REVIEW

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Unraveling the complex genetics of neural tube defects: From biological models to human genomics and back

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Abstract

Neural tube defects (NTDs) are a classic example of preventable birth defects for which there is a proven-effective intervention, folic acid (FA); however, further methods of prevention remain unrealized. In the decades following implementation of FA nutritional fortification programs throughout at least 87 nations, it has become apparent that not all NTDs can be prevented by FA. In the United States, FA fortification only reduced NTD rates by 28–35% (Williams et al., 2015). As such, it is imperative that further work is performed to understand the risk factors associated with NTDs and their underlying mechanisms so that alternative prevention strategies can be developed. However, this is complicated by the sheer number of genes associated with neural tube development, the heterogeneity of observable phenotypes in human cases, the rareness of the disease, and the myriad of environmental factors associated with NTD risk. Given the complex genetic architecture underlying NTD pathology and the way in which that architecture interacts dynamically with environmental factors, further prevention initiatives will undoubtedly require precision medicine strategies that utilize the power of human genomics and modern tools for assessing genetic risk factors. Herein, we review recent advances in genomic strategies for discovering genetic variants associated with these defects, and new ways in which biological models, such as mice and cell culture-derived organoids, are leveraged to assess mechanistic functionality, the way these variants interact with other genetic or environmental factors, and their ultimate contribution to human NTD risk.

KEYWORDS
anencephaly, genomics, in vitro models, mouse models, Myelomeningocele, spina bifida

1 | INTRODUCTION

Neural tube defects arise early in human embryogenesis when the neural tube, the precursor to the brain and spinal cord, fails to properly close during neurulation. Failure to complete or maintain posterior closure causes myelomeningocele or spina bifida (Lee & Gleeson, 2020). Incomplete cranial closure results in anencephaly (Avagliano et al., 2019), while failed closure along the entire neural tube is referred to as craniorachischisis. Fetuses with anencephaly and craniorachischisis die in utero or are stillborn, while most spina bifida (SB) patients can survive well into adulthood, yet are likely to suffer from severe, life-long disabilities, and are at risk for psychosocial maladjustment. NTD birth prevalence ranges from one in 3,000 to one in 100, depending on global location, making NTDs the second most common structural malformation in humans (Ross, Mason, & Finnell, 2017). Despite their relative frequency of NTDs, treatment paradigms for SB have not evolved significantly in over 30 years, and we are only capable of imperfectly managing the spectrum of

comorbidities associated with permanent and irreversible neurological damage resulting from these defects. As such, the efforts of public health research have generally focused on identifying the underlying risk factors and developing prevention strategies.

It is generally accepted that the origins of NTDs are multifactorial, in which both environmental (Brender, Suarez, Hendricks, Baetz, & Larsen, 2002; Brender & Suarez, 1990; Cabrera et al., 2019; Carmichael & Shaw, 2000; Carmichael, Shaw, Selvin, & Schaffer, 2003; Farley, Hambidge, & Daley, 2002; Lundberg, Wing, Xiong, Zhao, & Finnell, 2003; Sever, 1995; Shaw, Todoroff, et al., 1999; Shaw, Todoroff, Velie, & Lammer, 1998; Shaw, Velie, & Schaffer, 1996; Shaw, Wasserman, O'Malley, Nelson, & Jackson, 1999; Suarez, Cardarelli, & Hendricks, 2003; Waller et al., 2007; Wasserman, Shaw, Selvin, Gould, & Syme, 1998; White, Cohen, Sherman, & McCurdy, 1988) and genetic interactions contribute to the malformation (Figure 1) (Blom, Shaw, den Heijer, & Finnell, 2006; Campbell, Dayton, & Sohal, 1986; Shaw, Jensvold, Wasserman, & Lammer, 1994; Steele et al., 2019). Substantial evidence suggests that nonsyndromic NTDs are rarely monogenic disorders, and multiple pathological gene variants contribute to most human cases of failed neural tube closure (Chen, Lei, Cao, et al., 2018;

Ross, 2010; Wallingford, Niswander, Shaw, & Finnell, 2013). Indeed, the genetic heritability of human NTDs approximates 70% (Jorde, Fineman, & Martin, 1983). Despite this genetic complexity, periconceptional use of folic acid (FA) prevents a significant percentage of the population's burden of NTDs (Berry et al., 1999; Canfield et al., 2005; Czeizel & Dudas, 1992; De Wals et al., 2007; Honein, Paulozzi, Mathews, Erickson, & Wong, 2001; MRC Vitamin Study Group, 1991; Williams et al., 2002). Nevertheless, significant numbers of NTDs are not preventable by FA supplementation, and these FA-resistant NTDs occur at an apparent baseline rate of 1 per 2,000 live births (Heseker, Mason, Selhub, Rosenberg, & Jacques, 2009). Despite the success of national folate fortification programs in reducing the overall prevalence of these malformations, complete prevention remains unrealized, and these defects still affect up to 2,300 US births annually and some 166,000 SB patients currently live in the US, more than half of whom are now adults. Given this reality, more targeted interventions developed through precision medicine approaches may prove effective at further reducing NTD prevalence and developing such approaches will necessarily leverage the power of novel genetic models and human genomic initiatives.
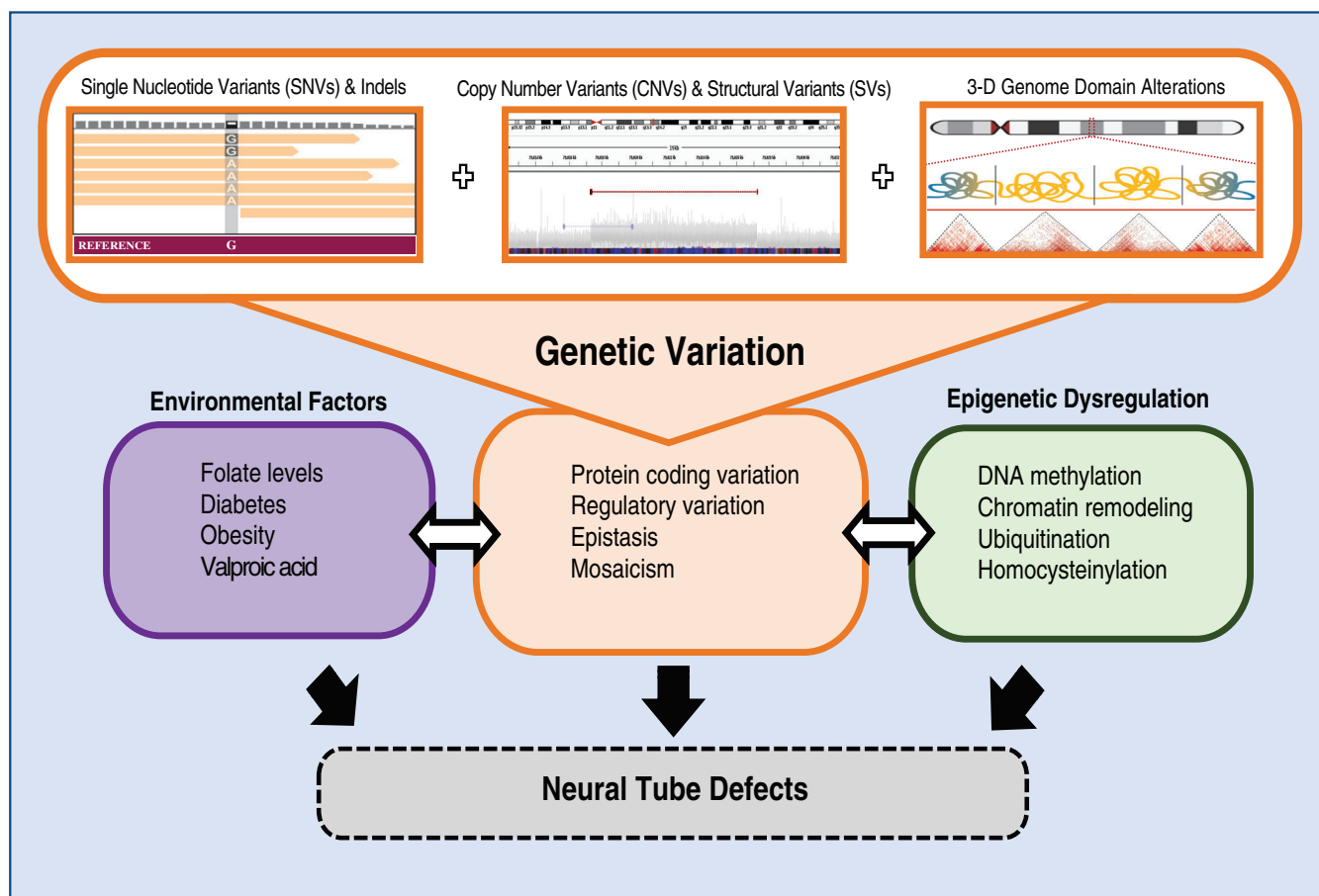

FIGURE 1 Potential mechanisms underlying NTD pathophysiology. Genetic variants contributing to NTDs may include single nucleotide variants (SNVs) and indels as well as copy number variants (CNVs) and structural variants (SVs). These may disrupt protein-coding regions of the genome or noncoding regions, which may dysregulate gene regulatory networks. NTD risk is comprised of the interplay between these genetic variants with environmental and epigenetic factors

In this review, we discuss advances in mouse model studies of genetic and biological mechanisms leading to NTDs and how these insights have influenced investigations of genetic factors contributing to NTDs in humans. Animal modeling is further illuminating mechanisms that underlie comorbidities of SB. These recent technical innovations in genomics and biological insights offer promise for developing novel and effective intervention strategies and therapeutics for SB patients.

2 | UTILIZING MOUSE KNOCKOUT MODELS TO ESTABLISH CANDIDATE NTD GENES

As an NTD model organism, the mouse has few rivals, as there are at least 250 NTD-causing mouse genetic mutations for hypothesis-generating investigations (Eppig et al., 2005; Harris & Juriloff, 2010), which underscores the genetic complexity underpinning these structural birth defects. The publications of mouse NTD models extend over three decades and are too extensive to comprehensively review here. However, studies that have, in aggregate, illuminated pathways that have guided human investigations deserve note. A number of clinical case–control studies focusing on one, or at most a few candidate genes, have found alleles suggesting an association with increased NTD risk. Many of the early studies were based on candidate genes identified in naturally occurring mouse models presenting with NTD phenotypes. The advent of gene editing and creation of transgenic mice with relative ease and efficiency has been critical to accelerating this gene discovery effort. The recognition that folic acid was a significant modifier of NTD prevalence (MRC Vitamin Study Group, 1991) drew attention to genes involved in the enzymatic biotransformation of folic acid as candidate genes for human NTDs. These models included gene inactivation for _Mthfr_, _Mtrr_, _CBS_, _Mthfd1_, _Shmt1_, _Bhmt_ and many others (Beaudin & Stover, 2007, 2009).

The Rozen Laboratory inactivated the _Mthfr_ gene and the nullizygous mutant mice presented with hyperhomocysteinemia and altered DNA methylation (Chen et al., 2001). Although these mice displayed an increased rate of resorptions and cardiac defects, the null mice lacked an NTD phenotype (Chen et al., 2001; Li et al., 2005). The frequency of neurological disorders in the _Mthfr_ mutant mice could be increased either by altering the genetic background of the null allele (Lawrance et al., 2011), or by administering human and murine teratogens such as valproic acid (Roy et al., 2008). Many subsequent studies have confirmed the importance of the genetic background on the penetrance of NTDs in individual mutant mouse lines and modifier loci that have been mapped in several lines (Juriloff et al., 2001; Korstanje et al., 2008). In parallel clinical studies, the thermolabile variant (C677T) in the _MTHFR_ gene was proposed to increase the NTD risk (Shields et al., 1999) in some, but not all human NTD cohorts. This was one of many candidate genes selected either based on mouse studies or initial trends gleaned from human genome sequences based on unfortunately small case numbers.

Rather than focusing on one-carbon metabolism (OCM) enzymes, other efforts pursued the idea that perhaps the link to NTDs was not so much insufficient dietary folate, or a defect in a critical metabolic enzyme, but rather that folate molecules were not being transported to the developing embryo due to defects in folate transport proteins (Finnell, Greer, Barber, & Piedrahita, 1998). By using gene targeting approaches to knockout the mouse ortholog (_Folr1_) of human folate receptor alpha gene (_hFRα_), highly penetrant anterior NTDs in the null embryos were observed (Barber, Lammer, Shaw, Greer, & Finnell, 1999; Piedrahita et al., 1999). The folate receptor 1 (_Folr1_) mutant mice have folate responsive NTDs (Piedrahita et al., 1999) and the folate-responsive gene expression profiles in the rostral portion of the neural tube of the mutant mice have been well described (Spiegelstein, Cabrera, Bozinov, Wlodarczyk, & Finnell, 2004). Previous work has emphasized the importance of considering the genetic background of mouse strains used in gene editing experiments, as it is likely to influence the development of diseases and congenital anomalies, including NTDs (Beck, 1999; Detrait et al., 2005). Leduc et al. (2017) demonstrated that background-dependent chromosomal linkage in _Cecr2_ mutants can impact the penetrance of NTD candidate genes.

To test the hypothesis that folate responsive defects are also largely influenced by gene–gene and gene-nutrient interactions, heterozygous _Folr1+/−_ mice were crossed onto _SWV-Fnn_ and _LM/Bc-Fnn_ background mouse strains utilizing a speed congenic approach (Grove, Eckardt, & McLaughlin, 2016). Cabrera et al. (2019) reported that while both strains demonstrated a dose response to folinic acid supplementation, mutant mice on the _SWV_ background had a significantly lesser reduction in NTD frequency at equal concentrations of folinic acid when compared to mutants on the _LM/Bc_ genetic background. It is apparent that modifier genes within the _LM/Bc_ strain contribute to the increased sensitivity of the _Folr1_ null embryos. Several examples show strain-dependent sensitivity to environmental factors observed in mouse NTD models, among them hyperthermia-induced NTDs. Pregnant dams were exposed for 10 min to a hyperthermic water bath during the critical window of neural tube closure to simulate maternal fever during pregnancy or exposure to occupational or environmental heat sources, such as dry cleaning facilities or recreational hot tubs. Swiss-Vancouver (_SWV_) offspring presented with a 44.3% occurrence of exencephaly, while less than 14% were affected in the other four strains tested (Finnell, Moon, Abbott, Golden, & Chernoff, 1986). Another example is exposure to the antiepileptic drug, valproic acid (VPA). SWV mice are similarly more sensitive to VPA than other highly inbred strains. For example, _DBA/2J_ mice appear entirely resistant to VPA, while about 20% of _LM/Bc_ and _C57/BL6_ embryos develop NTDs after VPA exposure at a particular dose during the critical window of neural tube closure. _SWV_ mouse embryos had the highest susceptibility, displaying a 35% occurrence of NTDs (Finnell, Bennett, Karras, & Mohl, 1988; Lundberg et al., 2004). This sensitivity has gradually been mapped to a specific locus in _SWV_ as genetic sequencing technologies have progressed over the years (Beck, 1999; Beck, 2001; Finnell et al., 1988; Finnell et al., 2000; Finnell, Wlodarczyk, Craig, Piedrahita, & Bennett, 1997;

Lundberg et al., 2004). Thus, sensitivity to NTDs in some strains suggests that there are permissive genetic backgrounds rendering individuals more susceptible for a higher individual risk for NTDs given particular environmental exposures. These permissive backgrounds may result from multigenic variant interactions across the global genomic landscape or single gene variants in critical pathways. Mouse models have shown that genetic impairment of folate transport (*Folr1* deficiency) or folate metabolism (*Mthfr* deficiency) increase NTD risk under conditions of maternal hyperglycemia in mouse models of diabetes (Lopez-Escobar et al., 2019).

Beyond an initial focus on OCM pathway genes, surprising connections between naturally occurring mouse NTD mutations and folate were exemplified in the Crooked tail mouse, for which folate supplementation protected homozygous animals from NTDs (Carter, Ulrich, Oofuji, Williams, & Ross, 1999). Positional cloning identified the mutation as a missense variant in the Lrp6 co-receptor needed for WNT signal transduction (Carter et al., 2005) and later work showed that while supplemental folate is protective against NTDs for $Lrp6^{Cd/Cd}$ mutants, excessive levels exacerbate embryopathy in the $Lrp6^{-/-}$, null, mice (Gray et al., 2010). Moreover, although both $Lrp6^{Cd/Cd}$ and $Lrp6^{-/-}$ mutant cells display reduced canonical WNT signaling, the non-canonical pathway is upregulated only in $Lrp6^{Cd/Cd}$ animals, evidenced by elevated RhoA-GTP activation and rescue from NTDs by ROCK inhibition in mouse embryo culture (Gray et al., 2013). Thus, not only is the gene association with NTDs important, but also the functional consequence of the mutation (gain or loss) is critical to predicting genetic interactions with the environment.

3 | PINPOINTING RELEVANT NTD PATHWAYS AND ASSOCIATED CHALLENGES

Investigators interested in developing murine NTD models were well aware of the role that Wnt signaling plays in neural development during early embryonic stages (Miller & McCrea, 2010). The planar cell polarity (PCP) (Curtin et al., 2003) genes in the non-canonical Wnt signaling pathway quickly became the focus of many genomic investigations for developing mouse models of neural tube closure defects. Multiple mouse PCP genes (*Fz*, *Dvl*, *Vangl* or *Celsr*) (Juriloff & Harris, 2012; Wallingford, 2012) result in an NTD phenotype when inactivated. Most of the NTDs associated with altered PCP pathway genes give rise to mice with craniorachischisis, secondary to defects in convergent extension during early embryogenesis (Wang, Hamblet, et al., 2006; Ybot-Gonzalez et al., 2007). By inactivating PCP genes, cells fail to interdigitate mediolaterally, and the resulting excessively wide neural plate tissue cannot properly fuse (Wallingford & Harland, 2001, 2002). Loss of function (LoF) alleles of the core components of the PCP pathway, including *Celsr1* (Curtin et al., 2003) and *Vangl2* (Greene, Gerrelli, Van Straaten, & Copp, 1998) result in craniorachischisis in mice. In addition to monogenic causes, double but not single knockouts of *Fzd3* together with *Fzd6* (Wang, Guo, & Nathans, 2006), as well as Dvls (Etheridge et al., 2008) also produce a craniorachischisis phenotype.

In addition to the many well described important developmental gene pathways, it is also important to interrogate mouse genes not intuitively relevant to neural tube closure. Such genes are often found by serendipity and will require functional analyses in models to document a potential value as human candidate NTD genes. One such example is *Fkbp8* (also known as *Fkbp38*) (Bai et al., 2007; Lam, Martin, & Wiederrecht, 1995), a member of the FK506-binding protein family that functions in cell size regulation (Rosner, Hofer, Kubista, & Hengstschläger, 2003) and was noted to be the cellular inhibitory target of rapamycin (mTOR) signaling (Bai et al., 2007). FKBP8 is also an important inhibitor of apoptosis, through anchoring the anti-apoptotic proteins BCL2 and BCLXL to the mitochondrial membranes (Shirane & Nakayama, 2004). During morphogenesis, *Fkbp8* controls neural cell fate through antagonism of SHH signaling, which is critical for proper neural tube closure (Bulgakov, Eggenschwiler, Hong, Anderson, & Li, 2004; Cho, Ko, & Eggenschwiler, 2008; Fong et al., 2003). *Fkbp8* also contains functional tetratricopeptide-repeat (TPR) domains, a leucine zipper repeat, and a C-terminal transmembrane (TM) domain (De Cicco, Milroy, & Dames, 2018). The C-terminal TM domain facilitates FKBP8 protein anchorage to mitochondrial membranes (Shirane & Nakayama, 2003). Additionally, Fkbp8 is a co-chaperone involved in the regulation of adenylyl cyclase trafficking to cilia as well as to various heat shock proteins (Walker, Atanasiu, Lam, & Shrier, 2007; Wang, Venable, et al., 2006).

Fkbp8 mouse mutants have an isolated and completely penetrant SB, which are both folic acid and inositol-resistant (Wong et al., 2008). Unlike other NTD mouse mutants, the *Fkbp8* nulls are not embryo lethal, enabling one to study both the evolution of the lesion from conception, past parturition into early life, as well as SB-associated comorbidities, such as urinary and fecal incontinence (Verhoef et al., 2004). The knockout mice share many features with human SB, including a dysplastic spinal cord, open neural canal and paralysis of limbs below the defect. There is increased apoptosis in the posterior neural tube, and expression of neural tube patterning genes is perturbed, suggesting that FKBP8 activity is critical in establishing the dorso-ventral patterning of the mouse neural tube. RNA sequencing on anterior and posterior tissues isolated from *Fkbp8*−/− and wild-type mice at E9.5 and E10.5 showed that *Fkbp8*−/− embryos have an abnormal expression profile within tissues harvested at posterior sites, thus leading to a posterior NTD. We found that *Fkbp8* knockout mouse embryos display abnormal *Wnt3a* and *Nkx2.9* expression during early stage neural tube development, perhaps accounting for the caudal location of its NTDs (Wong et al., 2008). Tsurubuchi et al. (2014) used neural stem cells from *FKBP8*−/− embryos and observed increased *Noggin* gene expression along with decreased *Msx2* expression and premature differentiation—neurogenesis. The authors considered that the inability of folic acid to protect *Fkbp8* nullizygous embryos from failed neural tube closure could be attributed in part to the increased noggin expression. In the presence of folic acid, noggin expression increases ventralization of the neural tube and further compromises proper fusion. These findings provide evidence that functional variants of *FKBP8* are risk factors for SB and suggest a novel mechanism by which *Fkbp8* mutations specifically cause SB in mice.

4 | FROM MOUSE MODELS TO HUMAN GENETICS OF NTDS

The translation of these insights from mouse models to assessment of human genetic contributions to NTDs have been pursued for several decades. While putative risk genes and alleles have been identified, it has not been possible to define patterns of multiple gene interactions that determine individual risk. If permissive genetic backgrounds could be identified, they could be compensated for by providing evidence-based counseling to at risk families such that pregnancy outcomes can be optimized. A number of challenges derive from the complexity of NTD genetics and the rarity of the condition compared to more prevalent complex disorders such as Type II Diabetes, Alzheimer's Dementia or Autism Spectrum Disorder (Table 1). Several arguments favor the view that genetic contributions to NTDs with the largest effect size are likely to be rare variants, including the very fact that NTDs have an estimated base prevalence of folate resistant NTDs of 1 per 2,000 births (Heseker et al., 2009), rather than highly prevalent diseases associated with common genetic variants (i.e., minor allele frequency or MAF >1–5%) like diabetes and Alzheimer's. Not only do rare variants carry greater effect sizes than common variants (Park et al., 2011), but for diseases that are under strong negative selection like NTDs, restricting the analyses to variants that are most rare has revealed strong enrichments for causal mutations (Bomba, Walter, & Soranzo, 2017; Petrovski et al., 2017).

TABLE 1 Recent point prevalence estimates of select complex genetic disorders

	United States	Worldwide
Autism spectrum disorder (ASD)	1 in 54	1 in 160
Type 2 diabetes (T2D)	1 in 10	1 in 17
Asthma	1 in 13	1 in 26
Alzheimer's disease (AD)[a]	1 in 9	1 in 14
Congenital heart Defects (CHD)[b]	1 in 125	1 in 100
Orofacial clefts (OFC)[b]	1 in 940	1 in 679
Spina bifida (SB)[b]	1 in 2,750	1 in 100 to 1 in 3,000

Source: ASD: https://www.cdc.gov/ncbddd/autism/data.html, https://www.who.int/news-room/fact-sheets/detail/autism-spectrum-disorders; T2D: https://www.cdc.gov/diabetes/basics/type2.html, https://www.who.int/news-room/fact-sheets/detail/diabetes; Asthma: https://www.cdc.gov/asthma/asthmadata.htm, https://www.who.int/news-room/q-a-detail/chronic-respiratory-diseases-asthma; AD: https://www.cdc.gov/aging/aginginfo/alzheimers.htm, https://www.who.int/news-room/fact-sheets/detail/dementia; CHD: GBD 2017 Congenital Heart Disease Collaborators (2020). https://doi.org/10.1016/S2352-4642(19)30402-X; OFC: https://www.nidcr.nih.gov/research/data-statistics/craniofacial-birth-defects/prevalence, Panamonta, Pradubwong, Panamonta, and Chowchuen (2015); SB: https://www.cdc.gov/ncbddd/spinabifida/data.html, Atta et al. (2016). https://doi.org/10.2105/AJPH.2015.302902.
[a]65 years and older.
[b]Reported as birth prevalence.

Indeed, multiple reported human gene associations with NTDs have used some version of a search among candidate genes for predicted deleterious rare variants. This has included direct targeted sequencing of a single gene or a selected list of candidate genes. Although the initial studies of small NTD cohorts failed to identify a link between variants in the human folate receptor gene (hFRα) and NTD prevalence (Barber et al., 1998; Barber et al., 1999; Trembath et al., 1999), subsequent studies of human folate transport genes described 12 novel variants in *FOLR1*, *FOLR2*, and *FOLR3* (Findley et al., 2017). This included four large insertion deletion variants in *FOLR3* as well as a single stop gain variant. Their findings support the utility of using murine NTD genes involved in folate transport and investigating their impact on human susceptibility to NTDs. Based on animal studies like those discussed above, robust efforts in ever larger human NTD cohorts have now identified mutations in several PCP genes (Chen, Lei, Cao, et al., 2018; Kibar et al., 2007; Lei et al., 2019; Tian, Lei, et al., 2020; Wang, Marco, Capra, & Kibar, 2019). As the databases grow with ever more genomic studies of complex birth defects, it is important to mine this genotype/phenotype data from animal studies for genes such as those in the PCP/Wnt pathway that share specific developmental functions as a means of identifying candidate genes to explain the population burden of human NTDs.

The candidate gene approach to find human counterparts of NTD associated mutations in mouse continues to yield translational insight. For example, the first paper interrogating *FKBP8* gene variants in human NTDs was only recently published (Tian, Cao, et al., 2020). These investigators used Sanger sequencing on genomic DNA samples from 472 SB and 565 control samples and identified five rare (MAF ≤ 0.001) deleterious variants in SB patients. Sequencing of the control cohort yielded no rare deleterious variants. One variant, p.Glu140*, was found to interfere with FKBP8 localization to the mitochondria as well as truncate the FKBP8 protein. The truncated protein was unable to interact with BCL2, resulting in an increase in cellular apoptosis. The p.Ser3Leu, p.Lys315Asn and p.Ala292Ser variants decreased the absolute concentration of the FKBP8 protein, while the p.Lys315Asn variant was shown to produce increased cellular apoptosis (Tian, Cao, et al., 2020). These functional assays, coupled with statistical argument for NTD association, remain a critically important avenue for the identification of relevant deleterious mutations.

A more recent strategy toward defining genetic contributions to NTD risk has employed whole exome sequencing (WES) for searches of protein coding regions of candidate genes or pathways coupled with gene collapsing strategies to detect putative NTD risk genes and variants. Examples include studies that examined 511 NTD cases for exon variants in glucose homeostasis/oxidative stress and OCM network (Hillman et al., 2020) or WNT signaling pathway genes (Hebert et al., 2020). Another study looked at the association between rare variants in specific functional pathways and in multiple sub-phenotypes of human neural tube defects (Zou et al., 2020). That study included 355 NTD cases manifesting a range of structural defects, including craniorachischisis, anencephaly, encephalocele, spina bifida aperta, spina bifida cystica and spina bifida occulta. Although the investigators reported potentially damaging rare variants

in genes functioning in different pathways with different NTD phenotypes (e.g., chromatin modifications in anencephaly but not SB), this preliminary work paves the way for refined phenotyping in NTD cohorts. It also underscores the utility of investigating rare variants across all NTDs.

5 | TOWARD GENOME-WIDE, UNTARGETED SEARCHES FOR HUMAN MUTATIONS ASSOCIATED WITH NTDS

It is increasingly apparent that if we are ever to understand the gene–gene interactions that interface with epigenetic and environmental influences contributing to human NTD risk, it will be necessary to devise means for looking at (a) the entire genome, not just the 2% represented by protein coding sequences, and (b) searching the genome in an untargeted manner. Whole genome sequencing (WGS) data provide an opportunity to interrogate DNA variants genome-wide and detect not only single nucleotide variants (SNVs) and indels, but also structural variants (SVs), whose breakpoints may fall in intergenic regions. Despite some known chromosomal abnormalities associated with NTDs, the effects of SVs on NTD susceptibility are not well understood. Rare SVs impact a greater number of nucleotides than rare SNVs and studies have shown that SVs can have numerous functional consequences, including disrupting the structure and dosage of genes (Ho, Urban, & Mills, 2020). Recent publications have also suggested that rare SVs may be causal at 3.5–6.8% of expression of quantitative trait loci (eQTL) sites (Chiang et al., 2017), which is much higher than previous estimates. Moreover, SVs are increasingly implicated in the genetic risk of several complex genetic disorders including schizophrenia (Caseras et al., 2021) and autism (Pavlides et al., 2016).

The difficulty with candidate gene or pathway interrogations is that the approach is inherently biased—one typically finds what one looks for. However, every genome differs from the reference on average at 4–5 million sites. Even if limited to rare variants with minor allele frequency (MAF) < 0.5% (0.005), the average genome contains 40,000–200,000 variants with respect to available databases. Our genome-wide investigations of exonic sequences established a threshold model of ultra-rare singleton loss of function variant (SloV) burden, replicated in three different populations, that found on average 9 SloVs per individual with SB, compared to 15 SloVs per genome in individuals with anencephaly (Chen, Lei, Zheng, et al., 2018). We recently reported an initial investigation of SVs through analysis of WGS from two ancestry balanced case–control cohorts (Wolujewicz et al., 2021). The study used a computational ensemble approach to identify copy number variants (CNV) with a MAF < 0.01%, relying on CNVs that met "high confidence" criteria. We found a statistically significant burden of rare gene disrupting CNVs among SB cases compared to controls, underscoring the importance of structural changes in intergenic regions to the genesis of human NTDs. These CNVs suggested new candidate genes within SB-associated pathways that are potential targets for biological investigation. However, none of the human genome studies mentioned in this section involve sufficient subject numbers to computationally identify statistically significant NTD associations at the single variant or gene level using an untargeted or completely unsupervised approach such as a genome-wide association study (GWAS). Nevertheless, these genome-wide, variant burden analyses illustrate why candidate gene approaches may fail to capture an adequate picture of the underlying mechanism and individual genetic predisposition to NTDs. There are several cautionary notes to consider for future genome-wide studies of NTDs, beyond a simple matter of accumulating larger cohorts. Population databases used for controls might not be representative and may skew frequency estimates for NTD risk alleles. Cohort stratification, especially by ancestral substructure, is a known confounder for genome-wide studies and rare variant interpretation (Jiang, Epstein, & Conneely, 2013; Ma & Shi, 2020; Mathieson & McVean, 2012). The prevalence of NTDs necessitates that large assembled cohorts will contain admixture and therefore must be adequately controlled through selection of unrelated control subjects of genetic background—based on ancestral markers, not geographic or self-identification—that is balanced with respect to cases (Elhaik & Ryan, 2019).

While amassing the thousands of NTD case and control data required to reach statistical significance in a GWAS, several computational strategies can be employed to minimize bias in a genome-wide search for novel genetic contributors to neurulation failure. One that we have recently explored is the use of a systems biology approach to NTD genetic factors (Aguiar-Pulido et al., in revision). Working with WGS data from 149 cases and 149 ancestry matched, unrelated controls, this strategy examined modestly rare single nucleotide variants (SNVs) in protein coding regions that represent a MAF < 1% that are predicted to be deleterious (stop loss/gain, frame shift, splice donor/acceptor, missense predicted to be deleterious by Sift/Polyphen/CADD). These variants were collapsed onto the gene level and submitted to training a random forest machine learning classifier to identify high discriminatory potential between SB cases from controls. 439 genes were then subjected to pathway analysis to obtain gene-enriched pathways that remained significant after FDR correction for multiple hypothesis testing. Remarkably, the pathways with highest significance were in Carbon Metabolism and Vitamin B12 Transport and Metabolism (adjusted p values 0.00081 and 0.00099, respectively). These genes, involved in glycolysis and fatty acid biosynthesis pathways, are linked to diabetes, obesity, metabolic syndrome and overlap with one-carbon transfer, reflecting major risk factors for NTD identified in epidemiological studies in the post-folate fortification era (Loeken, 2020). This result supports the validity of other pathways found to be significantly gene enriched in this study (Aguiar-Pulido et al., in revision). Further strategies to examine SNVs and SVs build a comprehensive picture of the entire genome landscape, including the 98% of the genome beyond protein coding sequences.

6 | TRIO VERSUS CASE–CONTROL ANALYSES

Exome and genome sequencing of case-parent trios allow for de novo mutation (DNM) detection toward discovering genes with large effect

size for neurodevelopmental disorders. The identification of DNMs from trio sequence data, requires comparing the observed rate of DNMs to the expected, and correlating the variant with phenotype based on biological function or pathway. Such analyses have identified new candidate NTD genes using 43 trios (Lemay et al., 2015).

Trio studies, aside from identifying DNMs, allow for use of transmission disequilibrium tests (TDTs) in order to assess whether alleles are overtransmitted to affected offspring, revealing patterns that may confer genetic risk. Furthermore, TDT results can be correlated with case–control association results using the ratio of the overtransmitted alleles compared to the undertransmitted alleles to serve as an approximation for the risk ratio. A recent study analyzed Irish and UK trios and identified a potential risk factor in dihydrofolate reductase 2 (*DHFR2*) with mixed results (Pangilinan et al., 2021). In their Irish cohort, which was comprised of 440 complete trios, the investigators observed a significant overtransmission of three alleles within *DHFR2* from parents to affected offspring. Since these alleles share a high degree of linkage disequilibrium, the authors report that this is likely one signal that is associated with an increased case risk for NTD. Interestingly, they did not observe a statistically significant overtransmission of these alleles within *DHFR2* in the UK trios, and it will be informative to see if these results are replicated in other cohorts.

A major advantage to conducting trio analyses is that they are generally resistant to confounding effects from population stratification. Moreover, extended family analyses can provide more statistical power (He et al., 2017). While the analysis of nuclear or extended families are critical to understanding the risk of de novo versus inherited variants in NTDs, there are certainly trade-offs. Statistical testing and power calculations between case–control and trio study designs should be considered at the time of recruitment. The statistical power of 500 trios is approximately the same as the p-value obtained from 500 cases and 1,000 controls, at considerably greater expense (Ahsan, Hodge, Heiman, Begg, & Susser, 2002; Hintsanen, Sevon, Onkamo, Eronen, & Toivonen, 2006). Given the challenges of trio recruitment, the statistical basis for a case–control study is appealing, provided that the controls are healthy and well matched in terms of population admixtures. Nevertheless, the judicious use of these study designs should help uncover genomic signals and variants that contribute risk toward NTD manifestation.

7 | HUMAN NTD ASSOCIATED GENE VARIANTS CAN BE GERMLINE OR SOMATIC

As DNA sequencing technologies improve and the analysis of the trove of data these technologies create get ever more instructive, the number of potential candidate genes that contribute to human NTDs has grown increasingly large. Within these candidate genes are similarly large numbers of variants—both somatic and germline—that require functional analyses in order to validate their pathogenicity. Gene variants in fetuses may be inherited from parents or can occur de novo. These de novo variants may arise at a very early stage of embryonic development, affecting virtually all cells. It is also possible for these de novo variants to occur at a later developmental stage, affecting cells confined in a specific organ. These are referred to as somatic variants, which are postzygotic changes that can alter more than one group of cells with different genotypes in an organism, rather than a change in the DNA in a single fertilized egg. While somatic variants are well documented in cancer patients, they are also known to occur in several neurodevelopmental disorders, such as McCune–Albright syndrome (Weinstein et al., 1991), Sturge–Weber syndrome (Shirley et al., 2013), Proteus syndrome (Lindhurst et al., 2011) and select brain malformations (Poduri et al., 2012). These studies confirm that the nervous system is highly susceptible to somatic mutation (Muotri et al., 2005). With respect to congenital abnormalities such as NTDs, the existing literature concerning any association between somatic variants with the development of an NTD is quite limited. Galea et al. (2018, 2021) reported that a somatic *Vangl2* deletion in murine neuroepithelial cells as well as in surface ectodermal cells causes SB in mice. In our recent publication, we described somatic variants in some key PCP genes (e.g., *VANGL1* and *FZD6*) in neural tissue that are associated with human NTDs, suggesting a potentially important role somatic variants can play in the occurrence of human NTDs (Tian et al., 2021).

The Mediator complex (MED) is known to have significant regulatory effects on WNT signaling, which represents many candidate NTD genes as this pathway has critically important signaling functions during neural tube closure (Rocha, Scholze, Bleiss, & Schrewe, 2010). We recently considered the possibility that genetic variants in the *Med12* and *Med13* genes which serve as subunits in the regulatory domain of the complex, may be associated with increased NTD risks. We interrogated an initial cohort that comprised 48 pairs of neural lesion site and umbilical cord tissues from NTD affected fetuses. We performed DNA sequencing to identify potential NTD-related somatic variants which was subsequently validated by Sanger sequencing. We identified a heterozygous somatic variant of *MED12* c.5344C>T (p.Arg1782Cys) in the neural lesion site tissue of a terminated craniorachischisis fetus. This variant was not found in any other normal tissues tested, indicating that this variant only occurred in neural tissue (Tian et al., 2021). This variant is not located within the functional domains of MED12, although the variant alters an amino acid at a tightly conserved position.

To examine germline mutations in the Med genes, we examined a second cohort comprised of 21 case-parent trios on which we performed WES to identify any de novo germline variants. As before, we also performed Sanger sequencing assays to validate any identified variants. In this cohort a heterozygous stop-gain variant of *MED13L* c.5278C>T (p.Arg1760*) was detected in an infant with myelomeningocele. Neither parent had this variant in the medPIWI domain, thus confirming its de novo nature. The mutant's affected amino acid is highly evolutionarily conserved among multiple species. With respect to functional studies, we performed subcellular localization on the MED12 p.Arg1782Cys variant by overexpressing it in MDCKII cell lines. Both the wild-type and MED12 mutant protein were expressed within the cell nucleus, indicating that the variant did not alter the subcellular location of MED12 protein, although it was determined that GFP signaling of mutated MED12 was reduced compared to the wild-type MED12

protein. The western blotting assay confirmed that the protein coded for by the MED12 variant p.Arg1782Cys was less abundant than wild-type MED12 ($p < .05$), indicating that this mutation may decrease MED12 expression or damage its protein stability.

We sought definitive evidence that the identified variant was related to NTDs by using CRISPR/Cas9 mutagenesis to generate knock-in mice with a homologous variant to that found in humans. Of the 192 injected embryos implanted into six recipients (each recipient carried 32 embryos), we collected 12 viable and 1 dead embryo. Of the 10 male embryos, 2 were MED p.Arg1784Cys knock-in hemizygotes that expressed NTD phenotypes (exencephaly and SB with curly tails), while the remaining viable embryos were normal. The result indicates that _Med12_ p.Arg1784Cys identified in a human NTD patient can also re-produce NTD phenotypes in mice.

8 | VALIDATING HUMAN NTD ASSOCIATED VARIANTS IN MOUSE MODELS AND ORGANOID SYSTEMS

Another excellent demonstration of the functional pathogenicity of genetic variants identified in candidate NTD genes involves DNA damage response (DDR) genes that function in DNA repair, cell cycle control, and are essential for the rapid proliferation of neural progenitor cells. Using whole-genome sequencing and targeted sequencing, we identified significant enrichment of rare deleterious _RAD9B_ variants in SB cases compared to unaffected control infants (8/409 vs. 0/298; $p = .0241$). This study yielded eight variants, and functional analyses determined that the two frameshift mutants and p.Gln146Glu affected _RAD9B_ nuclear localization. Of the other identified variants, the two frameshift mutants decreased the protein level of _RAD9B_. p.Ser354Gly, as well as the two frameshift variants adversely affected the cell proliferation rate, while the p.Ser354Gly, p.Ser10Gly, p.Ile112Met, p.Gln146Glu, as well as the frameshift variants were less able to activate JNK phosphorylation (Cao et al., 2020). To better appreciate the potential roles of RAD9B during embryonic development, embryoid bodies (Fong et al.) were derived from hESCs. We were able to achieve a 60% reduction in RAD9B expression using small interfering RNAs (siRNAs) in an Oct4-GFP-hESC cell line (Cao et al., 2020). We focused on OCT4 expression as it reflects the pluripotency of stem cells. We determined that _Oct4_ expression was significantly downregulated in the RAD9B-KD EBs compared to controls. Additionally, flow cytometry studies confirmed that the _Oct4_ positive cell population in RAD9B-KD EBs was decreased by twofold compared to
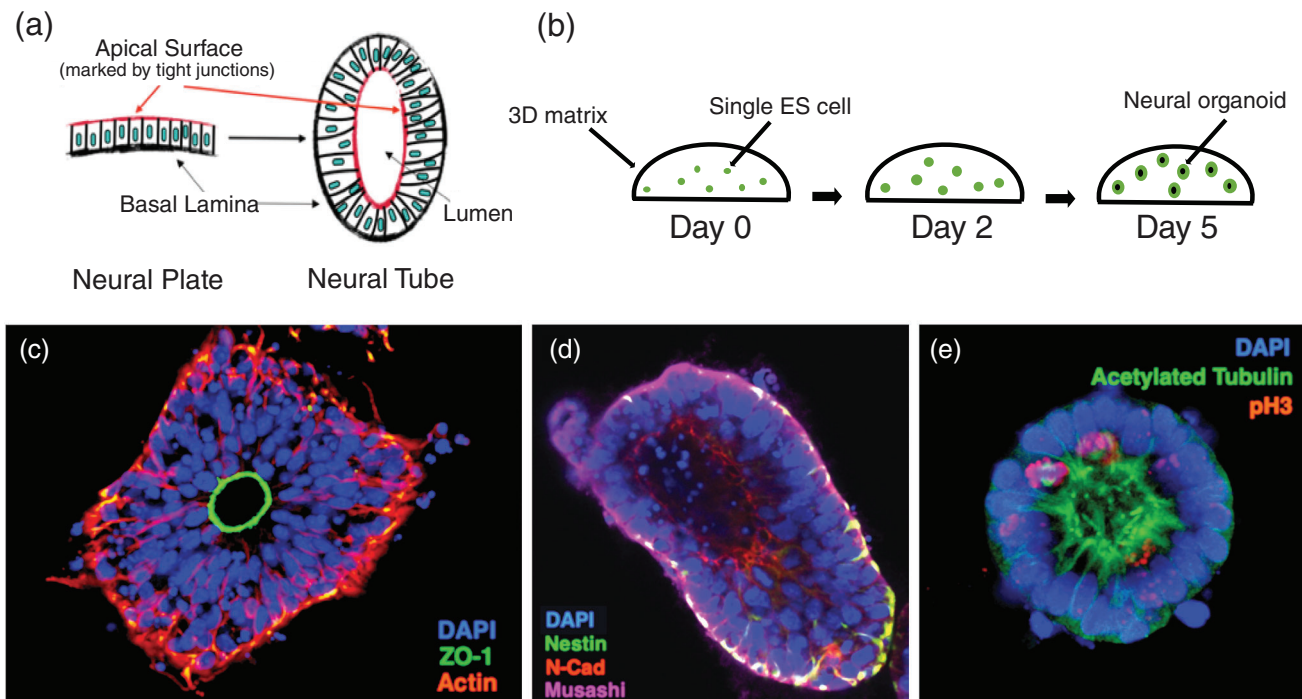

FIGURE 2 Cell culture derived organoids as a tractable NTD model system. One example is the "neurocyst" model developed by Meinhardt et al. (1). Neural organoids are from single mouse embryonic stem (ES) cells to recapitulate aspects of neuroepithelial architecture in the developing neural plate and neural tube, such as an apical and basal surface, cell–cell junctions, and a hollow central lumen (a). Individual mouse ES cells are plated inside a matrigel 3D matrix, and cultured in neural differentiation media (b). After 5 days, each individual cell has formed a neural organoid with a hollow lumen. Neuroepithelial cells are apically oriented toward the lumen, evidenced by tight junction marker (ZO-1) (c). They express neural cell markers Nestin, Musashi, and N-cadherin (d). They also have apically localized primary cilia marked with acetylated tubulin, and display interkinetic nuclear migration, so that cells are dividing in metaphase at the apical surface as evidenced by immunostaining with phospho-histone H3 (pH 3) (e). Each of these attributes recapitulates some aspect of neural tube development, making them suitable for studying certain mechanisms as an NTD model. However, this model does have some limitations. For example, they do not recapitulate tissue fusion of neural folds or dorsal-lateral hinge point formation (Meinhardt et al., 2014)

control EBs. Together, these data indicate that loss of *RAD9B* significantly impairs OCT4 expression in hESC derived EBs. Since *Pax6* is known to restrict Oct4 expression as well as other pluripotency factors (Zhang et al., 2010) we predicted, and found, that both mRNA and protein levels of PAX6 were significantly elevated in the RAD9B-KD hESCs. Together, these observations demonstrate that loss of functional *RAD9B* disrupts early cell fate specification in differentiating EBs through abnormal regulation of *PAX6* and *OCT4* circuitry. We believe that the inhibition of *RAD9B* compromised the formation of the neural organoids in vitro. These results represent the first demonstration of a DDR gene as an NTD risk factor in humans and validated the pathogenicity of the observed *RAD9B* gene variants.

Animal models are not without their limitations, and they cannot exactly replicate human NTDs, as one might expect given that their genomes differ from humans, especially in the intergenic, 3-D architecture of their respective genomes. Moreover, the morphogenetic processes involved in neural tube closure are not identical among different model species (O'Rahilly & Müller, 2002). It is possible to

carefully recapitulate human neural tube closure to better identify the underlying genetic architecture of NTDs by the use of three-dimensional in vitro cellular systems. In recent years, NTD studies have made excellent use of organoids, which are defined as 3-D cell aggregates generated from pluripotent stem cells or organ progenitor cells and consisting of organ-specific cell types that self-organize while maintaining restricted lineage commitment similar to that found in vivo (Lancaster & Knoblich, 2014; Runnels & Komiya, 2020). Organoids can be derived from embryonic stem cells (ESCs), adult stem cells (ASCs), and induced pluripotent stem cells (iPSCs). These cells, when provided the appropriate extracellular scaffolding and culture conditions with desired signaling factors, can be induced to self-organize and form distinct cell aggregates (Figure 2). Moreover, brain organoids can represent both the phenotypic and genetic characteristics of its in vivo counterparts and are capable of sustaining these features throughout their in vitro culturing process (Wu, Peng, Finnell, & Zheng, 2021). Thus, organoids represent a functional alternative to in vivo animal models as they can accurately recapitulate key elements
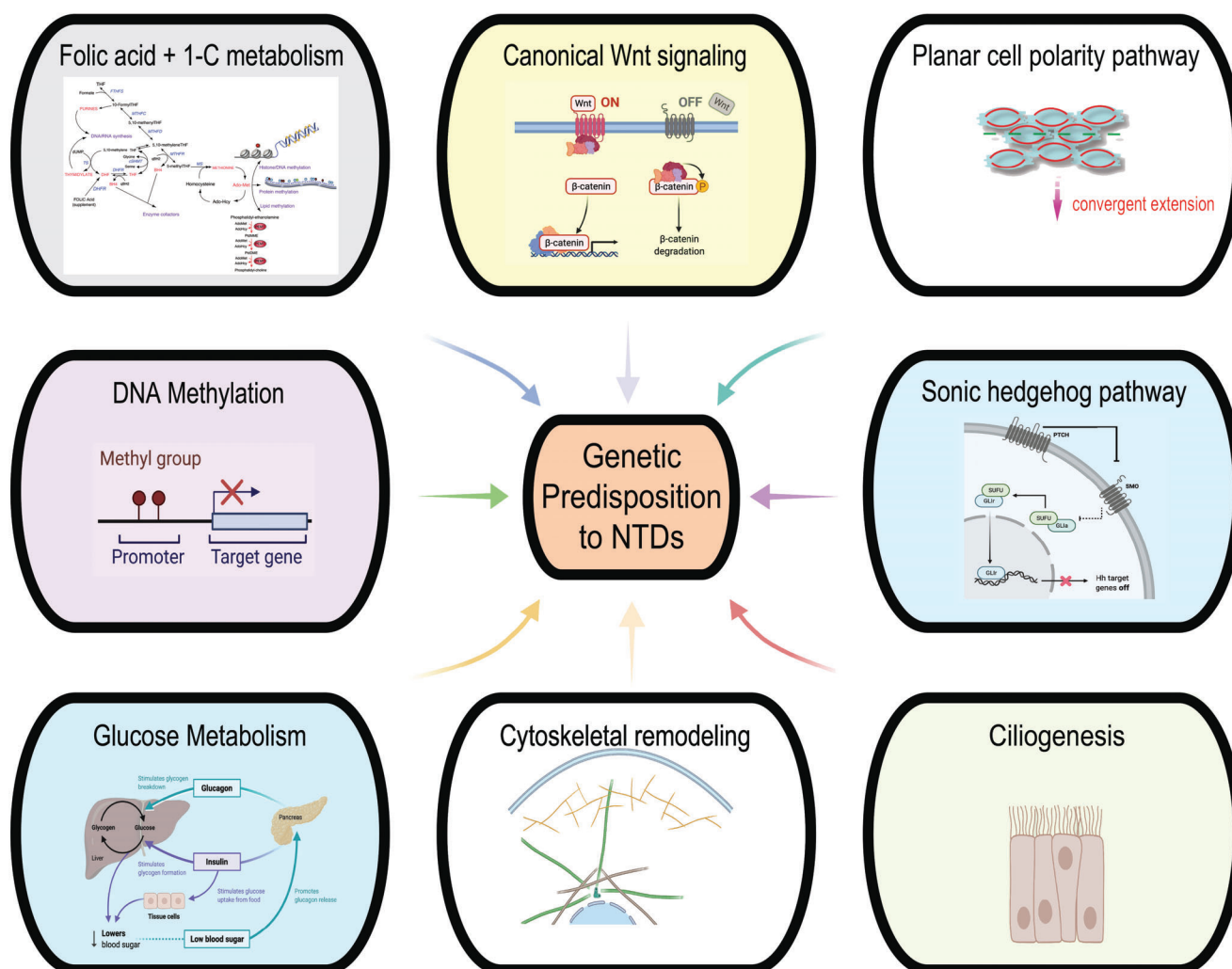

FIGURE 3 Genetic predisposition to NTDs arises from a number of implicated signaling and metabolic pathways

of normal embryonic development. Neural tube organoids are often compared with in vivo neural tubes on the basis of their overall structure, neural fate commitment, apical–basal polarity, and anterior–posterior and dorsal–ventral patterns. Most neural tube organoids reported in the literature have a cystic shaped cavity rather than the long columnar shape cavity found in neural tubes developing in vivo (Abdel Fattah & Ranga, 2020).

Consistent with in vivo morphogenesis many aspects of neural tube closure are time-dependent; therefore, temporal information is often emphasized when describing individual aspects of neural tube organoids, especially when considering various pathological processes leading to the development of NTDs. Unlike using in vivo animal models, it is relatively easy to expose the developing organoids in vitro to study the potential deleterious impact of exogenous compounds such as suspected environmental teratogens. Furthermore, intracellular developmental abnormalities can be studied by interfering with the cells used to create the organoids. It is also possible to use human patient derived iPSCs or iPSCs in which known genetic variants have been edited into these cells to create genetically compromised organoids. That is, hPSCs can be edited by CRISPR/Cas9 methodologies to reflect known variants in human NTD candidate genes, and these hPSCs can serve as the initiating cells for creating neural tube organoids (Liu et al., 2019). By comparing these organoids to the organoids created from control, unaffected hPSCs, we can gain a better understanding of the functional impact NTD associated genetic variants have on critical morphological/biomechanical aspects of neural tube closure. Thus, neural tube organoids are excellent in vitro models for studying neural tube development, pathogenesis, and treatment of NTDs.

9 | CONCLUSION

While prevention of thousands of NTDs through periconceptional FA supplementation and nationwide food fortification programs is a classic public health success story, full prevention of NTDs remains an unrealized goal. Undoubtedly, the underlying genetic complexity of these defects and the role of environmental interactions makes further progress in preventing the FA-non-responsive cases a daunting task. Future prevention strategies targeting these remaining non-FA responsive NTD cases will undoubtedly require modern precision medicine approaches that are capable of evaluating individual risks for mothers and infants using our increasing knowledge of the genomic landscape underlying these defects (Figure 3). Achieving such knowledge, while difficult, has become increasingly possible as the new boundaries in our technology have improved our ability to identify potentially damaging variants and dissect their mechanisms of action, or their interactions with other variants and environmental factors, by leveraging novel mouse and organoid models. In this review, we covered some examples of how the NTD research community has leveraged these technologies over recent years to continue advancing our knowledge of NTD etiology despite the challenges of working with such a rare and complex disease. We hope that by continuing to approach NTD prevention through these novel methodologies, and by developing new strategies for studying the genetic mechanisms of brain and spinal cord development, complete prevention of these debilitating defects can ultimately be achieved.

CONFLICT OF INTEREST

Dr. Finnell formerly held a leadership position in TeratOmic Consulting LLC, a now defunct organization. He also receives travel funds to attend editorial board meetings of the journal *Reproductive and Developmental Medicine*.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable as no new data were created or analyzed in this review.

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