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Pathogenesis of neural tube defects: the regulation and disruption of cellular processes underlying neural tube closure

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Abstract

Neural tube closure (NTC) is crucial for proper development of the brain and spinal cord and requires precise morphogenesis from a sheet of cells to an intact three-dimensional structure. NTC is dependent on successful regulation of hundreds of genes, a myriad of signaling pathways, concentration gradients, and is influenced by epigenetic and environmental cues. Failure of NTC is termed a neural tube defect (NTD) and is a leading class of congenital defects in the United States and worldwide. Though NTDs are all defined as incomplete closure of the neural tube, the pathogenesis of an NTD determines the type, severity, positioning, and accompanying phenotypes. In this review we survey pathogenesis of NTDs relating to disruption of cellular processes arising from genetic mutations, altered epigenetic regulation, and environmental influences by micronutrients and maternal condition.

1. INTRODUCTION

Neural tube defects

NTDs are devastating congenital defects which affect approximately 300,000 babies each year, 88,000 of which will be deadly, and the remaining cases often will experience significant impacts to the length and quality of life (Zaganjor et al., 2016). These statistics are most likely on the low side, as less than half of the 194 World Health Organization member states report data on NTDs. The outcome of an NTD depends heavily on where the neural folds fail to close. Failure to close in the developing brain causes exencephaly, which ultimately results in loss of brain tissue and anencephaly, which is lethal (Fig1 A,B)(Wilde et al., 2014). Failure to close more caudally leads to spina bifida or myelomeningocele (Fig 1 C,D), which has much better outcomes but the lifetime cost of care for spina bifida can be up to $700,000 (Grosse et al., 2016). Spina bifida occulta conversely is a mild defect caused by a gap in bone(s) of the spine and the neural tissue is not exposed

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Neural tube closure

This review emphasizes specific cellular processes and tissues that mediate the major steps of NTC, which are briefly covered here. Formation of the neural tube begins in humans at approximately day 18 and is completed by the fourth week of pregnancy (O'Rahilly & Müller, 1994). The process of NTC is similar in mice but starts at approximately embryonic day 8 and is completed by day 10 (Gray & Ross, 2011). Formation of the neural tube which will become the brain and most of the spinal cord is completed by a process called primary NTC, while neural tube formation below the sacral region is completed by secondary neural tube formation. The process of primary NTC begins with a flat sheet of epithelial cells overlying the notochord and mesoderm (Fig. 2A). Neural induction specifies the neuroepithelium (NE, green) from the non-neural ectoderm (NNE or surface ectoderm; blue). The first major morphogenetic event is cell intercalation which drives NE cells toward the midline resulting in medial-lateral narrowing and rostral-caudal lengthening of the NE. Morphogenesis continues through the elevation of the neural folds (Fig. 2B,C) which is driven by proliferation within the mesoderm and NE, and polarized actomyosin contractions which create hinge points at the medial and dorsolateral positions to shift the rising neural folds from a convex to concave orientation (Fig. 2C). As the neural folds approach each other, the NE and NNE cells separate, cell protrusions extend across the gap and each cell layer adheres with its partner tissue on the opposing fold (called fusion) to form a closed neural tube covered by NNE. Arising at the border between the NE and NNE are pre-migratory neural crest cells, which begin to delaminate during this last step of NTC and migrate to become neural crest cells. This process of NT closure does not occur simultaneously along the rostral-caudal axis. In humans, closure initiates at two discrete sites: one initiation site lies at the forebrain and zippers backwards towards the hindbrain, the other initiation point occurs at the hindbrain/ cervical boundary and zippers both rostrally towards the midbrain and caudally towards the sacral region where secondary neural tube formation will occur (O'Rahilly & Müller, 2002). Mice utilize a third closure point which lies between these two, and zippers both rostrally and caudally (Nikolopoulou et al., 2017). The following sections will analyze individual cellular processes which can fail and lead to an NTD. The overwhelming majority of examples are contextualized within the process of primary neural tube closure, as most NTDs result from defects in primary NTC. Secondary neural tube formation, which occurs in the most caudal region, is not accomplished by large morphogenetic changes, and instead relies on the condensation of tail-bud mesenchyme and subsequent migration and epithelialization to form a lumen (Catala, 2021).

2. CELLULAR PROCESSES UNDERLYING NTDS

NTC is a highly orchestrated process which requires coordination between a myriad of signaling pathways and cellular behaviors. NTC is driven by a multitude of genes, which presents many points of failure leading to NTDs. When attempting to understand the causes of neural tube defects, one can start by surveying the known genetic perturbations which can manifest as NTDs. Animal models that show NTD phenotypes have been crucial for elucidating mechanism of NTC and gene action. Indeed, over 300 genes in mice play critical roles in NTC and over 80 genes in human are associated with NTDs to date

(Kakebeen & Niswander, 2021; Wilde et al., 2014). However, most of the animal studies have investigated the effect of single gene, and often homozygous mutations, whereas the risk in humans of presenting with an NTD is multi-factorial and often polygenic, rather than simple mendelian inheritance due to single gene mutations (Lee & Gleeson, 2020; Neumann et al., 1994; Zohn, 2012). In other words, human NTDs are thought to be due to interactions between multiple gene variants and genetic modifiers. Nonetheless, we can learn much about the broad cellular processes required for NTC as highlighted by individual gene examples. Although there is not a strict correspondence between the genes associated with NTDs in mouse with those discovered from human NTD cases to date, there is excellent correspondence when considered in the context of the biological processes affected, such as outlined in the rest of this review. As we consider the genetic contributions towards NTDs, we will be drawing upon animal models to contextualize the cellular processes involved in NTC, each with their own tolerances to perturbations and failure points which may result in NTDs.

2.1 Proliferation

The process of NTC involves extensive morphogenetic changes to the embryo, accommodated in part by spatially and temporally directed increases in cell division. Too little proliferation can result in not enough material to form an intact neural tube, whereas too much proliferation can change the tissue geometry and inhibit closure. Proliferation defects often manifest during primary NTC, excluding *Xenopus* where proliferation is not an important component of early embryogenesis (Nikolopoulou et al., 2017). In chick and mouse models and human NTC, all embryonic cells are rapidly proliferating at this stage. Focusing on the tissues that contribute to neural tube formation (neuroepithelium, non-neural ectoderm, mesoderm), proliferation within these tissues needs to keep pace with one another for successful NTC.

Perturbation of proliferation within the NE cells of the closing neural tube is represented by many NTD models (Harris & Juriloff, 2010). This includes defects in canonical WNT/β-catenin signaling, which plays a key role in maintaining proliferative balance within the neural plate. Mouse lines which harbor *Pax3* mutations, a downstream effector of WNT/β-catenin, display both exencephaly and spina bifida - highlighting its importance spanning the rostro-caudal axis (Epstein et al., 1991). *Pax3* mutant mice display decreased proliferation and premature neuronal differentiation of dorsal NE cells - suggesting that WNT and PAX3 work to maintain the NE in a proliferative state (Fig. 3A) (Sudiwala et al., 2019). Loss of proliferation capacity in *Pax3* mutant mice prevents the neural folds from meeting, disrupting zippering of the mid and hindbrain neural folds (Fleming & Copp, 2000). Furthermore, the spatial restriction of PAX3 within the dorsal NE highlights the importance of regulated proliferation, even within similarly fated cells. This restriction of PAX3 dorsally is accomplished in part by the CDX family of proteins which interact with posteriorizing WNT signals and act as inducers for *Pax3* transcription, the loss of which results in a truncated anteroposterior axis, fused somites, and craniorachischisis (Ferras et al., 2012; Ramakrishnan et al., 2021; Savory et al., 2011). CDX2, which localizes with PAX3 dorsally, seems to be particularly important as it also regulates PTK7 which interacts with multiple WNT receptors to fine-tune canonical and non-canonical WNT signaling (Berger et al.,

2017; Hayes et al., 2013). The loss of *Ptk7* mimics phenotypes found in *Cdx1/2* conditional knockouts and results in 100% penetrance of craniorachischisis (Lu et al., 2004; Savory et al., 2011; M. Wang et al., 2015). *Pax3* restriction to the dorsal NE is also controlled by ventral SHH signaling, which attenuates WNT signaling through inhibition of ZIC2 (Sanchez-Ferras et al., 2014).

There is a gradient of proliferation along the dorsal-ventral axis of the NE at the time of NTC with higher proliferation on the dorsal side through WNT/PAX3 and lower rate of proliferation of the ventral side (Fig. 3A). PHACTR4 normally serves to restrict proliferation in the ventral NE at the time of NTC. *Phactr4* mutation in mice results in faster cell cycle in NE cells leading to too many cells, especially in the ventral neural tube, and exencephaly (Fig. 3B) (Kim et al., 2007). Similarly, mice harboring mutations of the *Nap1/2* genes experience increased mitotic events in neuronal precursors with exencephaly and spina bifida correlated with an overabundance of NE cells (Rogner et al., 2000).

Disrupted proliferation within the NE is also represented by NTD animal models whose mutations affect proliferation more indirectly by causing cells to prematurely differentiate and therein reducing their proliferative capacity. Several of these models fall within the Notch pathway, which works to ensure that NE specified cells retain proliferative capacity until NTC is complete (Hatakeyama & Kageyama, 2006). Notch itself is a marker of neuronal differentiation, however components of the Notch pathway act to inhibit Notch signaling and retain NE cells in a proliferative state (Engler et al., 2018). When components of the Notch pathway (Hes1, Hes3, Rbpj) are perturbed, Notch expression is increased, NE cells prematurely differentiate, resulting in either spina bifida or exencephaly (Harris & Juriloff, 2007; Ishibashi et al., 1995). Premature differentiation has also been proposed to cause NTDs through mechanical stiffening of the cells, which might disrupt dorsolateral hinge point bending, interfere with neural crest cell release, or inhibit adhesion between neural folds (Copp et al., 2003). An argument can be made that all three may be simultaneously possible. ZIC2 plays roles in both the roof plate cells and in regulating dorsolateral hinge point bending which is necessary for fold adhesion (Ybot-Gonzalez et al., 2007). Interestingly *Zic2* is regulated along with Notch by the transcription factor FOXD5, with feedback from Notch signaling regulating *Foxd5* expression itself (B. Yan et al., 2009). In disrupted Notch signaling mouse models, NTDs might occur from either premature neuronal differentiation alone, or alongside FOXD5 feedback associated with ZIC2 perturbation. This aligns with observations in *Zic2* mutant mice and zebrafish, which display lack of dorsolateral hinge point bending (Nyholm et al., 2009; Ybot-Gonzalez et al., 2007).

Looking beyond NE progenitor cells, NTD models associated with disrupted proliferation in other tissues are fewer in number. Within the mesoderm for example, cell migration seems to play a larger role, yet proper proliferative capacity is necessary to expand the mesoderm population and to form the mesenchyme bulges which will help elevate the neural folds (Fig. 4A) (Nikolopoulou et al., 2017). *Twist1* and *Cart1* knockout lines demonstrate reduced proliferative capacity within the mesenchyme, interfering with the formation of the biconvex neural folds and resulting in exencephaly (Fig. 4B) (Z. F. Chen & Behringer, 1995; Zhao et al., 1996, p. 1). It is noteworthy though that caudal NTC appears to be

less sensitive to disruption of the mesoderm, as demonstrated by physical depletion of the paraxial mesoderm still allowing subsequent NTC (van Straaten et al., 1993).

Proliferative defects in non-neural ectoderm cells (NNE) are even more rare, with cell structural changes and adhesion playing a larger role. Mutations which lengthen the cell cycle (*Gadd45a, TrP53*) can reduce overall cell numbers in both the NE and NNE leading to an NTD, albeit with relatively low penetrance (Harris & Juriloff, 2007). These mutations have also been proposed to reduce genomic stability, which could lead to cell death and reduced cell numbers (Hollander et al., 1999). In more caudal regions, reduction of *Grhl3* is associated with an imbalance of proliferation between the NE and caudal hindgut resulting in spina bifida (Gustavsson et al., 2007). These phenotypes associated with *Grhl3* mutation may actually be localized to uncommitted stem-like cells along the neural plate border as this phenotype can be rescued when paired with a deficiency of Dkk1/Kremen1 – canonical WNT signaling antagonists which promote NE specification within uncommitted neural plate border cells (Kimura-Yoshida et al., 2015).

2.2 Migration

Cell migration plays a key role in the processes of: convergent-extension which shapes the neural plate, mesoderm migration to help elevate the neural folds, clearance of neural crest cells, and lumen formation in secondary neural tube formation.

During early stages of primary NTC, the first key cell migration step involves intercalation of cells toward the midline, which narrows and lengthens the neural plate along the medial-lateral axis and the rostral-caudal axis, respectively (Fig. 5A,B). Animal NTD models in which these convergent-extension movements are disrupted display a wide neural plate and ultimately a large gap between the neural folds such that the folds cannot meet in the midline (Fig. 5C)(Harris & Juriloff, 2010). This cell migration is heavily dependent on planar cell polarity (PCP) signaling and the response seems to be tissue dependent (Shindo & Wallingford, 2014). In the mesenchyme, migration is accomplished by shortening of cell-cell junctions and cellular protrusions which exert traction to produce a crawling motion, with the orientation of the protrusions dependent on the distance from the midline mesoderm and notochord (Butler & Wallingford, 2018; Ezin et al., 2006). The positioning of the protrusions which drive intercalation is thought to be regulated by the non-canonical WNT-Frizzled PCP pathway, with extension of the protrusions regulated downstream of WNT in both a Dishevelled-dependent and independent manner (Keller, 2002). Mutations which disrupt the Dishevelled pathway in *Xenopus* affect convergent-extension and are marked by more but less stable and randomly oriented protrusions (Wallingford & Harland, 2002). In the neural plate, cell migration is also thought to be controlled by PCP signaling, but instead seems to be mainly accomplished by shortening of cell-cell junctions (Nishimura et al., 2012). This is accomplished by pulsed actomyosin contractions that are restricted to or enriched at mediolaterally oriented cell-cell junctions (Butler & Wallingford, 2018). These actomyosin contractions will be discussed further in the cell structure section but are dependent upon polarization within the cell. NTD mice deficient for PCP regulators PTK7 and Vangl2 display varying degrees of polarity loss, and NE cells in both cases fail to apically constrict (M. Williams et al., 2014), leading to the most severe form of NTD called

craniorachischisis in which the neural folds fail to meet in the midline along most of the rostral-caudal axis. Similarly in humans, mutations in the non-canonical WNT-Frizzled PCP pathway are associated with NTDs, including craniorachischisis (Kibar et al., 2001).

Completion of primary NTC requires that neural plate border cells, also referred to as pre-migratory neural crest cells – delaminate from the intact neural tube and NNE. Failure of neural crest cells to migrate can lead to NTDs, which might stem from inhibition of neural fold bending (Copp et al., 2003). The process of neural crest formation and migration requires neural plate border cells to undergo epithelial to mesenchymal transition (EMT). EMT is highlighted by loss of adherens junctions (specifically E-cadherin together with Catenins and actin rings), the loss of cell polarity, separation from surrounding epithelial tissue (delamination), and migration through extracellular matrix to adjacent tissues (Acloque et al., 2009). In mice, neural crest cells which overexpress the gap junction protein Connexin 43 fail to migrate which leads to exencephaly (Ewart et al., 1997). Furthermore, sequestration of chondroitin sulphate proteoglycans (a family of molecules important in regulating adhesion) in rats prevents the disruption of fibronectin-containing ECM necessary for neural crest migration, which delays dorsolateral bending of the neural folds (Morriss-Kay & Tuckett, 1989). As neural crest cells can be observed detaching from the neural folds before NTC is complete, it has been suggested that the failure of neural plate border cells to differentiate and thus migrate may inhibit the transition from biconvex to biconcave morphology of the neural plate (Copp et al., 2003).

In secondary neural tube formation, evidence also points towards the importance of cell migration and intercalation. Mesodermal cells undergo a mesenchymal to epithelial transition (MET) to form an epithelial tube that then fuses with the neural tube formed by primary NTC. When the mesodermal cells are prevented from undergoing epithelialization, the cells fail to participate in neural tube formation, and instead accumulate in the neural canal (Nakaya et al., 2004) Chick embryos in which SMAD3 is disrupted, a receptor involved in Transforming Growth Factor β (TGF-β) signaling, display disrupted secondary neural tube formation marked by multiple small lumen (Gonzalez-Gobartt et al., 2021). SMAD3 seems unique among other SMAD proteins in directing cell migration during secondary neural tube formation (Gonzalez-Gobartt et al., 2021). TGF-β signaling has been previously implicated in chick brain medial hinge point (MHP) formation (Nikolopoulou et al., 2017). Together these data have intriguing implications for possible cell migration & intercalation in the formation of the MHP in chick and mouse, as SMAD2 & SMAD3 are highly active at the MHP while other SMAD proteins involved in BMP signaling are down regulated (Amarnath & Agarwala, 2017). Furthermore, a role for cell migration in MHP formation may be obfuscated by the ability of dorsolateral hinge point bending to overcome the loss of MHP in mice (Ybot-Gonzalez et al., 2002).

2.3 Cell death

With NTC being a highly proliferative process, the subsequent death of some of these newly formed cells may seem counter-intuitive or even counter-productive. Indeed, the involvement of apoptosis in NTC is still a subject of debate, with evidence that it may be dispensable in the context of mammalian NTC (Massa et al., 2009). Still, cell death is observed and

may be necessary within the neural folds (Cecconi et al., 1998; Urase et al., 2003). Loss of apoptosis in mouse models has been observed to accompany disrupted neural fold bending and delayed NTC in the mid- and hind-brain (Yamaguchi et al., 2011). Regardless of the necessity of apoptosis for NTC, there is evidence that abnormally increased apoptosis can underlie NTDs in animal models, and this can result from genetic insult, nutritional deficiencies, or pharmacological insults (Kakebeen & Niswander, 2021).

Arguments for the necessity of apoptosis in NTC are supported by a myriad of mice NTD models (mutations in *Mapk8*/*Mapk9*, *Apaf1*, *Casp3*, *Casp9*, and *Cytc*) which result in decreased apoptosis and lead to midbrain exencephaly. Most of these mutant also show forebrain protrusion or cauliflower-like overgrowth not observed in other mouse NTD models (Harris & Juriloff, 2007). In *Xenopus*, the pro-apoptotic function of *Barhl2* has been demonstrated to be crucial for NTC, and is thought to normally limit the number of Chordin- and Shh-expressing cells in the prospective notochord and floorplate (Juraver-Geslin & Durand, 2015).

On the other hand, increased apoptosis can also increase the risk for NTDs. Analysis of human NTD autopsies found that hallmarks of anencephaly cases include higher amounts of apoptotic cells, higher expression of *p53*, and lower expression of *Pax3* (L. Wang, Lin, Yi, et al., 2017). In human spina bifida autopsies, *Pax3* is diminished, yet *p53* expression and the number of apoptotic cells resemble controls, suggesting that rostral NTC may be more sensitive than caudal NTC to increases in apoptosis (L. Wang, Lin, Yi, et al., 2017). Within animal NTD models, increased apoptosis is represented in *Bcl10* and *MEKK4* mutant mice (Harris & Juriloff, 2007). MEKK4 is specifically found in NE cells, and while *MEKK4* deficient cells demonstrate massively elevated apoptosis and highly penetrant NTDs, proliferation of NE cells seems to be largely unaffected (Chi et al., 2005). Alteration in the levels of micronutrients can lead to increased cell apoptosis and NTDs. For example, treating mouse embryos at the time of NTC with TPEN, a zinc chelator to model zinc deficiency, leads to stabilization of p53, increased apoptosis and failed NTC. The TPEN-induced NTD can be rescued by pharmacological inhibition of p53 or overexpression of a E3 ubiquitin ligase which targets p53 for degradation (H. Li, Zhang, & Niswander, 2018). These experiments not only demonstrated a mechanism for zinc-deficiency associated NTD risk, but also provide evidence that increased apoptosis can drive NTDs as opposed to simply accompanying them. Perhaps in a similar fashion, an excess of the drug all-trans-retinoic acid (RA) has also been demonstrated to induce caudal NTDs in mice and rats, which is accompanied by an increase in apoptosis (Kakebeen & Niswander, 2021; Wei et al., 2012). Notably, as discussed later, increased apoptosis is thought to be a driver of diabetes associated NTDs(Gao & Gao, 2007).

2.4 Structural Changes

NTC relies upon proper proliferation, cell death, and migration as previously discussed, but these cellular processes in turn are often dependent on cell structural changes. The directionality of cell migration, oriented cell division, actomyosin constriction, and cell shape is highly dependent on the cell polarity (Nikolopoulou et al., 2017). It is not surprising

then that a large number of NTD animal models are rooted in perturbed planar cell polarity (PCP) signaling (Harris & Juriloff, 2007; Juriloff & Harris, 2012).

In the context of the mesenchyme, we have already discussed disruptions to the Disheveled pathway which randomize cellular protrusions and prevent cell crawling (Wallingford & Harland, 2002). Mutations to *Ptk7* and *Vangl2* similarly disrupt convergent extension in both the mesenchyme and NE by disrupting cell-cell junction shortening required for cell migration (M. Williams et al., 2014). The proper cell-cell junction regulation in these mutants is dependent on restriction of actomyosin contractions to the mediolateral junctions. This restriction appears to be driven by the polarity of the cell imparted by asymmetric enrichment of PCP proteins Vangl2 and Prickle2 (Butler & Wallingford, 2018). Knockdown of *Prickle2* in *Xenopus*, which is spatiotemporally coordinated at shrinking junctions, disrupts convergent extension (Butler & Wallingford, 2018). Though Prickle2 is not associated with NTDs in human, it is noteworthy that the gene family member *Prickle1* has multiple documented mutations in human patients which are thought to pre-dispose to NTD risk (Bosoi et al., 2011). Though Vangl2 and Prickle2 may contribute to the actomyosin contractions, the directionality of this enrichment is thought to be influenced by the Fat/Dsch/Fjx1 pathway (Badouel et al., 2015). Fat1–4 and Dsch1/2 are atypical cadherins which are phosphorylated by Fjx1 to affect their binding affinity which is proposed to contextualize local cell behaviors to a larger axial orientation (Mangione & Martín-Blanco, 2018). The exact interaction between the Fat/Dsch/Fjx1 pathway and PCP signaling is still in question, however *Fat1*$^{-/-}$ mice display forebrain NTDs and *Fat4*$^{-/-}$ mice exhibit caudal defects and a broad spinal cord (Ciani et al., 2003; Nikolopoulou et al., 2017; Saburi et al., 2008).

In addition to the need for cell-cell junction shortening due to PCP signaling in the neural plate, NE cells contain unique points for cell structural regulation and failure. The formation of the dorsal-lateral-hinge point (DLHP) is an intersection of several such pathways (Fig. 6A). Bending of the neural plate in more rostral regions is dependent on actomyosin contractions, however, this regulation differs from NE cell-cell junction shortening (Haigo et al., 2003). The myosin contraction is carried out by the small GTPase RhoA (Arnold et al., 2018). Though there is no RhoA NTD model, Gef-H1 and RhoGap which act upstream in the neural folds both display severe exencephaly phenotypes accompanied by significant basal actin accumulation (Brouns et al., 2000; Itoh et al., 2014). Similarly, disruption of ROCK, LIMK, and Cofilin which act downstream of RhoA to regulate actin polymerization and turnover result in abnormal actin accumulation, failure of neuroepithelial folding, and NTDs (Fig. 6B) (Escuin et al., 2015). Interestingly Blebbistatin, a myosin inhibitor which blocks actomyosin cross-linking, rescues NTDs accompanied by abnormal actin accumulation. Conversely, the drug Jasplakinolide, which blocks actin depolymerization, significantly delays NTC (Cramer, 1999; Escuin et al., 2015). With the large influence that actin function and turnover has over DLHP bending, it is perhaps not a surprise that disrupted localization of ROCK would yield similar phenotypes to the previously discussed perturbations of its downstream signaling components. Indeed, disruption of Shroom3, which has been proposed to localize ROCK to apical cell junctions, results in failed neuroepithelial bending and severe exencephaly (Nishimura & Takeichi, 2008). A separate NTD model arising from failed neuroepithelial bending involves the gene encoding the

protein Noggin. Noggin normally works to inhibit BMP signaling at more caudal regions of the NE where its own expression is no longer repressed by Sonic Hedgehog signaling (Ybot-Gonzalez et al., 2007). *Noggin* knockout mice display spina bifida marked by increased BMP signaling activity and failure of neuroepithelial bending at the DLHP (Stottmann et al., 2006).

The NNE is unique in that NTD animal models that affect this tissue are represented almost exclusively by disruptions to cellular structure (Rolo et al., 2016). This is most clearly evident by the role that the NNE and its surface proteins play in neural fold fusion (Copp et al., 2003). As the neural folds undergo fusion or zippering along the rostral-caudal axis, the NNE cells exhibit protrusive behavior and this differs along the axis. Two sets of protrusions identified as important for NTC are long filopodia and ruffle-like lamellipodia, with filopodia thought to be important to cranial and upper spinal cord NTC, and lamellipodia more crucial to later most caudal closure (Rolo et al., 2016). The GRHL3 transcription factor is important in controlling some of these NNE cell structural elements. Knockout of *Grhl3* displays loss of sheet-like lamellipodia thought to be crucial for caudal neural fold fusion (Jaffe & Niswander, 2021). Loss of Rac1, more directly linked to lamellipodia production, mimics *Grhl3* loss phenotypes and suggests GRHL3 as an upstream regulator of Rac1 (Jaffe & Niswander, 2021; Rolo et al., 2016). This preferential role in the regulation of lamellipodia might be reflected in the infrequency of *Grhl3* mutants to produce cranial NTDs, whereas spina bifida is a completely penetrant phenotype (Nikolopoulou et al., 2017). The family member *Grhl2* is expressed uniformly within the NNE and upon loss demonstrates both cranial and spinal NTDs (Brouns et al., 2011; Rifat et al., 2010). GRHL2 regulates epithelial morphogenesis through its downstream targets Claudin4, Rab25, and E-cadherin. Although none of these downstream targets are represented by NTD models, this could be due to their critical roles during earlier stages of embryogenesis (Harris & Juriloff, 2010; Pyrgaki et al., 2011; Senga et al., 2012). Notably though, antisense oligonucleotide knockdown of E-cadherin leads to cranial NTDs, pointing towards a role in GRHL2 maintaining epithelial cohesion through tight junctions (B. Chen & Hales, 1995), and as seen by loss of NNE integrity in mouse *Grhl2* mutants (Ray & Niswander, 2016). It has been proposed the presence of N-cadherin at junctions may be sufficient to maintain neural identity (Punovuori et al., 2019), and a similar role for GRHL2 and E-cadherin in maintaining epithelial identity is supported by the observation that *Grhl2* loss is accompanied by mesenchymal markers in the NNE (Ray & Niswander, 2016).

3. EPIGENETICS OF NTDS

We previously defined individual genetic mutations which can produce an NTD, most often when gene function is largely ablated in animal models. We also discussed how a variety of genes regulate critical cellular processes. In humans it is thought that NTD penetrance results from a combination of genetic and environmental perturbations, which can be tolerated to a certain level, above which an NTD becomes a possibility. As epigenetic regulators act globally on the genome, it might be expected that mutations in epigenetic regulators could globally alter gene expression. Reflecting on the complexity of the cellular processes involved in NTC, it may seem that even small noise introduced by epigenetic disruption may lead to NTDs; however, variability in gene expression seems to be tolerated

and even a necessary aspect to maintain plasticity required in NTC (Pujadas & Feinberg, 2012). This partly points towards the extent of epigenetic disruption needed to induce an NTD, but perhaps also emphasizes the multi-factorial nature of NTDs and the interplay between epigenetic, genetic, and environmental factors. Of epigenetic regulators that act globally to modify gene expression, disruptions to DNA methylation, histone modifications, and chromatin structure are enriched in animal models of NTD (Kakebeen & Niswander, 2021; Wilde et al., 2014). The context that individual NTD epigenetic models provide will undoubtedly improve as researchers begin to cross-reference global epigenetic changes against the expanding knowledge created by the encyclopedia of coding elements project (ENCODE) which includes spatial and temporal profiles of regulatory elements within mouse development (J. E. Moore et al., 2020).

3.1 DNA methylation

The majority of the genome (70%−90%) harbors enzymatically added methyl groups at CpG dinucleotides which are established and maintained by DNA methyltransferases (DNMT) (Jones & Baylin, 2002). DNA methylation is essential for silencing retroviral elements, regulating tissue-specific gene expression, genomic imprinting, and X chromosome inactivation (L. D. Moore et al., 2013). CpG islands are defined as >500bp stretches with >55% GC content, and an observed/expected CpG ratio of 0.65 (Bird et al., 1985; Takai & Jones, 2002) Approximately 70% of gene promoters lie within CpG islands, and methylation in these regions is thought to contribute towards tissue specific transcriptional regulation through exclusion (Miranda & Jones, 2007) or attraction (Blattler & Farnham, 2013) of transcription factors and accessibility of DNA (L. D. Moore et al., 2013; Saxonov et al., 2006).

Disruption of methylation globally through depletion of the DNA methylation maintenance enzyme DNMT1 is detrimental to embryogenesis, however early embryonic death and ubiquity of function impedes the study of DNMT1 in the context of NTDs (E. Li et al., 1992; Wilde et al., 2014). Instances where disruption of global methylators result in NTDs are represented by mice lacking de novo methyltransferases DNMT3B and DNMT3L; however, mice lacking the other major de novo methyltransferase DNMT3A, die early in embryogenesis (Hata et al., 2002; Okano et al., 1999). Although these de novo methyltransferases can methylate DNA in a non-specific manner, they are also thought to methylate in a site-specific manner through protein complexes which contain tissue specific transcription factors (Hervouet et al., 2018; Yagi et al., 2020). These site-directed methylation behaviors have led to some insight to their role in NTC. It has been demonstrated that DNMT3A is required for methylation and proper transcription of *Polycomb* group target developmental genes, and has been proposed to act as switch mediating neural crest fate transition (Hu & Rosenfeld, 2012; Yagi et al., 2020). Knockdown of *DNMT3A* during NTC upregulates neural genes such as *Sox2* and down regulates neural crest specifier genes (*FoxD3*, *Sox10*, and *Snail2*), broadening the dorsal neural tube and leading to expansion of NE cells over neural crest cells (Hu & Rosenfeld, 2012). The NTD phenotype observed following loss of methylation of *Sox2* and neural crest genes points to the possible involvement of site directed methylation in other NTD animal models. Further evidence for specific methylation influencing NTDs can be found in human NTD cases

where increased methylation within the *Pax3* gene body has been observed (Lin et al., 2019; L. Wang, Lin, Zhang, et al., 2017). This is mirrored in mice treated with a benzoapyrene, which demonstrate hypermethylation of *Pax3* and NTDs (Lin et al., 2019).

3.2 Histone modifications

The ability of the nucleus to contain such large amounts of DNA is made possible by its organization and compaction into chromosomes. This organization is rooted in the wrapping of DNA around nucleosomes, composed of four homodimers of core histone proteins H2A, H2B, H3, and H4 (Rosenfeld et al., 2009). Post-translational modifications to the N-terminal tails of these histones are thought to regulate the interaction between DNA and nucleosomes, and subsequently the packaging and accessibility of the DNA to transcription factors (Kouzarides, 2007). There are eight different post-translational modifications that can affect more than 60 sites, with multiple modifications possible at each site (Kouzarides, 2007). This leads to a nearly endless combination of histone modifications. Moreover, the influence these marks exert over transcriptional regulation is also thought to be affected by neighboring proteins with their own combination of modifications. Overall, the influence of one or multiple histone states on transcription has been deemed the “histone code” (Jenuwein & Allis, 2001). Similarly to DNA methylation, histone modifiers act on a global scale, however the different marks and myriad of enzymes which produce local histone states lends itself to more direct interrogation of the roles of histone modifiers on NTC.

The most widely studied histone modifications are acetylation and methylation, a trend which is reflected in current NTD mice models (Harris & Juriloff, 2007). Histone acetylation is thought to decrease the affinity of DNA to nucleosomes, increasing the accessibility of the DNA and is associated often with increased transcription (Kouzarides, 2007). Mice harboring mutations in histone acetyltransferases (HATs) GCN5 and CBP/p300, which deposit acetyl marks, display exencephaly; and even mice heterozygous for CBP or p300 mutations display exencephaly (Bu et al., 2007; Partanen et al., 1999). These HAT mutations are thought to act through disruption of metabolic pathways such as glucose metabolism needed for NTC, which has interesting implications for pregnant people with diabetes who have a higher risk of NTD births (Wilde et al., 2014). Histone de-acetyltransferases (HDACs) serve the opposite role and loss of HDACs Sirt1 and HDAC4 leads to dysregulation of P53 and Runx2 respectively and are associated with exencephaly in mouse mutants (H.-L. Cheng et al., 2003; Vega et al., 2004). Treatment with HDAC inhibitor trichostatin A or anticonvulsant valproic acid are thought to produce NTDs through a similar pathway, as they display disruption of HDACs and hyperacetylation of histones (Göttlicher et al., 2001; Murko et al., 2013). Beyond specific HDACs, HDACs in general have been implicated in the regulation of differentiation, and have been demonstrated to be necessary to inhibit BMP2/4 signaling in the developing mouse forebrain (Shakèd et al., 2008; Wilde et al., 2014). Though there is much less evidence for histone methylation specifically affecting NTC, the loss of histone demethyltransferase FBXL10 in mice leads to early NE cell death and exencephaly (Fukuda et al., 2011).

4. ENVIRONMENTAL INFLUENCES

The impact that environmental factors exert on NTD risk is apparent when studying global statistics. NTD rates vary significantly by geographic location, and can be stratified even further by income status (Zaganjor et al., 2016). Many of these disparities can be tied to nutritional status as discussed below. Also the relatively high frequency of NTDs has allowed researchers and epidemiologists to make connections to a variety of maternal conditions and teratogenic agents.

4.1 Folic acid

The micronutrient most closely associated with NTD incidence is folate, found widely in the diet, especially in leafy green vegetables, and synthetically as Folic Acid (FA). This relationship was first recognized by a midwife named Catherina Schrader in 18th century Holland. Schrader's meticulous record keeping showed the correlation between poor crop years and higher incidence of NTDs (Michie, 1991). In 1965, a paper detailing a case-control study marked the recognition of an inverse relationship between deficient folate metabolism and increased NTD incidence (Hibbard & Smithells, 1965). Further clinical trials in the 1980's and 1990's confirmed the protective nature of FA in reducing NTD cases. Now, FA is widely used to fortify grains to avoid maternal folate deficiency ("Prevention of Neural Tube Defects," 1991). Starting in 1998 the United States enriched cereal grains with 140μg FA/100g, which has prevented an estimated 1,300 cased of NTDs annually (J. Williams et al., 2015). Eighty countries around the world also fortify their grains with FA. According to the Food Fortification Initiative, countries with higher FA supplementation have lower NTD incidence. However, this association, according to an epidemiological study done in 2020, may be confounded by socio-economic class (Qu et al., 2021). Still, women who had a previous NTD affected pregnancy are less likely to have a second NTD affected pregnancy after supplementing FA in the diet. Additionally, a randomized control trial in China showed that when pregnant women regularly took periconceptional FA, there was an 85% reduction in NTD risk (Berry et al., 1999). Thus, many epidemiological studies have established the efficacy of FA fortification on NTD prevention, although debate continues over the details of supplementation and public implementation.

Despite the focus on folate status, maternal folate deficiency itself is not enough to produce a NTD in mice, and instead requires a predisposing mutation in a folate-sensitive gene (Greene et al., 2009). Similarly, in humans it is believed that NTDs are multi-factorial in which genetic variants (inherited or de novo) along with environmental factors act in combination to affect NTD risk (Wilde et al., 2014). This has led to investigations to identify NTD gene mutations that are responsive to folate levels. Studies in mice have used FA deficient and FA supplemented regimes to begin to uncover the interplay between genetic and environmental factors. First, we will discuss genes within the folate one-carbon metabolism (OCM) pathway and then highlight that many of the FA responsive genes have no apparent roles in FA metabolism or other processes related to this pathway, indicating the broader impact of the OCM pathway on nucleotides, proteins, and lipids during NTC.

Folate is processed in the cell within the OCM pathway. Folic acid must be reduced to produce the body's biologically active form, tetrahydrofolate (THF). Further reduction

and the addition of a methyl group yields 5Me-THF. 5Me-THF can then be used as methyl carrier for transfer of one-carbon units to many molecules within the cell (Imbard et al., 2013). This pathway is essential for the biosynthesis of purines and thymidylate, remethylation of homocysteine to methionine, and from there making S-adenosylmethionine which is the universal methyl donor in the cell (Suh et al., 2001). It is thought that these key metabolites may be at suboptimal levels at critical closure time points in folate deficient mothers.

As highlighted earlier, the coordination of proliferation is critical for NTC. The folate pathway is necessary for production of the building blocks for DNA synthesis, and hence proliferation has been a focus of study in FA pathway mutants. Mutations that affect folate uptake and metabolism are a prime example of the correlation between folate levels and NTD incidence. Deletion of the Folate Receptor 1 (Folr1, Folbp1) in mouse causes NTD and the embryos are severely growth retarded (Piedrahita et al., 1999). The evidence in humans is less clear but there are reports of autoantibodies against folate receptors associated with NTD risk (Cabrera et al., 2008; Rothenberg et al., 2004). MTHFD1L is an enzyme in the mitochondria responsible for catalyzing the last step in the flow of one-carbon units (formate) from the mitochondria to the cytosol. Deletion of this gene in mice results in severe NTDs, including craniorachischisis, exencephaly, and a “wavy” neural tube (Momb et al., 2013). *Mthfd1L* mutant mice show reduced cellular proliferation in the brain after completion of NTC (Shin et al., 2019). Gene variants in the mitochondrial OCM pathway are associated with increased NTD risk in humans including *MTHFD1L*, *AMT*, and *GLDC*, and this increased NTD risk is borne out in studies in mice (Narisawa et al., 2012; Parle-McDermott et al., 2009). Within the cytosolic folate pathway, loss of serine hydroxymethyltransferase SHMT1, which utilizes serine to form one-carbon units, causes NTDs in mice, but only on a folate deficient diet, through decreased flux through the thymidylate biosynthesis pathway (Beaudin et al., 2011). It has been reported that SHMT1 C1420T parental alleles increase NTD risk (K Rebekah et al., 2017). Variants in MTHFD1, a tri-functional enzyme which catalyzes three steps in the folate cycle, are also associated with increased NTD risk (De Marco et al., 2002; Parle-McDermott et al., 2009; Zheng et al., 2015) and disease alleles show altered flux through thymidylate biosynthesis pathway (Field et al., 2015). These data link the importance of the folate OCM pathway in the regulation of proliferation and NTC.

Another proposed mechanism by which FA may affect the penetrance of NTDs is modulation of the epigenome through OCM metabolites, via the methionine cycle and methylation of substrates including DNA, RNA and proteins. In animal models, methionine cycle inhibitors or mutations in DNA methyltransferase proteins can cause NTDs (Dunlevy et al., 2006; Okano et al., 1999). The MTHFR enzyme catalyzes the synthesis of 5Me-MTHF, which is the methyl donor for homocysteine to regenerate methionine through the methionine synthase (MTR) and methionine synthase reductase (MTRR) enzymes. MTHFR has been studied for association with NTDs in human cases. In a case-control study within Italian families, it was determined that the families and mothers who have experienced an NTD are enriched in a MTHFR polymorphism A1298C (De Marco et al., 2002). Similarly, MTHFR polymorphism C677T is associated with a greater risk for NTDs and has been found to result in low plasma folate levels and higher levels of homocysteine, as well as

diminished DNA methylation, which could be improved by increased FA status (Friso et al., 2002; van der Put et al., 1995). MTR and MTRR variants are also associated with NTD (Cai et al., 2019; Ouyang et al., 2013) and altered homocysteine levels (Gaughan et al., 2001). These data indicate the importance of the methionine cycle in NTC. However, the interrelationship between the three OCM cycles – folate cycle, methionine cycle, and mitochondrial glycine cleavage cycle has been demonstrated; where, a mutation in one arm of the OCM pathway can alter the flux of one-carbon units in the other cycles. Thus, it may be an oversimplification to view the effect of a mutation as limited to an individual OCM cycle but instead to consider how it may affect the partitioning of one-carbon units between these three cycles that together are critical for NTC (Leung et al., 2017).

Epigenetic changes are also possible as FA supplementation over multiple generations exacerbates FA sensitive mutations (Marean et al., 2011). It has been demonstrated that there is a trans-generational effect of a mutation in the methionine synthase reductase gene. *Mtrr* deficiency in either maternal grandparent of offspring can result in developmental defects of the grandprogeny and global DNA hypomethylation (Padmanabhan et al., 2013). These data suggest that modulations in folate metabolism can be passed down epigenetically for many generations.

Notably, many of the FA responsive genes identified in mice have no apparent roles in FA metabolism or other processes related to this pathway. For example, PAX3 is necessary for successful NTC by regulating cell proliferation and suppressing neuronal differentiation. Although levels of individual folate cycle species are not altered in *Pax3* mutants (Sudiwala et al., 2019), supplementation with FA prevents NTD, whereas FA deficiency increases NTD incidence (Burren et al., 2008; Wlodarczyk et al., 2006). FA supplementation helps to drive cell cycle progression which is proposed to prevent NTD in *Pax3* mutants (Sudiwala et al., 2019). In most cases, however, how FA levels act to prevent or exacerbate NTD incidence in animal models of FA sensitive gene mutations is yet unclear. Additional processes impacted by FA levels but not directly associated with the OCM pathway have been uncovered. There is a correlation between ubiquitination of Histone 2A (H2A) under low FA conditions, and this can lead to modulation of the expression of NTC associated genes (Pei et al., 2019). Additionally, the formation of cilia can be modulated by FA supplementation (our lab unpublished data), and folate is thought to promote ciliogenesis by regulating the methylation of Septin2, an important ciliogenesis gene (Toriyama et al., 2017). The underlying mechanisms for how FA modulates NTC in the case of folate sensitive genes is likely a balance of all of the processes outlined above as well as yet to be discovered processes.

4.2 Non-FA micronutrients

In cases where FA does not protect against NTDs, other micronutrients have been examined. The quest for other factors that may prevent NTD has been driven in part by epidemiological studies and by an understanding of the biology underlying NTC and gene function in mouse models which do not respond to FA. Here we summarize evidence for an impact on NTD incidence by inositol, iron, zinc, and retinoic acid. Inositol is important in cell transduction and is an important component of phosphoinositol lipids. Inositol deficiency

can increase NTD risk and inositol supplementation can decrease NTD incidence in *curly tail* mouse mutants, which are FA-resistant, and in diabetic rodent models (Reece et al., 1993). Inositol supplementation was used as a preventative treatment for recurrent NTD in humans, with no detriment to the mother or the fetus (Cavalli et al., 2011). This led to a small clinical trial (PONTI: Prevention of Neural Tube Defects by Inositol) which showed inositol supplementation is safe and showed promising results relative to NTD risk (Greene et al., 2016). These results have led to speculation that combined inositol and FA treatment will have a more optimal effect among women who have had a NTD affected pregnancy.

Iron deficiency is the most common micronutrient deficiency among pregnant women, and the World Health Organization estimates that 40% of pregnant women are iron deficient or anemic. Despite the importance of iron in numerous cellular reactions and oxygen transport, its effect on NTC has been little studied. Epidemiological studies have differed as to whether there is a correlation between iron status and NTD risk (Felkner et al., 2005; Molloy et al., 2014). In mouse, mutation of the *Ferroportin1* (*Fpn1*) gene, which transports iron from the visceral endoderm to the embryo, causes a high NTD rate, as does culture of wildtype mouse embryos in an iron chelator (Mao et al., 2010). *Fpn1* mutants do not respond to FA but iron supplementation can reduce NTD incidence (Stokes et al., 2017). However, iron levels may also influence folate metabolism (Herbig & Stover, 2002). Indeed, in mouse it was found that iron supplementation can cause folate deficiency even in wildtype dams and embryos (Stokes et al., 2017). In a study of a mouse mutant for the *Lrp2* gene, which mediates endocytosis of the FA receptor as well as uptake of transferrin bound iron, high FA supplementation was able to prevent NTDs, while iron supplementation alone had no effect, or in combination iron worked against the positive effects of FA supplementation (Sabatino et al., 2017). This highlights the interrelationship between iron and folate metabolism and lends caution to an oversimplification of the effect of an individual nutrient without a broader look at other possible confounding effects, both genetic and environmental.

Zinc is another vital micronutrient and almost 3,000 proteins interact with zinc. Epidemiological studies indicate that zinc deficiency is associated with elevated NTD risk whereas zinc supplementation has been correlated with decreased risk as well as increased risk, perhaps due to intracellular versus peripheral levels of zinc depending on uptake (H. Li, Zhang, & Niswander, 2018). Knockout in the mouse of three zinc transporters (ZIP1, and double knockout of ZIP1 and ZIP3) on a zinc deficient diet leads to a 2–3 fold increase of risk for NTDs (Dufner-Beattie et al., 2006). This implies that zinc transporters are essential for the distribution and retention of zinc, and this is important for NTC. Zinc deficiency has been linked to p53 ubiquitylation, through the zinc binding protein, MDM2, which leads to p53 stabilization and abnormal apoptosis, resulting in NTDs (H. Li, Zhang, & Niswander, 2018). Conversely, experimentally induced zinc accumulation by exposure to zinc oxide nanoparticles cause NTDs in chickens and mice, likely due to abnormal apoptosis. This apoptosis is thought to result from endoplasmic reticulum related stress induced by intracellular calcium concentrations (Y. Yan et al., 2021). These studies indicate imbalance in the level of zinc may contribute to NTDs and that further studies of maternal and fetal zinc status in NTD cases are warranted.

Retinoic acid (RA) is another micronutrient implicated in NTDs. RA is a vitamin A derivative and a proper spatial gradient of RA is required for development of the hindbrain and spinal cord (Araya García, 2017). During normal development, RA suppresses BMP, another key factor in neural tube patterning. Cyp26a1 is an enzyme responsible for maintaining precise concentrations of RA, and in *Cyp26a1* knockout mice, NTC fails (Abu-Abed et al., 2001). FBXO30 ubiquitin ligase can promote ubiquitin-mediated degradation of Retinoic acid receptor gamma and hence can positively regulate BMP signaling. A case-control study in humans revealed that human NTD cases are enriched for mutations in vitamin A related processing genes (H. Li, Zhang, Chen, et al., 2018). In NTD cases with high levels of retinol, BMP target genes are downregulated and FBXO30 is dysregulated (X. Cheng et al., 2019). Together the data indicate that when the RA pathway is unbalanced due to deficiency or high dosage, the patterning of the neural tube cannot proceed as normal. Overall, micronutrients play a key and dynamic role during NTC, and the roles of these nutrients and how they influence each other requires further investigation in order to understand the causes of NTDs and the most effective types of therapies, depending on the genetic makeup of individuals.

4.2 Diabetes

Shifting to a consideration of maternal factors associated with NTD risk, maternal diabetes and obesity are significant risk factors for NTDs (Zabihi & Loeken, 2010). The embryo does not develop pancreatic function until week 7 of pregnancy, and thus relies on the metabolism of the mother during neural tube formation (Lupo et al., 2012). It is proposed that altered metabolism in the mother can detrimentally affect NTC. This is supported by correlation between SNPs in maternal glucose metabolism genes (*FTO, LEP, TCF7l2, LEPR, GLUT1, Glut2, and HK1*) and increased NTD risk(Loeken, 2020; Lupo et al., 2012; Wilde et al., 2014). The exact mechanism is still under debate, however animal models point towards decreased proliferation and increased apoptosis within the NE (Gao & Gao, 2007). Chemically induced diabetes in mice with streptozotocin has implicated several pathways including: increased oxidative stress and transcription of *Jnk1/2* which can trigger pro-apoptotic pathways (X. Li et al., 2012), upregulation of *miR-200b* and subsequent repression of its targets leading to ER stress (Gu et al., 2016), and activation of the *Foxo3a* - *Caspase8* pathway which upregulates apoptosis (Yang et al., 2013). Further evidence for the latter pathway is demonstrated through the reduction of NTDs in streptozotocin mice via knockout of *Foxo3a* (Xu et al., 2021). Evidence from another diabetic mouse model, the nonobese diabetic mouse strain which exhibits high rates of NTDs, points towards defects in cell migration when exposed to high amounts of glucose both in vitro, and in-vivo (Herion et al., 2019). This suggests convergent extension as another diabetic sensitive NTC process. The complexity in parsing diabetes associated contributions towards NTD risk is driven home by the observation that more than a third of genes involved in NTC have their expression altered in maternal diabetes models (Salbaum & Kappen, 2010).

Conclusion

Large-scale sequencing of NTD samples by researchers across the globe will be key in moving beyond testing of genes identified in animal studies to a more comprehensive

characterization of human risk alleles and to uncover the multigenic nature of NTDs in humans. Efforts to bring together cohort samples and sequencing data, such as the Spina Bifida Sequencing Consortium (https://sbseqconsortium.org), will greatly increase our ability to pinpoint candidate genes for future evaluation of NTD risk. Current sequencing studies include samples from NTD cases conceived post-FA fortification as well as cases prior to FA fortification or from countries that do not yet include FA fortification. This holds the possibility of identifying NTD risk alleles that differ in these populations, which may reflect FA sensitivity. It is speculated that the burden of de novo mutations and/or the genetic landscape may differ pre- and post-FA fortification, perhaps representing a shift in the threshold of severity or number of gene variants in NTD cases after decades of FA supplementation (Lee & Gleeson, 2020). Most sequencing efforts are focused on identifying germline mutations, but an additional challenge arises from the idea that somatic mutations at the NTD lesion site may contribute to NTD. Recent sequencing studies of the lesion site have discovered tissue mosaicism with somatic mutations in proteins of the Mediator complex as well as PCP genes(Tian et al., 2021). Moreover, experimental studies in mouse showed that as few as 16% of cells carrying a *Vangl2* mutation can non-autonomously prevent apical constriction in neighbouring cells to prevent NTC(Galea et al., 2021). Also, increased signatures of DNA mismatch repair deficiency have been associated with low folate status(H. Li et al., 2020). In the future, it is possible that single-cell sequencing may reveal the extent to which localized perturbations, rather than germline mutations, contribute to NTD. The overall goal will be to provide better genetic counselling for genetic risk factors and more personalized consideration of nutrient supplements for better outcomes of all births.

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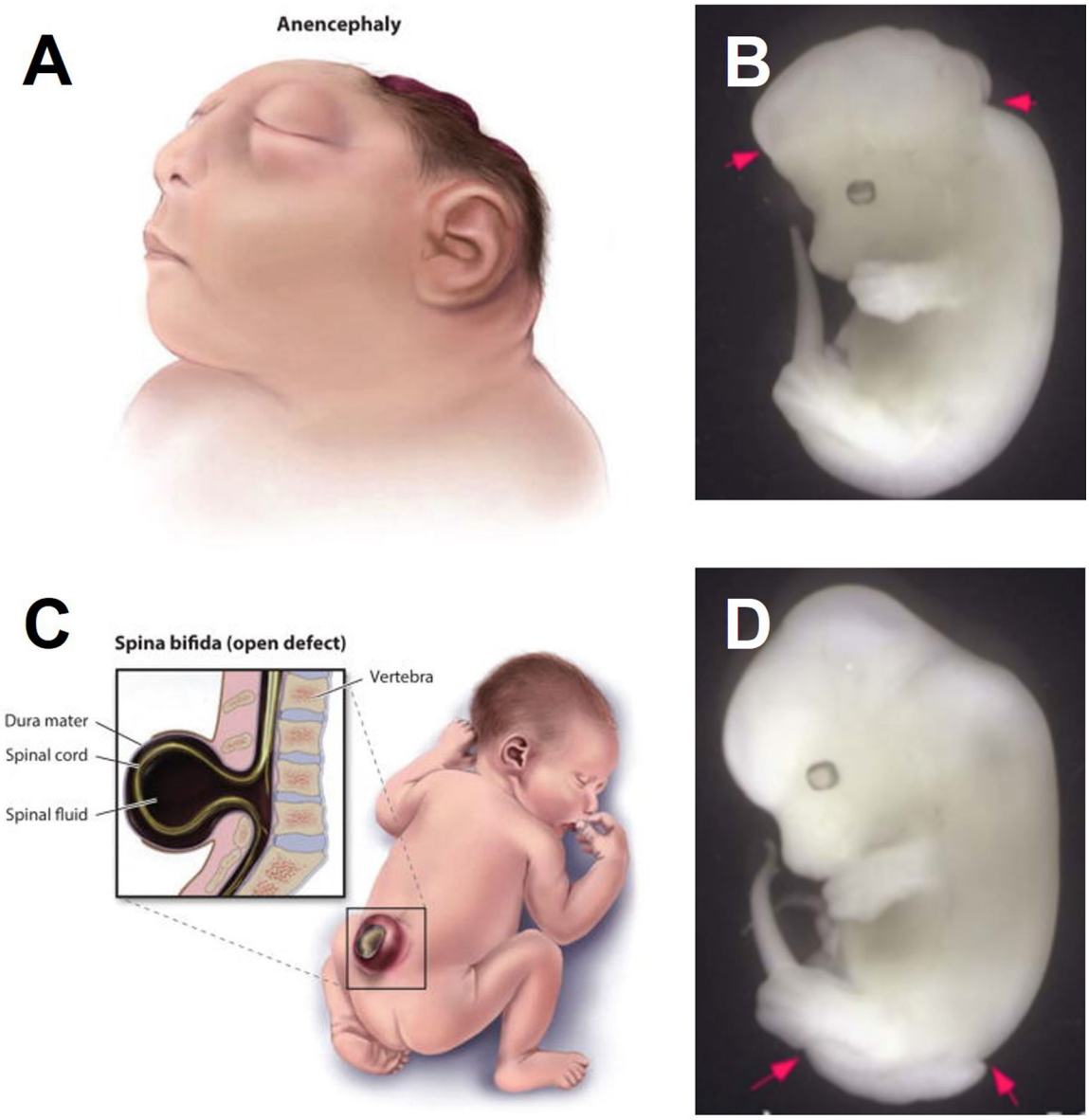

Figure 1: NTD phenotypes

A) Human anencephaly **B)** Mouse exencephaly **C)** Human spina bifida **D)** Mouse spina bifida

Fig 1A,C from Wilde, 2014

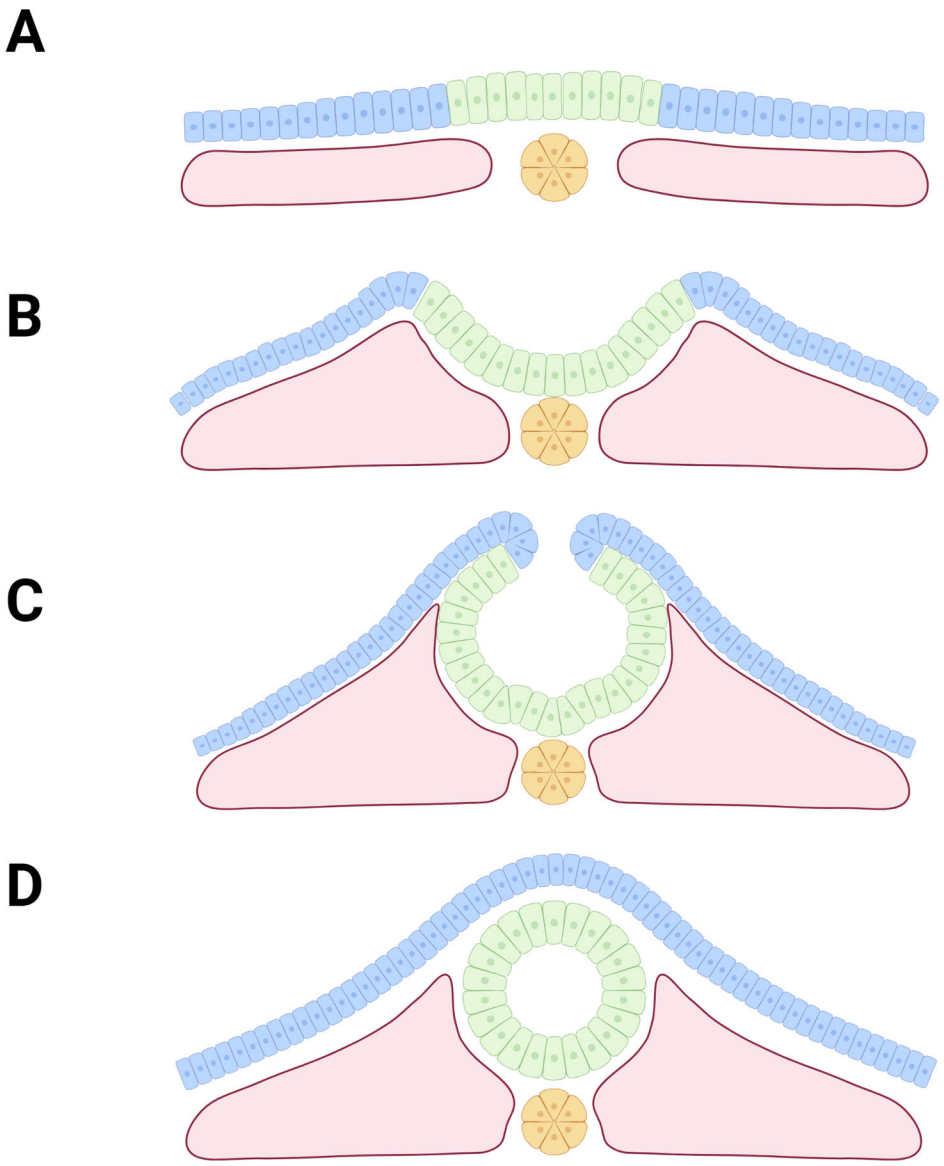

Figure 2: Process of NTC

A) NTC begins with a flat sheet of epithelial cells (NE cells shown in green, NNE cells shown in blue) overlaying the notochord (yellow) and mesoderm (pink). **B)** Convergent extension and proliferation drive the bulging of the mesoderm and formation of neural folds. **C)** Actomyosin contractions form medial and dorsolateral hinge points as neural folds bend and approach one another. **D)** Neural folds adhere to partner tissue and fuse, forming intact neural tube covered by NNE

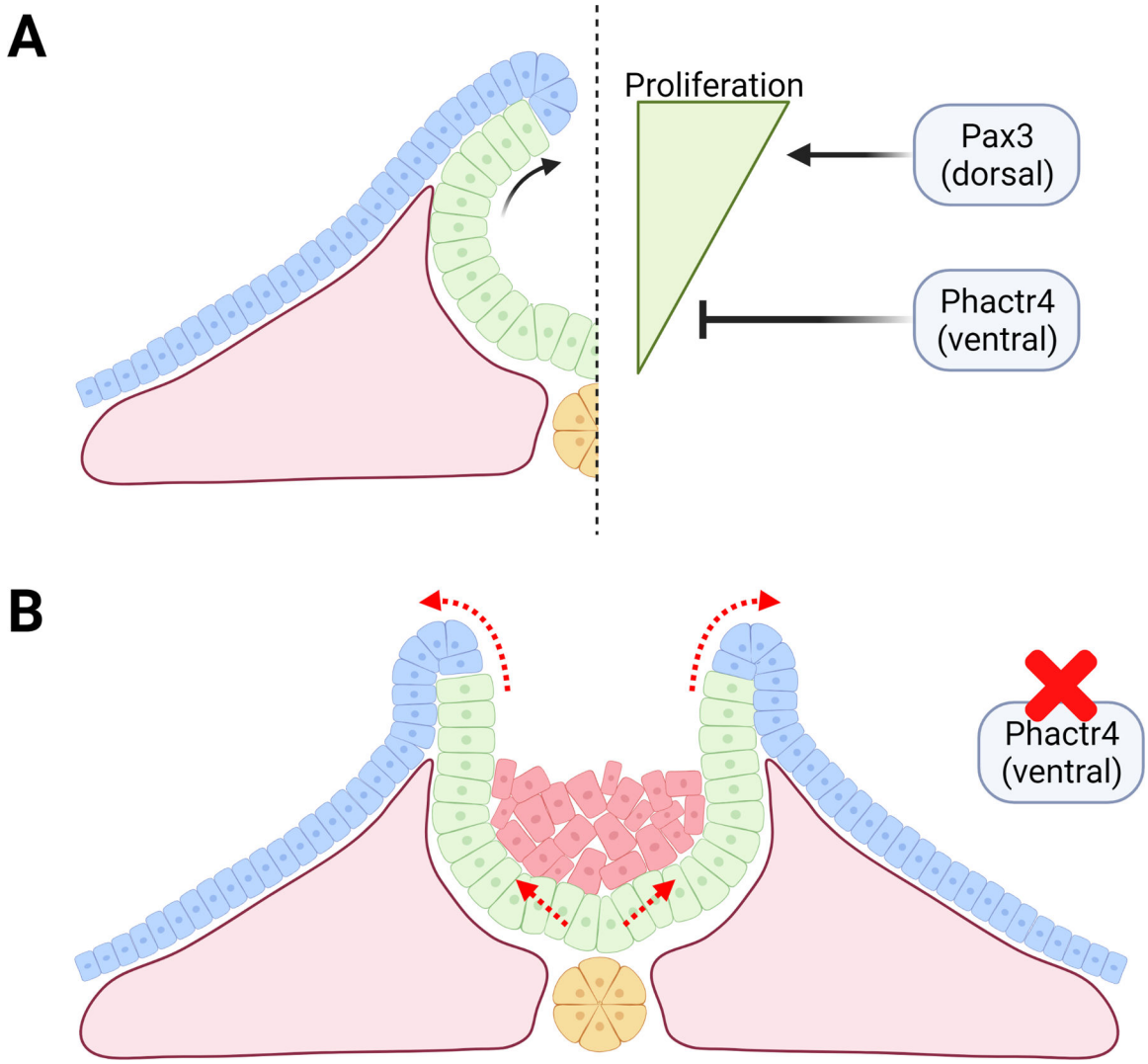

Figure 3: Disruption of NE proliferation

A) Illustration of proper balance of dorsal/ventral proliferation. Pax3 acts to increase proliferation dorsally, while Phactr4 acts to inhibit proliferation ventrally. **B)** Mice lacking Phactr4 display abnormal ventral NE proliferation (unexpected cells shown in red). Buildup of cells ventrally changes the geometry, which does not allow proper neural fold bending and inhibits closure.

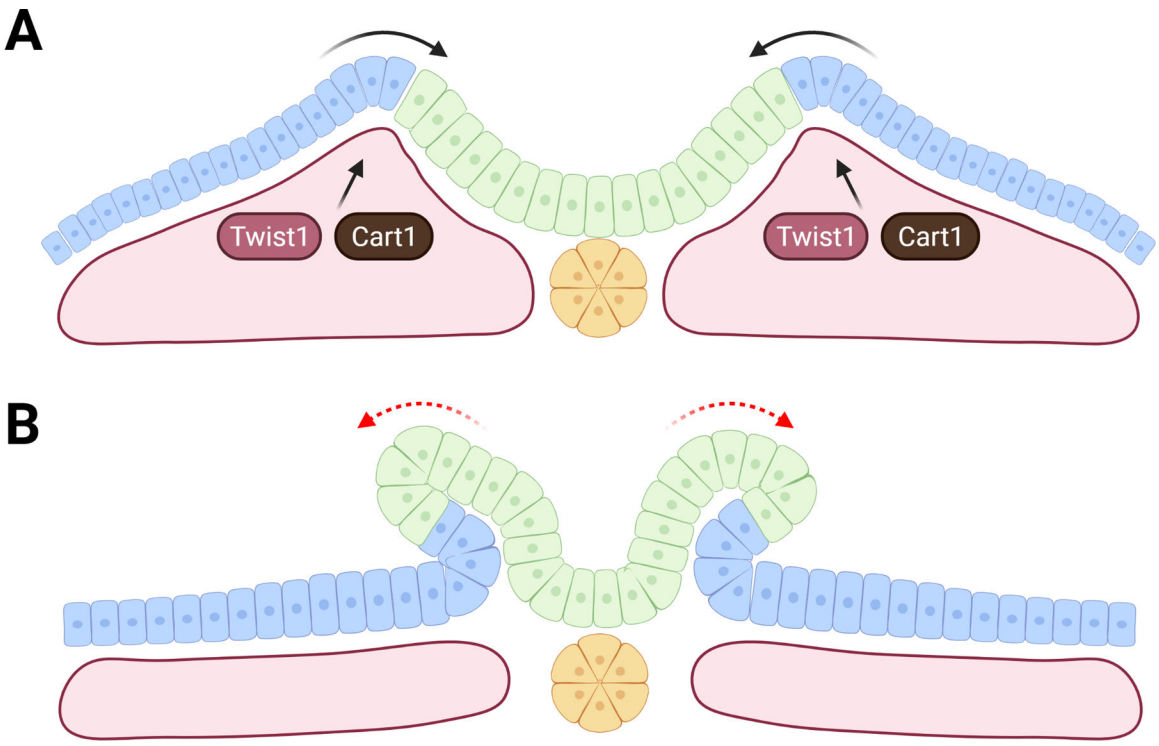

Figure 4: Disruption of mesoderm proliferation

A) Illustration of proper mesoderm proliferation and migration, highlighting *Twist1* and *Cart1* as necessary for bulging of mesoderm. **B)** Loss of *Twist1* or *Cart1* causes NE proliferation to outpace mesoderm growth, preventing the neural folds to adopt a concave orientation.

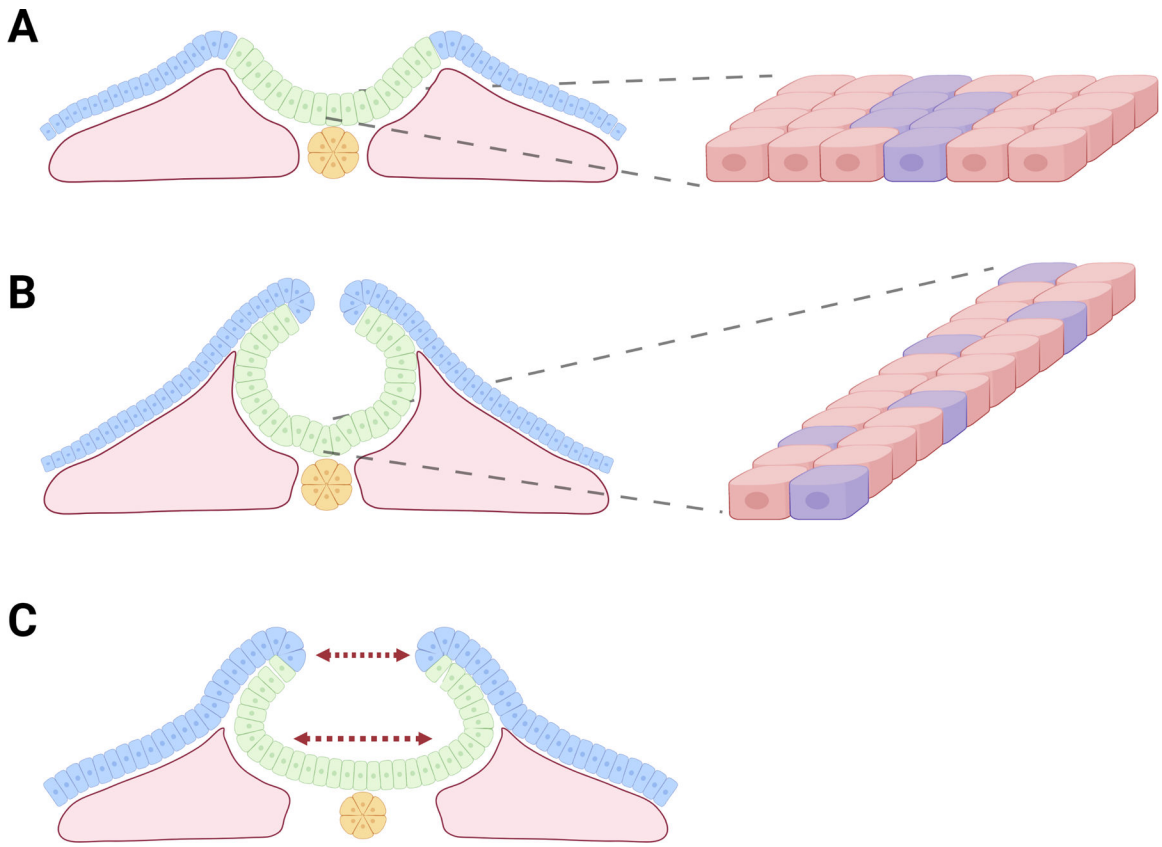

Figure 5: Disruption of cell migration & intercalation

A) Illustration of NE cell sheet with selected cells marked in purple. **B)** Convergent extensions movements drive the narrowing and lengthening of the cell sheet due to cell migration and intercalation. **C)** Disruptions to cell migration & intercalation result in a wide neural plate which prevents contact of neural folds.

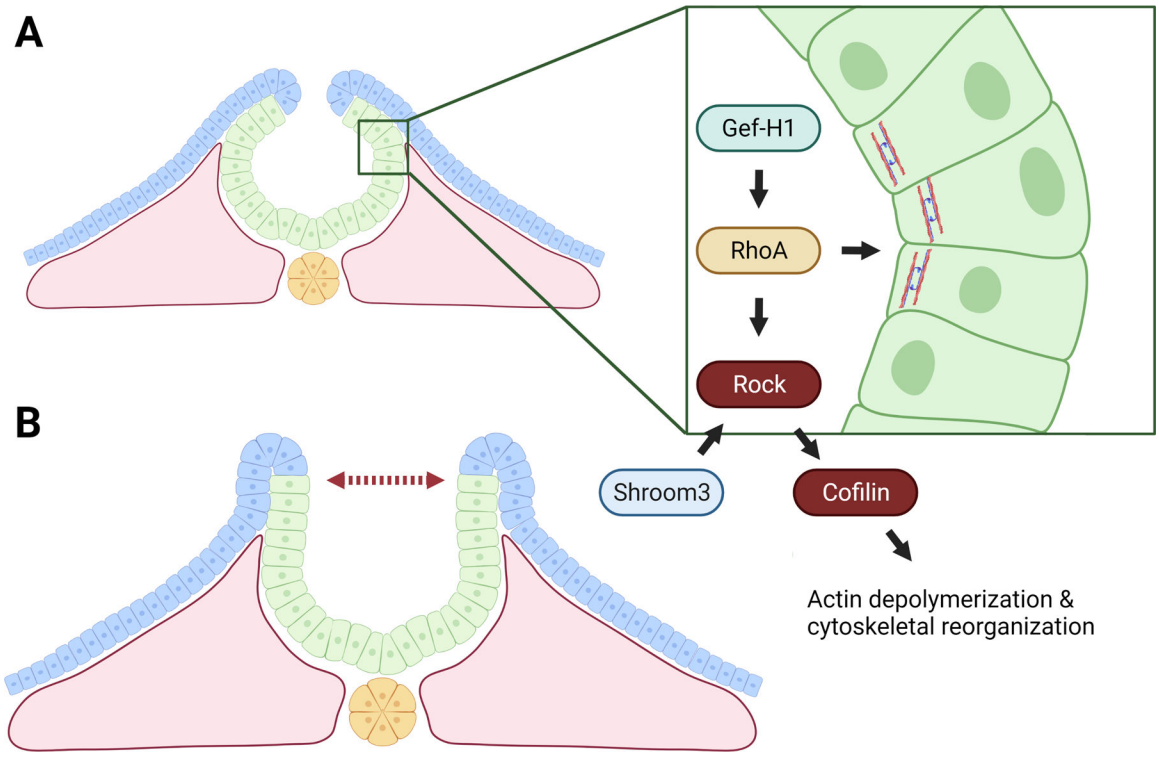

Figure 6: Disruption of actomyosin contraction

A) Illustration of components necessary for proper localization, contraction, and turnover of actomyosin. **B)** Disruption of actomyosin contraction inhibits formation of hinge points and neural fold bending.