Neurogenetics (2006) 7: 201–221
DOI 10.1007/s10048-006-0052-2

REVIEW ARTICLE

Patrizia De Marco · Elisa Merello · Samantha Mascelli · Valeria Capra

Current perspectives on the genetic causes of neural tube defects

Received: 11 April 2006 / Accepted: 29 May 2006 / Published online: 29 August 2006

Abstract Neural tube defects (NTDs) are a group of severe congenital abnormalities resulting from the failure of neurulation. The pattern of inheritance of these complex defects is multifactorial, making it difficult to identify the underlying causes. Scientific research has rapidly progressed in experimental embryology and molecular genetics to elucidate the basis of neurulation. Crucial mechanisms of neurulation include the planar cell polarity pathway, which is essential for the initiation of neural tube closure, and the sonic hedgehog signaling pathway, which regulates neural plate bending. Genes influencing neurulation have been investigated for their contribution to human neural tube defects, but only genes with well-established role in convergent extension provide an exciting new set of candidate genes. Biochemical factors such as folic acid appear to be the greatest modifiers of NTDs risk in the human population. Consequently, much research has focused on genes of folate-related metabolic pathways. Variants of several such genes have been found to be significantly associated with the risk of neural tube defects in more studies. In this manuscript, we reviewed the current perspectives on the causes of neural tube defects and highlighted that we are still a long way from understanding the etiology of these complex defects.

Keywords Neural tube defects (NTDs) · Neurulation · Genetic risk factors · Planar cell polarity (PCP) pathway · Folate metabolism

Abbreviations NTDs: Neural tube defects · AED: Antiepileptic drug · CRs: Cysteine-rich domains · BMP: Bone morphogenetic protein · FGF: Fibroblast growth factor · FGFR: Fibroblast growth factor receptor · IGF: Insulin-like growth factor · DV: Dorsoventral · AP: Anteroposterior · RA: Retinoic acid · Shh: Sonic hedgehog · b-HLH: Basic helix-loop-helix · RARs: Retinoic acid receptors · PCP: Planar cell polarity · MHP: Median hinge point · DLHPs: Dorsolateral hinge points · GCPS: Greig cephalopolysyndactyly syndrome · HPE: Holoprosencephaly · BM: Body mass · Hcy: Homocysteine · THF: Tetrahydrofolate · LDL: Low-density lipoprotein · PKC: Protein kinase C

P. De Marco · E. Merello · S. Mascelli · V. Capra (✉)
U.O. Neurochirurgia, Istituto G. Gaslini,
Largo G. Gaslini 5,
16148 Genova, Italy
e-mail: valeriacapra@ospedale-gaslini.ge.it
Tel.: +39-010-5636712
Fax: +39-010-3993159

Introduction

Neural tube defects (NTDs) are a group of birth anomalies resulting from failure of fusion of the neural tube around the 28th day after conception, at a time when most women do not know they are pregnant. NTDs are known to occur in 1 out every 1,000 pregnancies in the United States, with varying rates reported among the world's populations [1]. The most common NTDs is anencephaly, which results from failure of fusion of the cranial neural tube, and myelomeningocele (commonly called spina bifida), which results from the failure of fusion in the spinal region of the neural tube. Failure of closure that involves the entire body axis is known as craniorachischisis, which is an additional, relatively rare, form of dysraphism. Anencephaly and myelomeningocele are referred as "open" NTDs because the affected region is exposed to the body surface. There are also a number of closed or skin-covered conditions that involve the neural tube, including: encephalocele, meningocele, lipomeningocele, also referred to as spina bifida occulta, and sacral agenesis. All infants with anencephaly are stillborn or die shortly after birth, whereas many infants with spina bifida survive, usually as a result of extensive medical and surgical care. However, affected individuals are at risk for a range of physical and developmental disabilities (abnormal innervation beneath the level of lesion, varying degrees of muscle weakness, and sensory impairment, neurogenic bladder, and bowel). The majority of NTDs (approximately 70%) occur in isolation and show multifactorial inheritance.

Several lines of evidence suggest a genetic component of NTDs: firstly, NTDs are associated with known genetic syndromes, trisomies 13 and 18, and various chromosome rearrangements [2]; secondly, the estimated recurrence risk in siblings is 2–5%, representing up a 50-fold increased risk over that observed in general population [3]; and finally, the presence of family history in a number of affected ones [4]. There is a recent epidemiological evidence suggesting that genetic factors related to NTDs may be transmitted preferentially from the mother's side of the family [5]. Despite extensive efforts, no one single major gene has been implicated as a direct causal agent of these human defects, a common result in genetic studies of complex diseases.

Overview of human nervous system development

In human embryos, neurulation occurs in two phases: primary and secondary neurulation. Primary neurulation concerns the transformation of the flat ectodermal neural plate into the cylindrical neural tube. Secondary neurulation is limited to the tail bud and occurs by proliferation and condensation of stem cells that subsequently cavitates. The cavitation results in the formation of a tube making contact with the central canal of the portion of the neural tube formed during primary neurulation. The two processes have been reviewed elsewhere [6–8].

The closure of neural tube in the human embryo has been initially described as a continuous process that begins at the level of the future cervical region and proceeds both rostrally and caudally [9]. However, multiple initiation sites of neural tube closure have been demonstrated in mice and other species. In the mouse, neural tube closure begins at the boundary between the hindbrain and the cervical region (closure 1), with closure spreading from this site rostrally into the hindbrain, as well as caudally into the spinal region. A second point of de novo initiation of neural tube closure is in the vicinity of the forebrain–midbrain boundary (closure 2), and a third initiation site is at the rostral extremity of the neural plate (closure 3) [10]. Although the location of closures 1 and 3 appears to be uniform between mouse strains, the site of closure 2 is polymorphic and in some mouse strains (e.g., SELH/Bc) does not occur at all [11]. When closure site 1 fails, the entire neural tube from the midbrain to the lower spine remains open, which is a condition known as craniorachischisis. Embryos in which closure 2 is disrupted have exencephaly. The failure of closure 3 leads to anencephaly that is confined to the forebrain region, often in association with split-face malformation. When the caudal spread of fusion from closure 1 fails to be completed, the posterior neuropore remains open which results in open spina bifida or myelomeningocele. Based on a study of the type and frequency of human NTDs, Van Allen et al. [12] proposed a model in which five closure sites exist in human embryos. According to their model, closure 1 occurs in the cervical region and extends bidirectionally. Closure 2 takes place at the junction between prosencephalon and mesencephalon and also extends bidirectionally. Closure 3 initiates at the rostral tip of the neural groove and proceeds caudally to meet closure 2. Closure 4 covers the rhombencephalon and completes the closure of cranial neural tube. Closure 5 initiates from the caudal end of the neural groove and spreads cranially (Fig. 1). Although this model was attractive to explain human defects, examination of histological sections of human embryos leads to different models of neural tube closure. In fact, a study by Nakatsu et al. [13] described three sites of apposition, while O'Rahilly and Muller [14] found only two regions of fusion in humans, the first one extending bidirectionally from the rhombencephalic region and the second one that proceeding caudally from the prosencephalic region. The human closure events found by O'Rahilly and Muller have striking resemblance to mouse closures 1 and 3. Therefore, multisite neural tube closure may be a universal phenomenon, although the process appears to be not the same in the human and the other species. This implies, as remarked by Nakatsu et al. [13], that "care should be taken when extrapolating embryological data from laboratory animals to the human".

Molecular genetics of neurulation

Our ability to understand the pathological mechanisms leading to NTDs depends on the extent to which we understand the normal processes that take place during neurulation. Unfortunately, our current knowledge of these processes is limited, although this situation is changing rapidly because of the growing interest of biologists in neural development at a number of different levels: cellular studies of neurulation, studies of mutant mouse, and studies of the interactions responsible for inducing and patterning of neural plate and tube. This review dissects neurulation into distinct processes that, in turn, are further dissected at the molecular level. Moreover, we present the results of genetic analysis of human homologues of genes causing NTDs in animal models.

The earliest step in the formation of the nervous system is neural induction, the process by which ectodermal cells adopt a neural identity. Concomitantly with neural induction, the induced neural plate becomes patterned along the craniocaudal, dorsoventral, and mediolateral axes. While regional patterning is occurring, the neural plate undergoes morphogenesis: neural folds arise, approach one another in the dorsal midline, and fuse.

Neurulation is driven by redundant mechanisms both at the tissue and cellular level, as well as the molecular level of organization [7]. Nevertheless, even with both intrinsic and extrinsic forces acting redundantly, disruption of neurulation does occur, resulting in NTDs [7].

Neural induction

The amphibian embryo provides an excellent system for studying how the central nervous system is formed in a

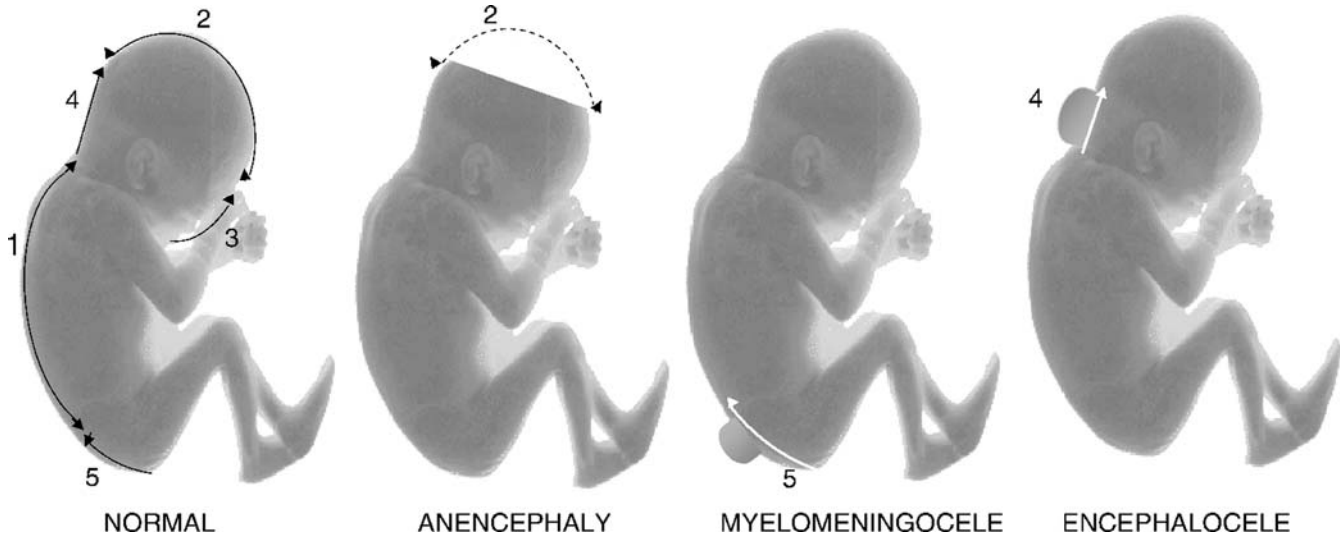

Fig. 1 Multisite closure of the neural tube in the human embryo based on the model proposed by van Allen et al. [12]. According to this model, five closures (four in the head and one in the lumbar region) exist in the neural tube of human embryos. The *arrows* indicate the direction of closure. The various types of human NTDs can be explained by failure of fusion of one of the site closures. Anencephaly results from the failure of closure 2; sacral myelomeningocele results from failure of closure 5; occipital cephalocele results from incomplete membrane fusion of closure 4

vertebrate embryo. Back in 1924, Spemann and Mangold [15] showed that grafts of the dorsal lip region ("organizer") of *Xenopus* embryos were able to induce twinning of neural tissue when transplated into an ectopic site. Extensive screens for organizer-specific genes have been performed and lead to the identification of genes that encode secreted proteins expressed specifically in Spemann's organizer [16]. Chordin is one of the most abundant proteins secreted by organizer tissue at the gastrula stage, reaching concentrations of 6–10 nM in the extracellular space. When microinjected into *Xenopus* embryos, chordin mRNA is able to induce twinning and neural induction, recapitulating Spemann's experiments [17, 18]. Chordin is a secreted protein with four cysteine-rich domains (CRs) of about 70 amino acids each. Each CR domain constitutes a bone morphogenetic protein (BMP)-binding module [19]. The chordin loss-of-function phenotype can be rescued by knockdown of BMPs, underscoring that chordin is a dedicated BMP antagonist [20]. BMPs normally prevent embryonic ectoderm from executing its natural "default" tendency to differentiate into neural tissue and instead instruct cells to form epidermis [21, 22]. Lack of BMP signaling in turn leads to the dorsalization of mesoderm and to the neuralization of ectoderm [23] (Fig. 2). Other BMP inhibitors include follistatin, noggin, cerberus, Xnr3 (*Xenopus* nodal-related 3), and TSG (twisted gastrulation) [21, 24–27]. Recent findings indicate that the neural ectoderm is specified in the blastula, before the Spemann's organizer even forms [28]. Fibroblast growth factor (FGF) signaling is required at this stage to enable later neural differentiation [29, 30]. FGFs are a large class of secreted diffusible glycoproteins that bind to four classes of extracellular receptors (FGF receptors, FGFR) to mediate their effects [31]. These transmembrane receptors consist of an extracellular FGF-ligand-binding domain, a transmembrane domain, and an intracellular signaling domain [31]. It has been demonstrated that overexpression of dominant negative forms of FGF receptors, which contain the FGF-binding domain but lack the intracellular domain, blocks the generation of neural tissue [32].

Canonical Wnt signaling has also been implicated in the selection of neural or epidermal fate in *Xenopus*. Wnt signaling molecules are a large class of highly conserved secreted glycoproteins, which participate in multiple developmental events during embryogenesis [33]. In *Xenopus*, Wnt signaling activates a transcriptional corepressor of the iroquois family (*Xiro1*), which in turn downregulates BMP expression [34].

Recently, the insulin-like growth factor (IGF) family has also been shown to act as neural inducer in *Xenopus* ectodermal explants [35]. IGFs signal through receptor tyrosine kinases; the phosphorylation and the consequent inhibition of a transcription factor called Smad1 leads to neural induction [36]. As proposed by Pera et al. [36], IGFs and BMP antagonists induce neural tissue by a common mechanism mediated by low levels of Smad1 activity.

Loss of function of noggin, chordin, follistatin, or cerberus 1 in the mouse does not lead to defects in neural induction [37–40]. Double homozygous mutants of chordin and noggin show loss of the prosencephalon [40]. In the zebrafish, loss of chordin in the *chordino* mutants leads to a decrease in the size of the neural plate [41]. By contrast, targeted null mutations in the mouse *Bmp2*, *Bmp4*, or *Bmp7* genes do not lead to changes in size of neural plate [42–45]. Mice lacking either Smad1 and Smad5 activity, two intracellular mediators of BMP signal-transduction components, die early in embryogenesis, but these mutations have no effect on neuroectodermal fate specification [46, 47]. Evaluation of *BMP4* gene and its inhibitor *Noggin* (*NOG*) has also been investigated as

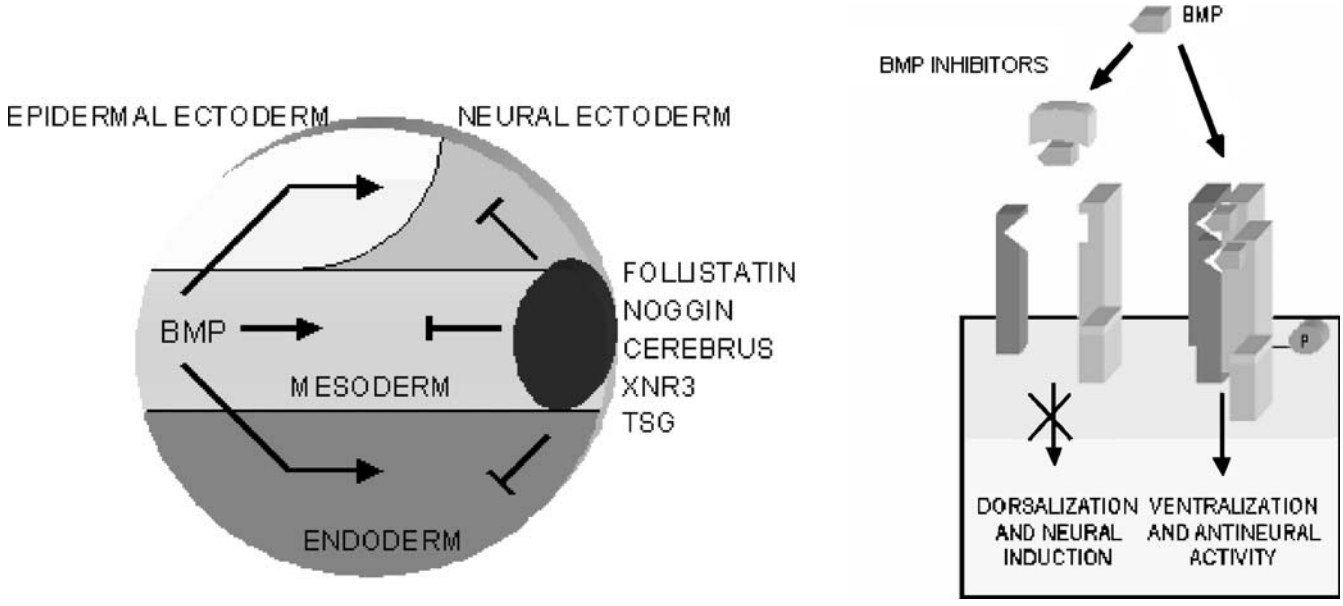

Fig. 2 Neural induction. Signals from the Spemann's organizer induce ectoderm to become anterior neural tissue. Secreted bone morphogenetic protein (BMP) causes ectodermal cells to become epidermis and prevents ectoderm from executing its natural "default" tendency to differentiate into neural tissue. Chordin, follistatin, noggin, cerberus, Xnr3, and TSG (produced by Spemann's organizer) block BMP signaling leading to the dorsalization of mesoderm and to neuralization of ectoderm. *Xnr3, Xenopus* nodal-related 3; *TSG,* twisted gastrulation

candidates in human NTDs. Four missense mutations in *BMP4* and one in *NOG* were found, but it is likely that these mutations act together with other gene variants in independently segregating loci [48].

Neural plate shaping

Increasing evidences suggest that the main driving force for neural plate shaping is convergence extension, a medially directed movement of the cells, with intercalation in the midline, which leads to narrowing and lengthening of the neural plate. In *Xenopus*, misexpression of *dishevelled* (*Xdsh*) produces disruption of convergence extension and NTDs because the broadened midline results in neural folds that are too far apart to meet [49]. These defects result from the failure of the planar cell polarity (PCP) pathway, which is instrumental in governing the polarization of cells within the plane of a cell sheet. PCP signals control cell polarity during convergent extension (CE), an essential process for vertebrate neural tube closure [49–55]. Very recently, Ciruna et al. [56] demonstrated a novel role for PCP signaling during neurulation. These authors demonstrated that PCP signaling polarizes neural progenitors along the AP axis. This polarity, which is transiently lost during cell division in the neural keel, is reestablished as daughter cells reintegrate into the neuroepithelium. The PCP pathway is also referred to as the noncanonical Wnt pathway, in contrast to the canonical Wnt pathway that acts via stabilization and nuclear translocation of armadillo (Arm, β-catenin in vertebrates) (Fig. 3). Members of Frizzled (Fz), a family of seven-transmembrane receptors, and Dishevelled (Dsh/Dvl), a cytoplasmic transducer protein, are essential for both the canonical and non-canonical PCP pathway [57, 58]. Drosophila genetics revealed several other signaling components that are specific to the PCP pathway, including Flamingo (Fmi), Strabismus (Stbm)/Van Gogh (Vangl), Prickle (Pk), Diego, RhoA, and Rho kinase [59–65]. A recent study has shown that the PCP pathway might also be associated with cilia morphogenesis. Disruption of *Xenopus laevis* orthologs of two fly PCP effector proteins, Inturned and Fuzzy, leads to defective neural tube closure [66]. The mutant embryos (Xint and Xfy) show failure of ciliogenesis as a consequence of microtubules incorrectly orienting themselves. Moreover, accumulation of Dsh and Int near the basal apparatus of cilia suggests that these proteins regulate ciliogenesis through a conserved PCP pathway [66]. The relationship between ciliary function and closure of the neural tube has been demonstrated in humans by the identification of two genes (*MSK1* and *MSK3*) responsible for Meckel syndrome, one of the major contributors to syndromic NTDs, and potentially involved in the formation of the cilia apparatus [67, 68].

Mutations in the mouse orthologs of the *Drosophila* PCP genes result in the failure of neural tube closure. Five mouse mutants, *loop-tail* (*Lp*), *circle-tail* (*Crc*), *crash* (*Crsh*), *PTK*, and *dishevelled1/dishevelled2*, harbor defects in PCP genes and fail to undergo closure 1, which leads to craniorachischisis [69–73]. The *Lp* gene, *Vangl2* (also known as *Lpp1* or *Ltap*), has been shown to encode a protein homologous to *Drosophila* strabismus [74]. *Vangl2* is expressed broadly in the neuroectoderm throughout early neurogenesis [75]. This and the fact that the gene was altered in two independent *Lp* alleles identified it as the likely basis for the *Lp* phenotype. The *Crsh* mouse harbors

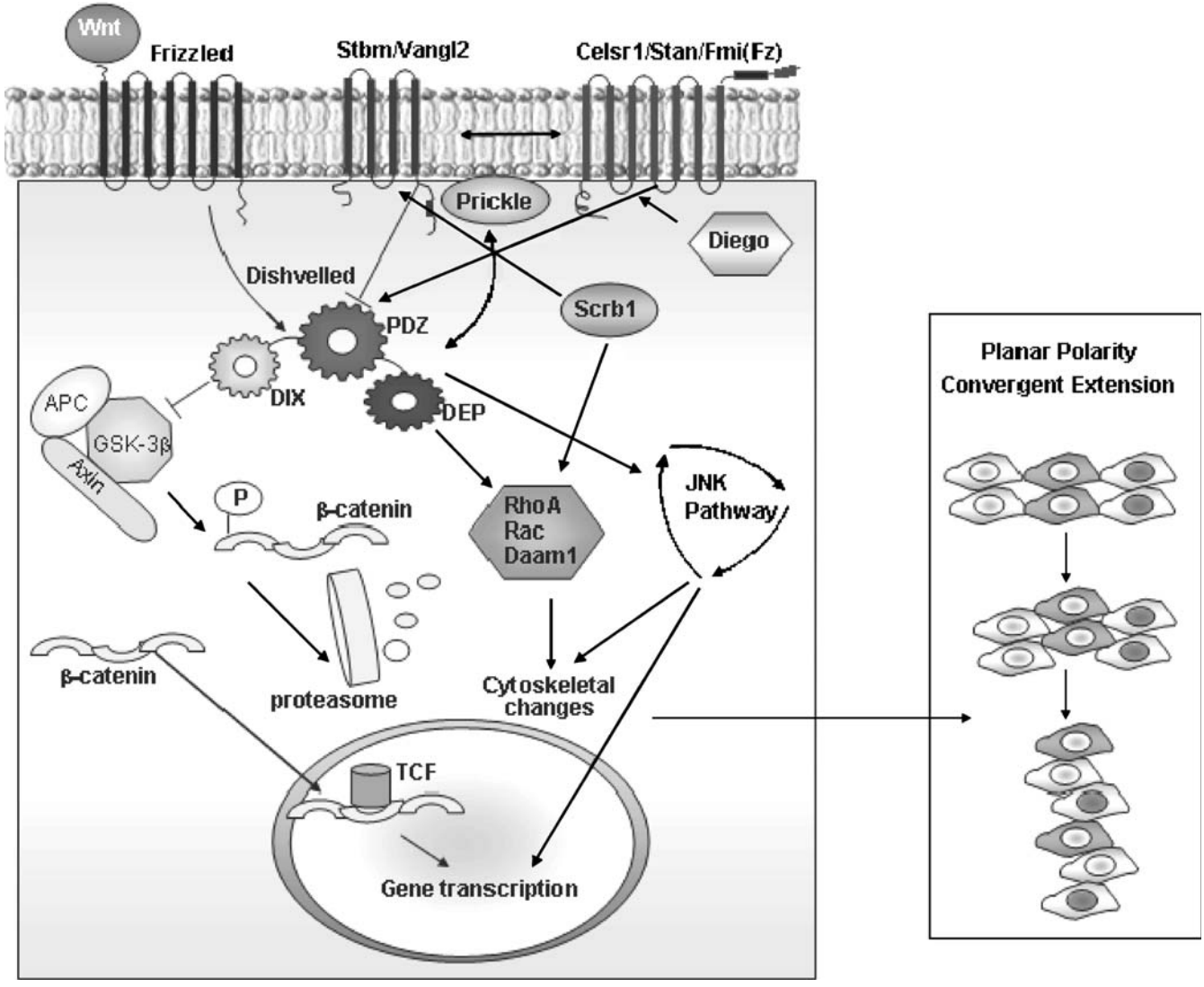

Fig. 3 Canonical (Wnt/β-catenin pathway) and noncanonical (PCP pathway) Wnt signaling. Both pathways require the receptor Frizzled (Fz) and the cytoplasmic transducer Dishevelled (Dsh/Dvl). Dsh contains three conserved domains: the DIX, which is required for canonical Wnt signaling, the PDZ domain, and the DEP domain, required for Dsh localization during PCP signaling. Canonical Wnt signaling is crucial in developmental processes, cell fate specification, and proliferation. In the absence of Wnt signals, a cytoplasmic protein complex, consisting of glycogen synthase kinase 3-β (GSK3-β), adenomatous polyposis coli (APC), and Axin, marks β-catenin for ubiquitin-dependent degradation. In response to a Wnt ligand, this degradation complex is inactivated, and stabilized β-catenin translocates to the nucleus, where it combines with the T-cell factor (TCF), and stimulates target gene expression. In contrast, the PCP pathway signals are transduced to the cytoskeleton through the activation of small Rho GTPases (RhoA/Rac/Daam1) and c-Jun N-terminal kinase (JNK). The PCP pathway requires a number of conserved proteins, including Flamingo, Strabismus, Diego, and Prickle whose interactions have not been defined. Scribble (Scrb1), a member of the LAP protein family, is implicated in the PCP pathway potentially by interaction with Vangl2 and with RhoA. In vertebrate embryos, similar molecular requirements have been established for convergent extension (CE) movements and neural tube closure. The CE movements involve convergence of cells toward the dorsal midline, mediolateral cell intercalations in the notochord, and neural tube leading to extension of the body axis

a mutation in *Celsr1*, a seven-pass transmembrane receptor that encodes a protein orthologous to *Drosophila* Flamingo, also known as *starry night* [71]. Like *Vangl2*, this gene also functions in the PCP pathway. In the *Crc* mouse, a point mutation was identified introducing a stop codon into the apical cell polarity gene *scribble* (*Scrb1*), a PDZ domain-containing gene that is the ortholog of *Drosophila scribble* [76]. *Scribble* was not known to be a PCP component in *Drosophila*. However, a polarity defect is observed in the inner ear of *Crc* mice, suggesting that *Scrb1* does function in establishment of polarity in vertebrates [77]. A mutation in the *protein tyrosine kinase 7* (*PTK7*) gene, which encodes a conserved transmembrane protein with tyrosine kinase homology, disrupts neural tube closure and stereociliary bundle orientation and shows genetic interaction with *Lp* [72]. These findings identify *PTK7* as a regulator of the PCP pathway. Embryos that are double null homozygous for both *dishevelled1* and *dishevelled2*

(*Dsh1*$^{-/-}$/*Dsh2*$^{-/-}$) also exhibit NTDs that closely resemble the craniorachischisis phenotype of *Lp* and *Crc* [73].

Components of the PCP pathway provide an exciting new set of candidate genes for the analysis of human NTDs. Combinations of mutations may act together to provide the genetic risk for human defects. Collectively, these data point to the need to conduct a full analysis of PCP genes to establish a role in the human population. Very recently, Doudney et al. [78] analyzed the coding and splice site regions of human *VANGL1* and *VANGL2* genes in a collection of 66 patients with NTDs. These included 21 patients with craniorachischisis, 24 with spina bifida, and 21 with anencephaly. Only the 346G→A variant in *VANGL1* resulted in a nonsynonymous change (A116T). This variant was found in craniorachischisis and spina bifida patients at a similar frequency with respect to European controls. An elevated frequency was observed in the anencephalic patients, although this is unlikely to be functionally significant. Overall, the data suggest that *VANGL* gene mutations are not a major cause of the severe human NTDs phenotypes.

Dorsoventral (DV) patterning of the neural tube

The neural tube of vertebrates develops a distinct DV pattern, with different cell types arising at different positions along the dorsoventral axis, including floor-plate cells at the ventral midline, motor neurons in ventral regions, and sensory neurons and neural crest in dorsal regions. The generation of DV pattern in the developing spinal cord takes place after neural induction. It has been recognized that the surrounding and underlying mesoderm is the major determinant of the DV structure of the neural tube, rather than it being due to an intrinsic self-organizing capacity (reviewed by Holtfreter and Hamburger [79]). Four classes of secreted factors have been implicated in the DV patterning: FGFs, retinoic acid (RA), sonic hedgehog (Shh), and BMP (Fig. 4). Firstly, FGFs, which are expressed by the caudal mesoderm, must be switched off [80]. Secondly, RA that is produced in the paraxial mesoderm diffuses into the neural tube where it induces differentiation in the neuroepithelium and inhibits FGF signaling [81, 82]. RA induces ventral genes such *Pax6*, *Irx3*, *Dbx1*, and *Dbx2*, a group of homeodomain transcription factors [82]. Thirdly, Shh acts both locally and distally in the control of cell fate in the ventral neural tube. Ectopic expression of Shh in vivo and in vitro can induce the differentiation of floor-plate cells, motor neurons, and ventral interneurons [83, 84]. Conversely, elimination of Shh signaling from the notochord by antibody blockade in vitro [83, 84], or through gene targeting in mice [85], prevents the differentiation of floor-plate cells. Recent studies have provided evidence that a group of homeodomain proteins expressed by ventral progenitor cells acts as intermediary factors in the realization of graded Shh signaling [86–88]. The differential expression of five class I (Shh-repressed) proteins, Pax7, Irx3, Dbx1, Dbx2, and Pax6, and two class II (Shh-induced) proteins, Nkx6.1 and Nkx2.2, subdivides the ventral neural tube into five cardinal progenitor domains [88–90]. Subsequently, cross-repressive interactions that occur between class I and class II proteins refine the initially imprecise pattern of homeodomain protein expression initiated by graded Shh signals [88].

Finally, neuronal patterning in the dorsal half of the spinal cord requires the inductive activities of BMPs produced in the overlying ectoderm and roof plate [91, 92]. BMP provides positional information in dorsal and intermediate regions by setting borders of expression of target genes, in a similar fashion to Shh [92, 93]. Initially, BMPs act in conjunction with Shh to set expression boundaries for the *Pax* genes. The *Pax6* expression boundary that marks the border between dorsal and intermediate cells is refined by the BMP-mediated activation of Msx1 in dorsal cells, which in turn represses Dbx2, a Dbx homeodomain protein that is expressed in the intermediate region of the neural tube [94]. This generates two pools of progenitor cells (intermediate and dorsal) with distinct developmental potentials. In intermediate cells, BMPs contribute to the generation of overlapping patterns of homeobox protein expression; their regulation of Dbx1, Dbx2, and Pax7 generates at least three distinct populations of progenitor cells [94]. In dorsal cells, the patterning information is provided in part by mutually exclusive expression of members of the basic helix-loop-helix (b-HLH) family of proteins (Math, Mash, and Ngn) and LIM homeodomain proteins (Lbx and Lmx) [94, 95] (Fig. 4).

Given the critical role of Pax proteins in nervous system patterning, it is important to study the effect of loss-of-function mutations in *Pax* genes in both human and mice. Except Pax1 and Pax9, all members of Pax family have spatially and temporally restricted expression pattern in the developing nervous system [96]. Thus far, mutations in four out of nine *Pax* genes have been associated to human diseases (*PAX1*, *PAX2*, *PAX3*, and *PAX6*). Remarkably, mutations in *Pax* genes demonstrate that they play a critical role in specification and maintenance of structures that are neural crest-derived [97]. Mutation of the *Pax3* gene causes the *Splotch* phenotype in mice (*Sp*) (reviewed by Chalepakis et al. [98]; Gruss and Walther [99]) and Waardenburg syndrome in humans [100–102]. *Sp/Sp* mice have malformations involving neural crest-derived tissues: NTDs (exencephaly and spina bifida), limb defects, and dysgenesis of spinal ganglia and heart structures. Loss of function of the *Pax6* gene causes the *Small eye* (*Sey*) phenotype in mice and the aniridia, a congenital disorder characterized by complete or partial absence of eye structures [103]. *Pax7* null mice exhibit a loss of craniofacial structures that are neural crest-derived, whereas mutations in regulatory elements of *Pax7* can lead to muscle deficiency and rhabdomyosarcoma [104]. The role of *PAX1*, *PAX3*, *PAX7*, and *PAX9* genes in NTDs pathogenesis has been investigated in 79 sporadic and 38 familial NTDs patients. In one patient with spina bifida, a mutation in the *PAX1* gene was detected changing a conserved amino acid in the paired domain of the protein.

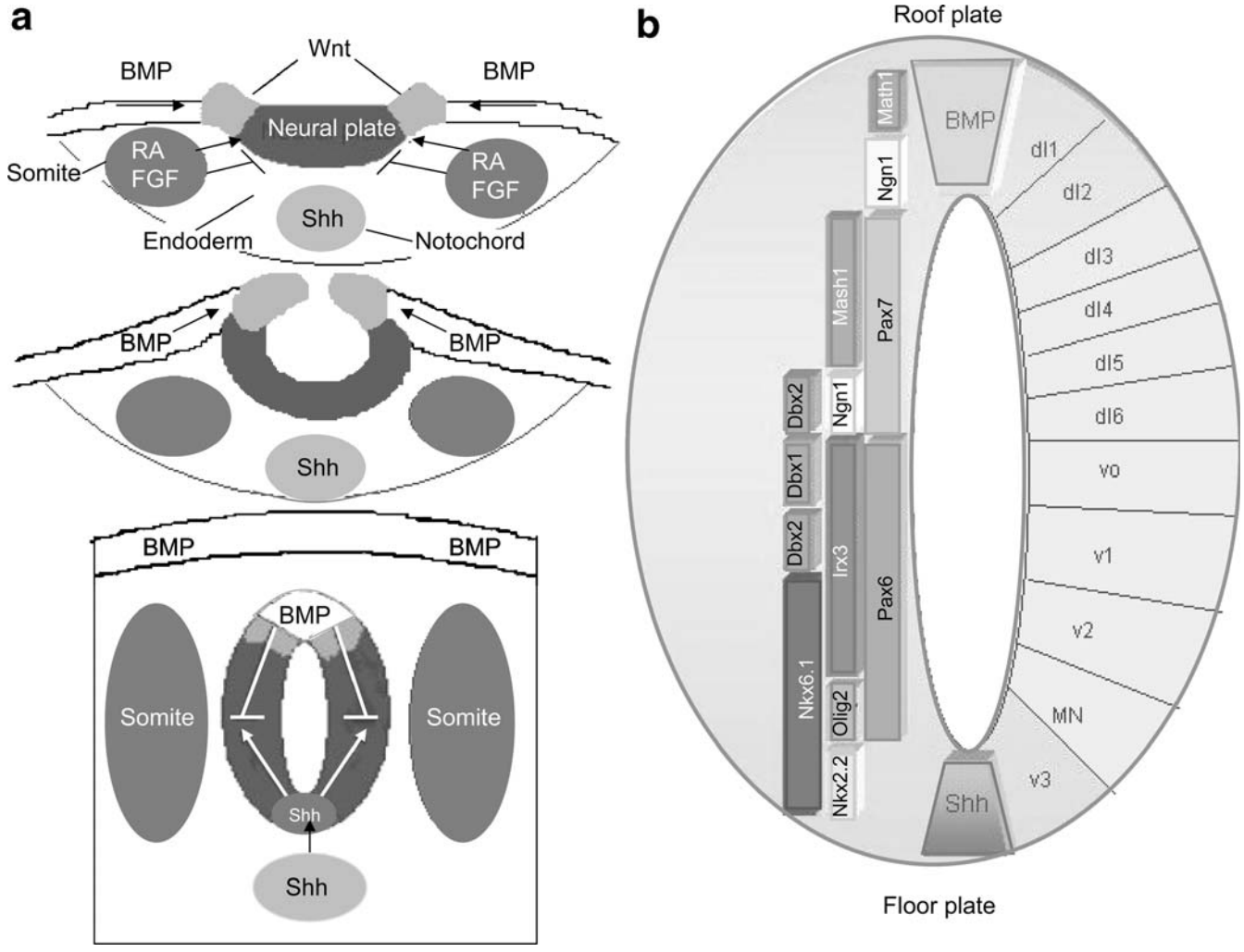

Fig. 4 Dorsoventral (DV) patterning of the neural tube. **a** Once the neural fate is specified, FGF (fibroblast growth factor), RA (retinoic acid), Shh (sonic hedgehog), and BMP (bone morphogenetic protein) signaling determine the DV pattern of the neural tube. FGFs from the underlying mesoderm prevent neural differentiation of the overlying neural plate. The somites express RA that antagonizes FGFs and induces a set of genes in the neural tube. The notochord differentiates and starts to express Shh. Shh is also expressed by floor-plate cells and spreads dorsally in a concentration-dependent gradient. BMP starts to be produced by the roof-plate cells and later spreads ventrally in a concentration gradient. Another extracellular signal which is present in the roof plate involves *Wnt*s. **b** The roof-plate BMP gradient and the floor-plate Shh gradient direct cells along the dorsoventral axis to specify their neuronal identity. There are six types of dorsal neurons (dI1–dI6) and five types of ventral neurons (V0–V3 and MN) in the developing neural tube. On the *left* are the protein markers which are used to identify the progenitor domains of the different DV regions

No sequence variation was observed in the paired domain of the *PAX7* and *PAX9* genes. So far, these findings do not support a major role of the *PAX* genes in the etiology of NTDs [105].

Antero-posterior (AP) patterning of the neural tube

A prevailing model for neural AP regionalization was formulated by Nieuwkoop who proposed that this process occurs in two steps: an "activation" step implying an initial induction of anterior neural structures through all presumptive neuroectoderm; subsequently, a second transforming signal is produced by posterior mesoderm [106] (Fig. 5). The major components of the activation signal are FGF and Wnt signals that act before gastrulation to induce the organizer to secrete inhibitors of BMP signaling such as noggin, chordin, cerberus, and follistatin (reviewed by Harland [107]). In turn, these BMP antagonists induce the neuroectoderm to adopt an anterior fate. Basic FGFs (bFGFs) may act as the second signal in the two-step model for induction of the AP neural pattern [108]. Two groups tested the effect of bFGFs on animal caps of *Xenopus* embryos treated with neuralizing agents like noggin or follistatin [109, 110]. Without bFGFs, these animal caps expressed only anterior neural markers (*Otx2* in forebrain and *En-2* at mid/hindbrain border). When bFGF was also added, both anterior and posterior neural markers (*Krox-20* and *Hoxb9*) were induced. In addition to FGFs, other factors like RA are involved in the AP patterning of the

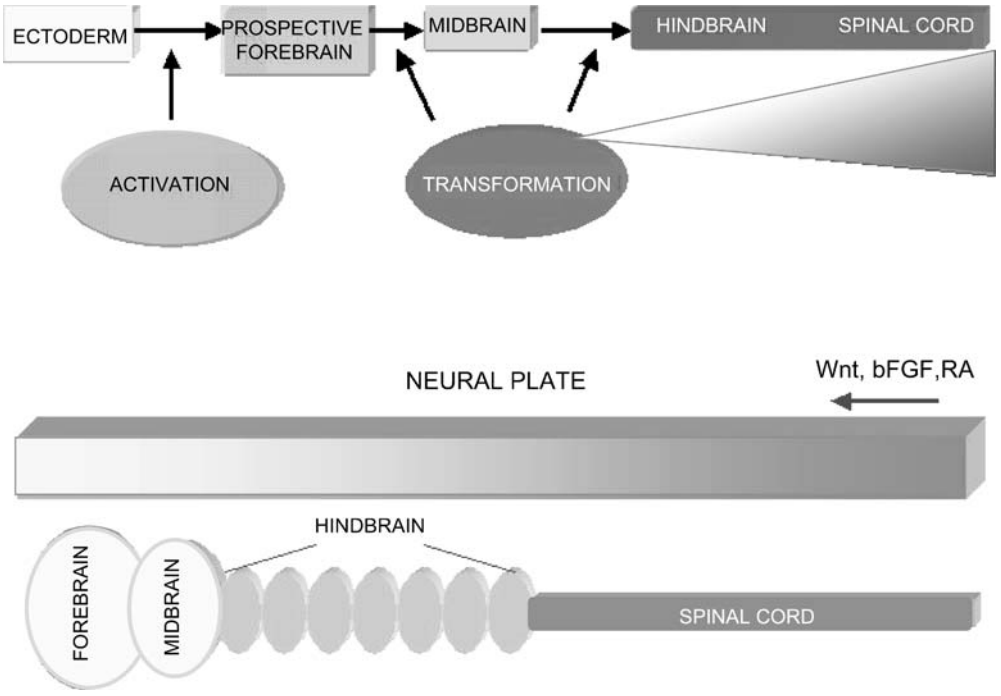

Fig. 5 Anteroposterior (AP) patterning of the neural tube. Neural AP regionalization occurs in two steps: an "activation" step implying an initial induction of anterior neural structures throughout all presumptive neuroectoderm, and a second transforming step that converts the neuroectoderm tissue to more posterior fates, including hindbrain and spinal cord. The major components of the transforming signals are Wnt, bFGF, and RA

neural tube. A series of experimental evidences show the critical role of RA signaling in positional patterning (Fig. 6). RA treatment of *Xenopus* embryos causes loss of forebrain, midbrain, and anterior hindbrain structures [111–116]. Overexpression of a dominant-negative form of RA receptors (RARs) showed that signaling through RARs is required for the RA-induced expression of the posterior markers Hoxb9, N-tubulin, and Xlim1 [117]. Moreover, overexpression of the *Xenopus* RA hydroxylase (Cyp26), which targets RA for degradation, leads to expansion of anterior structures [118, 119], whereas inhibition of Cyp26 expression induces expansion of posterior structures [120]. Overexpression of the RA biosynthetic enzyme Raldh2 leads to reduction of anterior structures [121], whereas Raldh2 loss of function results to a variety of axial defects in mice, including axial shortening and loss of posterior rhombomere identity and limb buds [122]. *Cyp26A1*-null mutant mice die during mid-late gestation and show a

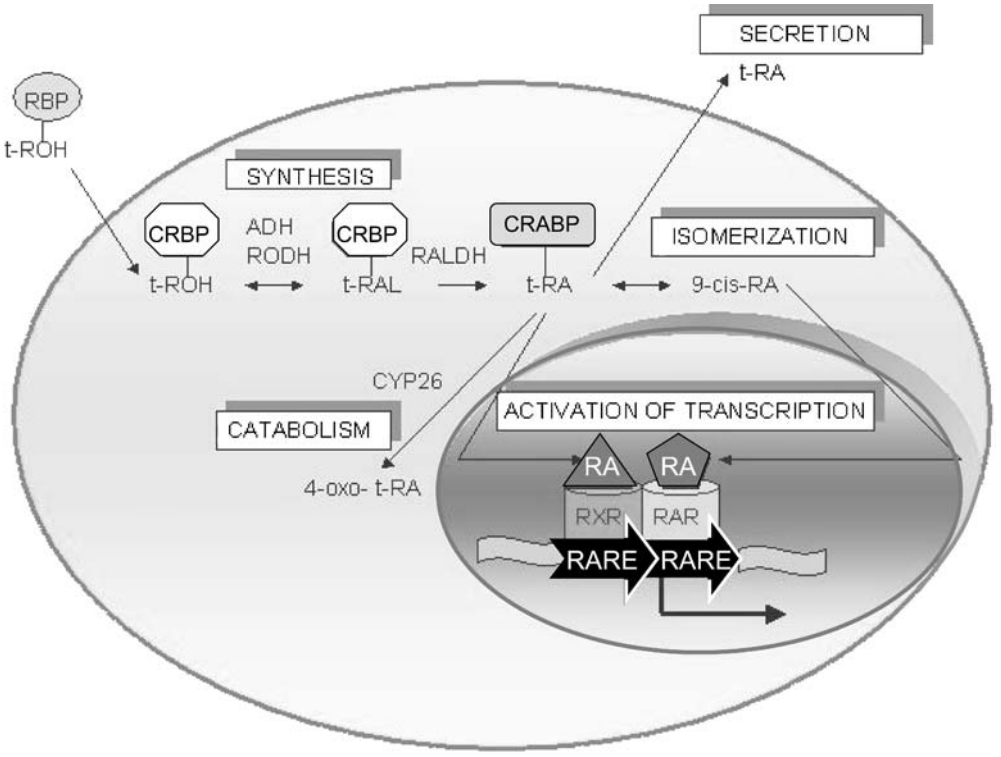

Fig. 6 The retinoic acid pathway. Trans-retinol (t-ROH) in the plasma is released from its carrier protein, retinol-binding protein (RBP), into the cell where it is bound by cellular retinol-binding protein (CRBP). The synthesis of retinoic acid (RA) is catalyzed by retinol dehydrogenase (RODH) and alcohol dehydrogenase (ADH) that form trans-retinaldehyde (t-RAL). This molecule is subsequently oxidized by retinaldehyde dehydrogenase (RALDH) to trans-RA (t-RA) which binds cellular retinoic acid-binding protein (CRABP). T-RA can (1) enter the nucleus and bind to retinoic acid receptors (RAR), activating transcription; (2) diffuse out of the cell (secretion); (3) isomerize to 9-*cis*-RA which binds to the RXR retinoic acid receptor; (4) oxidize to 4-*oxo*-t-RA catalyzed by the enzyme CYP26 (inactivation)

number of major morphogenetic defects: spina bifida and truncation of the tail and lumbosacral region (including abnormalities of the kidneys, urogenital tract, and hindgut) are the most conspicuous defects, leading in extreme cases to a sirenomelia phenotype. *Cyp26A1* mutants also show posterior transformations of cervical vertebrae and abnormal patterning of the rostral hindbrain, which appears to be partially posteriorly transformed [123].

It was also suggested that RA and FGF signaling can posteriorize anterior neural tissue, perhaps acting synergistically. These two pathways seem to converge on one common target gene, *Xdac3*, an homeobox gene, which has been implicated in the posteriorization pathway [124].

Because RA pathway genes have the potential for an involvement in human NTDs, human *ALDH*, *CYP26A1*, *CYP26B1*, and *CYP26C1* enzymes as well as *CRABP1* and *CRABP2* (cellular RA-binding proteins) have been studied in human NTDs cases. An association analysis using both allelic and genotypic single-locus tests revealed a significant association for NTDs risk only for three polymorphisms in the *ALDH1A2* gene [125].

A role for Wnt signaling in posteriorizing the embryonic axis has also been suggested by studies in *Xenopus* showing that overexpression of Wnt3A posteriorized anterior neuroectoderm [126, 127]. Moreover, blockade of Wnt8 signaling caused loss of posterior fates demonstrating that Wnt8 is an important transforming factor in zebrafish and *Xenopus* [128, 129]. Finally, Krumlauf and colleagues recently showed that the Wnt pathway posteriorizes *Xenopus* neural tissue via an indirect mechanism requiring FGF signaling. These findings suggest that the posteriorization pathway might be Wnt→FGF→Xcad3→ posterior HOX genes [130].

Bending of the neural plate

Neural folds form at the edges of the neural plate, then elevate and fuse in the dorsal midline to create the neural tube. In birds and mammals, bending of the neuroepithelium occurs at two specific sites in the neural plate [131, 132]: at the ventral midline (the median hinge point; MHP) and at paired lateral bending sites near the junction of the neural plate and surface ectoderm (the dorsolateral hinge points; DLHPs). Midline and dorsolateral bending are regulated by mutually antagonistic signals from the notochord and surface ectoderm.

Shh pathway is critical to neural tube bending (Fig. 7). In fact, DLHP formation is normally suppressed by Shh signaling at high levels of the spinal axis [133], although it is induced in cases of Shh deficiency [134]. Shh overexpression can produce NTDs, probably by inhibiting dorsolateral bending at levels of body axis at which it is essential for closure. Neural tube closure defects of both cranial and spinal regions are seen in mice with loss of *Patched 1* (*Ptc 1*) function [135, 136]. The Ptc receptor is responsible for maintaining Shh signaling in an "off" state in the absence of Shh ligand. Consequently, loss of function of Ptc 1 leads to constitutive Shh signaling, and like Shh overexpression, to severe NTDs. In contrast, mutations that directly or indirectly reduce the strength of Shh signaling, as seen in knockouts of *Gli1/2*, and *HNF3β* (hepatocyte nuclear factor 3β) (*Foxa2*) genes fail to develop NTDs presumably because of deinhibition of DLHP formation [137, 138]. Loss of function of Gli3, a negative regulator of Shh signaling in some tissues, leads to cranial NTDs in the *extra-toes* (*Xt*) mouse [139], suggesting a role for Shh signaling in cranial as well as spinal neurulation. Protein kinase A (PKA) is also known to be involved in neural tube formation, as it downregulates the Shh signaling. The PKA-deficient mouse model that retains only one functional catalytic subunit allele develops localized spinal NTDs. Decreased PKA activity causes dorsal expansion of the Shh signal through the thoracic to the sacral regions of the ventral tube, suggesting differential dependence on PKA activity along the AP axis [140].

NTDs are also seen in $Zic2^{-/-}$ and in the *open brain* (*opb*) mutants [141, 142]. *Opb* disrupts the *Rab23* gene, a member of small GTPase RAB proteins (a negative regulator of Shh), whereas *Zic2* encodes a zinc finger protein with anti-Shh activity. Both *opb* and $Zic2^{-/-}$ mutant embryos lack dorsal neural cell types and the ability to form DLHPs [141–143].

Mutations of genes involved in the molecular mechanisms controlling the neural plate bending have been looked for in human cases. Greig cephalopolysyndactyly syndrome (GCPS), characterized by craniofacial and limb anomalies, is associated with translocations as well as point mutations affecting one allele of the zinc finger gene *GLI3* [144, 145]. In addition to GCPS, Pallister-Hall syndrome and postaxial polydactyly type A are caused by *GLI3* mutations [146, 147]. NTDs are not a recognized feature of these syndromes. This is not surprising because NTDs in *Xt* mouse are of low penetrance, occur only in homozygotes, and are expected to be perinatally lethal [148]. A number of mutations in the *SHH* gene have been identified in cases of autosomal dominant holoprosencephaly (HPE), the most common congenital structural forebrain anomaly in humans [149, 150]. Mutations in *PATCHED-1*, the receptor for Shh, are also associated with HPE; these findings demonstrate that mutations in different components of a single signaling pathway can result in the same clinical condition [151]. A recent study identified notochord splitting and abnormal expression of *SHH* gene in the floor plate of human embryos with craniorachischisis and spina bifida [152]. A pilot study designed to identify an association between variations of the human *SHH* gene and spina bifida failed to uncover any disease-related mutation, although mutational screening was limited to the sequence encoding SHH-N protein and excluded the region coding for SHH-C protein that contains the autocatalytic cleavage site of SHH [153]. Genes encoding catalytic subunits of PKA and *ZIC1/ZIC2/ZIC3* were investigated for mutations that may predispose to NTDs with negative results [154, 155].

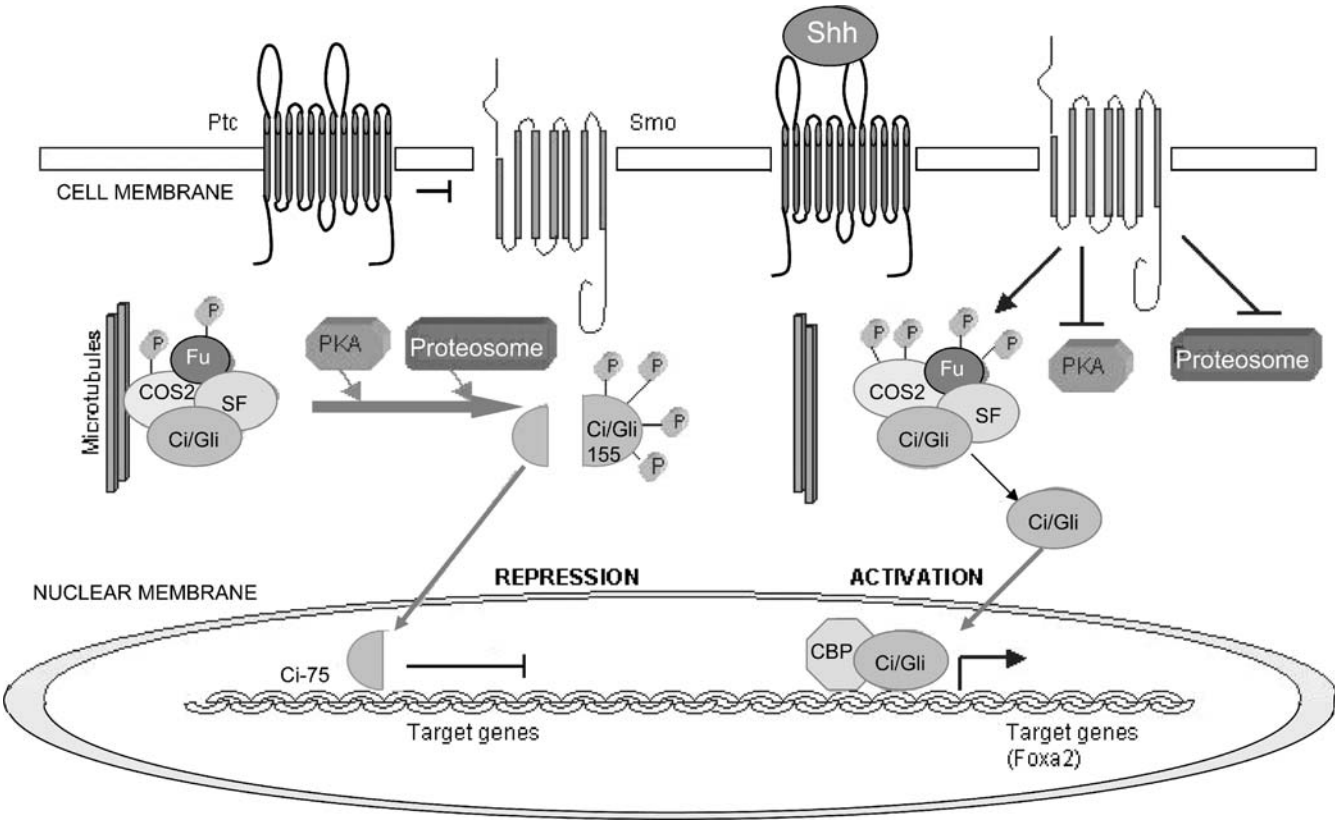

Fig. 7 Sonic hedgehog transduction (Shh) pathway. Shh binds the protein Patched (Ptc), which is a 12-pass membrane protein that acts as a receptor for Shh. In the absence of Shh, Ptc inhibits the protein smoothened (Smo), which is a seven-pass membrane protein that acts as a signal transducer. A transcription factor, called Cubitus interruptus (Ci) in *Drosophila* and Gli in vertebrates, acts as a transcriptional repressor. The nucleocytoplasmic shuttling of Ci/Gli proteins depends on a complex formed by the serine/threonine kinase Fused (Fu), suppressor of Fused (SF), and Costal 2 (COS2). Ci/Fu/SF/COS2 are bound together in a high weight molecular complex which is attached to microtubules. In absence of Shh stimulation, Ci/Gli is cleaved into a smaller N-terminal fragment (Ci-75) which enters the nucleus and represses target genes. Once Smo is freed of the inhibitory effects of Ptc, Smo signals causing hyperphosphorylation of COS2 and Fu and inducing the complex to loosen its hold on microtubules. This leads to the stabilization of full length Ci/Gli (Ci-155) that migrates into the nucleus where it binds the coactivator CBP/p300 and activates target genes. Also involved in Shh signaling are the proteosome and protein kinase A (PKA) that phosphorylates Ci/Gli at different residues

Apical constriction

Cell shape changes and cellular movements are required for correct neural tube formation. The process that converts columnar cells into wedge-shaped cells is called apical constriction. Apical constriction is coordinated both at the cellular and molecular level. More recent findings on genetic control of these processes have been reviewed by Wallingford [156]. Only very few molecules have been identified as regulators of apical constriction in the neural plate: p190RhoGAP and Shroom. P190RhoGAP is a negative regulator of the Rho GTPase, which is in turn a regulator of actin filament dynamics. Mice lacking a functional *p190RhoGAP* gene exhibit several defects in neural development, which include abnormalities in forebrain hemisphere fusion, ventricle shape, optic cup formation, neural tube closure, and layering of the cerebral cortex [157]. Cells of the neural tube floor plate in *p190RhoGAP* mutant mice present excessive accumulations of polymerized actin, suggesting a role for p190RhoGAP in the regulation of Rho-mediated actin assembly within the neuroepithelium [157]. The second molecule implicated in controlling apical constriction is the filamentous (F)-actin-binding protein, Shroom. Mice lacking *Shroom* function display 100% penetrant exencephaly with loss of apically localized actin in the neuroepithelium [158]. It has been demonstrated that ectopic Shroom can elicit apical constriction in undifferentiated epithelial cells of *Xenopus* [159]. Moreover, cells expressing Shroom undergo apical constriction and increase their apical surface [159]. Loss of function of other genes that regulate actin arrangement at the cell membrane, such as the protein kinase C targets *Macs* and *Mlp*, the actin-binding *vinculin*, the nonreceptor tyrosine kinases *Abl/Arg*, the cytoskeleton-related genes *Mena* (*Enah*)*/Profilin1* (*Pfn1*), results in cranial NTDs [160–164]. These findings suggest that actin-mediated mechanisms are crucial for closure of the cranial neural tube by allowing dorsolateral bending of the cranial neuroepithelium. *MLP* and *MACS* genes have been investigated as potential candidates for human NTDs, but no evidence of linkage disequilibrium has been found for flanking markers of these genes in 43 simplex NTDs families, suggesting that these genes are not major genes for NTDs in these families [165].

Fusion of the neural folds

Molecules that mediate neural fold adhesion and fusion are little known. Biological evidence supports a role for cell adhesion in neurulation, but few specific cell adhesion molecules have been identified. Inactivation of Eph-A5, a cell surface ephrin ligand, results in NTDs [166]. Inactivation of the Eph-A7 receptor also produces NTDs [166]. Eph tyrosine kinase receptors and their membrane-bound ephrin ligands mediate cell–cell interactions and participate in several developmental processes. Both Eph-A5 and Eph-A7 are expressed in the cranial neuroepithelium before and during neurulation, but not in spinal neuroepithelium, which accounts for cranial NTDs in these mice [166].

A recent study by Lee et al. [167] adds to the complexity of ephrin signaling. The authors showed that ephrinB1 acts through components of the PCP pathway to control the migration of cells during *Xenopus* development. The activity of the PCP pathway and CE movements require polarized clustering of Dsh at the cell membrane. As Eph–ephrin interactions lead to formation of large clusters at sites of cell contact, the interaction with ephrinB1 (and Eph receptors) is predicted to lead to clustering of Dsh and, therefore, to the activation of the PCP pathway. These data suggest that Eph receptors and ephrins may both contribute to the correct migration of neuronal progenitor cells.

The neural cell adhesion molecule (NCAM) is a prototypic immunoglobulin-like adhesion molecule and may play a role in neural tube formation and closure. Two members of another cell adhesion molecule family, the cadherins, have been found to be differentially expressed during neural plate formation. N-cadherin is expressed in the neural plate but not in the surrounding ectoderm, while E-cadherin shows an inverse pattern of expression [168]. *Xenopus* embryos in which the neural tube and surface ectoderm both express N-cadherin fail to separate the two tissues and develop NTDs [169]. However, mice with null mutations in *NCAM* or *N-cadherin* (*Cdh2*) genes undergo normal neural tube closure [170, 171]. Thus, these results indicate that these cell adhesion molecules do not play a major role in the normal process of neural tube formation. The role of *NCAM1* in the risk of human NTDs has been investigated in 132 spina bifida families. This study suggests that variations of *NCAM1* may contribute to NTDs risk; in addition, the authors confirmed the expression of *NCAM1* in the surrounding and late differentiating neurons of the central nervous system of human embryos [172].

Risk factors for human NTDs

Over the years, epidemiologic studies have been instrumental in elucidating the causes of NTDs in humans. Overall, these studies have suggested that environmental and genetic factors have a joint role in the causation of NTDs. However, many of the reported associations have been weak and have not been replicated.

Environmental risk factors

Maternal diabetes has long been associated with NTDs risk, while periconceptional glycemic control has been associated with a reduction in the risk. Maternal hyperinsulinemia and hyperglycemia have also been suggested to be putative risk factors for NTDs-affected pregnancies [173]. Abnormally, high glucose levels in maternal blood, which leads to increased glucose transport to the embryo, is responsible for teratogenic effects. In addition, maternal obesity increases the NTDs risk (1.5- to 3.5-fold higher risk) in women in the highest body mass (BM) index categories as compared to women with lower BM indices [174]. There is also strong evidence that maternal hyperthermia increases the risk of having a child with an NTDs by up to twofold [175].

Some specific pharmaceutical compounds have been associated with an increased risk for NTDs. During the first and second months of pregnancy, exposure to antiepileptic drugs (AEDs) is associated with a 10- to 20-fold increase in the frequency of NTDs-affected pregnancies [176]. The teratogenic mechanism is still unknown. Valproate and other AED are folic acid antagonists due to their ability to inhibit dihydrofolate reductase and decrease folate absorption. In addition to AEDs, association with NTDs has also been found with diuretics, antihistamines, and sulfonamides [177]. To date, the results on non-AED drugs are still debated and require further studies.

Other potential extrinsic factors that require further confirmation include: food contaminated with fumonisin (a class of mycotoxin), chlorination disinfection by products in drinking water, electromagnetic fields, pesticides, hazardous waste sites, maternal stress, lack of social support, and maternal diarrhea [178–184].

The most important epidemiological finding with respect to NTDs is the protective effect of maternal periconceptional folic acid supplementation [185, 186]. These studies demonstrated that maternal folic acid supplementation of at least 4 mg/day reduces the incidence of NTDs by up to 75%. This finding has been translated into public health policies, including educational campaigns and food fortification programs. However, public campaigns promoting the voluntary daily use of folic acid have not had an appreciable impact on the prevalence of NTDs. In contrast, data from countries that have implemented fortification programs indicate a 30–50% reduction in the prevalence of NTDs after fortification [187].

The role of folate in the etiology of human NTDs

Folate supplementation provides a well-documented benefit for the prevention of developmental defects in humans, most notably of craniofacial defects and NTDs. It has been estimated that 70% of human NTDs are preventable by adequate folate intake [185, 186].

Folate metabolism

Folic acid is an inactive water-soluble B vitamin that in mammalian tissues functions as substrate in series of interconnected metabolic cycles involving thymidilate and purine biosynthesis (adenosine and guanine) and methionine synthesis via homocysteine (Hcy) remethylation (Fig. 8). Thus, folate is directly or indirectly essential for cell function, division, and differentiation. There are over 25 proteins involved in the folate pathways. Several of the corresponding genes have been examined as risk factors for NTDs, but few have been associated with NTDs risk (Table 1). The 677C→T thermolabile isoform of *MTHFR* first emerged as a possible genetic risk factor for NTDs in some populations [188–190], even if other studies failed to demonstrate an association [191–193]. A recent meta-analysis found a pooled odds ratio for cases homozygous at 677C→T (677TT) of 1.7 (95% CI 1.4–2.2), with a pooled attributable fraction of NTDs cases of 6% for homozygosity [194]. A second mutation in the *MTHFR* gene (1298A→C) has also been associated with NTDs [195–197]. Given its important role in folate metabolism, the trifunctional enzyme methylenetetrahydrofolate dehydrogenase/methenyltetrahydrofolate cyclohydrolase/formyltetrahydrofolate synthetase (*MTHFD1*) may play a role in NTDs pathogenesis. Brody et al. [198, 199] demonstrated that the polymorphism 1958G→A (R653Q) within the *MTHFD1* gene resulting in the substitution of a conserved arginine by glutamine is a maternal risk factor for NTDs. A recent study by Rothenberg et al. [200] showed that some mothers with an NTDs pregnancy produce autoantibodies that bind to folate receptors on the placental membrane and therefore block the binding of folic acid. The authors suggest that folate supplementation would bypass autoantibody formation that mediates the placental folate receptor blockage. Mutations in genes for folate receptors are very rare and have been identified in only a few patients [201]. These receptors appear to be essential during embryogenesis such that their incorrect function leads to fetal death. On the other hand, a prevalent polymorphism (A80G) in

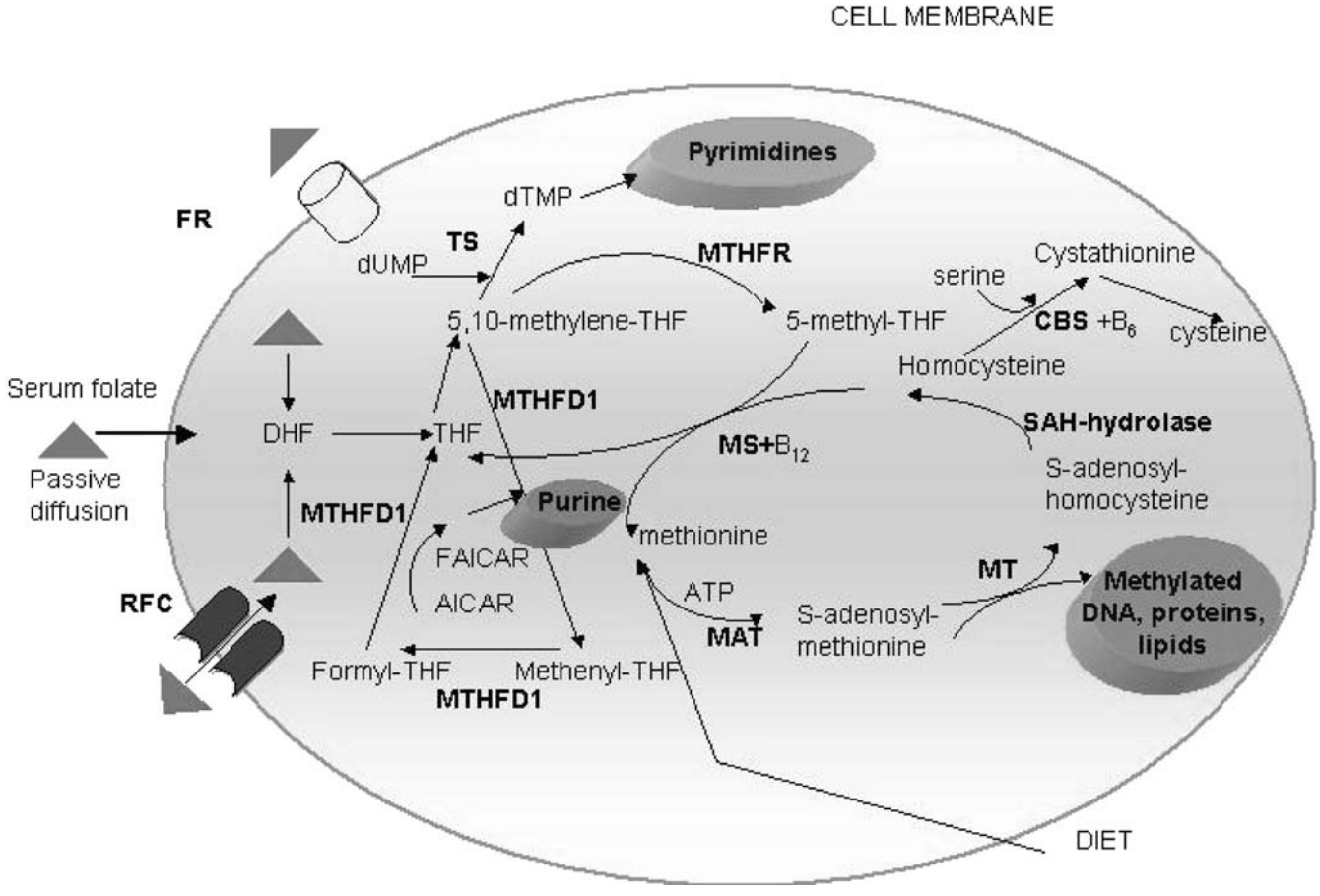

Fig. 8 Folate metabolism and the role of genes in the pathway. Folates: *DHF* dihydrofolate, *THF* tetrahydrofolate, *5,10-methylene-THF* 5,10-methylene-tetrahydrofolate, *5-methyl-THF* 5-methyl-tetrahydrofolate. Cofactors: B_{12} vitamin B_{12}, B_6 vitamin B_6. Folate genes (*bold characters*): *FR* folate receptor, *RFC* reduced folate carrier, *TS* thymidylate synthetase, *MTHFR* methylenetetrahydrofolate reductase, *MS* methionine synthase, *CBS* cystathionine-beta-synthase, *MTHFD1* methylene-tetrahydrofolate dehydrogenase/methenyltetrahydrofolate cyclohydrolase/formyltetrahydrofolate cyclohydrolase, *MAT* methionine adenosyltransferase, *MT* methyl transferase, *SAH hydrolase* *S*-adenosylhomocysteine hydrolase, *dUMP* deoxyuridine monophosphate, *dTMP* deoxythymidine monophosphate, *AICAR* 5-amino-4-imidazolecarboxamide ribonucleotide, *FAICAR* formyl-5-amino-4-imidazolecarboxamide ribonucleotide

Table 1 Candidates genes from folate/homocysteine metabolism

Human gene	Human locus	Polymorphism	Amino acid change	Association with NTDs risk
MTHFR	1p36.3	C677T	A222V	+/−
		A1298C	E429A	+/−
		C116T	P39P	+/−
		G1793A	R594Q	+/−
MTHFD1	14q24	G1958A	R653Q	+
RFC-1	21q22.3	G80A	R27H	+
GCPII	11p11.2	C1561T	H475Y	−
MS	1q43	A2756G	D919G	+/−
MTRR	5p15.2–15.3	A66G	I22M	+/−
		C524T	S175L	−
		A1049G	K350R	−
TCII	22q12.2	A67G	I23V	−
		G280A	G94S	−
		A701G	E234R	−
		C776G	P259R	−
		C1043T	S348F	−
		G1196A	R399E	−
SHMT	17p11.2	C1420T	L474F	−
BHMT	5q13.1–q13.2	G742A	R239Q	−
CBS	21q22.3	844ins68bp	Premature stop codon?[a]	+/−
TS	18p11.32	28 bp repeat	Promoter polymorphism	+/−

[a]This polymorphism creates an alternate splicing site, which eliminates not only the inserted intronic sequences but also the T833C mutation associated with this insertion. The net result is the generation of both quantitatively and qualitatively normal mRNA and CBS enzyme.
+, Positive association; −, negative association

the gene coding for the folate carrier (*RFC-1*) has been demonstrated as a genetic risk factor for NTDs [202, 203]. Additional studies demonstrated that this variant may interact with low folate status and *MTHFR* mutations to increase NTDs risk [204].

Homocysteine and vitamin B12 metabolism

Hcy participates in three essential reactions or sequences: (a) the transsulfuration pathway that leads via cystathionine to the formation of cysteine; (b) the cycling of intracellular folates; and (c) the catabolism of choline and betaine. Hyperhomocysteinemia is correlated with an increased risk for multifactorial diseases including NTDs. Several hypotheses have been advanced to explain the toxic effects of homocysteine. Exposure of neurons to homocysteine causes DNA damage through poly(ADP-ribose) polymerase activation and p53 induction leading to programmed cell death. There is also evidence that low maternal B_{12} levels represent risk factors for NTDs [205]. Investigation of an A-to-G transition at bp 2,756 and a C-to-A transversion at bp 5,094 of the methionine synthase (*MS* or *MTR*) gene, which converts folate and Hcy to tetrahydrofolate (THF) and methionine, yielded controversial conclusions depending on the population studied [206, 207]. Thus far, only few studies examined the role of the A66G polymorphism of the methionine synthase reductase (*MTRR*), the enzyme that activates MS. An increased prevalence of the *MTRR* 66GG homozygous genotype in NTDs patients and their mothers was observed. In combination with low vitamin B_{12} levels, the relative risk of the *MTRR* 66GG genotype increased by two- to fivefold in the NTDs patients and their mothers [208]. This may indicate that, especially when the cofactor of MS (vitamin B_{12}) is limited, a reduced activation of MS by MTRR could lead to disturbed remethylation of Hcy to methionine. However, a recent analysis of *MTRR* polymorphisms (A66G, C524T, and A1049G) in a large homogeneous Irish NTDs population did not support an important role for these variants in NTDs [209]. Two groups studied the role of some SNPs of the transcobalamin II (*TCII*) gene coding the protein involved in the transport of vitamin B_{12} within plasma, but none of these SNPs was associated with a significantly increased NTDs risk [210, 211]. Other genes of Hcy pathways have been investigated; among them are serine hydroxymethyltransferase (*SHMT*), betaine–homocysteine methyltransferase (*BHMT*), cystathionine beta-synthase (*CBS*), and thymidylate synthase (*TS*) [212–215]. So far, the results of these studies provide no direct evidences for a role of these genes in NTDs risk.

Gene–gene and gene–environment interactions

Given the interconnected nature of the folate-dependent Hcy pathway, it is possible that particular combinations epistatic of genetic variants may underlie NTDs suscepti-

bility. Multilocus analysis has investigated interactions between *MTHFR* 677C→T, *MTHFR* 1298A→C, *MTRR* 66A→G, *SHMT* 1420C→T, *CBS* 844ins68, *GCPII* 1561C→T, and *RFC-1* 80A→G. Collectively, the genes involved in folate biochemistry and their respective polymorphisms appear to produce only small increases of risk. It has been demonstrated that the penetrance of the *MTHFR* 677C→T mutation may be modified by dietary and supplemental folate [216]. Likewise, variations at the infant *RFC-1* 80A→G locus appear to modify the effects of folate supplementation on NTDs [204].

Folate supplementation in mouse models

The identification of mouse mutants with folate-sensitive NTDs greatly aids our understanding of genetic predisposition to folate-sensitive NTDs in the human population (Table 2). In mice, a number of genetic models exists in which there is an association between occurrence of NTDs and folate metabolism. In three folate-sensitive cases, the targeted or mutant gene encodes a protein that functions in transcriptional regulation: *Cart1* that encodes the homeobox gene, cartilage homeoprotein 1; *Cited 2* (known as *Mrg1/p35srj*) that encodes a member of the CITED (CBP7p300-interacting transactivators with glutamic acid (E)/aspartic acid (D)-rich C-terminal domain) family of transcriptional regulatory proteins; and *Splotch* mice that carry a mutated allele of *Pax3* [217–219]. Another folate-responsive mutant mouse model is *Folbp1*$^{-/-}$ [220]. Because Folbp1 (Folr1) is implicated in the transport of folate into cells, supplementation of the mutants corrects the cellular uptake deficiency possibly through redundant transport pathways. The NTDs in *Crooked tail* (*Cd*) mice is an excellent model of folate-responsive NTDs. Heterozygous *Cd* mice display a crooked tail whereas *Cd*/*Cd* animals have phenotypes that include early postimplantation lethality (20–30% of homozygotes) and exencephaly (20–30%) [221]. A missense mutation in *Cd* replaces a highly conserved amino acid in the low-density lipoprotein (LDL) receptor-related protein, Lrp6 [222]. Lrp6 is an LDL coreceptor for Wnt. Identification of an Lrp6 mutation in the *Crooked tail* mouse suggests that canonical Wnt pathway genes can be involved in folate-responsive NTDs.

Folate-resistant mouse models

Folate-resistant defects include cranial NTDs in *eph-A5* knockout mice and spinal NTDs in the *axial defects* (*Axd*) mice and *curly tail* mutants [166, 223, 224]. Spinal NTDs in *curly tail* mouse results from a defective proliferation of hindgut cells resulting in excessive ventral curvature of the caudal region of the embryo, thereby inhibiting closure of the neural folds. To date, a causative mutation in the *curly tail* mouse has not been reported; however, it has been proposed that the *curly tail* phenotype involves a mutation in the grainy head-like-3 (*Grhl3*) locus which encodes a member of the *grainy head-like* family of the transcription factors [225]. At present, the contribution of defective alleles of *Grhl3* to human NTDs has not been reported. Spinal NTDs in *curly tail* mouse can be prevented by supplementation with inositol either by administration to pregnant females or directly to embryo cultures [224]. The mechanism of action of inositol in preventing NTDs in *curly tail* mice involves protein kinase C (PKC), a family of serine/threonine kinases. The treatment of *curly tail* embryos with isoform-specific PKC inhibitors identified PKCβI and γ as essential isoforms for the normalizing action of inositol [226] (Fig. 9). These findings raise the possibility of developing novel clinical strategies for the prevention of human NTDs based on inositol. A recent study comparing the levels of myoinositol in serum from mothers that had normal pregnancies and mothers who had children with spina bifida supports the idea that inositol status may contribute to the susceptibility to NTDs. There was a 2.6-fold increased risk to have an affected child among mothers whose inositol levels were at the low end (tenth centile) of the range [227].

Table 2 Mouse models in which NTDs are preventable by exogenous agents

Mouse model	NTDs	Mechanism	Gene	Prevention by folic acid	Prevention by other agents
Cart1$^{-/-}$	Exencephaly	Excess of apoptosis	*Cart1*$^{-/-}$	Yes	–
Cited2$^{-/-}$	Exencephaly	Excess of apoptosis	*Cited2*	Yes	–
Crooked tail (*Cd*)	Exencephaly	–	*Cd*	Yes	–
Curly tail	Spina bifida, exencephaly	Defect in the hindgut proliferation	*Grhl3*	No	Inositol
Ephrin-A5$^{-/-}$	Spina Bifida, exencephaly	Adhesion defect	*Ephrin-A5*	No	–
Axial defects (*Axd*)	Spina bifida	–	*Axd*	No	Methionine
Folbp1$^{-/-}$	Exencephaly	Folate deficiency	*Folbp1*	Yes	–
Splotch^{2H}	Spina bifida, exencephaly	Excess of apoptosis	*Pax3*	Yes	Thymidine

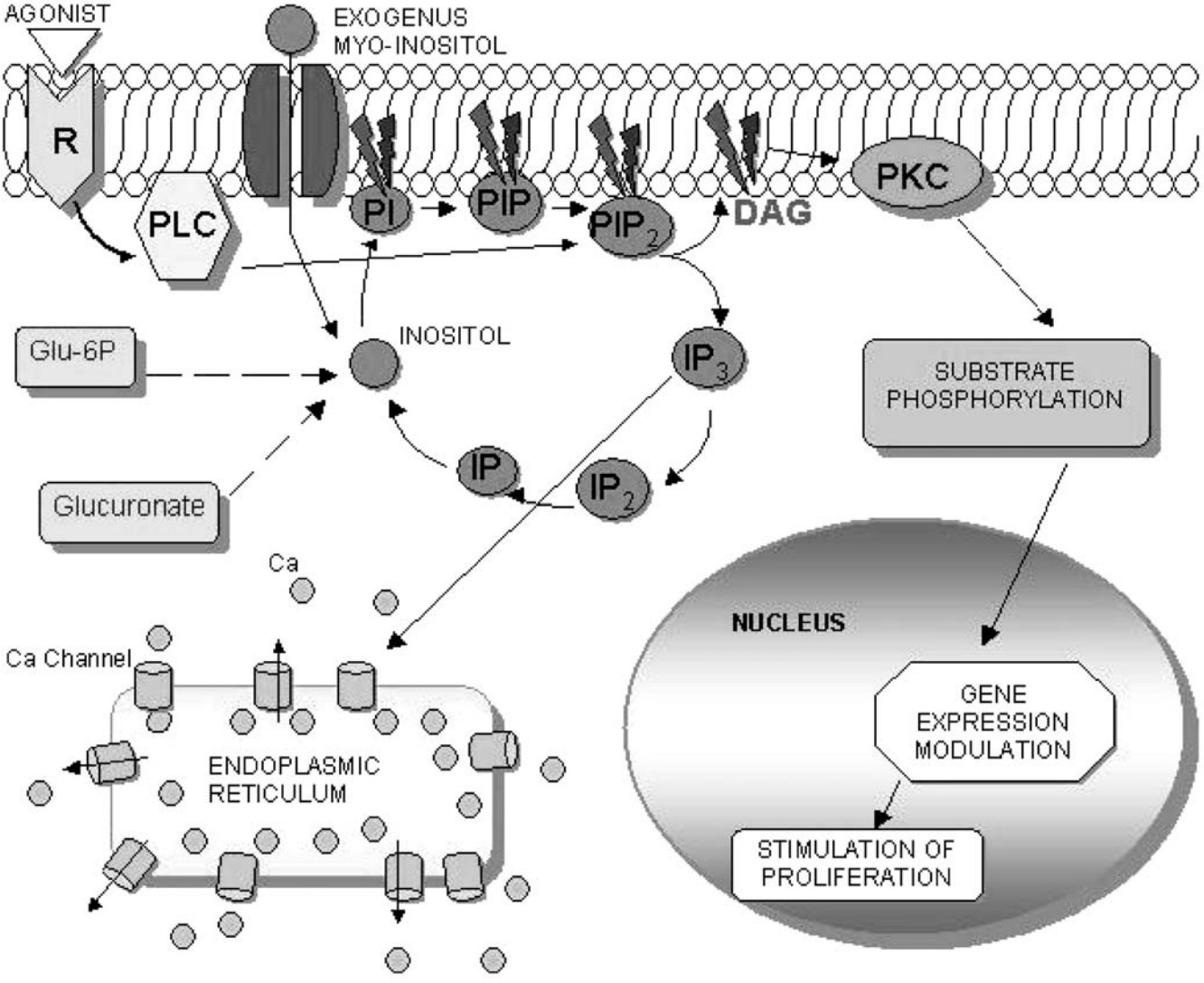

Fig. 9 Myoinositol metabolic pathway and inositol signaling system. Exogenous myoinositol enters the inositol phospholipid cycle and is incorporated into inositol phospholipids, including phosphatidylinositol (PI), phosphatidylinositol-4-phoshate (PIP), and finally phosphatidylinositol-4,5-bisphosphate (PIP_2). External signals, by binding to cell membrane receptors (R), induce the activation of phospholipase C (PLC) and hydrolysis of PIP_2 to diacylglycerol (DAG) and inositol triphosphate (IP_3), which are both downstream signaling molecules. IP_3 induces Ca^{2+} release from intracellular stores. Sequential dephosphorylation of inositol phosphates leads to the release of free inositol that combines with CDP-diacylglycerol to regenerate PI. DAG induces the activation of some isoforms of protein kinase C (PKC); the subsequent phosphorylation of specific substrates results in the modulation, at nuclear level, of gene expression and in the downstream correction of cell proliferation defects, thereby normalizing neural tube closure. *IP_2* Inositol bisphosphate, *IP* inositol monophosphate, *Glu-6P* glucose-6-phosphate, *R* cell membrane receptor

Conclusions

The identification of the relationship between folic acid and NTDs represents one of the most important successes of epidemiological research. Variants of several folate-related genes have been found to be significantly associated with the risk of NTDs. However, the identification of additional risk factors has proven to be difficult. So far, biochemical and developmental pathways as well as animal models have failed to yield definitive results. Given the multifactorial etiology of NTDs, interactions between environmental factors and target genes as well as additive or epistatic interactions should be considered. A concerted effort will be required to design adequately powered studies and to minimize the danger of bias and spurious results.

Acknowledgements The authors would like to thank A.J. Copp for his helpful discussion. P.D.M., E.M., and S.M. are supported by grants of Italian Ministry of Health. V.C. is supported by Gaslini Foundation.

References

1. Lary JM, Edmonds LD (1996) Prevalence of spina bifida at birth—United States, 1983–1990: a comparison of two surveillance systems. MMWR CDC Surveill Summ 45:15–26

2. Lynch SA (2005) Non-multifactorial neural tube defects. Am J Med Genet C Semin Med Genet 135:69–76
3. Risch N (1990) Linkage strategies for genetically complex traits. II. The power of affected relative pairs. Am J Hum Genet 46:229–241
4. Partington MD, McLone DG (1995) Hereditary factors in the etiology of neural tube defects. Results of a survey. Pediatr Neurosurg 23:311–316
5. Byrne J, Carolan S (2006) Adverse reproductive outcomes among pregnancies of aunts and (spouses of) uncles in Irish families with neural tube defects. Am J Med Genet A 140:52–61
6. Sadler TW (2005) Embryology of neural tube development. Am J Med Genet C Semin Med Genet 135:2–8
7. Colas JF, Schoenwolf GC (2003) Towards a cellular and molecular understanding of neurulation. Nat Rev Genet 4:784–793
8. Schoenwolf GC, Smith JL (2000) Mechanisms of neurulation. Methods Mol Biol 136:125–134
9. O'Rahilly R, Muller F (1994) Neurulation in the normal human embryo: neural tube defects. Ciba Found Symp 181:70–89
10. Copp AJ, Greene NDE, Murdoch JN (2003) The genetic basis of mammalian neurulation. Nat Rev Genet 4:784–793
11. Juriloff DM, Harris MJ, Tom C, MacDonald KB (1991) Normal mouse strains differ in the site of initiation of closure of the cranial neural tube. Teratology 44:225–233
12. van Allen MI, Kalousek DK, Chernoff GF, Juroff D, Harris M, McGillivray BC, Young S-L, Langlois S, MacLeod PM, Chitayat D et al (1993) Evidence for multi-site closure of the neural tube in humans. Am J Med Genet 47:723–743
13. Nakatsu T, Uwabe C, Shiota K (2000) Neural tube closure in humans initiates at multiple sites: evidence from human embryos and implications for the pathogenesis of neural tube defects. Anat Embryol (Berl) 201:455–466
14. O'Rahilly R, Muller F (2002) The two sites of fusion of the neural folds and the two neuropores in the human embryo. Teratology 65:162–170
15. Spemann H, Mangold H (2001) Induction of embryonic primordia by implantation of organizers from a different species. 1923. Int J Dev Biol 45:13–38
16. De Robertis EM, Larrain J, Oelgeschlager M, Wessely O (2000) The establishment of Spemann's organizer and patterning of the vertebrate embryo. Nat Rev Genet 1:171–181
17. Oelgeschlager M, Kuroda H, Reversade B, De Robertis EM (2003) Chordin is required for the Spemann organizer transplantation phenomenon in *Xenopus* embryos. Dev Cell 4:219–230
18. Sasai Y, Lu B, Steinbeisser H, Geissert D, Gont LK, De Robertis EM (1994) *Xenopus* chordin: a novel dorsalizing factor activated by organizer-specific homeobox genes. Cell 79:779–790
19. Larrain J, Bachiller D, Lu B, Agius E, Piccolo S, De Robertis EM (2000) BMP-binding modules in chordin: a model for signalling regulation in the extracellular space. Development 127:821–830
20. Hawley SH, Wunnenberg-Stapleton K, Hashimoto C, Laurent MN, Watabe T, Blumberg BW, Cho KW (1995) Disruption of BMP signals in embryonic *Xenopus* ectoderm leads to direct neural induction. Genes Dev 9:2923–2935
21. Hemmati-Brivanlou A, Kelly OG, Melton DA (1994) Follistatin, an antagonist of activin, is expressed in the Spemann organizer and displays direct neuralizing activity. Cell 77:283–295
22. Weinstein DC, Hemmati-Brivanlou A (1999) Neural induction. Annu Rev Cell Dev Biol 15:411–433
23. Linker C, Stern CD (2004) Neural induction requires BMP inhibition only as a late step, and involves signals other than FGF and Wnt antagonists. Development 131:5671–5681
24. Lamb TM, Knecht AK, Smith WC, Stachel SE, Economides AN, Stahl N, Yancopolous GD, Harland RM (1993) Neural induction by the secreted polypeptide noggin. Science 262:713–718
25. Bouwmeester T, Kim S-H, Sasai Y, Lu B, de Robertis EM (1996) Cerberus is a head-inducing secreted factor expressed in the anterior endoderm of Spemann's organizer. Nature 382:595–601
26. Hansen CS, Marion CD, Steele K, George S, Smith WC (1997) Direct neural induction and selective inhibition of mesoderm and epidermis inducers by Xnr3. Development 124:483–492
27. Chang C, Holtzman DA, Chau S, Chickering T, Woolf EA, Holmgren LM, Bodorova J, Gearing DP, Holmes WE, Brivanlou AH (2001) Twisted gastrulation can function as a BMP antagonist. Nature 410:483–487
28. Wessely O, Agius E, Oelgeschlager M, Pera EM, De Robertis EM (2001) Neural induction in the absence of mesoderm: beta-catenin-dependent expression of secreted BMP antagonists at the blastula stage in *Xenopus*. Dev Biol 234:161–173
29. Baird A, Bohlen P (1990) Fibroblast growth factors. In: Sporn MC, Roberts AB (eds) Peptide growth factors and their receptors. Springer, Berlin Heidelberg New York, pp 369–418
30. Streit A, Berliner AJ, Papanayotou C, Sirulnik A, Stern CD (2000) Initiation of neural induction by FGF signalling before gastrulation. Nature 406:74–78
31. Wilson SI, Graziano E, Harland R, Jessell TM, Edlund T (2000) An early requirement for FGF signalling in the acquisition of neural cell fate in the chick embryo. Curr Biol 10:421–429
32. Launay C, Fromentoux V, Shi DL, Boucaut JC (1996) A truncated FGF receptor blocks neural induction by endogenous *Xenopus* inducers. Development 122:869–880
33. Nelson WJ, Nusse R (2004) Convergence of Wnt, beta-catenin, and cadherin pathways. Science 303:1483–1487
34. Gomez-Skarmeta JL, de la Calle-Mustienes E, Modolell J (2001) The Wnt-activated *Xiro1* gene encodes a repressor that is essential for neural development and downregulates Bmp4. Development 128:551–560
35. Pera EM, Wessely O, Li SY, De Robertis EM (2001) Neural and head induction by insulin-like growth factor signals. Dev Cell 1:655–665
36. Pera EM, Ikeda A, Eivers E, De Robertis EM (2003) Integration of IGF, FGF, and anti-BMP signals via Smad1 phosphorylation in neural induction. Genes Dev 17:3023–3028
37. Matzuk MM, Lu N, Vogel H, Sellheyer K, Roop DR, Bradley A (1995) Multiple defects and perinatal death in mice deficient in follistatin. Nature 374:360–363
38. McMahon JA, Takada S, Zimmerman LB, Fan CM, Harland RM, McMahon AP (1998) Noggin-mediated antagonism of BMP signaling is required for growth and patterning of the neural tube and somite. Genes Dev 12:1438–1452
39. Simpson EH, Johnson DK, Hunsicker P, Suffolk R, Jordan SA, Jackson IJ (1999) The mouse *Cer1* (*Cerberus related or homologue*) gene is not required for anterior pattern formation. Dev Biol 213:202–206
40. Bachiller D, Klingensmith J, Kemp C, Belo JA, Anderson RM, May SR, McMahon JA, McMahon AP, Harland RM, Rossant J, DE Robertis EM (2000) The organizer factors Chordin and Noggin are required for mouse forebrain development. Nature 403:658–661
41. Schulte-Merker S, Lee KJ, McMahon AP, Hammerschmidt M (1997) The zebrafish organizer requires chordino. Nature 387:862–863
42. Winnier G, Blessing M, Labosky PA, Hogan BL (1995) Bone morphogenetic protein-4 is required for mesoderm formation and patterning in the mouse. Genes Dev 9:2105–2116
43. Zhang H, Bradley A (1996) Mice deficient for *BMP2* are nonviable and have defects in amnion/chorion and cardiac development. Development 122:2977–2986
44. Dudley AT, Lyons KM, Robertson EJ (1995) A requirement for bone morphogenetic protein-7 during development of the mammalian kidney and eye. Genes Dev 9:2795–2807
45. Luo G, Hofmann C, Bronckers AL, Sohocki M, Bradley A, Karsenty G (1995) BMP-7 is an inducer of nephrogenesis, and is also required for eye development and skeletal patterning. Genes Dev 9:2808–2820

46. Chang H, Huylebroeck D, Verschueren K, Guo Q, Matzuk MM, Zwijsen A (1999) *Smad5* knockout mice die at mid-gestation due to multiple embryonic and extraembryonic defects. Development 126:1631–1642
47. Tremblay KD, Dunn NR, Robertson EJ (2001) Mouse embryos lacking Smad1 signals display defects in extra-embryonic tissues and germ cell formation. Development 128:3609–3621
48. Felder B, Stegmann K, Schultealbert A, Geller F, Strehl E, Ermert A, Koch MC (2002) Evaluation of *BMP4* and its specific inhibitor *NOG* as candidates in human neural tube defects (NTDs). Eur J Hum Genet 10:53–56
49. Wallingford JB, Harland RM (2002) Neural tube closure requires Dishevelled-dependent convergent extension of the midline. Development 129:5815–5825
50. Wallingford JB, Fraser SE, Harland RM (2002) Convergent extension: the molecular control of polarized cell movement during embryonic development. Dev Cell 2:695–706
51. Wallingford JB, Rowning BA, Vogeli KM, Rothbacher U, Fraser SE, Harland RM (2000) Dishevelled controls cell polarity during *Xenopus* gastrulation. Nature 405:81–85
52. Keller R (2002) Shaping the vertebrate body plan by polarized embryonic cell movements. Science 298:1950–1954
53. Heisenberg CP, Tada M, Rauch GJ, Saude L, Concha ML, Geisler R, Stemple DL, Smith JC, Wilson SW (2000) lberblick/Wnt11 mediates convergent extension movements during zebrafish gastrulation. Nature 405:76–81
54. Tada M, Smith JC (2000) Xwnt11 is a target of *Xenopus* Brachyury: regulation of gastrulation movements via Dishevelled, but not through the canonical Wnt pathway. Development 127:2227–2238
55. Wallingford JB, Harland RM (2001) *Xenopus* Dishevelled signaling regulates both neural and mesodermal convergent extension: parallel forces elongating the body axis. Development 128:2581–2592
56. Ciruna B, Jenny A, Lee D, Mlodzik M, Schier AF (2006) Planar cell polarity signalling couples cell division and morphogenesis during neurulation. Nature 439:220–224
57. Katoh M (2005) WNT/PCP signaling pathway and human cancer (review). Oncol Rep 14:1583–1588
58. Saburi S, McNeill H (2005) Organising cells into tissues: new roles for cell adhesion molecules in planar cell polarity. Curr Opin Cell Biol 17:482–488
59. Usui T, Shima Y, Shimada Y, Hirano S, Burgess RW, Schwarz TL, Takeichi M, Uemura T (1999) Flamingo, a seven-pass transmembrane cadherin, regulates planar cell polarity under the control of Frizzled. Cell 98:585–595
60. Jenny A, Darken RS, Wilson PA, Mlodzik M (2003) Prickle and Strabismus form a functional complex to generate a correct axis during planar cell polarity signaling. EMBO J 22:4409–4420
61. Wolff T, Rubin GM (1998) *Strabismus*, a novel gene that regulates tissue polarity and cell fate decisions in Drosophila. Development 125:1149–1159
62. Gubb D, Green C, Huen D, Coulson D, Johnson G, Tree D, Collier S, Roote J (1999) The balance between isoforms of the prickle LIM domain protein is critical for planar polarity in Drosophila imaginal discs. Genes Dev 13:2315–2327
63. Feiguin F, Hannus M, Mlodzik M, Eaton S (2001) The ankyrin repeat protein Diego mediates Frizzled-dependent planar polarization. Dev Cell 1:93–101
64. Strutt DI, Weber U, Mlodzik M (1997) The role of RhoA in tissue polarity and Frizzled signalling. Nature 387:292–295
65. Winter CG, Wang B, Ballew A, Royou A, Karess R, Axelrod JD, Luo L (2001) Drosophila Rho-associated kinase (Drok) links Frizzled-mediated planar cell polarity signaling to the actin cytoskeleton. Cell 105:81–91
66. Park TJ, Haigo SL, Wallingford JB (2006) Ciliogenesis defects in embryos lacking inturned or fuzzy function are associated with failure of planar cell polarity and Hedgehog signalling. Nat Genet 38:303–311
67. Smith UM, Consugar M, Tee LJ, McKee BM, Maina EN, Whelan S, Morgan NV, Goranson E, Gissen P, Lilliquist S, Aligians IA, Ward CI, Pasha S, Punyashthiti R, Malik Sharif S, Batman PA, Bennett CP, Woods CG, McKeown C, Bucourt M, Miller CA, Cox P, Algazali M, Trembath RC, Torres VE, Attie-Bitah T, Kelly DA, Maher ER, Gattone VH, Harris PC, Jonhson CA (2006) The transmembrane protein meckelin (MKS3) is mutated in Meckel–Gruber syndrome and the *wpk* rat. Nat Genet 38:191–196
68. Kyttala M, Tallila J, Salonen R, Kopra O, Kohlschmidt N, Paavola-Sakki P, Peltonen L, Kestila M (2006) MKS1, encoding a component of the flagellar apparatus basal body proteome, is mutated in Meckel syndrome. Nat Genet 38:155–157
69. Smith LJ, Stein KF (1962) Axial elongation in the mouse and its retardation in homozygous *looptail* mice. J Embryol Exp Morphol 10:73–87
70. Murdoch JN, Rachel RA, Shah S, Beermann F, Stanier P, Mason CA, Copp AJ (2001) *Circletail*, a new mouse mutant with severe neural tube defects: chromosomal localization and interaction with the *loop-tail* mutation. Genomics 78:55–63
71. Curtin JA, Quint E, Tsipouri V, Arkell RM, Cattanach B, Copp AJ, Henderson DJ, Spurr N, Stanier P, Fisher EM, Nolam PM, Steel KP, Brown SD, Gray IC, Murdoch JN (2003) Mutation of *Celsr1* disrupts planar polarity of inner ear hair cells and causes severe neural tube defects in the mouse. Curr Biol 13:1129–1133
72. Lu X, Borchers AGM, Jolicoeur C, Rayburn H, Baker JC, Tessier-Lavigne M (2004) PTK7/CCK-4 is a novel regulator of planar cell polarity in vertebrates. Nature 430:93–98
73. Hamblet NS, Lijam N, Ruiz-Lozano P, Wang J, Yang Y, Luo Z, Mei L, Chien KR, Sussman DJ, Wynshaw-Boris A (2002) Dishevelled 2 is essential for cardiac outflow tract development, somite segmentation and neural tube closure. Development 129:5827–5838
74. Murdoch JN, Doudney K, Paternotte C, Copp AJ, Stanier P (2001) Severe neural tube defects in the *loop-tail* mouse result from mutation of *Lpp1*, a novel gene involved in floor plate specification. Hum Mol Genet 10:2593–2601
75. Jessen JR, Solnica-Krezel L (2004) Identification and developmental expression pattern of *van gogh-like 1*, a second zebrafish *strabismus* homologue. Gene Expr Patterns 4:339–344
76. Murdoch JN, Henderson DJ, Doudney K, Gaston-Massuet C, Phillips HM, Paternotte C, Arkell R, Stanier P, Copp AJ (2003) Disruption of *scribble* (*Scrb1*) causes severe neural tube defects in the *circletail* mouse. Hum Mol Genet 12:87–98
77. Montcouquiol M, Rachel RA, Lanford PJ, Copeland NG, Jenkins NA, Kelley MW (2003) Identification of *Vangl2* and *Scrb1* as planar polarity genes in mammals. Nature 423:173–177
78. Doudney K, Moore GE, Stanier P, Ybot-Gonzales P, Paternotte C, Greene ND, Copp AJ, Stevenson RE (2005) Analysis of the planar cell polarity gene *Vangl2* and its co-expressed paralog *Vangl1* in neural tube defects patients. Am J Med Genet A 138A:415
79. Holtfreter J, Hamburger V (1995) Embryogenesis: progressive differentiation. In: Willier H, Weiss PA, Hamburger V (eds) Analysis of development. Saunders, Philadelphia, PA, pp 230–296
80. Diez DC, Breitkreuz DN, Storey KG (2002) Onset of neuronal differentiation is regulated by paraxial mesoderm and requires attenuation of FGF signalling. Development 129:1681–1691
81. Diez DC, Olivera-Martinez I, Goriely A, Gale E, Maden M, Storey K (2003) Opposing FGF and retinoid pathways control ventral neural pattern, neuronal differentiation, and segmentation during body axis extension. Neuron 40:5–79
82. Novitch BG, Wichterle H, Jessel TM, Sockanathan S (2003) A requirement for retinoic acid-mediated transcriptional activation in ventral neural patterning and motor neuron specification. Neuron 40:81–95

83. Marti E, Bumcrot DA, Takada R, McMahon AP (1995) Requirement of 19K form of Sonic hedgehog for induction of distinct ventral cell types in CNS explants. Nature 375:322–325
84. Ericson J, Morton S, Kawakami A, Roelink H, Jessell TM (1996) Two critical periods of Sonic Hedgehog signaling required for the specification of motor neuron identity. Cell 87:661–673
85. Roelink H, Porter JA, Chiang C, Tanabe Y, Chang DT, Beachy PA, Jessell TM (1995) Floor plate and motor neuron induction by different concentrations of the amino-terminal cleavage product of sonic hedgehog autoproteolysis. Cell 81:445–455
86. Pierani A, Brenner-Morton S, Chiang C, Jessell TM (1999) A sonic hedgehog-independent, retinoid-activated pathway of neurogenesis in the ventral spinal cord. Cell 97:903–915
87. Briscoe J, Sussel L, Serup P, Hartigan-O'Connor D, Jessell TM, Rubenstein JL, Ericso J (1999) Homeobox gene *Nkx2.2* and specification of neuronal identity by graded Sonic hedgehog signalling. Nature 398:622–627
88. Briscoe J, Pierani A, Jessell TM, Ericson J (2000) A homeodomain protein code specifies progenitor cell identity and neuronal fate in the ventral neural tube. Cell 101:435–445
89. Ericson J, Rashbass P, Schedl A, Brenner-Morton S, Kawakami A, van Heyningen V, Jessell TM, Briscoe J (1997) Pax6 controls progenitor cell identity and neuronal fate in response to graded Shh signaling. Cell 90:169–180
90. Ericson J, Briscoe J, Rashbass P, van Heyningen V, Jessell TM (1997) Graded sonic hedgehog signaling and the specification of cell fate in the ventral neural tube. Cold Spring Harb Symp Quant Biol 62:451–466
91. Lee KJ, Jessell TM (1999) The specification of dorsal cell fates in the vertebrate central nervous system. Annu Rev Neurosci 22:261–294
92. Lee KJ, Mendelsohn M, Jessell TM (1998) Neuronal patterning by BMPs: a requirement for GDF7 in the generation of a discrete class of commissural interneurons in the mouse spinal cord. Genes Dev 12:3394–3407
93. Pierani A, Brenner-Morton S, Chiang C, Jessell TM (1999) A sonic hedgehog-independent, retinoid-activated pathway of neurogenesis in the ventral spinal cord. Cell 97:903–915
94. Timmer JR, Wang C, Niswander L (2002) BMP signaling patterns the dorsal and intermediate neural tube via regulation of homeobox and helix-loop-helix transcription factors. Development 129:2459–2472
95. Helms AW, Johnson JE (2003) Specification of dorsal spinal cord interneurons. Curr Opin Neurobiol 13:42–49
96. Stoykova A, Gruss P (1994) Roles of *Pax*-genes in developing and adult brain as suggested by expression patterns. J Neurosci 14:1395–1412
97. LaBonne C, Bronner-Fraser M (1999) Molecular mechanisms of neural crest formation. Annu Rev Cell Dev Biol 15:81–112
98. Chalepakis G, Fritsch R, Fickenscher H, Deutsch U, Goulding M, Gruss P (1991) The molecular basis of the *undulated/Pax-1* mutation. Cell 1(66):873–884
99. Gruss P, Walther C (1992) Pax in development. Cell 69:719–722
100. Tassabehji M, Read AP, Newton VE, Patton M, Gruss P, Harris R, Strachan T (1993) Mutations in the *PAX3* gene causing Waardenburg syndrome type 1 and type 2. Nat Genet 3:26–30
101. Baldwin CT, Lipsky NR, Hoth CF, Cohen T, Mamuya W, Milunsky A (1994) Mutations in *PAX3* associated with Waardenburg syndrome type I. Hum Mutat 3:205–211
102. Morell R, Friedman TB, Moeljopawiro S, Hartono, Soewito, Asher JH (1992) A frameshift mutation in the HuP2 paired domain of the probable human homolog of murine *Pax-3* is responsible for Waardenburg syndrome type 1 in an Indonesian family. Hum Mol Genet 1:243–247
103. Quiring R, Walldorf U, Kloter U, Gehring WJ (1994) Homology of the eyeless gene of Drosophila to the *Small eye* gene in mice and Aniridia in humans. Science 265:785–789
104. Mansouri A, Stoykova A, Torres M, Gruss P (1996) Dysgenesis of cephalic neural crest derivatives in *Pax7−/−* mutant mice. Development 122:831–838
105. Hol FA, Geurds MP, Chatkupt S, Shugart YY, Balling R, Schrander-Stumpel CT, Johnson WG, Hamel BC, Mariman EC (1996) *PAX* genes and human neural tube defects: an amino acid substitution in *PAX1* in a patient with spina bifida. J Med Genet 33:655–660
106. Nieuwkoop PD, Weijer CJ (1978) Neural induction, a two-way process. Med Biol 56:366–371
107. Harland R (2000) Neural induction. Curr Opin Genet Dev 10:357–362
108. Slack JM (1995) Developmental biology. Growth factor lends a hand. Nature 374:217–218
109. Lamb TM, Harland RM (1995) Fibroblast growth factor is a direct neural inducer, which combined with noggin generates anterior–posterior neural pattern. Development 121:3627–3636
110. Cox WG, Hemmati-Brivanlou A (1995) Caudalization of neural fate by tissue recombination and bFGF. Development 121:4349–4358
111. Sive HL, Hattori K, Weintraub H (1989) Progressive determination during formation of the anteroposterior axis in *Xenopus laevis*. Cell 58:171–180
112. Durston AJ, Timmermans JP, Hage WJ, Hendriks HF, de Vries NJ, Heideveld M, Nieuwkoop PD (1989) Retinoic acid causes an anteroposterior transformation in the developing central nervous system. Nature 340:140–144
113. Sive HL, Draper BW, Harland RM, Weintraub H (1990) Identification of a retinoic acid-sensitive period during primary axis formation in *Xenopus laevis*. Genes Dev 4:932–942
114. Altaba A, Jessell T (1991) Retinoic acid modifies mesodermal patterning in early *Xenopus* embryos. Genes Dev 5:175–187
115. Sive HL, Cheng P (1991) Retinoic acid perturbs the expression of *Xhox.lab* genes and alters mesodermal determination in *Xenopus laevis*. Genes Dev 5:1321–1332
116. Papalopulu N, Kintner C (1996) A posteriorising factor, retinoic acid, reveals that anteroposterior patterning controls the timing of neuronal differentiation in *Xenopus* neuroectoderm. Development 122:3409–3418
117. Blumberg B, Bolado J Jr, Derguin F, Craig AG, Moreno TA, Chakravarti D, Heyman RA, Buck J, Evans RM (1996) Novel retinoic acid receptor ligands in *Xenopus* embryos. Proc Natl Acad Sci USA 93:4873–4878
118. de Roos K, Sonneveld E, Compaan B, ten Berge D, Durston AJ, van der Saag PT (1999) Expression of retinoic acid 4-hydroxylase (CYP26) during mouse and *Xenopus laevis* embryogenesis. Mech Dev 82:205–211
119. Hollemann T, Chen Y, Grunz H, Pieler T (1998) Regionalized metabolic activity establishes boundaries of retinoic acid signalling. EMBO J 17:7361–7372
120. Kudoh T, Wilson SW, Dawid IB (2002) Distinct roles for Fgf, Wnt and retinoic acid in posteriorizing the neural ectoderm. Development 129:4335–4346
121. Chen Y, Pollet N, Niehrs C, Pieler T (2001) Increased XRALDH2 activity has a posteriorizing effect on the central nervous system of *Xenopus* embryos. Mech Dev 101:91–103
122. Niederreither K, Subbarayan V, Dolle P, Chambon P (1999) Embryonic retinoic acid synthesis is essential for early mouse post-implantation development. Nat Genet 21:444–448
123. Abu-Abed S, Dolle P, Metzger D, Beckett B, Chambon P, Petkovich M (2001) The retinoic acid-metabolizing enzyme, CYP26A1, is essential for normal hindbrain patterning, vertebral identity, and development of posterior structures. Genes Dev 15:226–240
124. Shiotsugu J, Katsuyama Y, Arima K, Baxter A, Koide T, Song J, Chandraratna RA, Blumberg B (2004) Multiple points of interaction between retinoic acid and FGF signaling during embryonic axis formation. Development 131:2653–2667

125. Deak KL, Dickerson ME, Linney E, Enterline DS, George TM, Melvin EC, Graham FL, Siegel DG, Hammock P, Mehltretter L, Bassuk AG, Kessler JA, Gilbert JR, Speer MC, NTD Collaborative Group (2005) Analysis of *ALDH1A2*, *CYP26A1*, *CYP26B1*, *CRABP1*, and *CRABP2* in human neural tube defects suggests a possible association with alleles in *ALDH1A2*. Birth Defects Res A Clin Mol Teratol 73:868–875
126. McGrew LL, Lai CJ, Moon RT (1995) Specification of the anteroposterior neural axis through synergistic interaction of the Wnt signaling cascade with noggin and follistatin. Dev Biol 172:337–342
127. McGrew LL, Hoppler S, Moon RT (1997) Wnt and FGF pathways cooperatively pattern anteroposterior neural ectoderm in *Xenopus*. Mech Dev 69:105–114
128. Bang AG, Papalopulu N, Goulding MD, Kintner C (1999) Expression of *Pax-3* in the lateral neural plate is dependent on a Wnt-mediated signal from posterior nonaxial mesoderm. Dev Biol 212:366–380
129. Fekany-Lee K, Gonzalez E, Miller-Bertoglio V, Solnica-Krezel L (2000) The homeobox gene *bozozok* promotes anterior neuroectoderm formation in zebrafish through negative regulation of BMP2/4 and Wnt pathways. Development 127:2333–2345
130. Domingos PM, Itasak N, Jones CM, Mercurio S, Sargent MG, Smith JC, Krumlauf R (2001) The Wnt/beta-catenin pathway posteriorizes neural tissue in *Xenopus* by an indirect mechanism requiring FGF signalling. Dev Biol 239:148–160
131. Schoenwolf GC, Smith JL (1990) Mechanisms of neurulation: traditional viewpoint and recent advances. Development 109:243–270
132. Shum AS, Copp AJ (1996) Regional differences in morphogenesis of the neuroepithelium suggest multiple mechanisms of spinal neurulation in the mouse. Anat Embryol (Berl) 194:65–73
133. Ybot-Gonzalez P, Cogram P, Gerrelli D, Copp AJ (2002) Sonic hedgehog and the molecular regulation of neural tube closure. Development 129:2507–2517
134. Echelard Y, Epstein DJ, Jacques B, Shen L, Mohler J, McMahon JA, McMahon AP (1993) Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. Cell 75:1417–1430
135. Goodrich LV, Milenkovic L, Higgins KM, Scott MP (1997) Altered neural cell fates and medulloblastoma in mouse *patched* mutants. Science 277:1109–1113
136. Milenkovic L, Goodrich LV, Higgins KM, Scott MP (1999) Mouse *patched1* controls body size determination and limb patterning. Development 126:4431–4440
137. Ding Q, Motoyama J, Gasca S, Mo R, Sasaki H, Rossant J, Hui CC (1998) Diminished Sonic hedgehog signaling and lack of floor plate differentiation in *Gli2* mutant mice. Development 125:2533–2543
138. Lee CS, Sund NJ, Behr R, Herrera PL, Kaestner KH (2005) Foxa2 is required for the differentiation of pancreatic alpha-cells. Dev Biol 278:484–495
139. Hui CC, Joyner AL (1993) A mouse model of greig cephalopolysyndactyly syndrome: the *extra-toes* mutation contains an intragenic deletion of the *Gli3* gene. Nat Genet 3:241–246
140. Huang Y, Roelink H, McKnight GS (2002) Protein kinase A deficiency causes axially localized neural tube defects in mice. J Biol Chem 277:19889–19896
141. Gunther T, Struwe M, Aguzzi A, Schughart K (1994) *Open brain*, a new mouse mutant with severe neural tube defects, shows altered gene expression patterns in the developing spinal cord. Development 120:3119–3130
142. Nagai T, Aruga J, Minowa O, Sugimoto T, Ohno Y, Noda T, Mikoshiba K (2000) Zic2 regulates the kinetics of neurulation. Proc Natl Acad Sci USA 97:1618–1623
143. Eggenschwile JT, Espinoza E, Anderson KV (2001) Rab23 is an essential negative regulator of the mouse Sonic hedgehog signalling pathway. Nature 412:194–198
144. Wild A, Kalff-Suske M, Vortkamp A, Bornholdt D, Konig R, Grzeschik KH (1997) Point mutations in human *GLI3* cause Greig syndrome. Hum Mol Genet 6:1979–1984
145. Kalff-Suske M, Wild A, Topp J, Wessling M, Jacobsen EM, Bornholdt D, Engel H, Heuer H, Aalfs CM, Ausems MG, Barone R, Herzog A, Heutink P, Homfray T, Gillessen-Kaessbach G, Konig R, Kunze J, Meinecke P, Muller D, Rizzo R, Strenge S, Superti-Furga A, Grzeschik KH (1999) Point mutations throughout the *GLI3* gene cause Greig cephalopolysyndactyly syndrome. Hum Mol Genet 8:1769–1777
146. Radhakrishna U, Wild A, Grzeschik KH, Antonarakis SE (1997) Mutation in *GLI3* in postaxial polydactyly type A. Nat Genet 17:269–271
147. Johnston JJ, Olivos-Glander I, Killoran C, Elson E, Turner JT, Peters KF, Abbott MH, Aughton DJ, Aylsworth AS, Bamshad MJ, Booth C, Curry CJ, David A, Dinulos MB, Flannery DB, Fox MA, Graham JM, Grange DK, Guttmacher AE, Hannibal MC, Henn W, Hennekam RC, Holmes LB, Hoyme HE, Leppig KA, Lin AE, Macleod P, Manchester DK, Marcelis C, Mazzanti L, McCann E, McDonald MT, Mendelsohn NJ, Moeschler JB, Moghaddam B, Neri G, Newbury-Ecob R, Pagon RA, Phillips JA, Sadler LS, Stoler JM, Tilstra D, Walsh Vockley CM, Zackai EH, Zadeh TM, Brueton L, Black GC, Biesecker LG (2005) Molecular and clinical analyses of Greig cephalopolysyndactyly and Pallister-Hall syndromes: robust phenotype prediction from the type and position of *GLI3* mutations. Am J Hum Genet 76:609–622
148. Winter RM, Huson SM (1988) Greig cephalopolysyndactyly syndrome: a possible mouse homologue (*Xt-extra toes*). Am J Med Genet 31:793–798
149. Nanni L, Ming JE, Bocian M, Steinhaus K, Bianchi DW, Die-Smulders C, Giannotti A, Imaizumi K, Jone KL, Campo MD, Martin RA, Meinecke P, Pierpont ME, Robin NH, Young ID, Roessler E, Muenke M (1999) The mutational spectrum of the sonic hedgehog gene in holoprosencephaly: *SHH* mutations cause a significant proportion of autosomal dominant holoprosencephaly. Hum Mol Genet 8:2479–2488
150. Wallis DE, Muenke M (1999) Molecular mechanisms of holoprosencephaly. Mol Genet Metab 68:126–138
151. Ming JE, Kaupas ME, Roessler E, Brunner HG, Golabi M, Tekin M, Stratton RF, Sujansky E, Bale SJ, Muenke M (2002) Mutations in *PATCHED-1*, the receptor for SONIC HEDGEHOG, are associated with holoprosencephaly. Hum Genet 110:97–301
152. Kirillova I, Novikova I, Auge J, Audollent S, Esnault D, Encha-Razavi F, Lazjuk G, Attie-Bitach T, Vekemans M (2000) Expression of the *sonic hedgehog* gene in human embryos with neural tube defects. Teratology 61:347–354
153. Zhu H, Barber R, Shaw GM, Lammer EJ, Finnell RH (2003) Is *Sonic hedgehog* (*SHH*) a candidate gene for spina bifida? A pilot study. Am J Med Genet A 117:87–88
154. Zhu H, Lu W, Laurent C, Shaw GM, Lammer EJ, Finnell RH (2005) Genes encoding catalytic subunits of protein kinase A and risk of spina bifida. Birth Defects Res A Clin Mol Teratol 73:591–596
155. Klootwijk R, Groenen P, Schijvenaars M, Hol F, Hamel B, Straatman H, Steegers-Theunissen R, Mariman E, Franke B (2004) Genetic variants in *ZIC1*, *ZIC2*, and *ZIC3* are not major risk factors for neural tube defects in humans. Am J Med Genet A 124:40–47
156. Wallingford JB (2005) Neural tube closure and neural tube defects: studies in animal models reveal known knowns and known unknowns. Am J Med Genet C Semin Med Genet 135:59–68
157. Brouns MR, Matheson SF, Hu KQ, Delalle I, Caviness VS, Silver J, Bronson RT, Settleman J (2000) The adhesion signaling molecule p190 RhoGAP is required for morphogenetic processes in neural development. Development 127:4891–4903
158. Hildebrand D, Soriano P (1999) Shroom, a PDZ domain-containing actin-binding protein, is required for neural tube morphogenesis in mice. Cell 99:485–497

159. Hildebrand R, Harland J, Wallingford JB (2003) Shroom induces apical constriction and is required for hingepoint formation during neural tube closure. Curr Biol 13:125–2137
160. Stumpo DJ, Bock CB, Tuttle JS, Blackshear PJ (1995) *MARCKS* deficiency in mice leads to abnormal brain development and perinatal death. Proc Natl Acad Sci USA 92:944–948
161. Chen J, Chang S, Duncan SA, Okano HJ, Fishell G, Aderem A (1996) Disruption of the *MacMARCKS* gene prevents cranial neural tube closure and results in anencephaly. Proc Natl Acad Sci USA 93:6275–6279
162. Xu W, Baribault H, Adamson ED (1998) *Vinculin* knockout results in heart and brain defects during embryonic development. Development 125:327–337
163. Koleske AJ, Gifford AM, Scott ML, Nee M, Bronson RT (1998) Essential roles for the Abl and Arg tyrosine kinases in neurulation. Neuron 21:1259–1272
164. Lanier LM, Gates MA, Witke W, Menzies AS, Wehman AM (1999) Mena is required for neurulation and commissure formation. Neuron 22:313–325
165. Stumpo DJ, Eddy RL, Haley LL, Sait S, Shows TB, Lai WS, Young WS, Speer MC, Dehejia A, Polymeropoulos M, Blackshear PJ (1998) Promoter sequence, expression, and fine chromosomal mapping of the human gene (*MLP*) encoding the MARCKS-like protein: identification of neighboring and linked polymorphic loci for *MLP* and *MACS* and use in the evaluation of human neural tube defects. Genomics 49:253–264
166. Holmberg J, Clarke DL, Frisen J (2000) Regulation of repulsion versus adhesion by different splice forms of an Eph receptor. Nature 408:203–206
167. Lee HS, Bong YS, Moore KB, Soria K, Moody SA, Daar IO (2005) Dishevelled mediates ephrinB1 signalling in the eye field through the planar cell polarity pathway. Nat Cell Biol 8:55–63
168. Rutishauser U, Jessell TM (1988) Cell adhesion molecules in vertebrate neural development. Physiol Rev 68:819–885
169. Detrick RJ, Dickey D, Kinter CR (1990) The effects of N-cadherin misexpression on morphogenesis in *Xenopus* embryos. Neuron 4:493–506
170. Cremer H, Chazal G, Goridis C, Represa A (1997) NCAM is essential for axonal growth and fasciculation in the hippocampus. Mol Cell Neurosci 8:323–335
171. Radice GL, Ferreira-Cornwell MC, Robinson SD, Rayburn H, Chodosh LA, Takeichi M, Hynes RO (1997) Precocious mammary gland development in *P-cadherin*-deficient mice. J Cell Biol 139:1025–1032
172. Deak KL, Boyles AL, Etchevers HC, Melvin EC, Siegel DG, Graham FL, Slifer SH, Enterline DS, Geoand Speer MC (2005) SNPs in the neural cell adhesion molecule 1 gene (*NCAM1*) may be associated with human neural tube defects. Hum Genet 117:133–142
173. Loeken MR (2005) Current perspectives on the causes of neural tube defects resulting from diabetic pregnancy. Am J Med Genet C Semin Med Genet 135C:77–87
174. Shaw GM, Velie EM, Schaffer D (1996) Risk of neural tube defect-affected pregnancies among obese women. JAMA 275:1093–1096
175. Lynberg MC, Khoury MJ, Lu X, Cocian T (1994) Maternal flu, fever and risk of neural tube defects: a population-based case-control study. Am J Epidemiol 140:244–255
176. Matalon S, Schechtman S, Goldzweig G, Ornoy A (2002) The teratogenic effect of carbamazepine: a meta-analysis of 1255 exposures. Reprod Toxicol 5:33–39
177. Myrianthopoulos NC, Melnick M (1987) Studies in neural tube defects. I. Epidemiological and etiologic aspects. Am J Med Genet 26:783–796
178. Ncayiyana DJ (1996) Neural tube defects among rural blacks in a Transkei district. A preliminary report and analysis. S Afr Med J 69:618–620
179. Bove F, Shim Y, Zeitz P (2002) Drinking water contaminants and adverse pregnancy outcome: a review. Environ Health Perspect 110:61–74
180. Shaw GM (2001) Adverse human reproductive outcomes and electromagnetic fields: a brief summary of the epidemiologic literature. Bioelectromagnetics 5:S5–S18 (Suppl)
181. Shaw GM, Wasserman CR, O'Malley CD, Nelson V, Jackson RJ (1999) Maternal pesticide exposure from multiple sources and selected congenital anomalies. Epidemiology 10:60–66
182. Croen LA, Shaw GM, Sanbonmatsu L, Selvin S, Buffler PA (1997) Maternal residential proximity to hazardous waste sites and risk for selected congenital malformations. Epidemiology 8:347–354
183. Suarez L, Cardarelli K, Hendricks K (2003) Maternal stress, social support, and risk of neural tube defects among Mexican Americans. Epidemiology 14:612–616
184. Felkner M, Hendricks K, Suarez L, Waller DK (2003) Diarrhea: a new risk factor for neural tube defects? Birth Defects Res A Clin Mol Teratol 67:504–508
185. MRC (1991) Prevention of neural tube defects: results of the Medical Research Council Vitamin Study. MRC Vitamin Study Research Group. Lancet 338:131–137
186. Czeizel AE, Dudas I (1992) Prevention of the first occurrence of neural-tube defects by periconceptional vitamin supplementation. N Engl J Med 327:1832–1835
187. Erickson JD (2002) Folic acid and prevention of spina bifida and anencephaly. 10 years after U.S. Public Health Service Recommendation. MMWR Recomm Rep 51:1–3
188. Van der Put NM, Steegers-Theunissen RP, Frosst P, Trijbels FJ, Eskes TK, van den Heuvel LP, Mariman EC, den Heyer M, Rozen R, Blom HJ (1995) Mutated methylenetetrahydrofolate reductase as risk factor for spina bifida. Lancet 346:1070–1071
189. Ou CY, Stevenson RE, Brown VK, Schwart CE, Allen WP, Khoury MJ, Rozen R, Oakley GP, Adams MJ (1996) *Methylenetetrahydrofolate reductase* genetic polymorphism as a risk factor for neural tube defects. Am J Med Genet 63:610–614
190. Christensen B, Arbour L, Tran P, Leclerc D, Sabbaghian N, Platt R, Gilfix BM, Rosenblatt DS, Gravel RA, Forbes P, Rozen R (1999) Genetic polymorphisms in methylenetetrahydrofolate reductase and methionine synthase, folate levels in red blood cells, and risk of neural tube defects. Am J Med Genet 84:151–157
191. Koch MC, Stegmann K, Ziegler A, Schroter B, Ermert A (1998) Evaluation of the *MTHFR* C677T allele and the *MTHFR* gene locus in a German spina bifida population. Eur J Pediatr 157:487–492
192. Gonzales-Herrera L, Garcia-Escalante G, Castillo-Zapata I, Canto-Herrera J, Caballos-Quintal J, Pinto-Escalante D, Diaz-Rubio F, Del Angel RM, Orozco-Orozco L (2002) Frequency of the thermolabile variant C677T in the *MTHFR* gene and lack of association with neural tube defects in the State of Yucatan, Mexico. Clin Genet 62:394–398
193. Revilla JIG, Hernandez FN, Martin MTC, Salvador MT, Romero JG (2003) C677T and A1298C *MTHFR* polymorphisms in the etiology of neural tube defects in Spanish population. Med Clin (Barc) 120:441–445
194. Botto LD, Yang Q (2000) 5,10-Methylenetetrahydrofolate reductase gene variants and congenital anomalies: a HuGE review. Am J Epidemiol 151:862–877
195. van der Put NM, Gabreels F, Stevens EM, Smeitink JA, Trijbels FJ, Eskes TK, van den Heuvel LP, Blom HJ (1998) A second common mutation in the methylenetetrahydrofolate reductase gene: an additional risk factor for neural-tube defects? Am J Hum Genet 62:1044–1051
196. Weisberg I, Tran P, Christensen B, Sibani S, Rozen R (1998) A second genetic polymorphism in methylenetetrahydrofolate reductase (*MTHFR*) associated with decreased enzyme activity. Mol Genet Metab 64:169–172

197. De Marco P, Calevo MG, Moroni A, Arata L, Merello E, Finnell RH, Zhu H, Andreussi L, Cama A, Capra V (2002) Study of *MTHFR* and *MS* polymorphisms as risk factors for NTD in the Italian population. J Hum Genet 47:319–324
198. Brody LC, Conley M, Cox C, Kirke PN, McKeever MP, Mills JL, Molloy AM, O'Leary VB, Parle-McDermott A, Scott JM, Swanson DA (2002) A polymorphism, R653Q, in the trifunctional enzyme methylenetetrahydrofolate dehydrogenase/methenyltetrahydrofolate cyclohydrolase/formyltetrahydrofolate synthetase is a maternal genetic risk factor for neural tube defects: report of the Birth Defects Research Group. Am J Hum Genet 71:1207–1215
199. Parle-McDermott A, Kirke PN, Mills JL, Molloy AM, Cox C, O'Leary VB, Pangilinan F, Conley M, Cleary L, Brody LC et al (2006) Confirmation of the R653Q polymorphism of the trifunction C1-synthase enzyme as a maternal risk for neural tube defects in the Irish population. Eur J Hum Genet 14:768–772
200. Rothenberg SP, da Costa MP, Sequeira JM, Cracco J, Roberts JL, Weedon J, Quadros EV (2004) Autoantibodies against folate receptors in women with a pregnancy complicated by a neural-tube defect. N Engl J Med 350:134–142
201. Barber RC, Shaw GM, Lammer EJ, Greer KA, Biela TA, Lacey SW, Wasserman CR, Finnell RH (1998) Lack of association between mutations in the *folate receptor-alpha* gene and spina bifida. Am J Med Genet 76:310–317
202. De Marco P, Calevo MG, Moroni A, Merello E, Raso A, Finnell RH, Zhu HP, Andreussi L, Cama A, Capra V (2003) Reduced folate carrier polymorphism (80A→G) and neural tube defects. Eur J Hum Genet 11:245–252
203. O'leary VB, Pangilinan F, Cox C, Parle-McDermott A, Conley M, Molloy AM, Kirke PN, Mills JL, Brody LC, Scott JM, Members of the Birth Defects Research Group (2006) Reduced folate carrier polymorphisms and neural tube defect risk. Mol Genet Metab 87:364–369
204. Shaw GM, Lammer EJ, Zhu HP, Baker MW, Neri E, Finnell RH (2002) Maternal periconceptional vitamin use, genetic variation of infant reduced folate carrier (A80G), and risk of spina bifida. Am J Med Genet 108:1–6
205. Kirke PN, Molloy AM, Daly LE, Burke H, Weir DG, Scott JM (1993) Maternal plasma folate and vitamin B12 are independent risk factors for neural tube defects. Q J Med 86:703–708
206. Afman LA, van der Put NM, Thomas CM, Trijbels JM, Blom HJ (2001) Reduced vitamin B12 binding by transcobalamin II increases the risk of neural tube defects. Q J Med 94:159–166
207. Morrison K, Papapetrou C, Hol FA, Mariman EC, Lynch SA, Burn J, Edwards YH (1998) Susceptibility to spina bifida; an association study of five candidate genes. Ann Hum Genet 62:379–396
208. Wilson A, Platt R, Wu Q, Leclerc D, Christensen B, Yang H, Gravel RA, Rozen R (1999) A common variant in methionine synthase reductase combined with low cobalamin (vitamin B12) increases risk for spina bifida. Mol Genet Metab 67:317–323
209. O'Leary VB, Mills JL, Pangilinan F, Kirke PN, Cox C, Conley M, Weiler A, Peng K, Shane B, Scott JM et al (2005) Analysis of methionine synthase reductase polymorphisms for neural tube defects risk association. Mol Genet Metab 85:220–227
210. Afman LA, Lievers KJ, van der Put NM, Trijbels FJM, Blom HJ (2002) Single nucleotide polymorphisms in the transcobalamin gene: relationship with transcobalamin concentrations and risk for neural tube defects. Eur J Hum Genet 10:433–438
211. Swanson DA, Pangilinan F, Mills JL, Kirke PN, Conley M, Weiler A, Frey T, Parle-McDermott A, O'Leary VB, Seltzer RR, Moynihan KA, Molloy AM, Burke H, Scott JM, Brody LC (2005) Evaluation of transcobalamin II polymorphisms as neural tube defect risk factors in an Irish population. Birth Defects Res A Clin Mol Teratol 73:239–244
212. Heil SG, van der Put NMJ, Waas ET, den Heijer M, Trijbels FJM, Blom HJ (2001) Is mutated serine hydroxymethyltransferase (*SHMT*) involved in the etiology of neural tube defects? Mol Genet Metab 73:164–172
213. Morin I, Platt R, Weisberg I, Sabbaghian N, Wu Q, Garrow TA, Rozen R (2003) Common variant in betaine–homocysteine methyltransferase (*BHMT*) and risk for spina bifida. Am J Med Genet 119A:172–176
214. Wilding CS, Relton CL, Sutton MJ, Jonas PA, Lynch SA, Tawn EJ, Burn J (2004) Thymidylate synthase repeat polymorphisms and risk of neural tube defects in a population from the northern United Kingdom. Birth Defects Res A Clin Mol Teratol 70:483–485
215. Volcik KA, Shaw GM, Zhu H, Lammer EJ, Laurent C, Finnell RH (2003) Associations between polymorphisms within the *thymidylate synthase* gene and spina bifida. Birth Defects Res A Clin Mol Teratol 67:924–928
216. Volcick KA, Shaw GM, Lammer EJ, Zhu H, Finnell RH (2003) Evaluation of infant methylenetetrahydrofolate reductase genotype, maternal vitamin use, and risk of high versus low level spina bifida defects. Birth Defects Res Part A Clin Mol Teratol 67:154–157
217. Zhao Q, Behringer RR, de Crombrugghe B (1996) Prenatal folic acid treatment suppresses acrania and meroanencephaly in mice mutant for the *Cart1* homeobox gene. Nat Genet 13:275–283
218. Barbera JP, Rodriguez TA, Greene ND, Weninger WJ, Simeone A, Copp AJ, Beddington RS, Dunwoodie S (2002) Folic acid prevents exencephaly in *Cited2* deficient mice. Hum Mol Genet 11:283–293
219. Conway SJ, Henderson DJ, Copp AJ (1997) Pax3 is required for cardiac neural crest migration in the mouse: evidence from the *splotch* (Sp2H) mutant. Development 124:505–514
220. Piedrahita JA, Oetama B, Bennett GD, van Waes J, Kamen BA, Richardson J, Lacey SW, Anderson RG, Finnell RH (1999) Mice lacking the folic acid-binding protein Folbp1 are defective in early embryonic development. Nat Genet 23:228–232
221. Carter M, Ulrich S, Oofuji Y, Williams DA, Ross ME (1999) *Crooked tail* (*Cd*) models human folate-responsive neural tube defects. Hum Mol Genet 8:2199–2204
222. Carter M, Chen X, Slowinska B, Minnerath S, Glickstein S, Shi C, Campagne F, Weinstein H, Ross ME (2005) *Crooked tail* (*Cd*) model of human folate-responsive neural tube defects is mutated in Wnt coreceptor lipoprotein receptor-related protein 6. Proc Natl Acad Sci USA 102:12843–12848
223. Haviland MB, Essien FB (1990) Expression of the *Axd* (*axial defects*) mutation in the mouse is insensitive to retinoic acid at low dose. J Exp Zool 256:342–346
224. Greene ND, Copp AJ (1997) Inositol prevents folate-resistant neural tube defects in the mouse. Nat Med 3:60–66
225. Ting SB, Caddy J, Hislop N, Wilanowski T, Auden A, Zhao LL, Ellis S, Kaur P, Uchida Y, Holleran WM, Elias PM, Cunningham JM, Jane SM (2005) A homolog of Drosophila *grainy head* is essential for epidermal integrity in mice. Science 308:411–413
226. Cogram P, Hynes A, Dunlevy LP, Greene ND, Copp AJ (2004) Specific isoforms of protein kinase C are essential for prevention of folate-resistant neural tube defects by inositol. Hum Mol Genet 13:7–14
227. Groenen PM, Peer PG, Wevers RA, Swinkels DW, Franke B, Mariman EC, Steegers-Theunissen RP (2003) Maternal myo-inositol, glucose, and zinc status is associated with the risk of offspring with spina bifida. Am J Obstet Gynecol 189:1713–1719