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One carbon metabolism and folate transporter genes: Do they factor prominently in the genetic etiology of neural tube defects?

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Abstract

Neural tube defects (NTDs) are a broad class of congenital birth defects that result from the failure of neural tube closure during neurulation. Folic acid supplementation has been shown to prevent the occurrence of NTDs by as much as 70% in some human populations, and folate deficiency in a pregnant woman is associated with increased risk for having an NTD affected infant. Thus, folate transport-related genes and genes involved in the subsequent folate-mediated one carbon metabolic pathway have long been considered primary candidates to study the genetic etiology of human NTDs. Herein, we review the genes involved in folate transport and one carbon metabolism thus far identified as contributing variants that influence human NTD risk, and place these findings in the context of our evolving understanding of the complex genetic architecture underlying these defects

Keywords

Neural tube defects; one carbon metabolism; folate transporters; genomics

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Author contribution

JWS, SEK, and RHF wrote and edited the manuscript.

Conflict of Interest

None declared.

Declaration of interests

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1. Introduction

Neural tube defects (NTDs) are a broad class of congenital malformations, including spina bifida and anencephaly, which are estimated to affect approximately 18.6 per 10,000 births globally[1]. While the etiologies of NTDs are multifactorial, encompassing both environmental and complex genetic components, it is well known that one carbon metabolism (OCM) is arguably the most critical modifier of risk associated with proper neural tube closure. OCM encompasses a range of single-carbon transfer reactions, mediated by the vitamin cofactor, folate, which are essential for various cellular processes including: proliferation, epigenetic regulation, metabolic homeostasis, and maintenance of cellular redox balance[2]. Knockout mouse models for various OCM genes result in NTD phenotypes; and in humans, improving maternal folate status through peri-conceptional folic acid supplementation or nutritional fortification with folic acid results in significantly decreased prevalence of NTDs in almost every practicing population[3]. Indeed, folic acid is the single greatest weapon in the arsenal of NTD prevention. Given the importance of folate and OCM in the biology of NTDs, genes involved in folate uptake, folate transport, and OCM have been considered prime candidates as genetic risk factors for these birth defects. While many variants of OCM-related genes have been identified in NTD genomic association studies and candidate gene analyses, we are far from truly understanding the roles of these variants in the genetic architecture underlying birth defects. Therefore, in this review we provide an overview of folate transport and one carbon metabolism and review the genes in this pathway thus far identified as contributing variants that increase human NTD risk. We then explain how these findings fit into our deepening understanding of the genetic components contributing to NTD etiology.

2. Folate transporters and NTDs

Humans, like all other animals, are not capable of biosynthesizing folates, and therefore must consume them in their diet[2]. Folates must then be distributed to critical tissues and transported into cells via folate transporters. We will discuss the role of known folate transporters and their association with NTDs in mice and in humans.

2.1. Folate Receptors

Folate Receptors (FRs) are cysteine rich glycosylphosphatidylinositol-anchored proteins that have a high binding affinity to folate and mediate the uptake of folate to cells via endocytosis-mediated internalization of receptor-folate complexes[4]. Folate is then released to the cytoplasm of cells from the acidified endosome followed by FR-mediated endocytosis with the help of the proton-coupled folate transporter (PCFT)[5]. It has also been proposed that FR1 may act as a nuclear transcription factor that regulates expression of pluripotency factors[6,7]. The expression of folate receptors is limited to the placenta, the neural tube, and the kidney during embryonic development, and the expression of FR1 is limited to the epithelial cells of the choroid plexus, lung, and renal tubular cells in adults. Three separate genes (FR1, 2, and 3) encode folate receptors in humans, whereas there are only two genes (*Folr1* and *Folr2*) in the mouse. *Folr1* deficiency in mice is associated with cranial neural tube defects along with heart defects, facial malformations, and early embryonic lethality by

embryonic day E10, whereas *Folr2* depletion in the mouse results in no significant phenotypic malformations[8]. The *Folr1* nullizygous phenotypes can be rescued with maternal folate supplementation in the form of folinic acid[8]. A higher concentration of FR autoantibodies in maternal serum has been suggested to be a risk factor in human NTDs[9] and a recent report implies the existence of a genetic association between FRs and myelomeningocele with the discovery of twelve novel variants in human cases[10]. Folate deficiency due to a brain specific loss-of-function mutation of *FOLR1* in humans is associated with cerebral folate transport defects that cause brain malformations and several neurological disorders including epilepsy, which can be partly reversed by folinic acid treatment[11].

2.2. Reduced folate carrier (*SLC19A1*)

Reduced Folate Carrier 1 (RFC1) is an anion antiporter mediating the intake of reduced folates at a neutral pH, such as 5-methyltetrahydrofolate (5-methyl-THF) or 5-formyltetrahydrofolate (5-formyl-THF); however, with relatively low affinity for folic acid[12]. *RFC1* is ubiquitously expressed during early development and is expressed in human tissues including the brush border of the small and large intestine, the basolateral membrane of renal tubular epithelium, hepatocytes, choroid plexus, and retinal pigment epithelium. Thus, it has been considered a primary folate transporter in cells. *Rfc1* gene inactivation in mice is embryonic lethal before E6.5[13]. Maternal folic acid supplementation of heterozygous dams with low dosage extended embryo survival until E9.5-E10.5, although they presented with severe neural tube defects, while high-dose folic acid supplementation rescued *Rfc1* null embryos until term (E18.5)[13]. A common human polymorphism of *RFC1* (A80G) is associated with several diseases including neural tube defects[14], and recently eight rare variants of *RFC1*, including one pathogenic variant, were found in myelomeningocele patients[10]. These findings suggest a close association between RFC genes and neural tube closure defects in both the human and mouse.

2.3. Proton coupled folate transporter (*SLC46A1*)

PCFT is an electrogenic folate transporter that is highly expressed in the duodenum and jejunum in humans[15]. It has the highest affinity for folate at pH5.5, and also has a high affinity for 5-methyl-THF and 5-formyl-THF under low pH conditions. PCFT mediates the folate absorption in the brush-border membrane of the small intestine, which has a low pH microenvironment. It is also a critical folate transporter in the central nervous system, as it mediates the folate absorption from blood to cerebrospinal fluid in the choroid plexus. Additionally, it has been reported that PCFT releases folates from acidic endosomes in cultured cells, suggesting a supportive role of PCFT in FR1 dependent folate transport[5]. Loss-of-function mutations in the *PCFT* gene in humans is associated with hereditary folate malabsorption (HFM) syndrome[15], which causes systemic cerebral folate deficiency (CFD). This condition responds well to therapeutic intervention with chronic high dose folinic acid. *Pcft* null mutant mice show a lack of folate uptake in the intestines and low folate concentration and subsequent increased homocysteine levels in serum and several organs. Therefore, the *Pcft* knockout mouse may serve as a murine HFM model. Parental folate supplementation was shown to rescue folate deficiency-induced anemia in *Pcft* mutant mice and increased the survival length of *Pcft* KO mice[16].

2.4. Mitochondrial Folate Transporter (*SLC25A32*)

Folates enter the mitochondria through the mitochondrial folate transporter (MFT), coded for by the gene *SLC25A32*. Inactivation of this gene in mice results in severe NTDs that can be partially rescued by maternal formate supplementation, but not 5-methyl-THF or other folate species[17]. Simulations have suggested that tetrahydrofolate (THF) is the predominant folate species transported into the mitochondria, and that transport of THF by MFT is dependent on π-cation interactions with residue Trp142, since site-specific mutagenesis of this residue in Chinese Hamster Ovary cells rendered MFT nonfunctional in terms of facilitating mitochondrial folate uptake[18]. One human NTD case has been identified with two heterozygous loss-of-function variants in *SLC25A32*, both with predicted disruption of the Trp142 residue[17]. Additionally, four singleton rare missense variants were identified in four separate NTD patients[17].

3. One Carbon Metabolism and NTDs

3.1. Dihydrofolate Reductase (*DHFR*)

After uptake, folic acid, the form commonly delivered in peri-conceptional multivitamins and fortified diets, must then be reduced to THF before it can participate in one carbon reactions. This reduction occurs through the enzyme, dihydrofolate reductase (DHFR). Impairment of this enzyme, as observed by the action of its inhibitor, methotrexate, results in failure of cells to utilize folic acid for OCM[2]. A 19 base pair deletion in *DHFR*, previously reported to be associated with unmetabolized folic acid in serum and decreased red blood cell folate[19], was found to correlate with increased risk for spina bifida birth outcomes[20]. This particular variant has been extensively studied in several populations where results have been variable, with some studies suggesting increased risk for NTDs[20,21], and others suggesting no correlation or possibly even a protective effect depending on the population being studied[22,23]. The conflicting results surrounding association of this mutant with NTD risk suggests the contribution of this 19 base deletion may depend on the genetic background of the populations under study.

3.2. Mitochondrial One Carbon Metabolism

Once reduced to the THF form, folates can then be loaded with carbon units derived from carbon donors such as serine, glycine, dimethylglycine, and sarcosine with serine being the predominant contributor *in vivo*. Those carbon units are then partitioned out of the folate cycle to various functions of OCM such as purine, thymidine, and methionine synthesis. The folate cycle itself, is physically compartmentalized between the cytosol, mitochondria, and nucleus[24]. Conventionally, it is understood that the majority of carbon units enter the cycle in the mitochondrial compartment, and through folate-mediated one carbon transfers, are increasingly oxidized to produce the single-carbon ion, formate[24]. The mitochondria-derived formate is then exported to the cytosol where it serves as the predominant carbon pool for cytosolic OCM[24]. The specific role of mitochondrial OCM is of interest to identifying NTD risk and prevention, since mouse knockout models of certain mitochondrial OCM genes result in defects that are not folate responsive and are instead rescued by formate supplementation. As mentioned earlier, while folic acid is the primary line of defense in NTD prevention, a baseline rate of 5 per 10,000 live births have proven to be unresponsive to

folate[25]. Therefore, variants of mitochondrial OCM genes may contribute to these unpreventable cases, thus increasing NTD risk regardless of maternal folate status.

In the mitochondria, THF can be loaded with carbon units donated from serine via the activity of SHMT2 (the mitochondrial serine hydroxymethyltransferase), from glycine via the Glycine Cleavage System, and dimethylglycine or sarcosine by DMGDH (dimethylglycine dehydrogenase) and SARDH (sarcosine dehydrogenase) respectively. While few variants in *SHMT2* and *DMGDH* have been identified in human patients, at least three *SARDH* polymorphisms (rs573904, rs2797840, and rs2873817) have been identified as nominally associated with NTD risk[26,27]. But of all mitochondrial gene products that contribute carbon units to the folate cycle, the Glycine Cleavage System is the most well studied and has the most identified human variants associated with NTDs. During glycine cleavage, the enzyme GLDC (glycine decarboxylase) transfers a carbon unit from glycine to the enzyme, AMT (amino-methyl transferase), which in turn transfers the carbon unit to THF. Knockout of either gene encoding these enzymes results in partially penetrant NTDs in mice[28,29], which, in *Gldc* mutants, could be rescued by formate[28]. Furthermore, variants in *AMT* and *GLDC* that may contribute to human NTD risk have been identified in several studies[28,29,30].

After carbon units are loaded onto THF, they are oxidized to formate by the actions of mitochondrial enzymes MTHFD2/2L (methylene tetrahydrofolate dehydrogenase2/2L) and MTHFD1L. MTHFD2 and MTHFD2L are bifunctional, performing both dehydrogenation of 5,10-methylene-THF to 5,10-methyneyl-THF and subsequent hydrolysis of that product to 10-formyl-THF. While mitochondrial 10-formyl-THF may have multiple fates, including synthesis of formyl-methionine for mitochondrial gene translation, MTHFD1L specifically converts the formyl group to a free formate ion, regenerating THF in the process. While no variants in *MTHFD2* or *MTHFD2L* have been associated with NTD risk, a common insertion/deletion polymorphism in *MTHFD1L* (rs3832406) was found to be loosely associated with NTDs in a human study[31]. Furthermore, knockout of this gene in mice results in NTD phenotypes similar to null *Slc25a32* phenotypes, with *Mthfd1l* knockouts being similarly unresponsive to folate supplementation and rescuable by formate[32].

3.3. Cytosolic One Carbon Metabolism

Once carbon units are exported to the cytosol in the form of mitochondrially-derived formate, and they re-enter the folate cycle through the activity of MTHFD1. MTHFD1 is trifunctional, performing all three reactions carried out by the combined efforts of MTHFD2/2L and MTHFD1L in mitochondria, although typically in the reverse direction given a favorable flux of carbon units entering the cytosol as formate. A meta-analysis of nine studies spanning 4,300 NTD cases suggested that one particular polymorphism of *MTHFD1* (rs2236225) increased the likelihood of neural tube defects by 15–30%[33].

Cytosolic 10-formyl-THF may contribute its carbon unit to purine synthesis via enzymes coded for by the genes *GART* and *ATIC*, both functioning at different steps in purine metabolism. The *GART* polymorphism, rs2070388, was determined to have an increased odds ratio of 1.89–1.96 in NTD cases compared to controls in an Irish study[34].

Cytosolic 5,10-methylene-THF could contribute carbon units to thymidine synthesis via thymidylate synthase (TYMS), to glycine and serine metabolism via the cytosolic serine hydroxymethyltransferase (SHMT1), or it could be reduced to 5-methyl-THF by the enzyme MTHFR (methylene tetrahydrofolate reductase) to feed carbon units into the methionine cycle. When examining single nucleotide polymorphisms associated with NTD risk under conditions of low maternal folate status, *SHMT1* variant, rs12939757, was associated with increased NTD risk in the infant[34]. Interestingly, that same study found some maternal variants of *TYMS* and *MTHFR,* as well one infant variant of *TYMS* to be slightly protective against NTDs[35]. That study also observed three variants of *MTHFD1* (rs2236224, rs2236225, and rs11627387) to increase NTD risk[35]. Of all OCM genes associated with human NTD risk, *MTHFR* mutant C677T is probably the most well-studied variant, being associated with a two to four-fold increase in NTD risk[36]. Other *MTHFR* variants, such as the A1298C mutant, have also been identified to slightly increase NTD risk[36].

3.4. The Methionine Cycle

Carbon units from 5-methyl-THF can be used to synthesize methionine from homocysteine through the methionine synthase enzyme (coded for by *MTR*) in conjunction with its cofactor, vitamin B12. Methionine synthase reductase (coded by *MTRR*) is also required to activate the methionine synthase enzyme. A maternal study demonstrated enhanced risk for mothers harboring the rs1808349 *MTRR* variant, as well *MTHFD1* rs2236225, *MTHFR* rs1801133, and *RFC1* rs1051226[37]. While studies have yielded conflicting results on the association of certain *MTR* variants on NTD risk, one meta-analysis looking at the common A66G mutant did not find increased NTD risk across 8 studies[38].

While methionine can be utilized in many aspects of amino acid metabolism, in the methionine cycle it is converted to S-adenosylmethionine (SAM) by methionine adenosyl transferase 1a (MAT1A). Carbon units from SAM are then unloaded by various methyl transferases for methylation of various substrates, including DNA, lipids, and proteins. The product of these demethylations of SAM is S-adenosylhomocysteine, which is converted back to homocysteine to regenerate methionine. Alternatively, homocysteine can be utilized for cysteine metabolism via the transsulfuration pathway to produce the critical antioxidant, glutathione. Increased homocysteine levels are a common risk factor for NTDs[39], likely because elevated homocysteine is a biomarker for impaired OCM.

4. Conclusion

Given the known importance of folate and OCM in neural tube closure, it is hardly surprising that variants associated with NTD risk have been found in several OCM related genes. However, it is important to consider that no OCM gene has been singularly identified as causative in any NTD case, and variants in OCM genes are not necessarily found in NTDs at a higher rate than variants in other pathways, such as the planar cell polarity pathway. In many studies, OCM genes were specifically targeted for sequencing and association analysis based on the already established understanding that OCM is a critical component of neural tube development. While more untargeted approaches do uncover novel variants that may be associated with NTD risk, the rare and diverse nature of these birth defects prevent genetic

association studies of NTD cases from gaining sufficient statistical power to properly assess risk of any particular variant. In some cases, meta-analyses have been performed to try to overcome this lack of power[33]. While some common variants are consistently linked to NTDs across multiple studies, such as *MTHFR* C677T and *MTHFD1* rs2236225, the association of other variants with NTDs is often inconclusive. At best, many of these studies are limited to only identifying candidate genes and variants that do not definitively translate into an informative understanding of the genetic architecture underlying NTDs.

All evidence collected over the last 30 years indicates that the genetic component of NTD pathology is best described by the omnigenic model of inheritance, whereby the genetic architecture of complex traits is spread across the genome[40], with the risk incurred by variants of "core" genes being determined by the interactions of those variants with other variants in all other genes. This hypothesis is supported by evidence that accumulation of singleton loss-of-function variants in any one individual may be more strongly determinative of NTD risk than any one variant in any particular gene[41]. Therefore, while OCM genes clearly represent a core mechanistic component of neural tube development, and damaging variants in this pathway may confer increased risk for human NTDs, deepening our understanding of the genetic etiology of NTDs will require understanding how these variants interact with an individual's global genomic landscape. As such, developing future NTD-prevention strategies will undoubtedly require a precision medicine approach.

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Abbreviations

NTD	Neural Tube Defect
OCM	One Carbon Metabolism
FR	Folate Receptor
PCFT	Proton-Coupled Folate Transporter
RFC	Reduced Folate Carrier
HFM	Hereditary Folate malabsorption
CFD	Cerebral Folate Deficiency THF: Tetrahydrofolate
DHFR	Dihydrofolate Reductase
SHMT1, 2	Serine Hydroxymethyltransferase 1, 2
SARDH	Sarcosine Dehydrogenase

DMGDH	Dimethylglycine dehydrogenase
GLDC	Glycine Decarboxylase
AMT	Amino-methyl transferase
MTHFD1, 1L, 2, 2L	Methylene Tetrahydrofolate Dehydrogenase 1, 1L, 2, 2L
TYMS	Thymidylate Synthase
MTHFR	Methylene Tetrahydrofolate Reductase
MTR	Methionine Synthase
MTRR	Methionine Synthase Reductase
MAT1A	Methionine Adenosyl Transferase 1A
SAM	S-Adenosylmethionine

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Highlights

1. We provide an overview of folate transport and one carbon metabolism, reviewing the genes in this pathway thus far identified as contributing variants to human NTD risk.
2. The rare and diverse nature of NTDs coupled with their complex, multifactorial etiology present a continuing challenge to elucidating their underlying genetic architecture.
3. Developing future prevention strategies will likely require a precision medicine approach by understanding how these variants influence NTD risk within an omnigenic context.

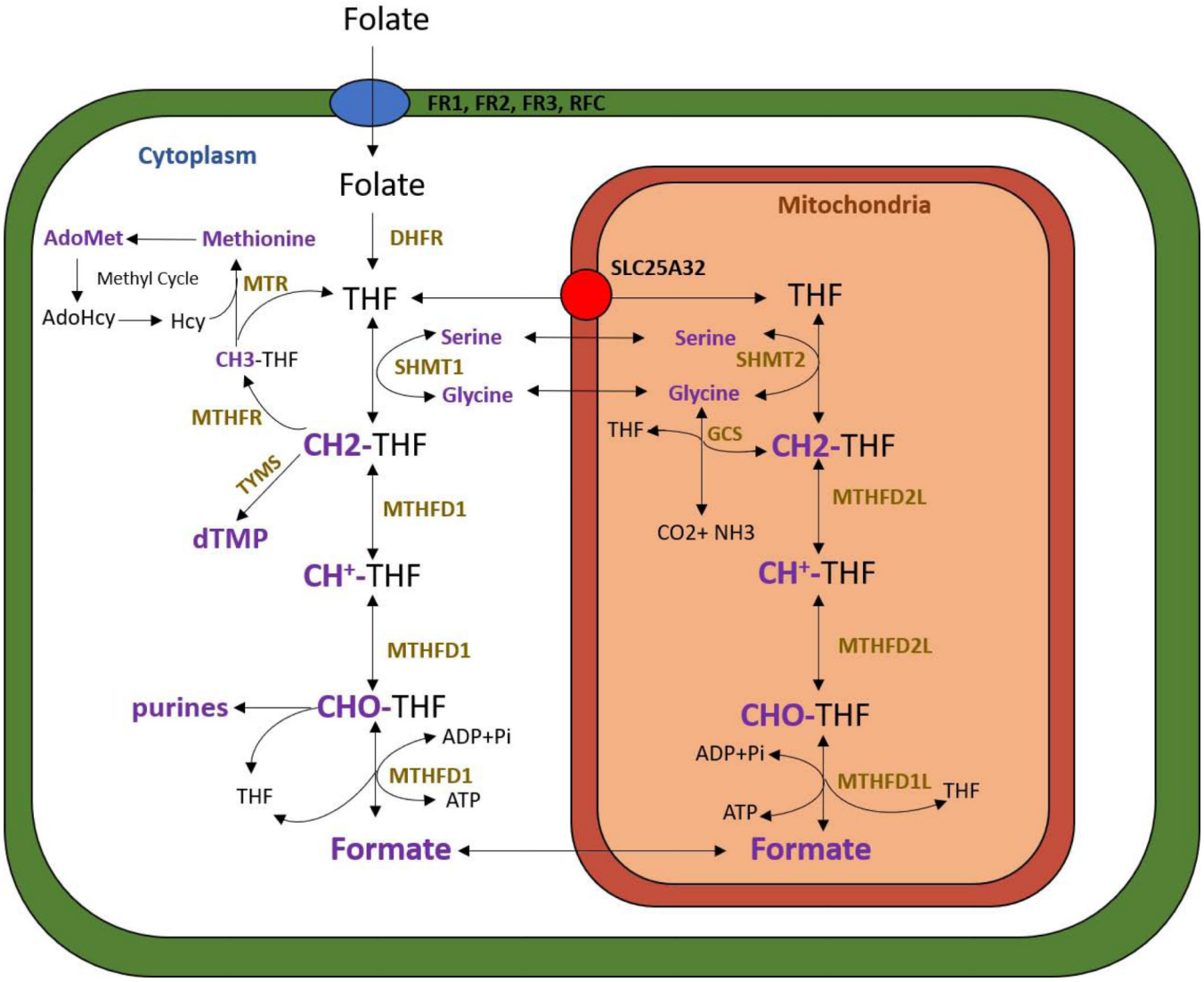

Figure 1. Overview of folate one carbon metabolism

Transport of folates into the cytosol and mitochondria are depicted by the blue and red circles respectively. Compartmentalization of one carbon metabolism between the cytosol and mitochondria is demonstrated, with key enzymes participating in one carbon reactions displayed in gold font. The Glycine Cleavage System (GCS) includes the enzymes AMT and GLDC. Carbon units, carbon donors, and carbon acceptors are displayed in purple font. CH3-THF = 5-methyl-THF, CH2-THF = 5,10-methylene-THF, CH^+-THF = 5,10-methenyl-THF, CHO-THF = 10-formyl-THF. **This figure was modified from our collaborators' in Momb et al. 2013*[32].

Table 1.

The phenotypes of folate transporters knock out mice.

Mouse gene	mouse phenotypes	References
Folr1	embryonic lethal at E10.0 cranial neural tube defects (E9.5) normal	Piedrahita et al. 1999[8]
Folr2	normal	Piedrahita et al., 1999[8]
Rfc (Slc19a1)	embryonic lethal at E6.5 cranial neural tube defects with EIIA-Cre (E9.5), Wnt1-Cre (E10.5) and TTR-Cre (E9.5)	Gelineau-van Waes et al., 2008[13] Toriyama et al., 2017[42]
Pcft (Slc46a1)	microcytic normochromic anemia/ pancytopenia systemic folate deficiency	Salojin et al., 2011[16]
Mft (Slc25a32)	exencephaly, craniorachischisis (E12.5)	Kim et al., 2018[17]

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