The search for genetic determinants of human neural tube defects

Paul Wolujewicz and M. Elizabeth Ross

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 world-wide. 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 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
birth defects, neural tube, spina bifida

INTRODUCTION

Neural tube defects (NTDs) refer to a group of often severe congenital malformations of the central nervous system (CNS) 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 300 000–500 000 new cases annually worldwide [1]. The most common NTDs, anencephaly and myelomeningocele, are often isolated and attributable to a multifactorial causation with an enlarging list of genes potentially conferring risk in humans. Recent evidence suggests that digenic or polygenic mutations result in NTDs whereas postneurulation 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 sonic hedgehog (SHH), β-catenin/WNT (canonical WNT) and noncanonical WNT/planar cell polarity (PCP), pathways (Table 1 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,

Center for Neurogenetics, Feil Family Brain and Mind Research Institute, Weill Cornell Medicine, New York, New York, USA

Correspondence to M. Elizabeth Ross, MD, PhD, Professor and Director, Center for Neurogenetics, Weill Cornell Medicine, 413 East 69th Street, Box 240, New York, NY 10021, USA. E-mail: mer2005@med.cornell.edu

Curr Opin Pediatr 2019, 31:000–000
DOI:10.1097/MOP.0000000000000817

KEY POINTS

- Complex interactions between genetic, environmental and epigenetic phenomena give rise to NTDs.
- The noncanonical 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.

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.

NEURAL TUBE DEFECT 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, whereas other mutant models that display deficits in formation or function of cilia – a structure that promotes neural tube 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

Table 1. 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 three 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	Noncanonical WNT/PCP signaling pathway
XIST	X inactive-specific transcript

PCP, planar cell polarity.

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 cyclic adenosine monophosphate signaling [10,11]. Kim *et al.* [9▪▪] 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. 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. 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 noncanonical 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 (Fig. 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 with unaffected controls, or search in child–parent trios, or multiplex families. One new study [17▪] analyzed WES data in eight 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 with 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 signaling and has an important role in facilitating long-range hedgehog 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 multihit 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), anenecephaly (125), encephalocele (46) and multiple (99) NTD phenotypes. The authors found evidence of digenic interactions, with individual NTD cases containing rare variants (less than 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

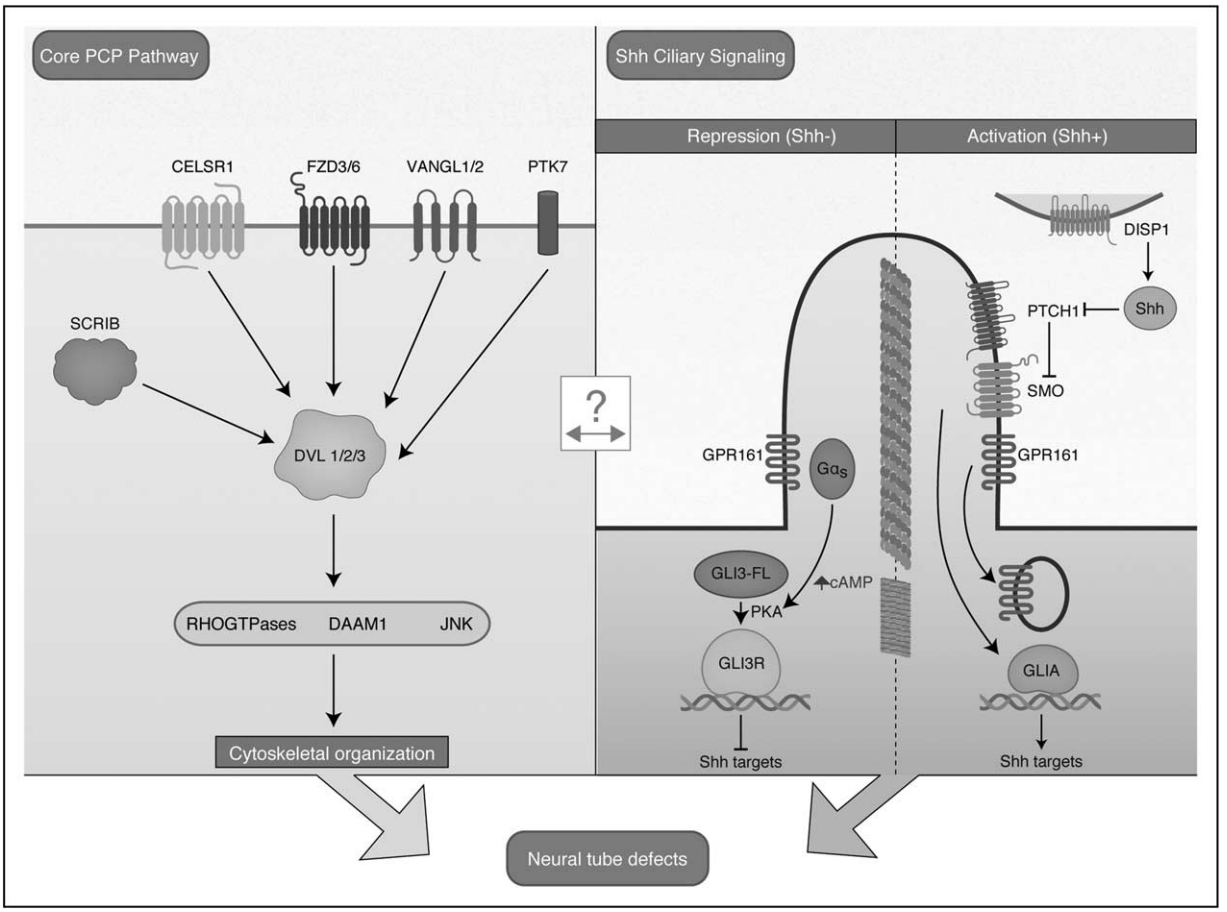

FIGURE 1. Noncanonical WNT/PCP and SHH signaling pathways. Digenic interactions in several core PCP genes are associated with neural tube defect risk. Genetic variants in *GPR161* may lead to neural tube defects associated with ciliary GPR161 mislocalization and dysregulated Wnt and Shh signaling. Molecular details of the cross-talk between PCP and Shh signaling remains to be elucidated. cAMP, cyclic adenosine monophosphate; 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; PCP, planar cell polarity; PTCH1, patched 1; SHH, sonic hedgehog; SMO, smoothened, frizzled class receptor; GPR161, G protein-coupled receptor 161.

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 proapoptotic 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 nonprotein coding, 98% of the genome. In that study, computational evaluations using two different population cohorts, conservatively compared the distributions of rare, likely LOF variants by restricting analyses to protein-coding regions and so avoid the challenges of in-silico pathogenicity prediction for noncoding variants. The authors found a significant statistical enrichment in rare singleton loss of function variants (SLoFVs) in NTD cases compared with controls from the 1000 Genomes Project and ExAC databases. The accumulation of SLoFVs in NTD affected individuals, compared with 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 and SNVs in nonprotein coding regions. Structural variants generally comprise at least 50 base pairs, and are investigated using both NGS and array-based platforms. Structural variants may arise as gains or losses in genetic material, for example copy number variants (CNVs), or as balanced rearrangements including inversions and translocations. Although there are more SNVs and small insertions/deletions (indels) compared with structural variants in any given genome, structural variants due to their range of sizes, account for more affected base pairs than SNVs and indels combined [27]. Moreover, structural variants have recently been predicted to be responsible for 3.5–6.8% of expression quantitative trait loci sites [28], which are increasingly associated with complex genetic disorders including diabetes and Alzheimer's disease. Despite the inherent challenges in structural variant 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 structural variants 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 structural variant 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

Although 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, S-adenosylmethionine, required for methylation of DNA, RNA, proteins and lipids (Fig. 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 individuals [36] and identified eight novel variants in a folate transporter (*SLC19A1*) as well as 20 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

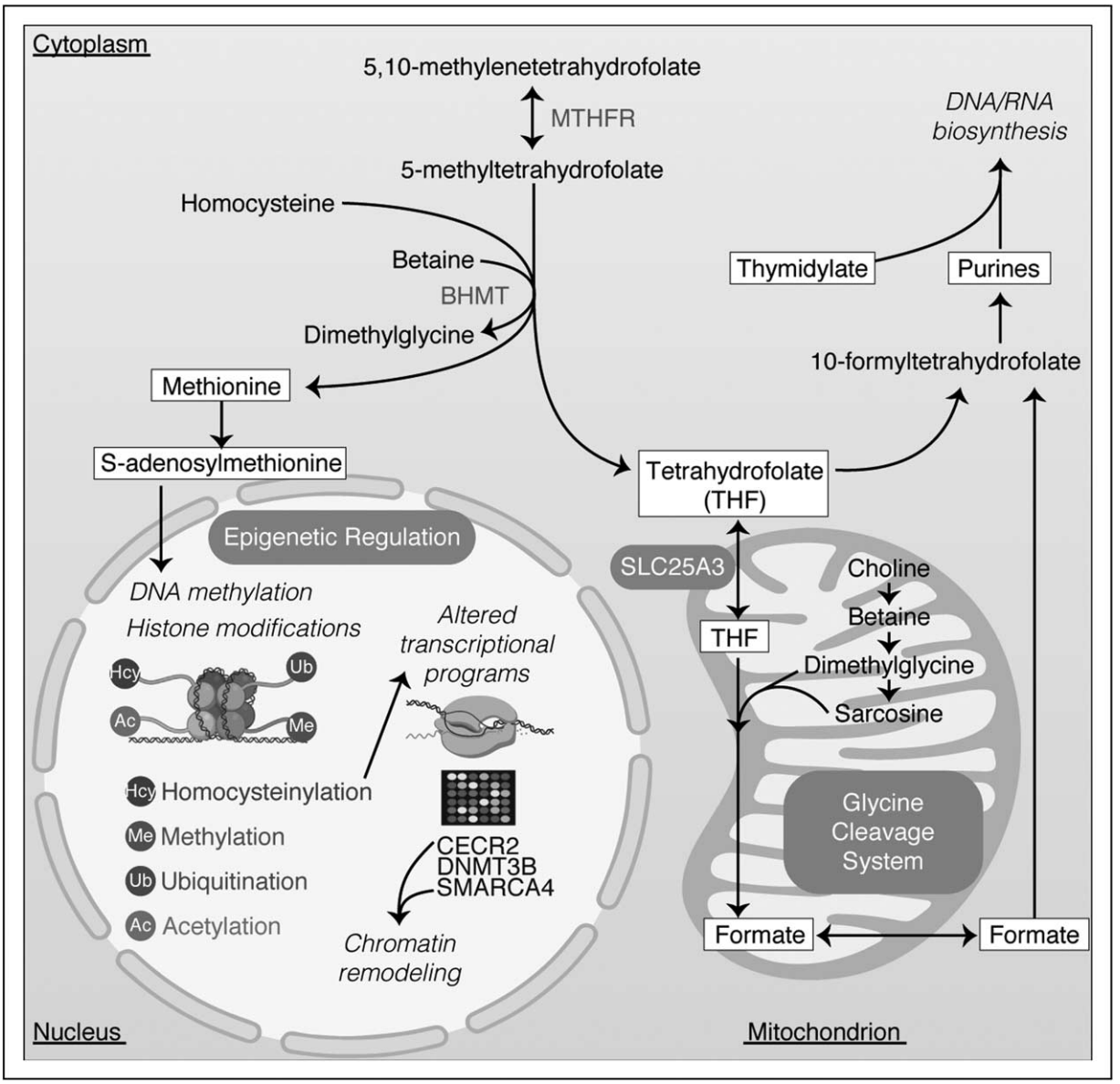

FIGURE 2. Key processes in folate one-carbon metabolism. Compartmentalized reactions in the cytosol and mitochondrion are depicted. Variants in several genes regulating these steps have been linked with neural tube defect risk and various epigenetic modifications – including DNA methylation requiring the primary methyl donor, S-adenosylmethionine 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 three beta; SMARCA4, SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily A, member 4.

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 with sex-matched 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 homocysteinylation 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 three (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 three-dimensional 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, three-dimensional 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.

Acknowledgements

We would like to thank Christina Usher for assistance with the figure design.

Financial support and sponsorship

The current study was supported by P01HD067244 (M.E.R.) and T32HD060600 (P.W.).

Conflicts of interest

There are no conflicts of interest.

REFERENCES AND RECOMMENDED READING

Papers of particular interest, published within the annual period of review, have been highlighted as:

- ▪ of special interest
- ▪▪ of outstanding interest

1. Copp AJ, Stanier P, Greene NDE. Genetic basis of neural tube defects. Textbook of pediatric neurosurgery. Cham: Springer; 2017; 1–28.
2. Rasmussen SA, Frias JL. Genetics of syndromic neural tube defects. In: Neural tube defects: from origin to treatment. Oxford: Oxford University Press; 2016. pp. 185–197.
3. López-Escobar B, Caro-Vega JM, Vijayraghavan DS, *et al.* The noncanonical WNT-PCP pathway shapes the mouse caudal neural plate. Development 2018; 145:1–15.
4. Juriloff DM, Harris MJ. A consideration of the evidence that genetic defects in planar cell polarity contribute to the etiology of human neural tube defects. Birth Defects Res A Clin Mol Teratol 2012; 94:824–840.
5. Harris MJ, Juriloff DM. An update to the list of mouse mutants with neural tube closure defects and advances toward a complete genetic perspective of neural tube closure. Birth Defects Res A Clin Mol Teratol 2010; 88:653–669.
6. Murdoch JN, Copp AJ. The relationship between sonic hedgehog signaling, cilia, and neural tube defects. Birth Defects Res A Clin Mol Teratol 2010; 88:633–652.
7. Wang Z, Wang L, Shangguan S, *et al.* Association between PTCH1 polymorphisms and risk of neural tube defects in a Chinese population. Birth Defects Res A Clin Mol Teratol 2013; 97:409–415.
8. Wu J, Lu X, Wang Z, *et al.* Association between PKA gene polymorphism and NTDs in high risk Chinese population in Shanxi. Int J Clin Exp Pathol 2013; 6:2968–2974.
9. ▪▪ Kim SE, Lei Y, Hwang SH, *et al.* Dominant negative GPR161 rare variants are risk factors of human spina bifida. Hum Mol Genet 2019; 28:200–208.

The article corroborates sound mouse data with human sequencing data in studying the impact variants in *GPR161* have on neural tube defect (NTD) risk through dysregulated SHH and WNT signaling.

10. Li BI, Matteson PG, Ababon MF, *et al.* The orphan GPCR, Gpr161, regulates the retinoic acid and canonical Wnt pathways during neurulation. Dev Biol 2015; 402:17–31.
11. Mukhopadhyay S, Wen X, Ratti N, *et al.* The ciliary G-protein-coupled receptor Gpr161 negatively regulates the Sonic hedgehog pathway via cAMP signaling. Cell 2013; 152:210–223.
12. Nikolopoulou E, Galea GL, Rolo A, *et al.* Neural tube closure: cellular, molecular and biomechanical mechanisms. Development 2017; 144:552–566.
13. Rosso SB, Sussman D, Wynshaw-Boris A, *et al.* Wnt signaling through Dishevelled, Rac and JNK regulates dendritic development. Nat Neurosci 2005; 8:34–42.
14. Shafer B, Onishi K, Lo C, *et al.* Vangl2 promotes Wnt/planar cell polarity-like signaling by antagonizing Dvl1-mediated feedback inhibition in growth cone guidance. Dev Cell 2011; 20:177–191. Erratum in: Dev Cell. 2011 Mar 15;20(3):407. PubMed PMID: 21316586.

15. Shi Z, Yang X, Li BB, *et al.* Novel mutation of LRP6 identified in chinese han population links canonical WNT signaling to neural tube defects. Birth Defects Res 2018; 110:63–71.
16. Lei Y, Kim SE, Chen Z, *et al.* Variants identified in PTK7 associated with neural tube defects. Mol Genet Genomic Med 2019; 7:e00584.
17. ▪ Lemay P, De Marco P, Traverso M, *et al.* Whole exome sequencing identifies novel predisposing genes in neural tube defects. Mol Genet Genomic Med 2019; 7:e00467.

The article illustrates the utility of whole exome sequencing in *de novo* and inherited genetic analyses and the discovery of novel NTD risk genes.

18. Beaumont M, Akloul L, Carré W, *et al.* Targeted panel sequencing establishes the implication of planar cell polarity pathway and involves new candidate genes in neural tube defect disorders. Hum Genet 2019; 138:363–374.
19. Yu Q, Lin B, Xie S, *et al.* A homozygous mutation p.Arg2167Trp in FREM2 causes isolated cryptophthalmos. Hum Mol Genet 2018; 27:2357–2366.
20. Jordan VK, Beck TF, Hernandez-Garcia A, *et al.* The role of FREM2 and FRAS1 in the development of congenital diaphragmatic hernia. Hum Mol Genet 2018; 27:2064–2075.
21. Tukachinsky H, Kuzmickas RP, Jao CY, *et al.* Dispatched and scube mediate the efficient secretion of the cholesterol-modified hedgehog ligand. Cell Rep 2012; 2:308–320.
22. Onishi K, Zou Y. Sonic Hedgehog switches on Wnt/planar cell polarity signaling in commissural axon growth cones by reducing levels of Shisa2. Elife 2017; 6:1–20.
23. ▪ Wang L, Xiao Y, Tian T, *et al.* Digenic variants of planar cell polarity genes in human neural tube defect patients. Mol Genet Metab 2018; 124:94–100.

This study shows strong evidence and support for the influence of digenic interactions toward NTD manifestation.

24. ▪▪ Delbridge ARD, Kueh AJ, Ke F, *et al.* Loss of p53 causes stochastic aberrant X-chromosome inactivation and female-specific neural tube defects. Cell Rep 2019; 27:442–454.e5.

The study provides robust data suggesting partial X inactivation as a mechanism in female-specific NTDs in mice and propose that loss of p53-induced apoptosis predisposes to NTDs.

25. Spellicy CJ, Norris J, Bend R, *et al.* Key apoptotic genes APAF1 and CASP9 implicated in recurrent folate-resistant neural tube defects. Eur J Hum Genet 2018; 26:420–427.
26. ▪▪ Chen Z, Lei Y, Zheng Y, *et al.* Threshold for neural tube defect risk by accumulated singleton loss-of-function variants. Cell Res 2018; 28:1039–1041.

The computational approaches in this study are comprised of unbiased genomic analyses and present a strong framework for a polygenic view of NTD risk.

27. Spielmann M, Lupiáñez DG, Mundlos S. Structural variation in the 3D genome. Nat Rev Genet 2018; 19:453–467.
28. Chiang C, Scott AJ, Davis JR, *et al.* The impact of structural variation on human gene expression. Nat Genet 2017; 49:692–699.
29. Kosugi S, Momozawa Y, Liu X, *et al.* Comprehensive evaluation of structural variation detection algorithms for whole genome sequencing. Genome Biol 2019; 20:117.
30. Chen X, Shen Y, Gao Y, *et al.* Detection of copy number variants reveals association of cilia genes with neural tube defects. PLoS One 2013; 8:e54492.
31. Goumy C, Gay-Bellile M, Eymard-Pierre E, *et al.* De novo 2q36.1q36.3 interstitial deletion involving the PAX3 and EPHA4 genes in a fetus with spina bifida and cleft palate. Birth Defects Res A Clin Mol Teratol 2014; 100:507–511.
32. ▪ Hofmeister W, Pettersson M, Kurtoglu D, *et al.* Targeted copy number screening highlights an intragenic deletion of WDR63 as the likely cause of human occipital encephalocele and abnormal CNS development in zebrafish. Hum Mutat 2018; 39:495–505.

This study supports their copy number screening findings with whole genome sequencing and CRISPR-Cas9-mediated functional modeling in zebrafish.

33. Furey CG, Choi J, Jin SC, *et al.* De novo mutation in genes regulating neural stem cell fate in human congenital hydrocephalus. Neuron 2018; 99:302–314.e4.
34. Au KS, Findley TO, Northrup H. Finding the genetic mechanisms of folate deficiency and neural tube defects-leaving no stone unturned. Am J Med Genet A 2017; 173:3042–3057.
35. Martiniova L, Field MS, Finkelstein JL, *et al.* Maternal dietary uridine causes, and deoxyuridine prevents, neural tube closure defects in a mouse model of folate-responsive neural tube defects. Am J Clin Nutr 2015; 101:860–869.
36. Findley TO, Tenpenny JC, O'Byrne MR, *et al.* Mutations in folate transporter genes and risk for human myelomeningocele. Am J Med Genet A 2017; 173:2973–2984.
37. Cai CQ, Fang YL, Shu JB, *et al.* Association of neural tube defects with maternal alterations and genetic polymorphisms in one-carbon metabolic pathway. Ital J Pediatr 2019; 45:37.
38. ▪ Kim J, Lei Y, Guo J, *et al.* Formate rescues neural tube defects caused by mutations in Slc25a32. Proc Natl Acad Sci U S A 2018; 115:4690–4695.

The artice provides interesting data on mitochondrial folate transporter variants in mice and partial rescue with formate supplementation.

39. Zhang H, Guo Y, Gu H, *et al.* TRIM4 is associated with neural tube defects based on genome-wide DNA methylation analysis. Clin Epigenetics 2019; 11:17.
40. ▪ Zhang Q, Bai B, Mei X, *et al.* Elevated H3K79 homocysteinylation causes abnormal gene expression during neural development and subsequent neural tube defects. Nat Commun 2018; 9:3436.

The study shows strong evidence for homocysteinylation as a histone modification and its subsequent potential influence on NTD risk.

41. Yang M, Li W, Wan Z, *et al.* Elevated homocysteine levels in mothers with neural tube defects: a systematic review and meta-analysis. J Matern Fetal Neonatal Med 2017; 30:2051–2057.
42. Lin Y, Yu J, Wu J, *et al.* Abnormal level of CUL4B-mediated histone H2A ubiquitination causes disruptive HOX gene expression. Epigenetics Chromatin 2019; 12:22.
43. Valensisi C, Andrus C, Buckberry S, *et al.* Epigenomic landscapes of hESC-derived neural rosettes: modeling neural tube formation and diseases. Cell Rep 2017; 20:1448–1462.