used lymphocytes isolated from fasting blood samples of healthy volunteers in methionine-free media and found a range of methionine-dependence, with approximately a third of participant's lymphocytes

growing at half the rate observed in full methionine media [26]. This study found that methionine-dependence did not relate to micronucleus frequency or mutations in either MTHFR or methionine synthase [26]. A more recent study found that the MTHFR C677T allele (rs1801133) significantly reduced the methionine-dependence index of lymphocytes, a measure of viable cell number between methionine-complete and methionine-free media [27]. Further evaluation of the methionine-dependent phenotype in lymphocytes, the underlying mechanisms, and the potential impact of a methionine restricted diet on lymphocyte growth *in vivo* is clearly warranted.

In animal models, dietary methionine restriction has been demonstrated to have antitumor activity against a broad spectrum of tumors *in vivo* and extend survival [1, 12, 15]. More recently, these findings have been extended to patient-derived xenograft models of cancer, which more faithfully capture the genetic heterogeneity of human cancer than established cancer cell line, including two chemotherapy-resistant KRas-driven colorectal cancer PDX models [28]. Methionine deprivation or restriction also inhibits metastasis in diverse murine models including sarcoma, triple-negative breast cancer and melanoma [20, 29, 30]. These antitumor effects of methionine restriction have been attributed to cell-cycle blockade and/or apoptosis induction in tumor cells [16, 17, 31, 32]. Dietary methionine restriction reduces methionine metabolite levels in serum, liver, and tumor cells [28, 33], however, some tissue-specific alterations in methionine cycle metabolites were found. One study using dietary methionine restriction in mice did not find a reduction in liver methionine levels [34]. Other studies found an initial decrease in liver methionine and methionine metabolites, which in one was maintained for the duration of the study [28] while in another recovered within 4 days [35]. A study evaluating sex difference in response to methionine restriction found liver methionine declined in female mice but not male mice, while liver SAM levels only decreased in male mice [36]. These studies suggest the impact of dietary methionine restriction on liver metabolites may depend on several factors, including time since diet initiation and gender. Considering the liver is normally the largest consumer of exogenous methionine, it is possible there are liver-specific compensatory mechanisms to maintain methionine levels

[5, 37]. It is also important to note that in most mammals, including rodents, SAM availability is regulated by threonine metabolism via threonine dehydrogenase (TDH), however in humans TDH is a non-functional pseudogene, suggesting human cells may be more reliant on methionine metabolism than other mammalian cells [8].

Given their dependence on exogenous methionine, many tumors have acquired mechanisms to take up methionine more efficiently than surrounding normal tissue in the tumor microenvironment [38]. Methionine and other essential amino acids are transported into cells by L-Type Amino Acid Transporters, LAT1, LAT2, LAT3, and LAT4 [39]. LAT1 is commonly overexpressed in various types of cancer [39, 40], and LAT1 expression is often associated with poor survival [40]. The expression levels of LAT1 also correlate with intracellular availability of SAM and the level of H3K9me3 within cells [39], suggesting LAT1 expression affects methionine availability or aspects of methionine metabolism. Moreover, LAT1 knockdown reduces the viability of cancer cell lines [40]. Intriguingly, overexpression of LAT4/SLC43A2 in tumor cells enables them to preferentially take up methionine in the tumor microenvironment and deplete methionine in T cells, leading to loss of key epigenetic drivers of cytokine signaling and impaired T cell immunity, while inhibition of LAT4 increased basal and checkpoint-induced tumor immunity [41]. Based on the increased uptake of methionine into rapidly growing tumor tissue, the radiotracer L-methyl-<sup>14</sup>C-methionine (<sup>14</sup>C-methionine) has also been used to measure treatment responsiveness [42] and to differentiate tumor margins from necrotic areas from previous radiation treatment to more accurately measure residual disease [43].

### **Mechanisms of methionine dependence in cancer: oxidative stress derails methionine recycling**

Rapidly proliferating cancer cells have an increased demand for methionine for protein synthesis, for maintenance and *de novo* methylation of DNA and chromatin, which requires SAM, and for the methionine cycle to generate intermediates and cofactors used in glutathione and nucleotide biosynthesis [2]. Methionine deprivation in transformed cells has been

found to lower available methionine and SAM [9, 44-48]. Methionine still appears to incorporate into newly synthesized proteins at a rate comparable to healthy cells, suggesting endogenous methionine is preferentially utilized or mobilized for protein synthesis in methionine limitation [1, 46, 49, 50]. Many enzymes within the methionine cycle, including MTR and AHCY, as well as enzymes involved in transmethylation, polyamine synthesis, and transsulfuration have been shown to be highly active in many types of tumor cells, suggesting many aspects of methionine metabolism may influence the growth capacity of cancer cells [49, 51]. In culture, methionine deprivation has also been found to induce apoptosis and increase p53-positive cells; however, this induction does not appear to fully depend on p53 status, as p53 knockout only led to a partial rescue of cell death in response to methionine deprivation [8, 17, 52]. Interestingly, wild-type p53 interacts with DNA (cytosine-5)-methyltransferase (DNMT1) to increase the *de novo* DNA methylation and gene silencing capacity of DNMT1, suggesting functional p53 may use DNMT1 and epigenetic plasticity to evade apoptosis in low methionine stress conditions [53].

A common feature of methionine-dependent cancer cells is their increased demand for exogenous methionine for transmethylation reactions [1, 19, 48, 51, 54]. When 18 tumor cell lines and 4 fibroblast cell lines were compared, all tumor cell lines showed elevated rates of transmethylation relative to fibroblasts in both normal and methionine-depleted media [55]. Methionine-dependent mammary adenocarcinoma and lymphoma cell lines were found to accumulate SAH five times faster than methionine-independent cell lines in response to AHCY inhibition, suggesting higher rates of transmethylation [49]. The same study isolated a methionine-independent-revertant from the mammary adenocarcinoma cell line and found the major difference between dependent and independent status was decreased transmethylation rates in the independent revertant [49]. Notably, the methionine-independent revertant also lost several hallmarks of malignancy, including colony-formation and increased anchorage-independence [46, 56, 57]. Moreover, methionine deprivation in cancer stem cells inhibits diverse histone methylation marks and these effects are largely abrogated by SAM or

methionine supplementation but not homocysteine [10, 44].

Many studies have also found a lower SAM:SAH ratio in methionine-dependent versus independent cells in response to methionine deprivation, with a methionine-independent-revertant exhibiting a normal SAM:SAH ratio [46, 49, 50]. Furthermore, SAM supplementation has also been found to rescue the effects of methionine deprivation in a variety of cell types [45, 58], including cancer stem cells [10, 44]. Interestingly, transmethylation and SAM may play a role in the development of cancer, as global DNA hypomethylation is commonly found in many types of cancer and pre-cancerous cells, and has been hypothesized to result from unbalanced global methylation, leading to the gradual loss of DNA methylation, particularly in genomic areas copied late in replication [46, 59, 60]. SAM is also used in the synthesis of polyamines, and various cancer cells have been found to have hyperactive polyamine synthesis to support rapid rates of growth and division; however, spermidine supplementation during methionine deprivation did not rescue cell growth defects [56].

While the transsulfuration pathway for glutathione synthesis is largely limited to the liver, pancreas, and kidneys in healthy adults, cancer cells appear to have a heightened dependency on transsulfuration of homocysteine to buffer oxidative stress that accumulates as a result of rapid cell growth paired with metabolic dysfunction [2, 61]. In humans, the conversion of homocysteine to cystathionine is reduced 20% under dietary methyl group restriction [5]. The methionine-dependent phenotype can be partially reversed by over-expression of the cystine transporter xCT, and loss of the xCT transporter leads to a significant decrease in intracellular glutathione [61, 62]. Indeed, oncogenic *PI3KCA* confers methionine dependence by reducing xCT expression and cystine import, thereby diverting homocysteine to the transsulfuration pathway to buffer oxidative stress rather than recycling methionine [61]. Moreover, our group and others have demonstrated that methionine deprivation transiently depletes glutathione levels and induces oxidative stress [28, 63-65], which is critical for the cytotoxic effects of methionine deprivation because antioxidants such as N-acetylcysteine (NAC) attenu-

ate its cytotoxicity [28, 65]. Collectively, these findings strongly suggest that at least part of the methionine dependence of cancer cells results from the need to divert homocysteine from the methionine cycle into the transsulfuration pathway to promote glutathione synthesis and buffer oxidative stress, thereby creating a dependence on exogenous methionine.

Methionine deprivation also activates nuclear factor erythroid 2-related factor 2 (NRF2) and enhances complex formation at antioxidant response elements (ARE) [66]. Interestingly, MAT II has been found to associate with MAFK-NRF2 and affect repression of target genes [67]. As noted above, the transient oxidative stress induced by methionine deprivation may partially result from glutathione depletion. One study found glutathione showed a time-dependent reduction during methionine deprivation, followed by an increase in nuclear NRF2 [68]. However, data on serum and intracellular glutathione levels in response to methionine deprivation have been variable [69-72], possibly due to ATF4-NRF2 activating multiple genes involved in glutathione biosynthesis [73] and likely reflecting temporal and/or tissue-specific differences. Indeed, a study of long-term methionine restriction in F344 rats found short-term methionine restriction depletes glutathione [74], while adaptive mechanisms, including diminished mitochondrial ROS production [75], lead to increased blood glutathione when methionine restriction is prolonged [74]. One consequence of diverting homocysteine away from the methionine cycle is reduced available intermediates for SAM synthesis needed to maintain methylation reactions and polyamine synthesis for cell growth and division [56]. Oxidative stress increases the activity of CBS while MAT I and MAT III activity is reduced, creating a further deficit in serum methionine and SAM availability [7, 76]. Increased uptake in exogenous methionine commonly seen in cancer may contribute to cancer cell dependence on transsulfuration through high intracellular sulfur load and the generation of cytotoxic sulfur intermediates that can interfere with cellular detoxification proteins and mitochondrial electron transport chain function [7, 38]. Interestingly when methionine-dependent and methionine-independent-revertant mammary carcinoma cell lines were compared, methionine-dependent cells showed an increase in total

SAH as well as increased conversion of homocysteine back to SAH compared to the revertant, further implicating oxidative stress with methionine dependence, as homocysteine itself directly contributes to oxidative damage [56]. Homocysteine also inhibits AHCY to further increase SAH levels [77]. SAH binds and inhibits methyltransferase activity [78], and intracellular SAH concentrations have been found to correlate with DNA hypomethylation [79]. In healthy year-old mice, methionine restriction decreased liver SAH levels 50% compared to control-fed mice; methionine restricted mice also maintained global DNA methylation in the liver comparable to young animals, while control-fed mice gradually lost global DNA methylation marks, similar to what is commonly seen in human aging [73]. Healthy cells in aging animals and humans may benefit from the reduced SAH burden seen during methionine restriction, while cancer cells, which have a generally heightened need for methionine, SAM, and redox buffering, may suffer from methyl shortage leading to cell cycle arrest or cell death.

Another factor evaluated for its role in methionine dependence is deletion of the methionine salvage enzyme gene, methylthioadenosine phosphorylase (*MTAP*). The *MTAP* gene is commonly deleted in a variety of cancer types, often due to co-deletion with the adjacent tumor suppressor gene *CDKN2A* which encodes p16 (INK4A) [1, 54]. *MTAP* uses MTA, a byproduct from the utilization of SAM in polyamine synthesis, to regenerate methionine. While *MTAP* is frequently mutated or deleted in cancer, cancer cell lines with intact methionine cycle enzymes can still be methionine dependent, suggesting *MTAP* deletion may facilitate, but is not crucial for, methionine-dependent status [80]. Furthermore, *MTAP* deletion or re-introduction in various human cancer cell lines did not affect methionine-dependent growth defects [81]. However, *MTAP* deletion does uncover a druggable dependence on the protein arginine methyltransferase PRMT5, which is directly inhibited by the resultant high levels of MTA [82, 83]. Moreover, *MTAP* deletion sensitizes tumor cells to depletion of MAT2A, which is needed to produce SAM for PRMT5-mediated methylation [82, 83]. PRMT5 has a much higher affinity for MTA than SAM, making its catalytic activity sensitive to the SAM/MTA

ratio [83], suggesting methionine restriction may further promote PRMT5 dependence in *MTAP*-deficient cells.

Another metabolic vulnerability of transformed cells exposed by methionine restriction is their intrinsic demand for nucleotide synthesis to fuel tumor growth. The methionine cycle is inextricably linked to the folate cycle via one-carbon metabolism (**Figure 2**) and perturbations of methionine availability impact folate cycle activity and outputs such as redox buffering and thymidine and purine synthesis for nucleotides [2]. Indeed, the folate cycle was the target of the first class of cytotoxic agents (e.g., 5-fluorouracil and methotrexate) that were developed [84]. Although the critical role of the pentose phosphate pathway (PPP) in producing NADPH for reductive synthesis and redox homeostasis is well established, the folate cycle was recently shown to be a key source of cellular NADPH synthesis from NADP via the conversion of methylene tetrahydrofolate to 10-formyl-tetrahydrofolate [85]. Intriguingly, the PPP also plays a key role in folate cycle activity: inhibition of PPP increases NADP levels which inhibit the folate cycle enzyme dihydrofolate reductase (DHFR) [86], thereby directly linking the PPP to one-carbon metabolism. In addition to redox buffering, the folate cycle plays a key role in nucleotide synthesis by generative thymidine and purine precursors. Notably, methionine restriction rapidly depletes multiple nucleotide precursors in the serum of humans and in murine tumors *in vivo*, while supplementation of nucleosides partly attenuates the cytotoxicity of methionine restriction in cultured cancer cells [28]. Metastatic melanoma cells are under greater oxidative stress than primary tumor cells and are readily suppressed by folate inhibition [87], suggesting that one-carbon metabolism may play a particularly prominent role in cancer progression/metastasis. Overall, these findings point to the interconnected nature of the folate and methionine cycles and the role of nucleotide depletion in the effects of methionine restriction.

### **Methionine-sensing and the cellular stress response: living with less (Methionine)**

The integrated stress response (ISR) is a highly conserved adaptive response to disparate cellular stressors mediated by protein kinase R

(PKR) in response to double stranded RNA, protein kinase R-like endoplasmic reticulum kinase (PERK) in response to endoplasmic reticulum (ER) stress, heme-regulated inhibitor (HRI) in response to heme depletion, and general control nonderepressible 2 (GCN2) in response to amino acid deprivation and ultraviolet (UV) damage [69, 88]. These kinases phosphorylate eukaryotic initiation factor 2 $\alpha$  (eIF2 $\alpha$ ), which blocks translation re-initiation by reducing GDP exchange for GTP on eIF2, inhibiting its function. Although global protein translation is suppressed, activating transcription factor (ATF) 4 mRNA is preferentially translated during times of cell stress when complete assembly of the translational machinery on ATF4 mRNA is slowed so that the 40S ribosomal subunit bypasses the upstream open reading frame, uORF2, that is translated in the absence of stress [89]. When the 60S ribosomal subunit attaches downstream of uORF2, the coding sequence of ATF4 is translated, allowing protein levels to rapidly increase in response to eIF2 $\alpha$  inactivation, including amino acid limitation [88, 90]. ATF4 is a member of the ATF/cyclic AMP response element binding protein (ATF/CREB) family that dimerizes and binds to DNA at CRE motifs. A component of the methionine deprivation-specific transcriptional response induced by ATF4 may depend on its dimerization partner [91-93].

Our lab observed that ATF4 and Sestrin2 (SESN2), an ATF4 target, are rapidly induced in triple-negative breast cancer cells in response to methionine deprivation [94]. Methionine deprivation also induced phosphorylation of eIF2 $\alpha$ ; however, this phosphorylation was not dependent on GCN2 or PERK, as knockdown of either or both did not eliminate phosphorylation of eIF2 $\alpha$  or induction of ATF4 or SESN2 in response to methionine deprivation [94]. The potential roles of PKR and HRI, other known kinases capable of phosphorylating eIF2 $\alpha$ , have not been investigated during methionine deprivation. However, ATF4 and the ISR can be activated without eIF2 $\alpha$  phosphorylation [95] and at least part of the methionine deprivation-specific response may be eIF2 $\alpha$  phosphorylation-independent [96]. Methionine deprivation in eIF2 $\alpha$ -knockout mouse embryonic fibroblasts (MEFs) resulted in an increase in ATF4 mRNA (albeit blunted response), and the authors postulated ATF4 is induced via lack of initiator tRNA<sup>met</sup> instead of phosphorylation of

eIF2 $\alpha$  [96]. Another group reported that hepatic ATF4-target gene induction in response to methionine restriction was not dependent on GCN2 or phosphorylation of eIF2 $\alpha$  [97]. They also found glutathione levels were decreased and NRF2 targets were strongly induced, suggesting ATF4-NRF2 may be induced regardless of phosphorylation of eIF2 $\alpha$  in response to methionine restriction [97]. GCN2- and eIF2 $\alpha$  phosphorylation-independent induction of ATF4 and ATF4-target genes in response to methionine restriction were also observed in mouse liver, along with glutathione depletion and NRF2 induction [98]. NRF2-ATF4 have been found to interact with each other at stress responsive elements [93], possibly in response to phosphoinositide 3-kinase (PI3K) [99] or protein kinase C (PKC) [100] pathway activation. Clearly, additional studies are needed to clarify the precise mechanisms by which methionine restriction activates the ISR and induces ATF4 transcription. Although GCN2 and phosphorylated eIF2 $\alpha$  play key roles in the canonical amino acid deprivation response [101], the methionine deprivation-specific response appears to be at least partly independent of GCN2 and eIF2 $\alpha$  phosphorylation to activate ATF4.

Another stress-activated pathway is autophagy, which functions to recycle macromolecules and organelles within a cell during stress or when nutrients are limited [102]. Methionine restriction has been found to extend the life- and health-span of various organisms [103, 104], and this effect has been attributed to increased autophagic flux [105, 106]. Interestingly, methionine restriction led to a decrease in Reactive Oxygen Species (ROS) [105], suggesting the increase in autophagy may promote mitochondrial function to prevent ROS leak. In line with these findings, a recent study found methionine restriction elevated mitophagy specifically, which is the autophagic recycling of mitochondria [107]. Furthermore, cells restricted of methionine no longer showed lifespan extension when key mitophagy genes were deleted or when mitochondrial function was blocked by the mitochondrial toxin, paraquat [107]. Mitochondrial peroxide production, indicative of mitochondrial dysfunction, was reduced approximately in half by methionine restriction compared to controls, and this reduction was not seen in cells with impaired mitophagy [107], suggesting increased mitophagy

agy may sustain functional mitochondria. Although methionine deprivation has been demonstrated to activate autophagy in cancer cells [108, 109], the role of autophagy in the antitumor effects of methionine restriction is poorly understood.

### **Methionine restriction and the cancer epigenome: methylation matters**

The bulk of genomic methylation is determined early in development to aid in cell differentiation and repress genes not needed for tissue-specific function. These methylation patterns often remain static throughout life, with changes mainly occurring in response to stress and injury [110, 111]. Epigenetic plasticity is seen in the normal cellular response to injury; however, when prolonged this plasticity can contribute to the hallmarks of cancer [112]. Gene mutations and truncations can also alter gene methylation patterns at specific loci, leading to heritable epigenetic alterations [110]. Global hypomethylation has been implicated in the acquisition of senescent cells, aging, and various age-related diseases [113]. Cancer cells in general exhibit genome-wide loss of epigenetic stability, often exhibiting global hypomethylation [114, 115] and promoter-specific hypermethylation [116], which has been implicating in oncogene activation and tumor suppressor silencing respectively [117, 118]. These epigenetic alterations are also observed in precancerous cells [112, 119], normal tissue adjacent to tumor tissue [120, 121], and hereditary human cancers [122]. Loss of heterochromatin, particularly loss of H3K9me<sub>2</sub>, has also been associated with the epithelial-to-mesenchymal (EMT) transition used by cancer cells for migration and metastasis, further implicating epigenetic dysfunction with malignant progression [123-125]. Multiple studies evaluating the cancer cell epigenome have found a correlation between epigenetic dysfunction and tumor progression, particularly for sustained growth in suboptimal conditions [123, 126, 127].

Methionine deprivation, through reduced available SAM, decreases the histone methylation marks H3K4me<sub>2</sub>, H3K4me<sub>3</sub>, H3K9me<sub>2</sub>, and H3K27me<sub>3</sub> *in vitro* and *in vivo* [8, 34, 45]. Bulk screening for methylation at CpG island promoters or long interspersed nuclear element-1 (LINE-1) repetitive DNA sequences found no significant alterations, indicating cells respond

to methionine deprivation by salvaging methyl groups preferentially from existing histone marks instead of DNA methylation [45]. Widespread DNA hypomethylation in cancer is associated with gene silencing due to the formation of repressive chromatin structures at these sites, suggesting cancer cells with epigenetic dysfunction may rely more heavily on histone methylation than DNA methylation to maintain viability [59, 128]. DNA methylation marks are also generally more stable than histone methylation [129-131], implicating histone methylation as more dynamic and/or replaceable for cell survival than DNA methylation [110, 112, 132]. Interestingly, MAT II directly interacts with histone methyltransferases and this interaction can alter histone methylation dynamics [133]. Further work evaluating the allele-specific methylation response to methionine deprivation over time may better elucidate the role DNA methylation plays in methionine deprivation, as bulk methylome sequencing may overlook changes in individual allele methylation patterns.

“Epigenetic persistence” is defined as the ability of a cell to reestablish its epigenetic signature following environmental alterations or metabolic stress [134]. In response to methionine deprivation both di- and tri-methyl histone marks are preferentially removed while H3K9 mono-methylation marks are preserved to maintain heterochromatin stability during times of low SAM availability [134]. When H3K9 mono-methylation is blocked and cells are subjected to low SAM availability, they are no longer capable of epigenetic persistence when returned to full methionine media [134], suggesting H3K9 mono-methylation is necessary for a cell to maintain its heterochromatin state. The specific role of epigenetic persistence, and H3K9 mono-methylation in mediating the effects of methionine deprivation on transformed cells will require further investigation.

Additionally, SAM is utilized for RNA modifications to generate functional mRNA, control mRNA degradation, and aid in tRNA base-pairing under stress conditions [135-137]. The most abundant modification of eukaryotic mRNA is methylation of adenosine by guanine 7-methyltransferase (RNMT), which uses SAM to create the 5'-methyl cap on pre-mRNA, where eukaryotic initiation factor 4 $\alpha$  (eIF4 $\alpha$ ) binds and recruits the 40S ribosomal subunit

to begin translation and control m<sup>6</sup>A-dependent mRNA decay [136, 138, 139]. In response to genotoxic stress, the uridine of tRNAs that encode lysine, glutamine, and glutamic acid is modified to enhance the wobble of the third position for less exact base pairing in translation, allowing base-pair mutated transcripts to be translated [2, 137]. Both uridine modifications, methoxycarbonylmethyluridine and thiolation of uridine, consume SAM, and the abundance of tRNA uridine thiolation correlates with sulfur amino acid availability [106, 137]. Lysine, glutamine, and glutamic acid codons are prevalent in ribosome biogenesis genes, suggesting altered tRNA thiolation may impact general translation under reduced SAM conditions [106]. Ribosome thiolation also alters complex I ROS production, suggesting reducing thiolation may alter cellular redox status [140]. This may be another mechanism by which methionine deprivation preferentially affects cancer cells that require mRNA methylation to generate mature mRNA transcripts and may depend more heavily on tRNA uridine modifications to translate damaged mRNA transcripts, both of which may contribute to translation inhibition in cancer cells in response to methionine limitation.

### **Cancer stem cells: a dependency on SAM biosynthesis**

Pluripotent embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) both appear more methionine-dependent than differentiated cells, and self-renewal capacity correlates with SAM concentration [8]. Methionine deprivation in ESCs and iPSCs primes cells for differentiation, and once differentiated, total cellular methionine demand decreases [8]. This suggests that part of the methionine-dependence of transformed cells may be due to the stem-like characteristics of some cancer cells. It also suggests that normal stem cell maintenance may be adversely affected by prolonged methionine deprivation. However, a study by Altundag *et al.* [141] comparing bone marrow mesenchymal stem cells, umbilical cord stem cells, and cancer stem cells found differing responses of each cell type to varying levels of methionine. Notably in cancer stem cells OCT3/4 and NANOG were nearly undetectable at 0 and 10  $\mu$ M methionine, while levels of both were maintained in other stem cells, suggesting cancer stem cells may have a selective vulnerability to low methionine conditions.

Cancer stem cells or tumor-initiating cells commonly exhibit resistance to a variety of cytotoxic agents and undergo latent growth periods, making them treatment-resistant and critical to eliminate to reduce the risk of recurrence [142]. Redox state contributes to cancer stem cell therapeutic resistance, with quiescent cancer stem cell populations exhibiting low ROS levels due to enhanced antioxidant pathways and reliance on glycolysis [143-145]. Tumorspheres, cancer cells grown on ultra-low-attachment plates, are a common method to propagate tumor-initiating and cancer stem cells [142, 146]. Work from our lab using triple-negative breast cancer (TNBC) tumorspheres showed methionine deprivation robustly decreased the number of tumorspheres as well as the population of cancer cells with CD44<sup>hi</sup>/CD24<sup>low</sup> cancer stem cell immunophenotype [10]. These effects were partly rescued by SAM supplementation, underscoring that SAM, rather than methionine *per se*, is the critical metabolite for cancer stem cell survival. Methionine deprivation also increased mRNA and protein levels of MAT2A as previously reported [8, 9] and led to H3K4me3 demethylation and suppression of Sox9 expression [10], a regulator of plasticity in cancer stem cells [147]. Moreover, inhibition of MAT2A by siRNAs and/or the amino acid analogue cycloleucine [148] augmented the effects of methionine deprivation on tumorspheres and these molecular markers, respectively [10]. These results underscore that methionine metabolism, specifically SAM biosynthesis, plays a critical role in cancer stem cell survival, although the functional role of these epigenetic alterations in the observed effects of methionine deprivation on cancer stem cells has not been delineated.

Lung cancer tumorspheres are also enriched for SAM and SAH as well as methionine cycle activity and intermediates relative to isogenic, adherent cells, suggesting cancer stem cells may have a heightened requirement for methionine and SAM when compared to their more differentiated counterparts [44]. Following 48 hours of methionine deprivation, lung cancer tumorspheres showed reduced methionine cycle activity and a 30-fold reduction in SAM accompanied by decreased histone methylation, suggesting exogenous methionine deprivation greatly affects cancer stem cell methionine metabolism [44]. Furthermore, when tumorspheres were injected into NOD Scid

Gamma (NSG) mice following methionine deprivation they greatly lost their tumor-forming abilities, decreasing the tumor burden 94% compared to mice injected with tumorspheres grown in control media, and this effect was specific to methionine deprivation [44]. Inhibition of MAT2A with the small molecule FIDAS-5 [149] phenocopied many of the cellular and *in vivo* effects of methionine deprivation and these effects were attenuated by SAM supplementation [44]. Collectively, these studies underscore that cancer stem cells rely on methionine and SAM biosynthesis for their self-renewal and tumor-forming capacity, pointing to this metabolic vulnerability as a druggable target to eliminate these chemotherapy-resistant cells.

### **Methionine restriction: priming cancer cells to die**

Despite preclinical activity against diverse malignancies, methionine restriction has been largely disappointing in clinical trials to date (see section below). We postulated we could enhance the activity of methionine restriction by targeting molecular vulnerabilities uncovered by low methionine stress, a strategy we have termed “metabolic priming”. To this end, we performed a proteomics analysis of triple-negative breast cancer cells cultured in methionine-free media [150]. One of the proteins induced by methionine deprivation is the proapoptotic TRAIL receptor-2 (TRAIL-R2/DR5). Methionine deprivation, either through methionine-free media or the addition of methioninase, sensitized cells to the agonistic TRAIL-R2 monoclonal antibody lexatumumab by increasing cell surface expression of TRAIL-R2 [150]. At least part of this increase in TRAIL receptor expression in response to methionine deprivation is attributed to a loss of MAGE family member D2 (MAGED2) protein, which normally inhibits TRAIL receptor expression [150]. Interestingly, non-transformed breast epithelial cells were largely resistant to the combination of methionine deprivation and lexatumumab likely due to the lack of cell surface induction of TRAIL-R2. Mice fed a methionine-free diet and treated with lexatumumab for 3 weeks had smaller primary tumors and reduced lung metastases compared to mice treated with a methionine-deficient diet or lexatumumab alone in a triple-negative mCherry-MDA-MB-468 tumor model [150]. Similar results were

recently reported for oral methioninase and the agonistic TRAIL-R2 mAb tigatuzumab in a mouse model of pancreatic cancer [151].

A second intriguing protein identified in our proteomic screen is MAT2A [150], the widely expressed enzyme that converts methionine into SAM. Methionine deprivation increases MAT2A mRNA stability [139] as well as protein and mRNA levels in response to declining SAM availability [10, 152]. The MAT inhibitor cycloleucine [148] augmented the effects of methionine deprivation on tumorsphere formation and demethylation of H3K4me3 [10]. Moreover, the combination methionine deprivation with cycloleucine was more effective than either treatment alone at suppressing lung metastases in immunodeficient mice with orthotopic triple-negative breast tumors [10]. These effects are unlikely to be off-target effects of cycloleucine because siRNAs specifically targeting MAT2A phenocopy the effects of cycloleucine on tumorspheres [10]. Both lexatumumab and cycloleucine had previously been abandoned as anti-cancer treatments due to lack of efficacy [153-157] and unacceptable toxicity [158, 159] respectively in clinical trials. When paired with methionine deprivation, both drugs showed enhanced cytotoxicity at much lower doses, suggesting methionine deprivation may be a useful strategy to enhance efficacy and lower toxicity of various anti-cancer drugs [10, 150]. Additionally, newer MAT2A inhibitors such as FIDAS-5 [149] and the allosteric inhibitor PF-9366 [37] may have improved therapeutic indices.

Another potentially attractive biological target that emerged from the initial proteomics screen of triple-negative breast cancer cells treated with methionine deprivation is thioredoxin reductase 1 (TXNRD1) [150]. TXNRD1 is the cytosolic isoform of the enzyme that catalyzes the rate-limiting step in the thioredoxin antioxidant pathway [160]. Tumor cells rely on the glutathione and thioredoxin pathways to buffer and survive the high levels of ROS they encounter in the tumor microenvironment [161]. Although tumor cells can survive defects in either single pathway, dual inhibition of both antioxidant pathways is synthetic lethal due to overwhelming oxidative stress [162-164]. Consistent with methionine being metabolized to glutathione via the transsulfuration of homocysteine, methionine restriction tran-

## Cancer's methionine addiction

**Table 1.** Methionine restriction or deprivation in combination with various chemotherapies in preclinical models

Methionine restriction to metabolically prime cancer cells		
Chemotherapy	Drug Target	Reference(s)
Lexatumumab	Monoclonal agonist antibody for the death receptor TRAIL receptor 2	Strekalova <i>et al.</i> 2015 [150]
Cycloleucine	Methionine adenosyltransferase (MAT) inhibitor	Strekalova <i>et al.</i> 2019 [10]
Auranofin	Thioredoxin reductase (TXNRD) inhibitor	Malin <i>et al.</i> 2021 [65]
Methionine restriction and standard of care chemotherapy		
Chemotherapy	Drug Target	Reference(s)
5-Fluorouracil	Thymidylate synthase (TS) inhibitor	Gao <i>et al.</i> 2019 [28] Yoshioka <i>et al.</i> 1998 [52]
Cisplatin	Binds DNA and interferes with repair	Poirson-Bichat <i>et al.</i> 2000 [63] Tan <i>et al.</i> 1999 [210]
Doxorubicin	Binds and inhibits DNA Topoisomerase II	Poirson-Bichat <i>et al.</i> 2000 [63] Stern <i>et al.</i> 1986 [16] Nagahama <i>et al.</i> 1998 [170]
Vincristine	Interacts with tubulin to prevent mitotic spindle formation	Stern <i>et al.</i> 1986 [16] Nagahama <i>et al.</i> 1998 [170]
Decitabine	DNA demethylating agent	Higuchi [168]
Azacitidine	DNA demethylating agent	Higuchi [169]
Gemcitabine	Pyrimidine antagonist	Kawaguchi [171]

siently depleted reduced glutathione, triggering oxidative stress and inducing TXNRD1 mRNA/protein via a NRF2/ATF4-dependent mechanism [65]. Strikingly, methionine restriction primed triple-negative breast cancer cells, but not untransformed breast epithelial cells, to respond to TXNRD1 silencing or the pan-TXNRD inhibitor Auranofin [65], a gold-containing anti-rheumatic drug [165]. Notably, methionine restriction or TXNRD inhibition alone had modest or no effects on cell viability. Furthermore, the synergy between methionine restriction and TXNRD inhibition was dependent on oxidative stress and was abrogated by N-acetylcysteine treatment [65]. Dietary methionine restriction also increased TXNRD1 expression in mammary tumors and enhanced the antitumor activity of Auranofin in metastatic and Patient-derived Xenograft (PDX) models of breast cancer in mice [65]. Overall, these studies provide proof-of-concept preclinical evidence that methionine restriction can be used to metabolically prime tumor cells to respond to rationally selected agents that target vulnerabilities exposed by methionine stress.

### Methionine restriction in combination with cytotoxic agents

Many studies have examined combining methionine deprivation or restriction with various

cytotoxic agents to enhance their therapeutic activity in preclinical models (**Table 1**). Methionine deprivation induces a reversible growth arrest in late S/G<sub>2</sub> phase of the cell cycle [46, 166] and/or G<sub>1</sub> arrest [19, 167], sensitizing cancer cells to cell cycle phase-specific chemotherapies such as DNA-damaging and microtubule-targeted agents [1, 16]. Furthermore, DNA and chromatin methylation have been implicated in resistance to DNA-damaging agents by limiting access to DNA [38]. Methionine deprivation or restriction reduces various histone methylation marks [10, 34, 44, 134], potentially facilitating DNA access to enhance the activity of DNA-damaging drugs such as doxorubicin and cisplatin. Indeed, the methylation inhibitor decitabine enhanced the antitumor activity of enzymatic methionine depletion in a soft tissue sarcoma PDX model [168], while another methylation inhibitor (azacitidine) augmented the activity of enzymatic methionine depletion in a PDX model of osteosarcoma [169]. Moreover, multi-drug-resistant colon cancer xenografts were sensitive to the combination of cisplatin, methionine deprivation, and the methionine analog ethionine to potentiate methionine deprivation [63]. Similarly, the combination of doxorubicin, methionine deprivation and ethionine resulted in 51% growth inhibition and 1.7-fold longer survival in a xenograft model of small cell lung cancer resistant to doxorubicin

alone [63]. Similarly, methionine deprivation in combination with doxorubicin and vincristine prolonged survival of Yoshida sarcoma-bearing rats despite only partially depleting circulating methionine (18.4  $\mu$ M in methionine-depleted TPN-treated rats versus 87.7  $\mu$ M in control rats) [170]. Notably, the combination of methionine deprivation with doxorubicin and vincristine has tumor-selective cytotoxic effects on a broad spectrum of cancer cell types, including methionine-independent revertant cancer cells, with minimal effects on cocultured fibroblasts [16]. These preclinical findings support the combination of methionine deprivation with DNA-damaging and microtubule-targeted agents.

Some of the earliest chemotherapy drugs that were developed target folate metabolism (e.g., 5-fluorouracil (FU) and methotrexate) [84], suggesting that they may act synergistically with methionine restriction to disrupt one-carbon metabolism. Indeed, a recent study demonstrated that doses of 5-FU that were inactive against a colorectal cancer patient-derived xenograft model in mice synergized with dietary methionine restriction to cause oxidative stress, disrupt nucleotide metabolism and inhibit tumor growth [28]. Notably, the antiproliferative effects of methionine restriction and 5-FU were partially rescued by N-acetylcysteine and/or nucleosides [28], pointing to the critical role of ROS and disrupted nucleotide metabolism, respectively, in the observed effects. Similarly, the combination of the nucleoside analog gemcitabine with methionine depletion inhibited tumor growth and induced regression in a mouse model of gemcitabine-resistant pancreatic cancer [171]. The synergistic effects of methionine restriction with antifolate agents may at least partly reflect the reduced protein level and activity of thymidylate synthase (TS) observed during methionine deprivation [172]. SAM supplementation has been found to increase resistance to 5-fluorouracil through up-regulation of DNMTs [173], suggesting methionine deprivation may synergize with 5-fluorouracil administration by maintaining low intracellular SAM levels. Other chemotherapy drugs that target the folate cycle, such as methotrexate and pemetrexed, inhibit dihydrofolate reductase, leading to accumulation and diversion of 5,10-methylene tetrahydrofolate into thymidylate synthesis, reducing available intermediates for one-carbon biosynthesis and to

re-methylate homocysteine into methionine [174]. Methotrexate has also been reported to inhibit MAT2A expression and activity [175]. Additionally, methionine restriction enhances the antitumor activity of radiation in a sarcoma mouse model and alters redox and nucleotide metabolism [28]. Overall, these and other studies point to the ability of methionine restriction to augment the activity of chemotherapy drugs that target folate metabolism.

Methionine contributes to the production of glutathione via its transsulfuration to cysteine [176] and elevated glutathione has also been implicated in the development of drug resistance [17]. Since methionine restriction lowers intracellular glutathione levels in tumor cells [28], methionine restriction may further prove to be a useful strategy to enhance the sensitivity of cancer cells to various chemotherapeutic agents that augment oxidative stress [17, 63, 65]. Many commonly used classes of chemotherapy, such as anthracyclines, alkylating agents and platinum compounds as well as radiation generate oxidative stress via production of ROS and superoxide radicals [177, 178]. However, moderate levels of oxidative stress induced by these drugs can decrease drug effectiveness by activating cytoprotective antioxidant systems [177]. Hence, the combined use of these chemotherapy drugs with methionine restriction may enhance ROS by diminishing glutathione levels, resulting in lethal oxidative stress. Consistent with this idea, studies have demonstrated that the enhanced activity of chemotherapy drugs in combination with methionine deprivation or restriction is dependent on oxidative stress and that this synergy is abrogated by various antioxidants [28, 63]. Additionally, we recently demonstrated that methionine restriction depletes glutathione, activates TXNRD1 by an NRF2/ATF4-dependent mechanism, and renders tumors dependent on TXNRD1 [65], strongly implicating this protein as a promising therapeutic target in combination with methionine restriction. Consistent with this idea, TXNRD1 expression in diverse malignancies correlates with tumor grade and/or poor prognosis [179-181].

### **Methionine restriction in humans and clinical trials**

Human fasting plasma methionine concentration ranges from 3-30 micromolar and corre-

lates with SAM levels [34]. This variation in plasma methionine is partially explained by diet (~30%), as well as clinical variables such as age, gender and body composition (~30%), and genetic variables [34]. A study with six healthy, middle-aged individuals on a low methionine diet (approximately 2.92 mg/kg/day) resulted in lower serum methionine and methionine cycle metabolites, as well as diminished reduced glutathione and N-acetyl cysteine (NAC) [28]. Dietary methionine restriction (2 mg per kg per day) in 12 patients with diverse metastatic solid tumors led to a 58% reduction in plasma methionine by 2 weeks, with only mild losses in body weight that were regained upon return to a full methionine diet [182]. However, another study of twenty-six obese adults with metabolic syndrome randomized to a diet restricted to 2 mg methionine/kg body weight per day and were provided capsules containing placebo or methionine (33 mg/kg body weight per day) resulted in only a 13.8% reduction in plasma methionine concentrations and both groups had similar weight loss and improved metabolic indices (decreased plasma insulin and triglycerides), while the methionine restricted group had significantly higher fat oxidation and reduced hepatic lipid content [183]. In each of these studies, dietary methionine restriction was accomplished by providing 75% of total protein through a methionine-free medicinal beverage (Hominex-2, Abbott Nutrition) with the remaining 25% of protein through low-methionine foods such as fruits, vegetables, and grains [28, 182, 183]. These studies support the tolerability and feasibility of methionine restricted diets even in patients with advance malignancies, but they also suggest that variability in dietary adherence due to poor diet palatability or other variables [184] may affect the impact on systemic methionine levels, an important consideration when assessing its potential antitumor effects in clinical trials.

To overcome the palatability issue of existing methionine restriction diets, Fang *et al.* [185] explored the option of subjecting protein to oxidizing conditions to reduce methionine and cysteine while still leaving the protein intact. To do this, casein was oxidized by hydrogen peroxide to remove essentially all methionine and cysteine without impacting other amino acids (tryptophan and lysine were impacted but were added back) [185]. Mice were fed either an

elemental methionine restriction diet, an oxidized casein methionine restriction diet or one of the respective control diets for 8 weeks, and several behavioral, physiological, endocrine and transcriptional endpoints were evaluated [185]. Both methionine restriction diets performed comparably in almost every measure studied including body weight, fat mass, energy expenditure, lipogenic gene expression, and gene expression signatures in line with ISR activation [185]. While these results are promising, future work is necessary to evaluate the feasibility of subjecting other proteins to oxidizing conditions as well as the palatability of these oxidized proteins in humans.

Based on preclinical studies demonstrating that a methionine-free diet [63] or methionine-free total parenteral nutrition (TPN) [186] sensitized drug-refractory solid tumors to alkylating chemotherapies, clinical studies investigating methionine deprivation in combination with the alkylating agent, cysteamine were initiated. In phase I and phase II clinical trials of glioma and melanoma patients on 24-hour methionine deprivation in combination with cysteamine (every two weeks), adherence to the diet was acceptable, there was no significant loss of body weight, and blood and inflammation markers remained stable, while plasma methionine fell by 41% or 53% on the day of the dietary intervention in these studies [187, 188]. In the phase I study, 8 of 10 patients showed disease stabilization after the second cycle of treatment, 2 of 7 showed stabilization after the fourth cycle of treatment, and the median overall survival was 6.5 months [187]. In the phase II trial, of the 22 patients treated, 3 showed disease stabilization and the median survival time was 4.6 months [188]. There were no complete or partial responses.

Methionine deprivation in preclinical models inhibits O(6)-methylguanine-DNA methyltransferase (MGMT), an enzyme linked to resistance to alkylating agents [189, 190]. Consistent with these observations, methionine deprivation in combination with cysteamine in 6 glioma and melanoma patients resulted in a 13% decrease in MGMT activity in peripheral blood mononuclear cells [191]. The decreases in MGMT activity as well as plasma methionine were not significantly different when methionine deprivation was extended beyond 24-hours, suggesting brief methionine deprivation may be suffi-

cient to re-sensitize cancer cells to chemotherapy [191]. The purpose of this study was only to evaluate MGMT activity and plasma methionine levels, so response was not evaluated [191].

Due to the success of methionine deprivation in combination with 5-fluorouracil and platinum agents [28, 38, 63, 150, 172, 192, 193] the FOLFOX combination of 5-fluorouracil, leucovorin, and oxaloplatin, was tested in combination with three consecutive days of dietary methionine restriction in 11 metastatic colorectal cancer patients, and found to lower plasma methionine by 58% while maintaining good compliance and tolerance to the diet [194]. Of the 11 patients, 4 were evaluated for a response, 3 of whom showed a partial response and the other one showed stabilization [194].

Based on preclinical data [195] and small clinical studies showing synergy between a methionine-depleting TPN and 5-FU [196, 197], a methionine- and cystine-free TPN infusion, was given in combination with 5-FU to 7 advanced gastric cancer patients and the results compared to 7 control gastric cancer patients given standard TPN and 5-FU [196]. The activity of thymidylate synthase, the primary molecular target of 5-fluorouracil, was reduced in the methionine-free TPN group (1.12 pmol/g) compared to control (2.35 pmol/g) [196]. Methionine-free TPN also enhanced necrotic area within tumors, shown by higher histological grades, without any major complications [196]. Since then, other groups have evaluated the combination of a methionine-depleting TPN and 5-FU on cancer [198, 199], including a clinical study that found this combination suppressed proliferation of tumor cells in gastric cancer patients [200].

Overall, these small clinical studies have shown dietary methionine restriction lowers circulating methionine and is well tolerated even in combination with various chemotherapy drugs in patients with advanced cancer. To date, none of these studies have demonstrated objective response. Considering many are pilot studies of small patient size, larger trials are necessary to more accurately evaluate anti-cancer outcomes in response to methionine restriction. However, given the lack of clear efficacy in any of these studies, we have proposed to study methionine restriction in combination with agents that target pathways activated by low

methionine stress ("metabolic priming"), while others have combined methionine restriction with cytotoxic agents that act synergistically with methionine deprivation.

There are currently multiple clinical trials ongoing and/or recently ended that may help to better elucidate the therapeutic potential of methionine restriction in cancer (**Table 2**) [201-208]. One trial (NCT03733119) was designed to examine whether methionine restriction metabolically primes metastatic triple-negative breast cancer to respond to a novel TRAIL agonist (ONC201) based on preclinical findings from our lab and others that methionine deprivation induces expression of TRAIL-R2 and sensitizes tumors to TRAIL-R2 agonists [150, 151]. Another trial (NCT03186937) was designed to evaluate the impact of methionine restriction on TRAIL receptor expression and its impact on cancer stem cells based on preclinical findings that methionine deprivation targets many properties of cancer stem cells [10, 150]. Methionine restriction was also tested in combination with radiation therapy in lung, prostate and breast cancer patients (NCT03574194) to determine if dietary methionine restriction can be used to radio-sensitize tumors. Another study (NCT00508456) in patients with glioblastoma multiforme was conducted to test the safety and efficacy of combination methionine restriction with temozolomide, an alkylating agent commonly used to treat glioblastoma. To better understand the impacts of methionine metabolism on life- and health-span, two studies were conducted to evaluate energy and glucose metabolism in overweight or diabetic adults using dietary methionine restriction (NCT00640757) or dietary methionine and cysteine restriction (NCT03629392), and a new study using sulfur amino acid (SAA) restriction has recently started recruiting (NCT04701346). One study also evaluated metabolic changes during combination methionine-cysteine restriction in healthy adults (NCT02192437). It remains to be determined what has been learned from these small studies in patients with diverse malignancies and/or metabolic disorders. Moreover, many of these studies have been hindered by difficulties recruiting patients and challenges with the tolerability of the methionine restricted diet for more than a few cycles, a shortcoming addressed by using non-dietary approaches to deplete methionine

## Cancer's methionine addiction

**Table 2.** Recent clinical trials utilizing methionine restriction

Identifier	Status	Recruiting Location	Study Title	Condition(s)	Study Goals
NCT03186937	Terminated	University of Wisconsin Madison Carbone Cancer Center	A window of opportunity study of methionine deprivation in triple negative breast cancer	Triple negative breast cancer (TNBC)	<ul style="list-style-type: none"> <li>• Effect of methionine restriction on TRAIL R2 cell surface expression</li> <li>• Effect on cancer stem cells and metabolic health</li> </ul>
NCT03574194	Suspended	West Virginia University Cancer Institute	Methionine-restricted diet to potentiate the effects of radiation therapy	Lung cancer Prostate cancer Breast cancer	<ul style="list-style-type: none"> <li>• Safety and adherence of methionine-restricted diet to radio-sensitize tumors</li> </ul>
NCT03733119	Terminated	University of Wisconsin Madison Carbone Cancer Center	ONC201 with and without methionine-restricted diet in patients with metastatic triple negative breast cancer	Metastatic triple negative breast cancer (TNBC)	<ul style="list-style-type: none"> <li>• Phase II trial combination methionine restriction and ONC201 (Akt/ERK inhibitor) for response rate</li> </ul>
NCT00508456	Terminated	U.T.M.D. Anderson Cancer Center Houston Texas	Dietary Methionine Restriction Plus Temozolomide for Recurrent GBM	Glioblastoma Multiforme	<ul style="list-style-type: none"> <li>• Safety and tolerability of methionine restriction in combination with temozolomide</li> <li>• Correlate response and survival with serum methionine and peripheral blood lymphocyte methylation</li> </ul>
NCT00640757	Completed	Pennington Biomedical Research Center	Methionine-Restriction Diet (MRD) in Obese Adults with Metabolic Syndrome	Metabolic syndrome	<ul style="list-style-type: none"> <li>• Impact of methionine restricted diet on weight loss and glucose metabolism/tolerance</li> </ul>
NCT02192437	Completed	Penn State University State College Milton S. Hershey Medical Center	Dietary Methionine and Cysteine Restriction in Healthy Adults	Healthy Individuals	<ul style="list-style-type: none"> <li>• Evaluate short- and long-term metabolic changes associated with health-span in response to dietary methionine or methionine-cysteine restriction</li> </ul>
NCT03629392	Completed	University of Oslo, Norway	Methionine and Cysteine Restriction in Humans	Overweight Individuals	<ul style="list-style-type: none"> <li>• Effect of methionine and cysteine restriction on energy and macronutrient metabolism in overweight humans</li> </ul>
NCT04701346	Recruiting	University of Oslo, Norway	Sulfur Amino Acids, Energy Metabolism and Obesity (STAY)	Overweight and Obese Individuals	<ul style="list-style-type: none"> <li>• Effect of dietary SAA restriction on body weight/ composition and energy expenditure in humans</li> <li>• Comparison of high SAA and low SAA diet</li> </ul>

(discussed in the next paragraph). Clearly, methionine restriction is likely to have direct antitumor effects by depleting tumor methionine levels as well as indirect systemic effects by improving metabolic health that may be challenging to tease apart in clinical studies. Additionally, the potential adverse effects of methionine restriction on immune cells [209] in the tumor microenvironment, which are out-competed for methionine uptake by tumor cells when methionine levels are limiting [41] will have to be investigated.

### Alternatives to dietary methionine restriction

L-Methionine- $\gamma$ -lyase or methioninase, a bacterially derived enzyme that degrades sulfur-containing amino acids including methionine, depletes methionine in both media and serum and may be a more readily translatable approach to clinical methionine restriction [54, 210, 211]. Co-culture of human embryonic lung fibroblasts and acute lymphoblastic leukemia cells treated with methioninase plus homocysteine eliminated malignant cells but had little impact on fibroblasts. Serum methionine levels in mice were > 95% depleted following a single injection of 300 units methioninase and this decrease was maintained for 8 hours [52]. Methioninase also depleted serum homocysteine, showing it effectively reduces methionine cycle intermediates [212]. Additionally, methioninase acts synergistically with 5-FU and other cytotoxic drugs in preclinical models [52, 210]. Methioninase did not significantly alter body weight following 21 days of treatment and showed minimal antigenicity in immune-competent mice following repeat exposures [52]. However, bacterial methioninase has a short half-life in macaque monkeys (approximately 2 hours) and resulted in anaphylactic reactions [213]. In humans, methioninase depleted serum methionine within 30 minutes and was maintained for up to 4 hours after infusion had stopped without outward signs of toxicity [214].

More recently, *oral* recombinant methioninase has been demonstrated to have antitumor activity in diverse murine models of cancer, including PDX models [215, 216]. In an orthotopic model of TNBC, oral recombinant methioninase reduced recurrent tumor weight and the number of metastatic lung nodules (5.3 in control versus 1.3 in treated mice) [217]. Oral methioninase has also been showed to act

synergistically with 5-FU, platinum agents and other cytotoxic drugs in preclinical models [218, 219]. Additionally, three patients with advanced prostate cancer have been treated with oral methioninase resulting in reduction [220] or stabilization [221] of PSA levels.

To enhance immune tolerance and prolong catalytic activity, bacterial methioninase has also been encapsulated in human erythrocytes [222]. Human erythrocyte encapsulated methioninase maintained lower serum methionine levels with 34% depletion compared to control at 9 days after administration [223]. Human erythrocyte encapsulated methioninase inhibited the growth of human glioblastoma tumor xenografts by 85% compared to control at day 45 [223]. Additionally, human cystathionine- $\gamma$ -lyase, which normally catalyzes the conversion of cystathionine to cysteine, has been engineered to degrade methionine instead of cystathionine by three amino acid substitutions and shown to robustly reduce methionine levels for 72 hours, exerting antitumor effects in murine models without overt toxicity [108, 224]. This engineered human methioninase depleted methionine and SAM, enhanced ROS, induced autophagy and activated apoptosis in cultured cancer cells [108]. Collectively, these studies suggest methioninase, particularly humanized or encapsulated versions, may be a safe and effective way to reduce circulating and intracellular methionine levels without altering diet, thereby potentially accelerating clinical translation of methionine restriction.

### Conclusions

Due to their intrinsic hyperproliferation and oxidative stress fueled by oncogenic alterations, cancer cells and cancer stem cells have a high demand for methionine for protein synthesis and SAM generation, which is used for RNA, DNA, and histone methylation, as well as glutathione and nucleotide synthesis. When this augmented demand for methionine is paired with limited availability of methionine, the ensuing oxidative stress and deficiency of nucleotide precursors exposes a metabolic vulnerability termed "methionine-dependence" that is a hallmark of transformed cells. Although the underlying molecular mechanisms of this metabolic vulnerability are incompletely understood, numerous groups have explored its therapeutic potential in diverse preclinical models of can-

cer and small clinical studies in patients with advanced malignancies. Rationally designed combination therapies with synergistic cytotoxic agents such as antifolates or exposed metabolic liabilities (e.g., TRAIL or thioredoxin pathways) are likely to be needed to enhance the clinical activity of methionine restriction. Moreover, the tolerability of dietary methionine restriction particularly for prolonged therapy as well as other barriers to patient recruitment have hindered clinical investigation to date. The use of methionine-degrading enzymes, especially humanized or encapsulated versions, or the use of methionine oxidation of protein-rich foods to deplete methionine levels may partly address this issue in future studies. Finally, the elucidation of biomarkers of methionine-dependent tumors such as key epigenetic signatures as well a more complete molecular understanding of the cellular response to methionine restriction in tumor cells may point to improved strategies to target this unique cancer metabolic vulnerability.

### Acknowledgements

This work was supported in part by grants from the V Foundation for Cancer Research (VLC), Wisconsin Partnership Program (VLC) and Breast Cancer Research Foundation (VLC).

### Disclosure of conflict of interest

None.

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