

## REVIEW

# Toward a better understanding of folate metabolism in health and disease

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**Folate metabolism is crucial for many biochemical processes, including purine and thymidine monophosphate (dTMP) biosynthesis, mitochondrial protein translation, and methionine regeneration. These biochemical processes in turn support critical cellular functions such as cell proliferation, mitochondrial respiration, and epigenetic regulation. Not surprisingly, abnormal folate metabolism has been causally linked with a myriad of diseases. In this review, we provide a historical perspective, delve into folate chemistry that is often overlooked, and point out various missing links and underdeveloped areas in folate metabolism for future exploration.**

## Introduction

In 1931, Lucy Wills reported that an unusual form of anemia, macrocytic anemia, could be cured by yeast extracts, suggesting that the disease is a nutritional deficiency (Carpenter, 2003a,b). Soon thereafter, many groups were racing to identify the active nutrient, later named folic acid or folate. This was at a time when modern chromatography techniques were yet to be developed, yet folic acid was successfully isolated by two independent groups using ingenious methods, including using activated charcoal to adsorb and concentrate folic acid and using a microbiological assay to rapidly detect its activity (Hutchings et al., 1941; Mitchell et al., 1941). These intense efforts culminated in 1948, when E.L. Robert Stokstad and colleagues at Lederle Laboratories elucidated the chemical structure of folic acid by chemical degradation and total synthesis (Stokstad and Jukes, 1987).

Having learned of the discovery of folic acid, Sidney Farber recruited a cohort of leukemic children to test whether folic acid might be able to restore normalcy to the blood cells in leukemia, just as it does in macrocytic anemia (Farber et al., 1947). Although this trial was quickly stopped, as folic acid was found to actually exacerbate the disease, it led to a revised idea that folic acid antagonists might stop leukemia. This revised idea was borne out in Farber's next trial. As described in his landmark paper in 1948 (Farber and Diamond, 1948), one of the tested folic acid antagonists, aminopterin, produced temporary remission in children with acute lymphoblastic leukemia. Thus, the era of cancer chemotherapy had begun.

What also ensued was a "golden era" for enzymology in general (1950–1960; Huennekens, 1996), during which many enzymes involved in folate metabolism were purified from crude cell or tissue extracts and characterized, and their reactions

merged into coherent pathways. Among these enzymes, dihydrofolate reductase (DHFR) was identified as the major target of aminopterin and its more widely used close analogue, methotrexate (Werkheiser, 1961; Huennekens, 1994). In the following decades, rapid development of molecular biology techniques allowed cloning of the genes encoding the enzymes, and Robert Schimke discovered that amplification of the *DHFR* gene confers tumor resistance to methotrexate. Remarkably, this was the first known case of gene amplification in mammalian somatic cells (Schimke, 1989).

Many excellent reviews on folate metabolism have appeared over the past decade, some of which cover metabolic compartmentation (Tibbetts and Appling, 2010; Scotti et al., 2013), folate transport (Zhao et al., 2011), and the roles of folates in redox homeostasis (Ducker and Rabinowitz, 2017), embryonic development (Momb and Appling, 2014), cancers (Locasale, 2013; Yang and Vousden, 2016), and plants (Hanson and Gregory, 2011).

It is sometimes claimed in the current literature that all the components in the metabolic pathways have been identified, and the challenge now is to understand how the metabolic fluxes are regulated. While we wholeheartedly agree with the latter point, we share with others the opinion that many components in mammalian folate metabolism remain to be identified, and their functions remain to be understood. Recently introduced disciplines, such as comparative genomics (Gabaldón and Koonin, 2013), functional genomics (Wang et al., 2014; Shalem et al., 2015; Horlbeck et al., 2016), and organelle proteomics (Chapel et al., 2013; Pagliarini and Rutter, 2013; Calvo et al., 2016), have opened up new opportunities for solving these remaining puzzles. Thus, in this review, we emphasize missing links and underdeveloped areas and discuss potential approaches to illuminate them. We

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begin with a survey of folate chemistry, an important aspect of folate metabolism that is sometimes overlooked.

## Structure and chemistry of folates

### Nomenclature

By convention, “folates” is a generic term, referring to a large family of compounds consisting of a 2-amino-4-hydroxy-pteridine ring, linked by a methylene ( $\text{CH}_2$ ) group to a *p*-aminobenzoyl moiety, which is in turn linked through amide bond to the  $\alpha$ -amino group of a monoglutamate or poly- $\gamma$ -glutamate (Fig. 1 A; Blakley, 1987). One-carbon (1C) units can be attached to N5, N10, or both. On the other hand, the name “folic acid” is reserved for the synthetic form with the fully oxidized pteridine ring and no 1C substitution (Fig. 1 B).

The number of distinct molecular species within the folate family could be as many as 150, due to the combinatorial effect of varying 1C and pteridine oxidation states and varying polyglutamate chain lengths (Krumdieck et al., 1983). Nevertheless, typically <50 species are detectable in natural animal and plant sources (Gregory, 1989), with the pteridine ring predominantly in the most reduced, tetrahydro state (Fig. 1 B), N5 and N10 either unsubstituted or substituted with one of six different 1C units (Fig. 1 C), and the polyglutamate chain length being predominantly five to eight (Lin et al., 1993).

### Folates as 1C carriers or electron carriers

In most folate-mediated reactions, tetrahydrofolate (THF) serves as a 1C carrier, which obtains the 1C unit from a donor (e.g., serine) and then transfers it to an acceptor, typically a biosynthetic intermediate. The attached 1C units exist in three different oxidation states that are equivalent to methanol, formaldehyde, and formate (Fig. 1 C) and can be interconverted by cellular enzymes using nicotinamide adenine dinucleotide phosphate (NADP) or nicotinamide adenine dinucleotide (NAD) as a cosubstrate.

Although the pteridine ring in folates is capable of undergoing redox reactions, this capability is rarely used. A notable exception occurs in the thymidylate synthase-catalyzed reaction (Fig. 1 B), in which the folate pteridine ring undergoes a two-electron oxidation. To sustain THF levels, therefore, DHFR is required to act downstream of thymidylate synthase to recycle DHF back to THF.

### Chemical lability of reduced folates

Naturally occurring, reduced folates are chemically labile. In fact, the first identified folate, folic acid, was likely an artifact due to the lengthy isolation procedures, during which natural folates were largely decomposed by reacting with molecular oxygen, and a minor component among the oxidized products was folic acid (Krumdieck et al., 1983). The chemical lability is an important aspect of folates, not only presenting a technical challenge for their chemical analysis, but also reflecting their tendency to break down *in vivo*. Antioxidants, ascorbate in particular, are effective in minimizing folate decomposition and have proved useful in various analytical methods for folates (Quinlivan et al., 2006).

Different classes of folates, defined by the pteridine oxidation state and the 1C identity, display markedly different stabilities and follow distinct degradation pathways. We describe the pathways for each class, roughly in the order of increased stability.

**THF.** In the absence of antioxidants, the half-life of THF in aqueous solutions at 37°C is typically <30 min (Pollock and Kaufman, 1978). The pathway of THF decomposition is mediated by quinoid DHF (qDHF), generated via a free radical mechanism (Fig. 2 A). qDHF is unstable and readily cleaves into 7,8-dihydropyrimidin, formaldehyde, and *p*-aminobenzoylglutamate (pABG; Reed and Archer, 1980; Burgos-Barragan et al., 2017). Formaldehyde is a potent cross-linker for proteins and nucleic acids (Burgos-Barragan et al., 2017).

**10-Formyl-THF.** In the absence of antioxidants, 10-formyl-THF is readily oxidized to 10-formyl-DHF (half-life, typically <30 min at 37°C), and on prolonged incubation, is further oxidized to 10-formyl-folic acid (Baggott et al., 1995; Baggott, 2000).

**5,10-CH<sub>2</sub>-THF and 5,10-CH<sup>+</sup>-THF.** There appear to be few reports on the stability of 5,10-CH<sub>2</sub>-THF and 5,10-CH<sup>+</sup>-THF, presumably because 5,10-CH<sub>2</sub>-THF is in equilibrium with THF and formaldehyde (Kallen, 1971), and 5,10-CH<sup>+</sup>-THF would hydrolyze to 10-formyl-THF and, to a much lesser degree, 5-formyl-THF at neutral pHs (Stover and Schirch, 1993).

**DHF.** DHF is much more stable than THF, with a half-life of 4.5 h at 30°C, pH 7.0 (Reed and Archer, 1980). DHF can undergo either pteridine ring oxidation to folic acid or C9-N10 cleavage to pABG and 6-formyl-dihydropterin or dihydroxanthopterin (Chippel and Scrimgeour, 1970; Reed and Archer, 1980). The partition into these three pathways seems to depend on the pH and the type of buffer (Chippel and Scrimgeour, 1970; Reed and Archer, 1980).

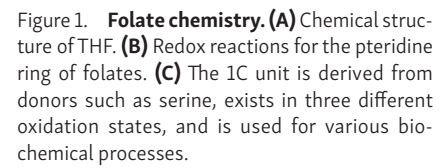
**5-Methyl-THF.** 5-Methyl-THF can be readily oxidized to 5-methyl-5,6-DHF. In the presence of thiols or ascorbate, 5-methyl-5,6-DHF readily reverts to 5-methyl-THF. Conversely, when subject to more oxidative conditions, 5-methyl-5,6-DHF either rearranges to a 5-methyl-dihydro-pyrazino-s-triazine compound or cleaves into an unidentified pterin and pABG (Gregory, 1989).

**Folic acid and 5-formyl-THF.** These two species are stable to oxidation, except under harsh conditions such as in the presence of  $\text{KMnO}_4$  (Maruyama et al., 1978).

### Paradoxical stability of folates *in vivo*

Given the intrinsic tendency of reduced folates to decompose, it is surprising that folates were found to be extremely slowly turned over in human whole body or in cultured mammalian cells (Krumdieck et al., 1978; von der Porten et al., 1992; Lawrence et al., 2014). For example, using a <sup>2</sup>H-folic acid tracer, folate breakdown rates in adult humans were estimated to be ~0.5% per day (half-life, ~19 d; von der Porten et al., 1992). This is in stark contrast to the situation in plants, where folate breakdown is very rapid, at a rate of ~10% per day (Orsomando et al., 2006).

A series of excellent reviews (Linster et al., 2013; Lerma-Ortiz et al., 2016; Sun et al., 2017) emphasized two important facets of metabolism that were largely overlooked in classic textbooks of biochemistry. First, enzymes are not always exquisitely specific and efficient, and many of them also catalyze unwanted side reactions. Moreover, many metabolites are chemically labile and reactive, and thus prone to damage. To counter such metabolite damage, cells have often evolved dedicated metabolite repair enzymes. In an example related to folate metabolism, 5-formyl-THF, a dead-end metabolite formed from 5,10-CH<sup>+</sup>-THF by a side reac-



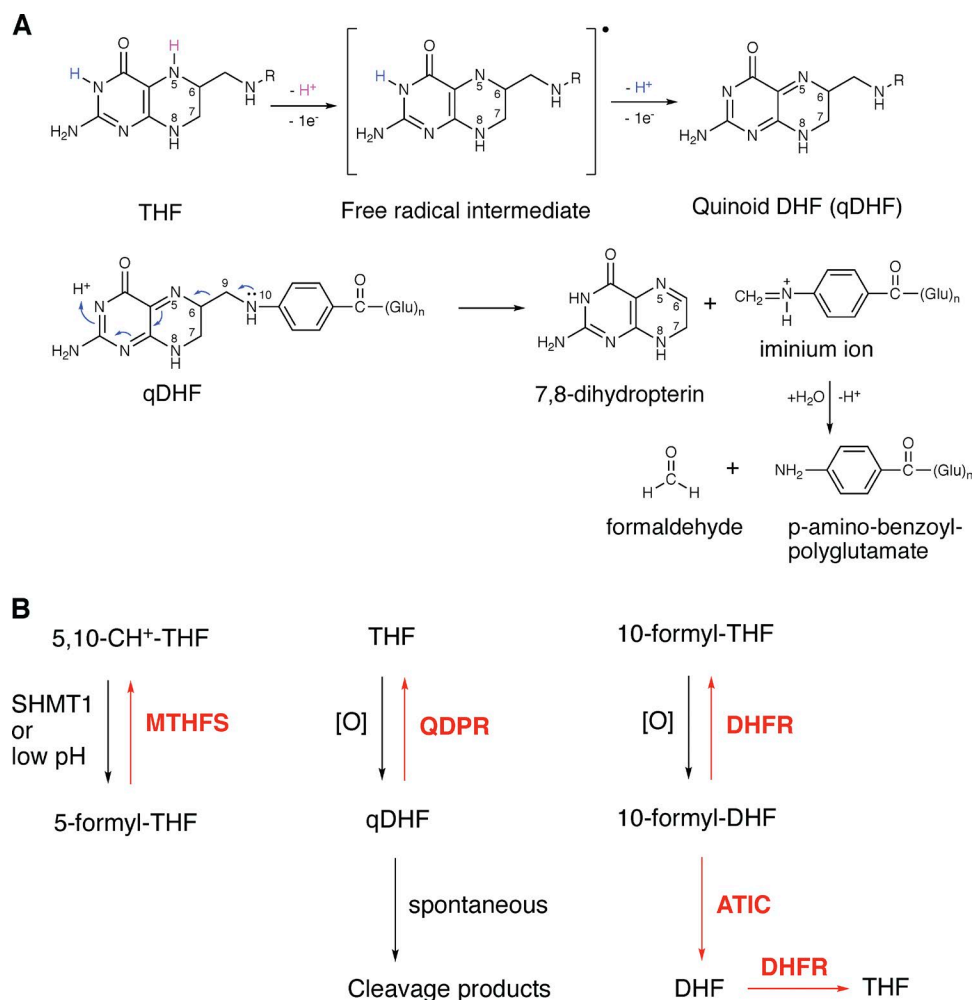


Figure 2. **Folate decomposition and damage repair.** (A) Chemical pathway of THF decomposition. (B) Cellular enzymatic systems for folate damage repair.

tion of serine hydroxymethyltransferases (SHMT1/2; [Stover and Schirch, 1992](#)) or spontaneously at mildly acidic pHs ([Baggott, 2000](#)), is salvaged by methenyl-THF synthetase (MTHFS) back to 5,10-CH<sup>+</sup>-THF in eukaryotes and most prokaryotes ([Fig. 2 B](#); [Linster et al., 2013](#)).

We recently found that cellular THF decomposition can be prevented by an enzyme, quinoid dihydropteridine reductase (QDPR); specifically, the qDHF intermediate of THF decomposition can be reduced by QDPR back to THF at the expense of two reducing equivalents from NADH ([Fig. 2 B](#); [Zheng et al., 2018](#)). Moreover, the oxidation product of 10-formyl-THF, 10-formyl-DHF, can be reduced back to 10-formyl-THF by DHFR, or to THF by tandem actions of ATIC and DHFR ([Fig. 2 B](#); [Zheng et al., 2018](#)). Thus, the oxidative damage to the two least stable folates, THF and 10-formyl-THF, can be enzymatically repaired in mammalian cells.

It is likely that other factors also contribute to folate stability in vivo. For example, folates can be stabilized through protein binding ([Wittwer and Wagner, 1981](#); [Cook and Wagner, 1982](#); [Min et al., 1988](#); [Jones and Nixon, 2002](#)). In addition, the reduced intracellular milieu maintained by glutathione- and thioredoxin-mediated systems may play an indispensable role as well.

### Folate pathways: Identified and unidentified components

Among the three processes we discuss in this section, folate uptake and catabolism typically occur on a much slower timescale than most 1C transfer reactions, consistent with the cell's differential needs for folate and 1C. Folate is needed only in catalytic amounts to function as a cofactor, whereas 1C must be rapidly transferred via heavy-traffic pathways to support demanding processes such as nucleotide synthesis.

### Folate uptake and intracellular accumulation

**Folate uptake at the cell surface.** At the plasma membrane, two transmembrane carriers (SLC19A1 and SLC46A1) and three glycosylphosphatidylinositol-anchored receptors (FOLR1-3) can mediate folate uptake ([Fig. 3 A](#); [Zhao et al., 2011](#)). The substrate of these transporters and receptors is folate monoglutamate, consistent with the fact that the circulating folate is predominantly 5-methyl-THF monoglutamate ([Ratanasthien et al., 1974](#)). SLC19A1, also known as the reduced folate carrier, is a widely expressed antiporter that exchanges a reduced folate for another anion substrate. Candidates for the counter substrate of SLC19A1, including thiamine phosphates and 5-aminoimidazole-4-carboxamide ribonucleotide monophosphate (ZMP or AICAR), have been suggested ([Visentin et al., 2012](#)). ZMP is an intermediate



in purine biosynthesis and is known to accumulate in folate deficiency, in both bacteria (Stetten and Fox, 1945; Bochner and Ames, 1982) and mammal cells (Herbert et al., 1964; McGeer et al., 1965). Therefore, using ZMP as a counter substrate for SLC19A1 would represent an elegant feedback mechanism for regulating intracellular folate homeostasis.

SLC46A1, also known as the proton-coupled folate transporter, is most active at acidic extracellular pHs. Consistent with its low pH optimum, SLC46A1 catalyzes symport of protons with the folate substrate, notably in the acidic, upper region of the small intestine, where it is highly expressed. Loss-of-function mutations in SLC46A1 are the only known cause of the rare disease hereditary folate malabsorption (Qiu et al., 2006; Zhao et al., 2007).

The folate receptors, strongly expressed in certain epithelial tissues and some gynecological cancers, mediate folate uptake through an endocytic pathway, but the exact mechanism is a matter of debate (Paulos et al., 2004; Chadda et al., 2007). Whatever the mechanism, to support cellular folate-dependent functions, the internalized folate monoglutamate must be effluxed from an internal vesicular compartment into the cytosol (Fig. 3 A). It has been suggested that SLC46A1 could mediate this efflux (Zhao et al., 2009), yet the same authors also pointed out that FOLR1 can function normally in some cells that lack SLC46A1 expression, indicating that there is at least one alternative transporter (Zhao et al., 2011).

**Folate uptake into the mitochondria.** At the mitochondrial inner membrane, SLC25A32, a member of the mitochondrial carrier family that consists of ~50 proteins, imports reduced folate monoglutamates into the mitochondrial matrix (Fig. 3 A). Surprisingly, however, human *SLC25A32* cDNA was able to complement the growth defects of a yeast mutant lacking a homologous gene, *FLX1*, that encodes a putative mitochondrial flavin adenine dinucleotide (FAD) transporter (Spaan et al., 2005). Recently, compound heterozygous mutations in *SLC25A32* were found in a patient with riboflavin-responsive exercise intolerance, further supporting a link between SLC25A32 and FAD metabolism (Schiff et al., 2016). Thus, these studies have raised the question of whether mammalian SLC25A32 is a carrier for folate, FAD, or both.

The virtual absence of mitochondrial folates in *SLC25A32* mutant Chinese hamster ovary (CHO) cells strongly argues that folate is a bona fide substrate for SLC25A32 (Titus and Moran, 2000; McCarthy et al., 2004). In contrast, *FLX1*-deficient yeast still has sizable mitochondrial FAD (Tzagoloff et al., 1996). Moreover, mammalian mitochondria (Barile et al., 2000; Calvo et al., 2016), and possibly yeast mitochondria as well (Bafunno et al., 2004), can synthesize FAD from riboflavin using riboflavin kinase and FAD synthetase, so it is not apparent why FAD needs to be imported from the cytosol. Given that the transport mechanism of SLC25A32 remains to be further delineated (Lawrence et al., 2011), it is possible that SLC25A32 is an antiporter that couples folate import with FAD export (Fig. 3 A). Consistent with this possibility, yeast *FLX1* has been suggested to be a mitochondrial FAD export carrier (Bafunno et al., 2004).

#### **Cytosolic and mitochondrial folate retention by polyglutamation.**

Folate polyglutamation, catalyzed by folylpolyglutamate synthetase (FPGS), plays an indispensable role for intracellular folate

accumulation. In cells lacking FPGS, folate monoglutamates that have been taken up will readily efflux out of the cell, resulting in only ~10% of normal folate accumulation (Osborne et al., 1993). Thus, polyglutamation adds multiple negative charges to the imported folates and thereby dramatically enhances their intracellular accumulation. Moreover, polyglutamation markedly increases the affinity of most folate-dependent enzymes for their folate substrate (Matthews et al., 1987).

DHF, THF, and 10-formyl-THF are efficient substrates for FPGS, whereas 5-methyl-THF, 5-formyl-THF, and folic acid are poor substrates (Cichowicz and Shane, 1987). Because 5-methyl-THF is the major circulating folate, its conversion by methionine synthase (MTR) to THF is thought to be the first metabolic step in cells that precedes polyglutamation (Cook et al., 1987).

FPGS is expressed in most tissues as at least two isoforms that result from alternative splicing and alternative translational start sites, one of which targets to the mitochondrial matrix (Lawrence et al., 2014). This enables the establishment of distinct, nonmiscible cytosolic and mitochondrial folate pools that operate in two parallel pathways, described next.

#### **Parallel cytosolic and mitochondrial 1C pathways**

Parallel 1C pathways exist in the cytosol and in mitochondria, respectively, each composed of a core set of serine hydroxymethyltransferase,  $\text{CH}_2$ -THF dehydrogenase/ $\text{CH}^+$ -THF cyclohydrolase, and 10-formyl-THF synthetase activities (Fig. 3 B; Shin et al., 2014). These two pathways are connected by small metabolites that can readily traverse the mitochondrial inner membrane, such as serine, glycine, and formate. The consensus for the interrelationship of the two pathways is that the mitochondrial 1C pathway produces formate, which in turn is used in the cytosolic pathway for purine, dTMP, and methionine biosynthesis, or excreted out of the cell if in excess (Christensen and MacKenzie, 2006; Tibbetts and Appling, 2010; Meiser et al., 2016; Ducker and Rabinowitz, 2017).

Recently, SFXN1 has been identified as a mitochondrial serine transporter (Kory et al., 2018). SLC25A38 has been suggested to be a mitochondrial glycine transporter (Fernández-Murray et al., 2016; Lunetti et al., 2016). However, the genes encoding mitochondrial transporters for formate, sarcosine, and dimethylglycine remain unknown (Fig. 3 A).

Another well-recognized role for the mitochondrial 1C pathway is glycine synthesis (Titus and Moran, 2000; Christensen and MacKenzie, 2006; Tibbetts and Appling, 2010; Jain et al., 2012; Ducker and Rabinowitz, 2017). A long-standing question is why the cytosolic SHMT1 cannot produce sufficient glycine. To generate glycine continuously via SHMT1, THF has to be continuously regenerated. For the cytosolic 1C pathway operating in the direction of glycine synthesis, THF regeneration is impeded by the unfavorable NADP/NADPH and ADP/ATP ratios in the cytosol (Veech et al., 1979; Christensen and MacKenzie, 2006). Our recent work showed that overexpression of *ALDH1L1* can overcome this thermodynamic barrier by bypassing the unfavorable 10-formyl-THF synthetase step, thereby allowing continuous THF regeneration and glycine synthesis in the cytosol (Zheng et al., 2018).

The mitochondrial 1C pathway also supports mitochondrion-specific functions. It is well known that mitochondrial

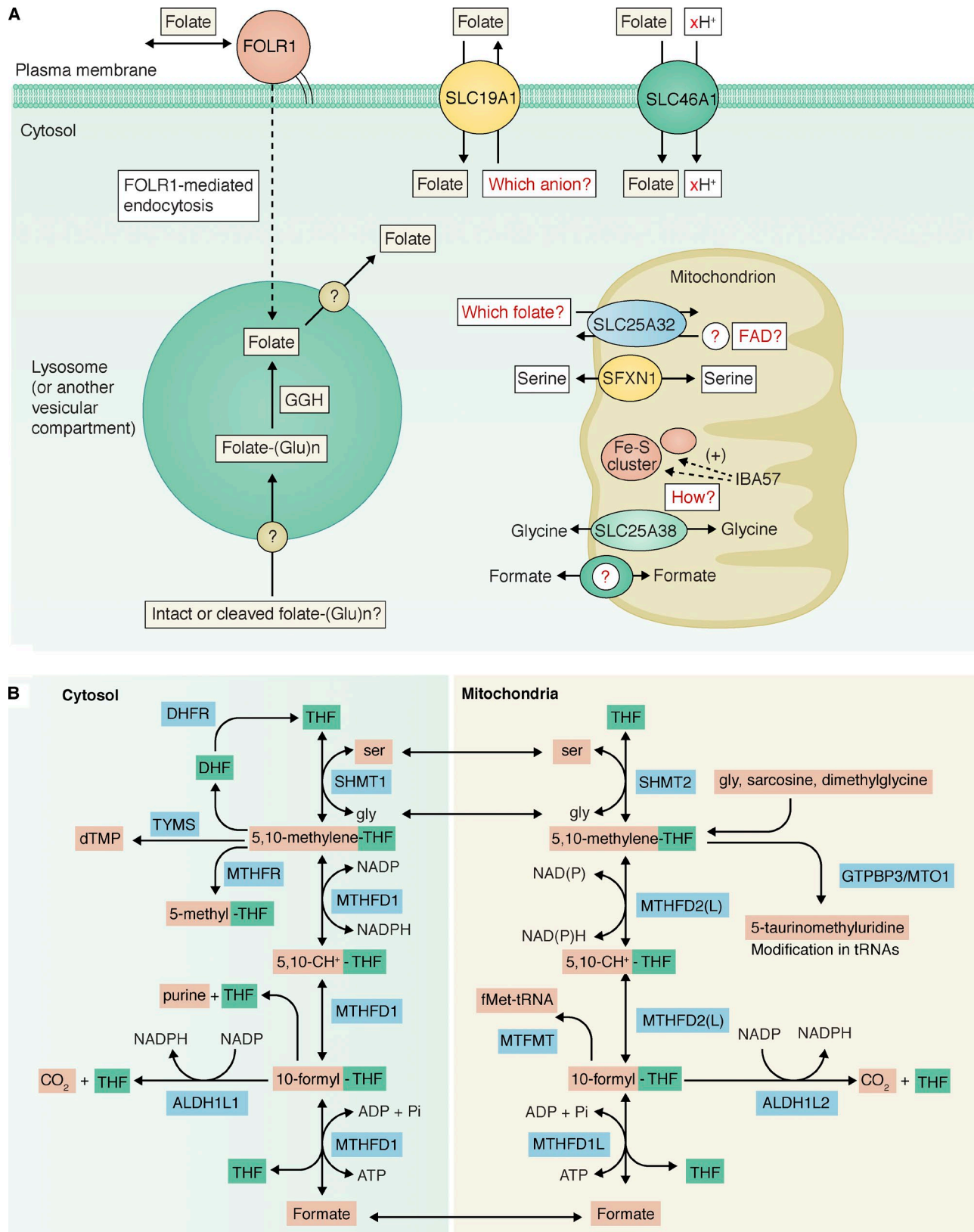


Figure 3. **Folate transport and metabolic compartmentation.** (A) Knowledge gaps in cellular folate metabolism. (B) Parallel cytosolic and mitochondrial folate pathways.

10-formyl-THF is used for N-formylation of the initiator methionine in mitochondrial methionyl-tRNA<sup>Met</sup>. Loss-of-function mutations in the human formyltransferase gene, *MTFMT*, have been causally linked to Leigh syndrome, a mitochondrial disease (Tucker et al., 2011). Unexpectedly, a recent study showed that *SHMT2*-knockout HCT116 cells contained normal levels of formylmethionine in mitochondrially translated peptides (Morscher et al., 2018). In contrast, another recent study found severe depletion of mitochondrial formyl-methionyl-tRNA<sup>Met</sup> in *SHMT2*-knockout Jurkat cells (Minton et al., 2018). It remains to be clarified whether these discrepancies can be accounted for by the differences in the cell line context, and if so, what the context-specific modulators are.

Recent research is also beginning to reveal a surprising role for mitochondrial 5,10-CH<sub>2</sub>-THF in mitochondrial protein translation. These studies, first performed on prokaryotic systems, highlight the synergy between genetic, structural, and biochemical approaches. It was first established, through genetics, that two bacterial proteins, MnmE and MnmG, are involved in modifying uridine at the wobble position of certain bacterial transfer RNAs (tRNAs) to 5-methyluridine derivatives (Elseviers et al., 1984; Hagervall et al., 1987), yet the biochemical understanding was lagging behind.

A breakthrough came when x-ray crystallography revealed that MnmE contains a folate-binding fold strikingly resembling that in several known folate-binding enzymes (Scrima et al., 2005; Scrutton and Leys, 2005). This revelation enabled the long-awaited in vitro reconstitution experiments, in which various THF derivatives were tested (together with other known cosubstrates) for the ability to stimulate the reaction of the MnmE/MnmG complex with tRNAs (Moukadi et al., 2009; Armengod et al., 2012). Indeed, 5,10-CH<sub>2</sub>-THF was found to be active and was suggested to be an essential cofactor for MnmE/MnmG.

The human orthologues of MnmE and MnmG, GTPBP3 and MTO1, have been predicted to be responsible for the occurrence of 5-taurinomethyluridine at the wobble position in certain mitochondrial tRNAs (Suzuki et al., 2011; Suzuki and Suzuki, 2014). To date, successful in vitro reconstitution for this putative activity has not been published. However, compelling genetic evidence has been provided by a recent study: knocking out either *MTO1* or *SHMT2* (which supplies mitochondrial 5,10-CH<sub>2</sub>-THF) in HCT116 cells resulted in no detectable levels of 5-taurinomethyluridine in mitochondrial tRNAs (Morscher et al., 2018). Consistent with the crucial role of this wobble uridine modification in stabilizing codon-anticodon pairing, these knockout cells were defective in mitochondrial protein translation (Morscher et al., 2018). Thus, the mitochondrial folate pathway provides the 5,10-CH<sub>2</sub>-THF substrate for GTPBP3/MTO1-mediated mitochondrial tRNA modification in human cells, an important finding with relevance to the mitochondrial disorders myoclonic epilepsy with ragged red fibers and mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes, which are caused by defective 5-taurinomethyluridine modification (Suzuki et al., 2011; Suzuki and Suzuki, 2014).

Our understanding of mitochondrial folate metabolism is not yet complete, however. Like MnmE, another bacterial protein YgfZ contains a folate-binding fold in its crystal structure, and

its folate-binding activity was confirmed by biophysical experiments (Teplyakov et al., 2004). YgfZ homologues (named IBA57 for the human gene) occur in all domains of life and, through a poorly understood mechanism, are essential for the assembly or maintenance of certain iron-sulfur proteins in bacteria (Waller et al., 2010, 2012) and in yeast (Gelling et al., 2008) and human (Sheftel et al., 2012) mitochondria (Fig. 3 A). At least in bacteria, the function of YgfZ in iron-sulfur cluster metabolism is folate dependent (Waller et al., 2010, 2012). Further investigation into IBA57 is much needed to fully decipher the mitochondrial respiration defects caused by impaired mitochondrial folate metabolism.

### Folate catabolism

Folate catabolism is extremely slow under normal conditions, but can be greatly accelerated with methotrexate treatment (Zheng et al., 2018). The bulk of folate catabolism in vivo is accounted for by the appearance of *p*-acetamidobenzoylglutamate (pAcABG) and pterins, products of folate C9-N10 bond cleavage, in urine (Murphy et al., 1976; Krumdieck et al., 1978). Whereas the C9-N10 bond cleavage is probably spontaneous, reflecting folate intrinsic instability, formation of pAcABG from the direct cleavage product pABG requires an enzyme, the NAT1 acetyltransferase (Minchin, 1995; Wakefield et al., 2007).

The cellular pathways for folate catabolism are poorly understood. Because plasma membrane efflux is prohibited for polyglutamated folates, pABG, or pAcABG, conversion of folate polyglutamate into folate monoglutamate, or deglutamation, is required for cellular clearance of folate catabolites. The only known enzyme capable of folate deglutamation is  $\gamma$ -glutamyl-hydrolase (GGH), which is either contained within lysosomes or secreted into the extracellular space (Hugonnet, 2012). Consistent with the role of GGH in folate deglutamation, GGH up-regulation was associated with a shift in intracellular folate distribution to shorter polyglutamate species in a hepatoma cell line (Yao et al., 1995). To access GGH, polyglutamated folates, pABG, or pAcABG have to be imported into the lysosome. A lysosomal carrier specific for polyglutamated rather than monoglutamated methotrexate was reported in the 1990s (Barrueco et al., 1992; Sirotiak and Tolner, 1999), but many questions remain unanswered, such as its gene identity and whether it preferentially imports cleaved folates (Fig. 3 A). The acidic pH (~4.5) of lysosomal lumen is expected to precipitate some spontaneous reactions of folates, such as the conversion of 5,10-CH<sup>+</sup>-THF to 5-formyl-THF (Baggott, 2000). Finally, it remains to be determined whether the monoglutamate product of GGH is directly secreted via lysosomal exocytosis (Settembre et al., 2013) or first released into the cytosol via a lysosomal carrier.

### Approaches to identify missing components

Historically, many components involved in folate metabolism were discovered by biochemical purification to chase down the protein responsible for an activity of interest (Tan et al., 1977; Huennekens, 1996) and by forward genetic screens followed by cDNA complementation (Dixon et al., 1994; Titus and Moran, 2000). The use of radioactive folic acid tracer in rats also greatly accelerated the discoveries of folate transporters/receptors and



folate-binding enzymes for which folate was not an expected substrate but later proved to be an integral component of their catalysis (Wagner, 1982; Garcia et al., 2016). More recently, searching for paralogs in the then just-completed human genome database allowed identification of missing genes for known activities (Prasanna et al., 2003; Bolusani et al., 2011).

It is possible that saturation has not been reached in traditional forward genetic screens where chemical-induced mutagenesis could have target bias. Moreover, in some cases, the causal gene remains elusive. For example, glycine auxotrophic mutants of CHO cells discovered in the 1970s (Chasin et al., 1974; McBurney and Whitmore, 1974) fall into four complementation groups: *GLYA* (= *SHMT2*), *GLYB* (= *SLC25A32*), *AUXB1* (= *FPGS*), and *GLYC/AUXB2*. Although the growth defect of the *GLYC* mutant was complemented by human *DHFR2* (also named *DHFR1L1*) cDNA (Anderson et al., 2011), the *DHFR2* gene does not exist in the rodent from which the CHO line was derived. Thus, the *GLYC/AUXB2* gene remains to be identified, for example by RNA or whole-exome sequencing.

The radioactive folic acid tracer approach had been limited to folate binders that are highly abundant and show high affinity (dissociation constant = submicromolar or lower) toward its folate ligand (Min et al., 1988). The same limitations would be encountered if a folate affinity column (Wittwer and Wagner, 1980) were used to pull down potential binders (Burdine and Kodadek, 2004). Although protein abundance is less an issue with the advent of highly sensitive mass spectrometry instruments, the requirement for tight binding remains a hurdle. Thus, new proteomic approaches such as thermal proteome profiling (Martinez Molina et al., 2013; Franken et al., 2015) and limited proteolysis–small molecule mapping (Piazza et al., 2018), developed to capture both strong and weak protein–ligand interactions in cells, can be powerfully complementary.

If an activity is known to localize to an intracellular organelle (e.g., lysosomes or mitochondria), then the search for the gene can be dramatically narrowed down to dozens or fewer candidates by referring to published inventories of genes encoding proteins for that organelle (Chapel et al., 2013; Pagliarini and Rutter, 2013; Calvo et al., 2016). A caveat is that these inventories have incomplete coverage and finite false discovery rates (Pagliarini and Rutter, 2013), and moreover, for the lysosome, it might be difficult to distinguish resident proteins from cargo proteins delivered there for degradation (Chapel et al., 2013). Nevertheless, recent success stories, such as the discoveries of the mitochondrial pyruvate carrier (Bricker et al., 2012; Herzig et al., 2012) and the mitochondrial calcium uniporter (Baughman et al., 2011), highlight the excellent potential for this approach.

The above organelle proteomics-based approach correlates an activity with a list of proteins on the basis of subcellular colocalization. Yet there are other ways to infer correlations. For example, genes with unknown functions can be linked to genes with well-defined functions on the basis of coevolution, coexpression, protein–protein interactions, suppression and synthetic interactions, and for microbial orthologues of a candidate human gene, gene clustering and gene fusion (<https://string-db.org/> and <https://thebiogrid.org/>; Pagliarini and Rutter, 2013; Niehaus et al., 2015). To further understand the function of a gene of inter-

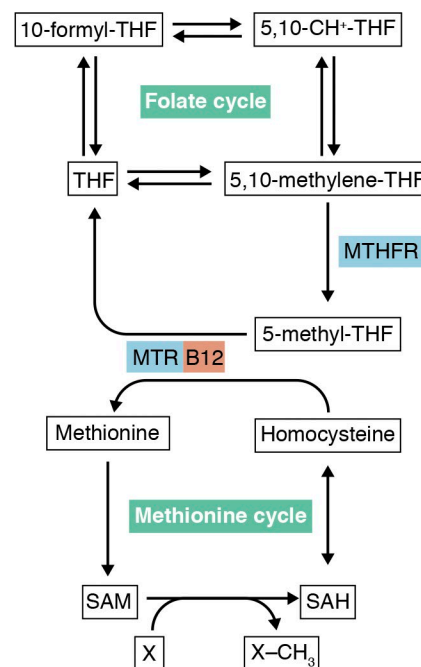


Figure 4. The folate cycle is linked to the methionine cycle via MTHFR and vitamin B12—using MTR.

est, one may zero in on short stretches of protein-encoding or promoter sequences and correlate the protein or DNA sequences with well-defined protein kinase phosphorylation or transcription factor binding motifs, as detailed in previous reviews (Frame and Cohen, 2001; Manning and Cantley, 2007) and exemplified by some recent studies (Ben-Sahra et al., 2013, 2016; Ye et al., 2014; DeNicola et al., 2015; Lee et al., 2017). Ultimately, the hypotheses thus generated need to be tested using genetic, biochemical, and pharmacological approaches.

#### Interaction of the folate cycle with the methionine cycle

ATP, NAD, and NADP are universal cofactors in metabolism. Therefore, folate pathways are connected to almost every other metabolic pathway through shared ATP, NAD, and NADP pools. Although folate pathways can contribute to the production of NADPH and ATP, their fluxes are also directly affected by compartment-specific ATP/ADP, NAD/NADH, and NADPH/NADP ratios. Recently it has been proposed that NADPH produced by ALDH1L2 in the mitochondrial 1C pathway is important to limit mitochondrial ROS (Piskounova et al., 2015; Yang and Vousden, 2016). Here, we further discuss the methionine cycle, which is linked to folate cycle via a more “specialized” metabolite, 5-methyl-THF (Fig. 4).

The link between folate and methionine cycles has long been recognized, since a deficiency in either folate or vitamin B12 (an essential cofactor for MTR) produces megaloblastic anemias that are hematologically indistinguishable (Shane and Stokstad, 1985). The megaloblastic anemia in both cases is ascribed to defective DNA synthesis and can be reversed by large doses of folates. Thus, vitamin B12 deficiency causes functional folate deficiency, and this is best explained by the methyl trap model (Shane and Stokstad, 1985). Methylenetetrahydrofolate



reductase (MTHFR)-catalyzed conversion of 5,10-CH<sub>2</sub>-THF to 5-methyl-THF is irreversible in cells, due to the large negative standard free-energy change and high cytosolic NADPH/NADP ratios (Matthews and Drummond, 1990). In addition, methionine synthase is the only enzyme that can remove the methyl group from 5-methyl-THF. Thus, a lack of vitamin B12 and thus MTR activity would irreversibly trap folates into 5-methyl-THF, at the expense of 5,10-CH<sub>2</sub>-THF and 10-formyl-THF, both of which are crucial to DNA synthesis.

The original methyl trap model has been expanded to include two subsequent developments. First, MTHFR is allosterically inhibited by S-adenosyl-methionine (SAM) with an inhibition constant of 2.8  $\mu$ M (Shane and Stokstad, 1985). Thus, large doses of methionine can increase intracellular SAM, block the methyl trap, and thereby ameliorate both folate and B12-deficient megaloblastic anemia. Second, as discussed above, the MTR-catalyzed conversion of 5-methyl-THF to THF is likely the first folate metabolic step in cells that precedes polyglutamation, so vitamin B12 deficiency would also compromise intracellular folate polyglutamation and retention. This explains the clinically observed, diminished total cellular folate content in vitamin B12 deficiency.

It is currently unresolved to what extent the folate cycle contributes to cellular methylations in normal physiology and diseases such as cancers. *MTHFR*-knockout mice are viable, and some clinical symptoms of human MTHFR deficiency may be ascribed to hyperhomocysteinemia rather than alterations in methylation (Schwahn et al., 2003). Many cultured cell lines are unable to proliferate when the medium methionine is replaced with homocysteine (Borrego et al., 2016; Lien et al., 2017), and indeed stable isotope labeling and mass spectrometry analysis indicated that for many cell lines, the contribution of serine and folate pathways to the active methyl group is insignificant compared with medium methionine (Pike et al., 2010; Shyh-Chang et al., 2013; Ducker et al., 2016; Maddocks et al., 2016). Understanding the mechanistic bases for such methionine dependency in cancer cell lines should be an important future direction, especially in light of the recent discoveries that many oncogenic mutations directly impact DNA and histone methyltransferases and demethylases (Garraway and Lander, 2013).

### Functions of folate metabolism and disease connections

The biochemical outputs of folate metabolism include nucleotide synthesis, serine-glycine interconversion, mitochondrial tRNA modification, and methyl group biogenesis, which in turn supports cellular functions such as proliferation, mitochondrial respiration, and epigenetic regulation. Abnormal folate metabolism has been causally linked with a myriad of diseases. However, for each disease, it is often unclear which biochemical processes and cellular functions are culprits.

The earliest known folate-related disease is probably megaloblastic anemia. Systemic folate deficiency, whether dietary, alcohol or drug induced, or due to germline mutations in *DHFR* or *SLC46A1* (Qiu et al., 2006; Banka et al., 2011; Cario et al., 2011), invariably results in megaloblastic anemia. The molecular basis for megaloblastic anemia has been ascribed to deranged DNA synthesis, which results in chromosome breakage, and continued cell growth without division during red blood cell production

(Shane and Stokstad, 1985). In some other cell types, folate deficiency can cause uracil misincorporation into DNA, leading to chromosome breakage (Blount et al., 1997). It has been proposed that such breaks could contribute to the increased risk of cancer (Blount et al., 1997).

Cerebral folate deficiency, caused by folate receptor autoantibodies or germline mutations in *FOLR1* or *SLC46A1*, is manifested in neurological impairments and shows some resemblance to severe MTHFR deficiency (Watkins and Rosenblatt, 2014). Thus, folate metabolism plays a crucial, yet poorly defined, role in the brain.

Insufficient folate intake around and during pregnancy has been associated with increased risk of neural tube defects, which is the basis for the mandatory folate fortification of foods in the US since 1998 (Momb and Appling, 2014). Strong evidence for the role of the mitochondrial folate pathway in neural tube development has been provided by a *MTHFDIL*-knockout mouse model, which presents neural tube defects with 100% penetrance while on a folate-proficient diet (Momb et al., 2013). In addition, mice deficient in the *AMT* or *GLDC* gene, encoding a subunit of the glycine cleavage system, also developed neural tube defects with partial penetrance (Narisawa et al., 2012; Pai et al., 2015). It was further shown with the *MTHFDIL* or *GLDC*-knockout mice that supplementation with formate rescued the neural tube defects (Momb et al., 2013; Pai et al., 2015). In a recent study, further deletion of *MTHFR* in *GLDC*-null mouse embryos resulted in significant protection from neural tube defects, suggesting that folate and formate-supported nucleotide synthesis, rather than MTHFR-mediated methyl group biogenesis, is crucial for neural tube development (Leung et al., 2017).

Conversely, excess folate intake may enhance proliferation of malignant tumors (Strickland et al., 2013). Moreover, genes encoding enzymes in serine biosynthesis and the mitochondrial folate pathway are often amplified or overexpressed in cancers (Locasale et al., 2011; Possemato et al., 2011; Nilsson et al., 2014). It has been suggested that metabolic activities under oncogenic control in cancers can be categorized based on whether they are transforming, enabling, or neutral (Vander Heiden and DeBerardinis, 2017). As attested by the successes of methotrexate and other antifolates in treating cancers, it is likely that serine biosynthesis and mitochondrial folate metabolism belong to the first two categories, and therefore are potential therapeutic targets (Mullarky et al., 2016; Pacold et al., 2016; Ducker et al., 2017). On the other hand, restriction of dietary serine and glycine has been proposed to treat cancers that lack up-regulation of de novo serine biosynthesis (Maddocks et al., 2017).

### Future perspectives

Throughout this review, we have highlighted areas that await further development and discussed how to explore them using existing technologies. Yet it is hard to predict where upcoming technologies will lead us. From a biochemical perspective, we envision a future where the entire human proteome is structurally solved (Petsko, 2014) and the folate binding fold (Scrutton and Leys, 2005) might be revealed in many more proteins, thus pushing forward a new level of understanding of folate metabolism in human health and disease.

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