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The Search for Genetic Determinants of Human Neural Tube Defects

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Abstract

Purpose of Review: An update is presented regarding neural tube defects (NTDs) including spina bifida and anencephaly, which are among the most common serious birth defects worldwide. Decades of research suggest that no single factor is responsible for neurulation failure, but rather NTDs arise from a complex interplay of disrupted gene regulatory networks, environmental influences and epigenetic regulation. A comprehensive understanding of these dynamics is critical to advance NTD research and prevention.

Recent Findings: Next-generation sequencing (NGS) has ushered in a new era of genomic insight toward NTD pathophysiology, implicating novel gene associations with human NTD risk. Ongoing research is moving from a candidate gene approach toward genome-wide, systems-based investigations that are starting to uncover genetic and epigenetic complexities that underlie NTD manifestation.

Summary: Neural tube closure is critical for the formation of the human brain and spinal cord. Broader, more all-inclusive perspectives are emerging to identify the genetic determinants of human NTDs.

Keywords

Neural tube; spina bifida; birth defects

Introduction

Neural tube defects (NTDs) refer to a group of often severe congenital malformations of the central nervous system that arise from a failure in neural tube closure in the first month of human embryonic development. This failure can occur during the processes of primary or secondary neurulation or postneurulation skeletogenesis. With a prevalence of 0.5 – 10 per 1000 live births and considerable variability among population geographic regions and ancestral background, NTDs are estimated to result in 300,000 to 500,000 new cases annually worldwide [1]. The most common NTDs, anencephaly and myelomeningocele, are

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often isolated and attributable to a multi-factorial causation with an enlarging list of genes potentially conferring risk in humans. Recent evidence suggests that digenic or polygenic mutations result in NTDs whereas post-neurulation defects (e.g. encephalocele) are more often either monogenic or associated with chromosomal rearrangement and tend to be syndromic, affecting multiple organs [2].

Several developmentally relevant signaling processes critical to neural tube closure include SHH, β -catenin / WNT (canonical WNT) and non-canonical WNT / PCP, pathways (Table I for nomenclature). These pathways direct cellular differentiation and convergent extension of the neural plate to elongate the rostral-caudal axis of the embryo. Disruption of these signaling processes have been observed in both mouse and human NTDs [3, 4]. Guided by over 300 gene associations with NTDs in mouse models to date [5], next generation sequencing (NGS) efforts in humans are starting to shed light on clinically relevant risk genes. Given the polygenic nature of many NTDs, various genome-wide approaches from a systems biology perspective are needed to unravel the genetic architecture of this complex disorder.

Following the successes of maternal folic acid supplementation in reducing NTD incidence, considerable attention in research has been trained on genes involved in folate metabolism. This metabolic pathway supports a broader set of chemical steps involving the transfer of a single carbon unit, collectively known as one-carbon metabolism, which is directly involved in several significant physiologic processes of purine and thymidine biosynthesis, glycine, serine and methionine homeostasis and epigenetic balance. Therefore, deficits or dysfunction in the context of one-carbon metabolism can have a wide-ranging impact on NTD formation. Nevertheless, at least 30% of NTD cases are folate resistant, occurring despite prenatal folic acid supplementation. Here we review findings from recent studies that probe mechanistic aspects of NTD pathophysiology and highlight key gene regulatory networks as well as epigenetic processes that lead to failed neural tube closure.

NTD Risk Genes in Key Signaling Pathways

SHH signaling is critical for many aspects of early embryonic CNS development including dorsoventral patterning within the neural tube and regulation of neural plate bending. Numerous mouse models have shown that increased activation of SHH signaling activity promotes NTD formation, while other mutant models that display deficits in formation or function of cilia— a structure that promotes NT closure – exhibit decreased SHH signaling in the spinal cord. The mechanisms through which SHH pathway activity may influence NTDs include disrupted cell proliferation, differentiation or apoptosis [6]. Numerous mouse mutants that harbor genetic variants in *Shh* related genes display NTD phenotypes. In humans, variants in a few negative regulators of SHH (e.g. *PTCH1* and *PKA*) have been linked with spina bifida [7, 8], although additional potential risk genes are anticipated.

Kim *et al* [9**] reported agreement between mouse and human data suggesting that deleterious *GPR161* variants carry increased risk in humans, particularly for spina bifida. As a ciliary G-protein coupled receptor, GPR161 negatively regulates canonical WNT and retinoic acid signaling pathways during neurulation in mouse models, and also suppresses

the SHH pathway via cAMP signaling [10, 11]. Kim and colleagues found two novel, rare *GPR161* missense single nucleotide variants (SNVs) in their human NTD cohorts that were predicted *in silico* to be deleterious and studied the SNV impact by expressing mutant protein in 293T cells [9**]. They observed mislocalization of the mutant GPR161 receptors, increased SHH activity and reduced WNT activity, consistent with the transcriptional dysregulation that they found by RNA sequence analysis of *Gpr161* knockout mice at embryonic day 9.5 (E9.5). These findings add further insight into the largely unresolved interplay of SHH and WNT signaling during neurulation.

Recent studies have interrogated candidate genes in other NTD implicated pathways including PCP, a pathway directly involved in aspects of neurulation and neural tube closure. This strongly conserved non-canonical WNT pathway has a well-established role in controlling vertebrate convergent extension to shape the neural plate for closure [12]. Initiating the pathway, ligand binding triggers complex formation and subcellular localization of several interacting transmembrane (FZD, VANGL, CELSR1–3) and cytoplasmic (DVL, DIVERSIN, PK, SCRIB) proteins. Downstream signaling involves RHO-GTPases and activation of JNK kinase and scaffold protein, DAAM1, which further leads to a variety of transcriptional and cytoskeletal changes in both actin and microtubule cytoskeletal components [FIGURE 1]. The WNT / PCP pathway has been implicated in axon guidance and regulating dendritic development among other functions [13, 14].

Variants in PCP genes are increasingly implicated to confer risk for NTDs [15, 16]. Recent candidate gene approaches have interrogated PCP genes using specifically targeted DNA sequencing to find novel SNVs enriched in human NTD cases that are ripe for follow-up and functional analyses. At the same time, whole exome sequencing (WES) has begun unbiased surveys across the genome to detect the frequency of predicted deleterious mutations in NTD cases compared to unaffected controls, or search in child-parent trios, or multiplex families. One new study [17*] analyzed WES data in 8 families having two or more members affected by NTD and reanalyzed 43 trios, pooling 18 affected individual data from multiplex families and 43 affected singletons from trios. They screened for *de novo* and inherited mutations, using both candidate gene and genetic burden approaches. The authors found four novel loss-of-function (LOF) variants in three genes previously associated with NTDs (*MTHFR*, *DLC1* and *ITGB1*), and also found enrichment in NTD cases of variants in *MYO1E*, a novel candidate gene involved in cytoskeletal remodeling [17*]. These results that further implicate cytoskeletal regulators like *DLC1*, *ITGB1* and *MYO1E* underscore the need for further investigation into genes modulating actin-myosin dynamics and will surely involve crosstalk between cytoskeletal regulators and the WNT / PCP genes.

Another targeted panel sequencing approach [18] conducted in a cohort of 52 patients identified variants in novel genes *FREM2* and *DISP1* along with a relatively high prevalence of Wnt / PCP SNVs, compared to previous studies. *FREM2* encodes an integral membrane protein and associates with *FRAS1* forming a self-stabilizing complex in the extracellular matrix that is disrupted in Fraser syndrome and congenital diaphragmatic hernia [19, 20]. *DISP1* is essential for vertebrate Hedgehog (Hh) signaling and has an important role in facilitating long-range Hh signaling [21]. Additional evidence implicating SHH in NTD suggests that SHH may help determine the temporal and spatial characteristics of cell

polarity signaling via PCP as well as modulate gene expression through canonical, β -catenin / Wnt pathway activity [22].

An emerging hypothesis regarding NTD risk invokes a multi-hit or digenic interaction requirement for neurulation to fail, and while there are compelling data supporting this in mouse models, evidence is sparse in human genomic studies. The paucity of evidence for digenic and polygenic interactions in human studies is in part attributable to challenges posed by heterogeneous NTD subtypes, so that case phenotypic stratification reduces statistical power, as well as the exclusion of more common variants within genetic control databases that may nevertheless contribute to risk. A recent approach employed by Wang *et al* [23*] used targeted NGS to sequence Wnt / PCP genes in a cohort of 510 NTD patients, including spina bifida (232), anencephaly (125), encephalocele (46) and multiple (99) NTD phenotypes. The authors found evidence of digenic interactions, with individual NTD cases containing rare variants (<0.01 mean allele frequency) found in *CELSR1* and *SCRIB*, *CELSR1* and *DVL3*, or *PTK7* and *SCRIB* genes. These findings indicate that NTD pathophysiology can result from digenically determined risk and suggest that grouping all NTD phenotypes together initially may be a fruitful approach to precede granular genotype-phenotype investigation.

In an elegant mouse genetic study, an insight into the recognized female predominance in NTD prevalence has further implicated digenic interactions. Delbridge *et al* [24**] made the surprising observation that p53 is required for Xist expression, it binds to enhancers in the X-inactivation centers, and p53 loss promotes stochastic biallelic expression of X-linked genes. Furthermore, digenic interactions were illustrated in that the 17%–60% penetrance of NTDs in p53^{-/-} female mouse embryos increased to 100% when *BIM* – a pro-apoptotic gene – was also lost. These data should spark further investigation into X-linked gene interactions with autosomal variants. Intriguingly, variants in other apoptotic genes (*APAF1* and *CASP9*) have been found in two families with folate-resistant NTDs [25], recapitulating phenotypes observed in knockout mouse models, and providing the first published report linking variants in apoptosis genes with human NTD risk. These recent contributions to the field provide strong genetic evidence supporting the hypothesis that loss or disruption of key programmed cell death genes is a significant risk factor for NTDs.

From Digenic to Polygenic and Genome-wide Modeling

A comprehensive interrogation of the genetic underpinning of NTDs must move from a candidate gene approach toward a genome-wide investigation of DNA variation, its impact on transcriptomics, as well as epigenetic modifications that underlie gene-environment interactions. Chen *et al* [26**] have made progress toward genome-wide investigations utilizing whole genome sequencing (WGS) toward a polygenic view of NTD risk. WGS covers not only the protein coding exons (exome) but also the other non-protein coding, 98% of the genome. In that study, computational evaluations using two different population cohorts, conservatively compared the distributions of rare, likely loss of function (LOF) variants by restricting analyses to protein-coding regions and so avoid the challenges of *in silico* pathogenicity prediction for non-coding variants. The authors found a significant statistical enrichment in rare singleton loss of function variants (SLoFVs) in NTD cases

compared to controls from the 1000 Genomes Project and ExAC databases. The accumulation of SLoFVs in NTD affected individuals, compared to matched controls, was similar across two different ancestral populations thereby indicating that NTD risk in humans rises with the enrichment of deleterious variants in an individual. With polygenicity in mind, computational analyses utilizing genomic variant thresholds or polygenic risk scores in NTD biology will likely prove to be useful.

Protein encoding, exomes, though important, represent only around 3% of the genome. The range and diversity of genomic variation in NTD affected individuals must include structural variants (SVs) and SNVs in non-protein coding regions. SVs generally comprise at least 50 base pairs, and are investigated using both NGS and array-based platforms. SVs may arise as gains or losses in genetic material, e.g. copy number variants (CNVs), or as balanced rearrangements including inversions and translocations. Although there are more SNVs and small insertions/deletions (indels) compared to SVs in any given genome, SVs due to their range of sizes, account for more affected base pairs than SNVs and indels combined [27]. Moreover, SVs have recently been predicted to be responsible for 3.5–6.8% of expression quantitative trait loci (eQTL) sites [28], which are increasingly associated with complex genetic disorders including diabetes and Alzheimer's disease. Despite the inherent challenges in SV detection from WGS data, recent approaches utilizing a combinatorial algorithmic strategy are showing great promise [29]. Accurate detection of this underexplored variation form can, in principle, help refine our understanding of the gene regulatory networks that are essential for proper neural tube closure.

Little is yet known about the contributions of SVs to human NTD risk. Array-based analyses have found enrichment of CNVs encompassing ciliogenic genes in a cohort of 85 NTD-affected embryos [30] and have highlighted haploinsufficiency of human *PAX3* as a potential for a single gene cause of NTD [31]. More recently, Hofmeister *et al* [32*] described likely pathogenic CNVs in an NTD cohort using high-resolution copy number screening, including an intragenic deletion spanning exons 14–17 in the *WDR63* gene. They supported the claim that this deletion is the likely cause of human occipital encephalocele by CRISPR/Cas9 mediated introduction of this deletion into a zebrafish model that also subsequently exhibited abnormal CNS development. In a neural tube-associated malformation, human congenital hydrocephalus, exome sequencing identified *de novo* variants, including CNV duplications at the *SHH* locus [33]. Overcoming the inherent technical challenges in SV detection from NGS data promises to enhance statistical power and reveal novel genes and networks relevant to NTD physiology.

One-Carbon Metabolism and Epigenetic Regulation

While NTDs can be resistant to folic acid supplementation, folate deficiency and variants in a growing number of genes involved in folate and one-carbon metabolism have an established risk [34]. Through coupled reactions that take place in the mitochondria and cytosol, folate metabolism supports one-carbon transfer events, which contribute purine and thymidine biosynthesis and the most ubiquitous methyl-donor, SAM, required for methylation of DNA, RNA, proteins and lipids [FIGURE 2]. The demand for one-carbon units is highest during fetal development. Disruption of DNA synthesis, required for cell

proliferation, and DNA or histone methylation dependent epigenetic regulation of gene expression likely contribute to NTDs [35]. Human NTD-associated variants that impair folate one-carbon metabolism are coming to light. A recent study sequenced the exons and flanking introns from a cohort of 348 myelomeningocele subjects [36] and identified eight novel variants in a folate transporter (*SLC19A1*) as well as twenty novel variants in folate receptors (*FOLR1*, *FOLR2*, *FOLR3*), thus strengthening associations between folate intracellular transport and NTD risk. Another study [37] found additional polymorphisms in folate metabolic pathway genes (*MTHFR*, *MTHFD1*, *MTRR*, *RFC1*) as maternal NTD risk factors.

Kim *et al* [38*] showed that inactivating the mitochondrial folate transporter *Slc25a3* in mice induces folate-unresponsive cranial NTDs that can be mostly prevented with formate supplementation. These investigators corroborated their findings in mice by finding loss of function *SLC25A3* variants in humans with NTDs. Further studies may enable determination of individual variant specificity that would indicate superiority of glycine or formate supplementation in certain individuals for prevention of folic acid resistant NTDs.

How one-carbon metabolism feeds into epigenetic regulation to further influence NTD formation deserves greater attention. As the major source of methyl donor for enzymatic reactions, dietary folate intake and metabolism comprise an important environmental influence on gene expression through DNA and/or histone methylation, but the mechanistic details relating to NTD risk are incompletely understood. Zhang *et al* [39] found that spinal cord tissues from human NTD fetuses had aberrant genome-wide methylation patterns compared to sex- and gestational week-matched controls. That study found hypomethylation patterns in *TRIM4* associated with increased TRIM4 mRNA and protein expression that was postulated to influence NTD pathogenesis via immune-driven pathways.

Epigenetic modifications beyond methylation should be considered in NTD pathogenesis. A recent study [40*] illustrates histone homocystenylation as influencing the expression of NTD critical genes. Homocysteine is an intermediate of methionine metabolism and has been associated with NTDs in a number of studies [41] though largely unknown mechanism(s). Investigators have observed that chromatin modulator, histone 3 (H3), is homocystenylated on lysine 79 (H3K79Hcy) when cellular homocysteine levels are elevated in human fetal brains. Chromatin immunoprecipitation followed by DNA sequencing (ChIP-seq) and RNA sequence analyses showed that elevated H3K79Kcy levels lead to decreased expression of several genes involved in NT closure (*CECR2*, *SMARCA4*, *DNMT3B*), suggesting that higher homocysteine levels contribute to NTD onset by this mechanism. *SMARCA4* and *CECR2* both function in chromatin remodeling and *DNMT3B* is a DNA methyltransferase, which further implicates crosstalk between homocysteine and methylation reactions. Other recently reported epigenetic mechanisms, such as histone ubiquitination and disruption of *HOX* gene expression, are emerging as potential NTD risk factors [42]. Such studies underscore the importance of genome-wide epigenetic investigations to illuminate the gene expression mechanisms underpinning of NTDs.

Genetic and epigenetic mechanisms influencing NTD pathophysiology have recently been investigated using three-dimensional neural cysts derived from human embryonic stem cells

to recapitulate human NT formation *in vitro*. Valensisi *et al* [43] showed that 3-D neural cysts offer a distinct transcriptional and epigenomic landscapes that differ from two-dimensional neural stem cell cultures. These model systems can help bridge the gap between the environmentally influenced epigenetic responses to folate and their association with gene regulatory networks in human NTDs.

Conclusion

The advances in NGS technologies position us on the frontier of emerging reliable genetic tests with predictive value for NTD risk, insight into NTD polygenicity and opportunities for interrogating the diversity of genome variation. Studies are elucidating the complex roles that epigenetic modifications have in NTD causation. Increasing evidence for one-carbon transfer deficits implicate dysregulation of epigenetic mechanisms including, but not limited to, DNA methylation. Lastly, 3-D *in vitro* modeling using human cells can help disentangle the genetic and epigenetic landscape and further support computational genomic approaches toward understanding the complexities underlying NTD mechanisms.

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Key Points

- Complex interactions between genetic, environmental and epigenetic phenomena give rise to NTDs.
- The non-canonical WNT / PCP pathway contributes to NTD formation via cytoskeletal organization and potential crosstalk with SHH signaling.
- Understanding the genetic basis of NTDs necessitates investigating both digenic and polygenic interactions as well as a broader range of genome variation.
- Folate one-carbon metabolism carries a well-known NTD risk and bridges genetic and environmental processes through its critical regulation of epigenetic phenomena.

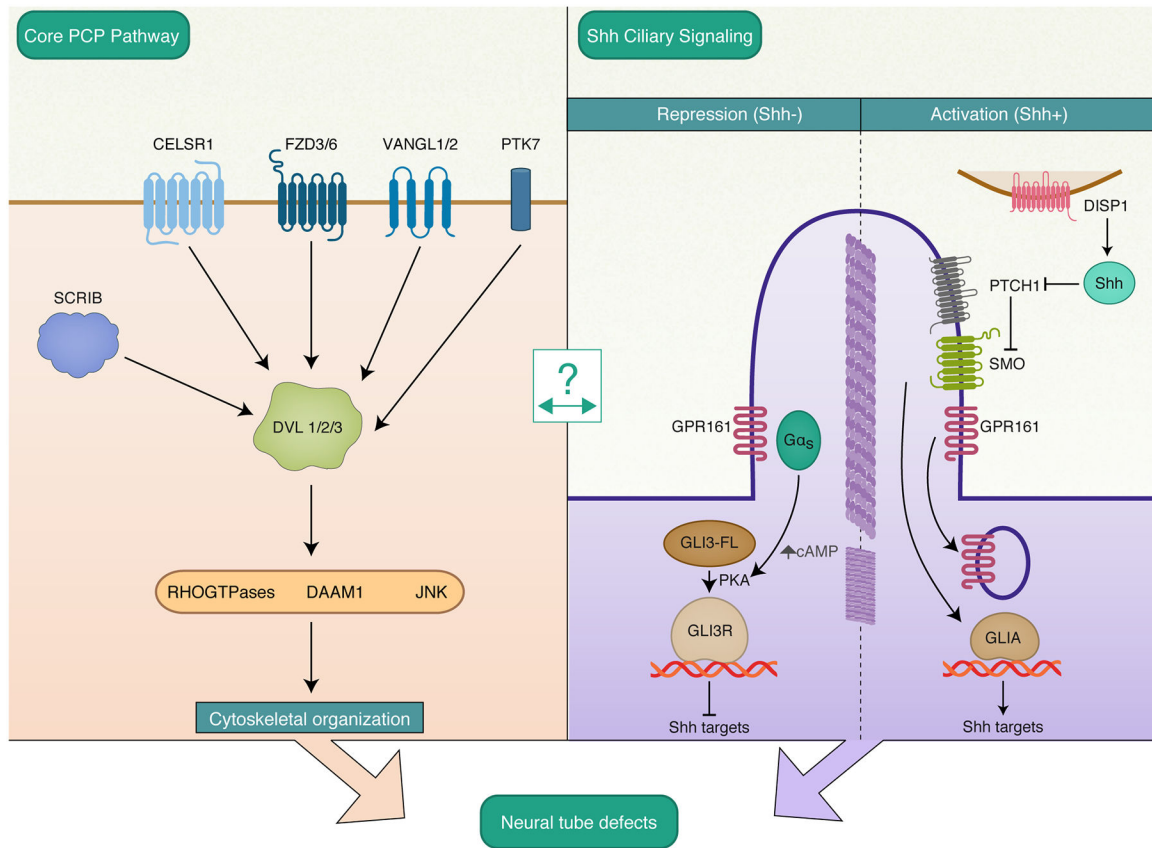


Figure 1. Non-canonical WNT / PCP and SHH signaling pathways.

Digenic interactions in several core PCP genes are associated with NTD risk. Genetic variants in *GPR161* may lead to NTDs associated with ciliary GPR161 mislocalization and dysregulated Wnt and Shh signaling. Molecular details of the cross-talk between PCP and Shh signaling remains to be elucidated. Abbreviations: CELSR1, Cadherin EGF LAG Seven-pass G-type Receptor 1; FZD3/6, Frizzled Class Receptor 3/6; VANGL1/2, Vangogh-Like Planar Cell Polarity Protein 1/2; PTK7, Protein Tyrosine Kinase 7; SCRIB, Scribble Planar Cell Polarity Protein; DVL 1/2/3, Dishevelled Segment Polarity Protein 1/2/3; DAAM1, Dishevelled Associated Activator of Morphogenesis 1/2/3; JNK, c-Jun N-terminal kinase; DISP1, Dispatched RND Transporter Family Member 1; PTCH1, Patched 1; SMO, Smoothened, Frizzled Class Receptor; GPR161, G Protein-Coupled Receptor 161.

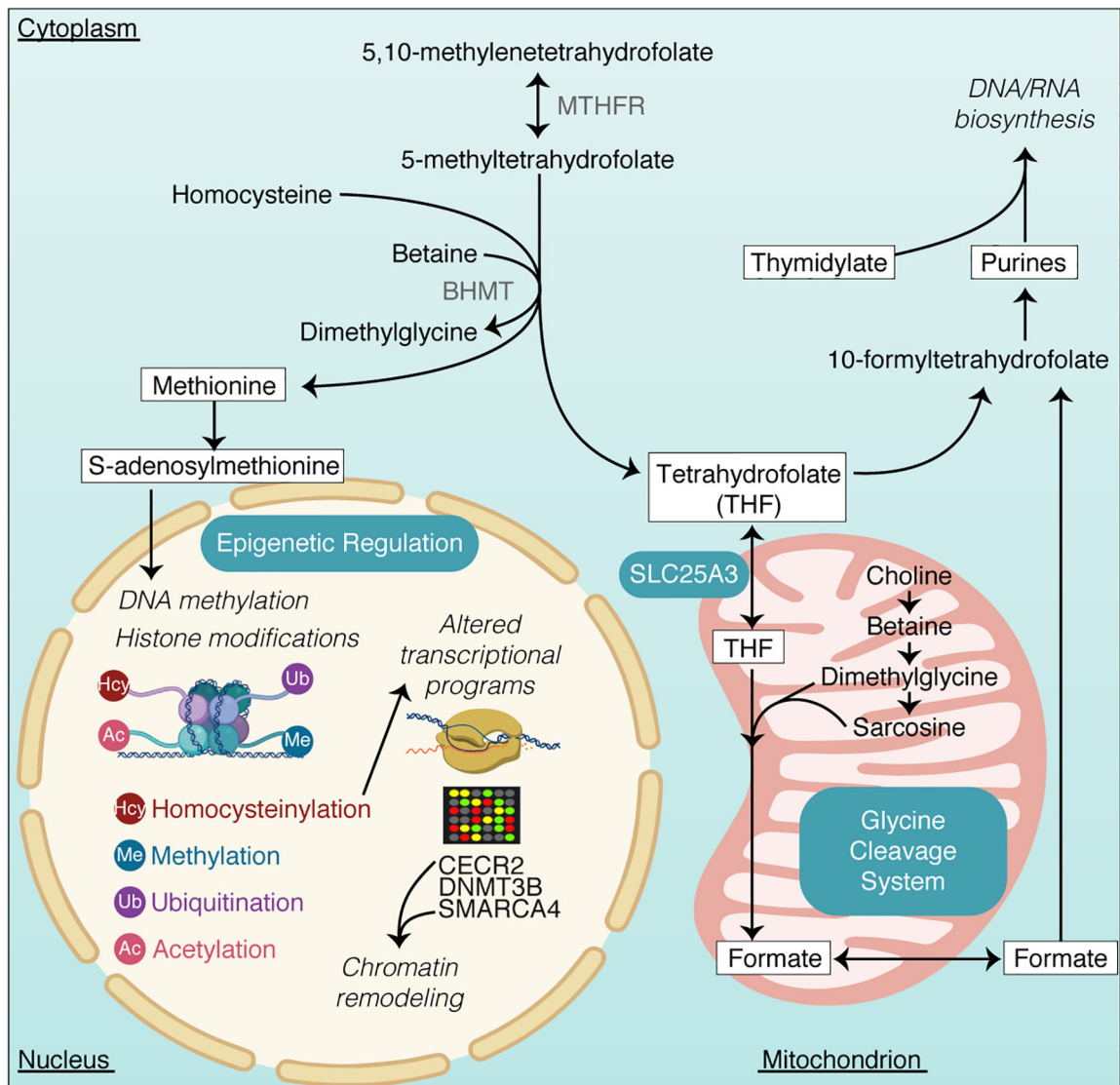


Figure 2. Key processes in folate one-carbon metabolism.

Compartmentalized reactions in the cytosol and mitochondrion are depicted. Variants in several genes regulating these steps have been linked with NTD risk and various epigenetic modifications - including DNA methylation requiring the primary methyl donor, S-adenosylmethionine (SAM) and histone homocysteinylation - have been shown to influence neural tube closure through aberrant expression of genes critical for neurulation. MTHFR, Methylenetetrahydrofolate Reductase; BHMT, Betaine-Homocysteine Methyltransferase; SLC25A3, Solute Carrier Family 25 Member 3; CECR2, CECR2 Histone Acetyl-Lysine Reader; DNMT3B, DNA Methyltransferase 3 Beta; SMARCA4, SWI/SNF Related, Matrix Associated, Actin Dependent Regulator of Chromatin, Subfamily A, Member 4.

Table I.

Gene / Pathway Symbols and Corresponding Names

APAF1	Apoptotic Peptidase Activating Factor 1
β -catenin / WNT	Canonical β -catenin-dependent Wnt Signaling Pathway
BIM	BCL2 Like 11
CASP9	Caspase 9
CECR2	CECR2 Histone Acetyl-Lysine Reader
CELSR1-3	Cadherin EGF LAG Seven-Pass G-Type Receptor 1-3
DAAM1	Dishevelled Associated Activator of Morphogenesis 1
DISP1	Dispatched RND Transporter Family Member 1
DLC1	DLC1 Rho GTPase Activating Protein
DNMT3B	DNA Methyltransferase 3 Beta
DVL 1/2/3	Dishevelled Segment Polarity Protein 1/2/3
FOLR1/2/3	Folate Receptor Alpha / Beta / Gamma
FRAS1	Fraser Extracellular Matrix Complex Subunit 1
FREM2	FRAS1 Related Extracellular Matrix 2
FZD 3/6	Frizzled 3/6
GPR161	G Protein-Coupled Receptor 161
ITGB1	Integrin Subunit Beta 1
JNK	c-Jun N-terminal kinase
MTHFD1	Methylenetetrahydrofolate Dehydrogenase, Cyclohydrolase And Formyltetrahydrofolate Synthetase 1
MTHFR	Methylenetetrahydrofolate Reductase
MTRR	5-Methyltetrahydrofolate-Homocysteine Methyltransferase Reductase
MYO1E	Myosin IE
PAX3	Paired Box 3
PK	Prickle Planar Cell Polarity Protein
PTCH1	Patched 1
PTK7	Protein Tyrosine Kinase 7
RFC1	Replication Factor C Subunit 1
SCRIB	Scribble Planar Cell Polarity Protein
SHH	Sonic Hedgehog
SLC19A1	Solute Carrier Family 19 Member 1
SLC25A3	Solute Carrier Family 25 Member 3
SMARCA4	SWI/SNF Related, Matrix Associated, Actin Dependent Regulator of Chromatin, Subfamily A, Member 4
TRIM4	Tripartite Motif Containing 4
VANGL1/2	Vangogh-Like Planar Cell Polarity Protein 1/2
WDR63	WD Repeat Domain 63
WNT / PCP	Non-canonical WNT / PCP Signaling Pathway
XIST	X Inactive Specific Transcript