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The relationship between Sonic hedgehog signalling, cilia and neural tube defects

Jennifer N. Murdoch^{1,*} and Andrew J. Copp²

¹Centre for Biomedical Sciences, School of Biological Sciences, Royal Holloway University of London, Egham, Surrey, TW20 0EX, UK.

²UCL Institute of Child Health, 30 Guilford Street, London WC1N 1EH, UK.

Abstract

The Hedgehog signalling pathway is essential for many aspects of normal embryonic development, including formation and patterning of the neural tube. Absence of Shh ligand is associated with the midline defect holoprosencephaly, while increased Shh signalling is associated with exencephaly and spina bifida. To complicate this apparently simple relationship, mutation of proteins required for function of cilia often leads to impaired Shh signalling and to disruption of neural tube closure. In this manuscript, we review the literature on Shh pathway mutants and discuss the relationship between Shh signalling, cilia and neural tube defects.

Keywords

Neural tube defects; Hedgehog Signalling Pathway; Cilia; Exencephaly; Spina bifida; Intraflagellar Transport; Neural Tube Patterning

INTRODUCTION

The Hedgehog signalling pathway

The Hedgehog signalling pathway was originally defined in *Drosophila*, and many of the core components are conserved between species. A number of excellent recent reviews have elaborated the details of the pathway (Jiang and Hui, 2008; Varjosalo and Taipale, 2008; Wong and Reiter, 2008; Simpson et al, 2009) which is summarised in Figure 1. In outline, Hedgehog signalling involves multiple inhibitory interactions. Binding of Hedgehog ligand to its receptor, Patched1 (Ptc1), removes the inhibitory action of Ptc1 on the transmembrane protein Smoothened (Smo). The activated Smo protein then initiates an intracellular signalling cascade that leads to inhibition of formation of Gli transcriptional repressors, and stimulation of the Gli transcriptional activators. Of course, the intracellular signalling mechanism is much more complex than this brief summary reveals, and new components of the pathway continue to be identified. The precise intracellular process has diverged between species, involving differing mechanisms and protein components. In particular, vertebrate Hedgehog signalling involves a specialized organelle, the primary cilium (Figure 1).

Formation and maintenance of the cilium requires movement of proteins from the cell into the cilium, since no protein synthesis occurs within the cilium itself. This movement is termed intraflagellar transport (IFT), and involves many proteins. The B complex of IFT

* Author for correspondence Tel: 01784 276289 Fax: 01784 414224 Jenny.Murdoch@rhul.ac.uk.

proteins is involved in transport into the cilium (anterograde transport), along microtubules within the ciliary axoneme, in association with kinesin motor proteins. Another complex of IFT proteins (complex A) is involved in transport out of the cilium (retrograde transport), in association with dynein motors. The requirement for the cilium in Hedgehog signalling was first realised following the identification of mouse mutants that disrupt the intraflagellar transport proteins, IFT88 and IFT172 (Huangfu et al, 2003). These mutants exhibit morphological defects and evidence of disturbed Hh-regulated patterning. Since then, a number of regulators of intraflagellar transport have been shown to be required for Shh signalling (see (Eggenchwiler and Anderson, 2007; Wong and Reiter, 2008) for recent reviews).

Several components of the Shh pathway components are present within the cilium. Smoothened localises to the distal tip of primary cilia following pathway stimulation and this is an essential, though not sufficient, requirement for activation of the downstream signalling cascade (Corbit et al, 2005; Rohatgi et al, 2007; Rohatgi et al, 2009). Relocation of Smo to the cilium is inhibited by Ptch1 (Rohatgi et al, 2007) and involves β -arrestin2 (Kovacs et al, 2008). Activated Smoothened is phosphorylated by adrenergic receptor kinase beta1 (Adrbk1, previously named Grk2), and this promotes the physical interaction of Smo with β -arrestin2, triggering the internalization of Smo by endocytosis in clathrin-coated pits and stimulating Gli activity (Chen et al, 2004; Meloni et al, 2006; Philipp et al, 2008). The Gli proteins are also detected at the distal tips of cilia (Haycraft et al, 2005), and recent reports show that this localisation is dependent on pathway activation and ciliary accumulation of activated Smo (Chen et al, 2009; Kim et al, 2009). Accumulation of Gli in the cilia is not sufficient for downstream pathway activation, however, as functional retrograde transport is also required (Kim et al, 2009). Indeed, activated Gli2 must subsequently enter the nucleus in order to function as a transcriptional activator. In unstimulated cells, Gli2 is observed in faint cytoplasmic puncta, often in association with microtubules, and disruption of cytoplasmic microtubules can also block pathway activation (Kim et al, 2009).

There are three Gli proteins; Gli2 and Gli3 are direct effectors in the Hedgehog pathway, whereas Gli1 is induced as a later event. Gli2 and Gli3 proteins are processed in three different ways. Full length proteins can be activated, to function as transcriptional activators. The precise step of "activation" is not yet defined, although Unc-51-like kinase 3 (ULK3) has recently been demonstrated to phosphorylate Gli2, increasing its transcriptional activity (Maloverjan et al, 2010). Full length Gli3 is converted to an activator following nuclear localisation, phosphorylation and destabilization (Humke et al, 2010). Gli2 and Gli3 can also be proteolytically cleaved to form transcriptional repressors, with Gli3 being the more significant repressor in vivo. Formation of Gli repressors is well characterised, in molecular terms: phosphorylation by protein kinase A (PKA) primes Gli3 for phosphorylation by Glycogen synthase kinase 3 β (GSK3 β) and casein kinase 1 (CK1). Phosphorylation of Gli3 promotes binding to β -TRCP which stimulates proteolytic processing of Gli3. The third molecular path for Gli proteins involves degradation. This involves Spop, which promotes ubiquitination of Gli2 and Gli3 by the Cullin3 E3 ubiquitin ligase, and subsequently complete degradation through the 26S-proteasome.

Suppressor of Fused (Sufu) is a key negative regulator of the Hedgehog pathway, functioning genetically downstream of Smo. *Sufu* mutant MEFs show constitutive Gli activity, as determined using a luciferase reporter with Gli binding sites (Svard et al, 2006), and Sufu reduces pathway activity following Gli1 or Gli2 expression (Jia et al, 2009). Sufu interacts with all three Gli proteins (Dunaeva et al, 2003; Jia et al, 2009) and proposed actions include sequestering Gli1 and Gli2 in the cytoplasm (Kogerman et al, 1999; Dunaeva et al, 2003), or inhibiting Gli transcriptional activation via recruitment of sin3-

associated polypeptide 18 (SAP18) and the histone deacetylation machinery (Barnfield et al, 2005). Sufu also promotes the proteolytic cleavage of Gli3 to form the Gli3 repressor, by recruiting GSK3 β and augmenting its ability to phosphorylate PKA-primed Gli3 (Kise et al, 2009). Most recently, examination of endogenous interactions has demonstrated that Sufu interacts with full length Gli3 (Gli3FL) in the absence of Shh signalling, and this complex dissociates following Shh stimulation (Humke et al, 2010). Precisely how Shh stimulation triggers Sufu-Gli3FL dissociation is unclear, although this event can be inhibited by PKA activation, and requires kinesin family member Kif3a (Humke et al, 2010). Notably, Sufu and Gli3FL must dissociate in order to allow Gli3FL to translocate into the nucleus where it is phosphorylated, destabilized and converted to a transcriptional activator (Humke et al, 2010).

Although Sufu can be detected in the cilia, there is conflicting evidence as to whether cilia are required or not, in order to repress pathway activity; Sufu function is dependent on *Ift172* and *Dync2h1* (Ocbina and Anderson, 2008) yet acts genetically downstream of *Kif3a* and *Ift88* (Chen et al, 2009; Jia et al, 2009). *Sufu* mutants exhibit a dramatic decrease in the endogenous levels of both Gli2 and Gli3 proteins (Chen et al, 2009; Jia et al, 2009; Kise et al, 2009), as Sufu normally prevents the proteasomal degradation of Gli2 and Gli3 by inhibiting the ubiquitination promoter Spop (Chen et al, 2009). The paradoxical increase in pathway activity in Sufu mutants, despite the low level of Gli2 and Gli3 expression, is thought to reflect an increase in the proportion of Gli that is active (Humke et al, 2010); indeed, Gli2R is a potent antagonist of Gli2 activator (Pan et al, 2009). The cyclin dependent kinase 11b (Cdk11b, previously named Cdc211) forms a complex with Sufu and prevents Sufu from interacting with Gli1 (Evangelista et al, 2008). In fact, Cdk11b is necessary and sufficient for activation of the Hh pathway, by inhibiting the negative effect of Sufu.

A number of additional negative regulators act between Smo and Gli, including Ras-related protein 23 (Rab23), FK506 binding protein 8 (Fkbp8), Tectonic and Tubby-like protein 3 (Tulp3). The Rab family of proteins regulate the fusion and docking of membranes between specific vesicles and organelles. Tulp3 may also affect protein movement within the cell, since a related Tubby-protein family member has been implicated in vesicular trafficking in ciliated photoreceptors (Hagstrom et al, 2001; Xi et al, 2005; Xi et al, 2007). Tectonic functions genetically downstream of *Rab23*, and is required for maximal pathway activation in the presence of Hh ligand, and repression in the absence of ligand, consistent with a role in formation of both Gli activators and repressors (Reiter and Skarnes, 2006).

Neural tube closure

The embryonic neural tube is the developmental precursor of the brain and spinal cord. It forms by the rolling up and fusion of a plate of specialised ectodermal cells along the dorsal midline of the embryo. The process of neural tube closure occurs with a characteristic sequence of events along the embryonic axis (Figure 2A) (Greene and Copp, 2009). Initiation of closure first occurs at the future cervical-hindbrain boundary, around the 5-somite stage in mouse, and continues both rostrally (into the hindbrain) and caudally (into the spine) from this site, termed Closure 1. Initiation of closure also occurs within defined regions of the developing brain, at the forebrain/midbrain boundary (termed Closure 2) and at the most rostral extremity of the forebrain (termed Closure 3). Continuation of closure between these initiation sites is responsible for complete cranial neurulation, by about the 17-somite stage in the mouse. Neural tube closure within the spinal region continues for a further day of mouse gestation, as the embryo grows. Spinal closure is finally completed at around the 30-somite stage, with closure of the posterior neuropore.

Neural tube defects

Disruption of the normal process of neural tube closure results in neural tube defects (NTDs). These are a set of major congenital malformations that have devastating consequences on affected individuals. NTDs are classified according to the region of the brain and spinal cord that is affected (Figure 2A-D) (Copp and Greene, 2010).

Craniorachischisis is the most severe form of NTD, in which almost the entire brain and spinal cord remains open. This defect results from failure of Closure 1 and is invariably fatal. Anencephaly is another fatal defect in which development of the brain is disrupted, and this is caused by failure to complete neural tube closure in the cranial region. Spina bifida most commonly affects the lumbo-sacral spinal region, and results from failure to complete closure at the posterior neuropore. Many cases of spina bifida are compatible with postnatal survival, yet patients often have challenges in mobility, pain sensation and genitourinary control as the result of damage to the exposed neural tissue. The severity of the disorders and the common prevalence of NTDs (affecting 0.5-2/1000 established pregnancies) mean that they are a significant clinical problem. Understanding the causes of NTDs is important, therefore, since knowledge of the basic etiology is required in order to be able to make additional progress towards the amelioration of these devastating disorders.

Neural tube patterning

The Shh signalling cascade plays an essential role in patterning the dorsoventral axis of the neural tube (Altaba et al, 2003; Wilson and Maden, 2005; Stamatakis et al, 2005; Dessaud et al, 2007; Ribes and Briscoe, 2009). Shh is expressed first in the notochord, underlying the ventral neural tube. This induces expression of *Foxa2* in the ventral midline, which itself initiates expression of Shh within the ventral midline, the prospective floor plate. The Shh signal from the notochord and floor plate acts to specify the ventral marker domains, in a concentration- and time-dependent manner. High levels of Shh stimulate the most ventral marker domains, *Nkx2.2* in V3 progenitor cells, while lower levels stimulate progressively more dorsal precursor markers and neuronal subtypes such as motoneurons and ventral interneurons. Dorsal patterning involves BMP signals from the surface ectoderm and roof plate. Dorso-ventral (DV) patterning is also modulated by the retinoic acid and fibroblast growth factor pathways. The essential requirement for Shh signalling in the specification of ventral marker domains is often used as a way of assessing the level of activity of the Shh pathway.

MUTATION OF SHH PATHWAY COMPONENTS AND THE EFFECT ON NEURAL TUBE CLOSURE

Cranial defects in mutants with a complete loss of Shh signalling

The Shh signalling cascade is essential for many aspects of cranial development, but it is not required for neural tube closure (Table 1). Complete absence of Shh signalling, as in the null mutant for Shh itself (Chiang et al, 1996) results in a dramatic effect on DV patterning within the neural tube, with absence of ventral cell types and failure of floor plate formation. Embryos are smaller than wild-type littermates, and exhibit holoprosencephaly (Figure 2E). In this defect, the cranial neural tube is closed, but with a failure of midline development such that the forebrain develops as a single structure, rather than the normal paired cerebral hemispheres. The eye fields are abnormally close together or fused in the midline, a condition termed cyclopia.

Smoothened is the transmembrane protein that is required for all Hedgehog pathway activation, resulting from stimulation with Sonic Hedgehog, or the other mammalian ligands, Desert Hedgehog and Indian Hedgehog. Thus, loss of Smo results in more severe embryonic defects than seen in *Shh* mutants. *Smo* mutants are small and arrest at or before

E9.5, likely owing to heart defects; mutants exhibit abnormal heart looping, and enlarged pericardial sacs, plus also defects in embryonic turning (Zhang et al, 2001; Caspary et al, 2002; Ma et al, 2002). *Smo* mutants exhibit dramatic effects on DV patterning within the neural tube, with loss of the Shh-dependent ventral markers, *Foxa2*, *Nkx2.2* and ventral expansion of the dorsal marker *Pax6* (Zhang et al, 2001; Caspary et al, 2002; Wijgerde et al, 2002). In the head, *Smo* mutants exhibit a reduction of midline tissues in the forebrain, and incomplete separation of the developing optic vesicles (Ma et al, 2002); these anomalies would presumably result in holoprosencephaly and cyclopia in later embryos, if they survived. Despite these multiple developmental defects, neural tube closure occurs in *Smo* embryos.

Reduced Shh signalling is also seen in the mutants for *Dispatched1* (*Disp1*). *Disp1* is required for release of cholesterol-modified Shh ligand from the cells in which it is synthesised (Ma et al, 2002; Kawakami et al, 2002). *Disp1* mutants exhibit a phenotype very similar to *Smo* mutants, presumably since *Disp1* is required for release of all Hedgehog ligands. *Disp1* mutants exhibit developmental arrest at around E9.5, with an enlarged pericardial sac, defective embryonic turning and abnormal heart looping. Patterning within the neural tube is disrupted, with loss of ventral markers including Shh itself, *Foxa2*, *Nkx2.2* and *Nkx6.1* and expansion of *Pax6* and *Pax7* expression (Caspary et al, 2002; Ma et al, 2002; Kawakami et al, 2002). *Disp1* mutants exhibit similar HPE-like head defects to *Smo*, including reduced forebrain midline tissues, and presence of single optic vesicle evagination (Caspary et al, 2002; Ma et al, 2002; Kawakami et al, 2002). Also like *Smo*, *Disp1* mutants exhibit no abnormality in neural tube closure.

Cell adhesion molecule-related/down-regulated by oncogenes (*Cdon*) and biregional *Cdon* binding protein (*Boc*) are cell surface proteins of the immunoglobulin/fibronectin superfamily. These proteins interact physically with each other, and also bind Shh ligand with high affinity. *Cdon* and *Boc* are required for maximal Shh pathway activity, perhaps by facilitating ligand presentation to *Ptch1* (Tenzen et al, 2006). Growth arrest specific 1 (*Gas1*) is another cell surface protein that is required for maximal Shh pathway activation. Loss of *Boc* does not affect neural tube closure or patterning, although *Boc* mutants do have defects in commissural axon guidance (Okada et al, 2006). Mutation of *Gas1* or *Cdon* results in reduction of ventral markers, eye defects and craniofacial abnormalities resembling mild forms of holoprosencephaly, yet no abnormalities in neural tube closure are observed (Cole and Krauss, 2003; Tenzen et al, 2006; Zhang et al, 2006; Seppala et al, 2007; Allen et al, 2007; Martinelli and Fan, 2007; Zhang et al, 2009).

Mutants for the secreted protein Tectonic exhibit loss of ventral markers and reduced expression of *Ptch1* and *Gli1*, indicating decreased activation of the Shh pathway. These mutants exhibit holoprosencephaly, but not NTDs (Reiter and Skarnes, 2006). Loss of another protein that has a positive role in the Shh signalling pathway, *Adrbk1* (*Grk2*), leads to heart failure but not NTDs (Jaber et al, 1996) while disruption of downstream kinase *Cdk11b* (*Cdc211*) causes embryonic death during the blastocyst stage, owing to cell cycle arrest (Li et al, 2004). Disruption of *Gli2*, the major transcriptional regulator that activates downstream genes, leads to reduced ventral marker expression in the neural tube, but not NTDs. Over-expression of *Ptch1* can reduce pathway activity, with expansion of dorsal markers further ventrally (but no loss of ventral markers), and some embryos exhibit an open region of neural tube in the forebrain, hindbrain or along the spine. However, this has been suggested to result from a re-opening event rather than a defect of closure; embryos also have a defect resembling holoprosencephaly (Goodrich et al, 1999).

Thus, evidence from a number of mutants indicates that loss of Shh signalling is associated with defects in cranial development, specifically holoprosencephaly and cyclopia, yet does not prevent closure of the neural tube.

Exencephaly in mutants with activation of the Shh pathway

In contrast to the absence of NTDs in mutants that reduce Shh pathway activity, activation of the Shh pathway is commonly associated with NTDs (Table 2). A number of negative regulators of the Hedgehog pathway are known, and these are characterized by expansion of the ventral marker domains within the spinal cord. *Ptch1* is a key negative regulator of Shh signalling, serving to inhibit activation of Smoothened by preventing translocation into the cilium in the absence of Hedgehog ligand (Rohatgi et al, 2007). Mutation of *Ptch1* therefore allows constitutive activation of Smo. *Ptch1* null mutants show dramatic ventralization of the spinal neural tube, with expansion of ventral markers across the entire DV extent of the neural tube and loss of dorsal markers (Goodrich et al, 1997; Caspary et al, 2002). *Ptch1* null mutants die at around E9.5, and exhibit almost complete failure of neural tube closure; inspection of mutants indicates that Closure 1 occurs, but closure fails in the head and spinal cord (Goodrich et al, 1997; Caspary et al, 2002).

Failure of cranial neural tube closure has also been observed in a conditional null allele of *Ptch1* that was recombined using a ubiquitous Cre driver. The authors refer to complete failure of neural tube closure, although this is not evident in the images provided (Ellis et al, 2003). Transgenic expression of *Ptch1* under the control of the metallothionein promoter partially rescues development of *Ptch1* mutants, and these *Ptch1*^{-/-}; *MT-Ptch1* embryos survive to E14 (Milenkovic et al, 1999). However, they retain NTDs and, in the presence of relatively low levels of transgenic *Ptch1* expression, exhibit both exencephaly and severe spina bifida. Only the region of Closure 1 appears to close successfully (Milenkovic et al, 1999). Examination of spinal cord patterning reveals partial rescue of normal patterning; in particular, the more severely affected embryos have the more ventralized neural tube.

Sufu is another key negative regulator of Shh signalling, and mutation of *Sufu* results in major developmental anomalies with some similarities to *Ptch1* mutants (Cooper et al, 2005; Svard et al, 2006). *Sufu* null embryos, like *Ptch1* mutants, die at around E9.5, likely due to defects in heart development. *Sufu* mutants exhibit failure of cranial neurulation with complete penetrance; some embryos have failure of neural tube closure in the spine, or along the entire axis. Loss of *Sufu* results in DV patterning defects similar to *Ptch1*^{-/-}, with dramatic expansion of ventral markers over the entire DV extent of the neural tube, and virtual absence of dorsal markers Pax6 and Pax7 (Cooper et al, 2005; Svard et al, 2006).

A caveat applies to the interpretation of neural tube closure defects in *Ptch1* and *Sufu* mutants, however. The early death of both *Ptch1* and *Sufu* null embryos (at E9.5) may preclude neural tube closure merely because the appropriate stage for completion of closure is not reached. This is unlikely to apply, however, to the *MT-Ptch1*-rescued *Ptch1*^{-/-} embryos which survive well beyond neurulation stages and still exhibit NTDs (Milenkovic et al, 1999). Further evidence in this regard comes from a group of other mutants: *PKA*, *Rab23*, *Tulp3* and *Fkbp8*, in which there is partial activation of Shh pathway activity. These mutants exhibit some ventralization of the neural tube, though only partially along the DV axis, and also have regional effects along the anterior-posterior axis. Notably, in these mutants, survival is well beyond neurulation stages, and yet closure of the neural tube is affected, albeit with variable phenotypes.

Rab23 mutants were first identified from their exencephalic phenotype, and were named *open brain* (Gunther et al, 1994). These mutants exhibit failure of neural tube closure in the midbrain and hindbrain, as well as failure of posterior neuropore closure. An allelic

mutation, *opb*², was identified from an ENU screen and showed similar phenotypes (Kasarskis et al, 1998). Additionally, these mutants exhibit eye defects and polydactyly, plus a dorsal expansion of the spinal cord in the thoraco-lumbar region (Kasarskis et al, 1998). Positional cloning of *open brain* identified *Rab23* as the mutant gene (Eggenchwiler et al, 2001), and showed that mutant embryos exhibit ectopic expression of *Ptch1*, even in the absence of Shh ligand, indicating that Rab23 functions as a negative regulator of the Shh pathway (Eggenchwiler et al, 2001). This is reinforced by the observation that *Rab23* expression in chick neural tubes induces expression of Pax3 and Pax7. The degree of neural tube ventralization in *Rab23*^{opb} mutants is less than observed in *Sufu* and *Ptch1* mutants: Nkx2.2 is expressed in the ventral half of the neural tube in *Rab23*^{opb} mutants (Eggenchwiler et al, 2001). The precise role of Rab23 remains undefined, although genetic experiments demonstrate that it acts downstream of Smo and upstream of Gli2, and *Rab23*^{opb} mutants have defective processing of Gli3 (Eggenchwiler et al, 2006). The *Rab23*^{opb} mutant phenotype also requires the presence of cilia, since genetic ablation of IFT complex B proteins (Ift172 or Ift88) prevents the *Rab23*^{opb} mutant defects (Huangfu et al, 2003). It is thought that Rab23 is involved in vesicular trafficking, although the target molecule remains to be determined.

Tulp3 mutants exhibit many similarities to *Rab23* mutants. Both a targeted null allele and an ENU-induced strong hypomorph (*hitchhiker*, *Tulp3*^{hhkr}) demonstrate exencephaly and/or spina bifida in a proportion of homozygous mutants (Figure 2D) (Ikeda et al, 2001; Patterson et al, 2009; Norman et al, 2009; Cameron et al, 2009). Like *Rab23*, *Tulp3* mutants exhibit defects in eye formation, preaxial polydactyly, and expansion of the spinal cord in the thoraco-lumbar region. Given the similarity in gross morphology between *Tulp3* and *Rab23* mutants, it is perhaps not surprising that the DV patterning of *Tulp3* mutants resembles that of *Rab23* mutants, with expansion of ventral markers and reduction of dorsal markers (Ikeda et al, 2001; Patterson et al, 2009; Norman et al, 2009; Cameron et al, 2009). The role of *Tulp3* is not clear, although there are further similarities to Rab23: it acts genetically between Smo and Gli2, with a requirement for cilia. It is tempting to suggest that Rab23 and *Tulp3* have similar molecular roles, particularly since related Tubby family members are postulated to be involved in vesicular trafficking (Xi et al, 2005; Xi et al, 2007). However, while *Rab23* mutants show reduced processing of Gli3, no detectable change is found in *Tulp3* mutants, indicating that there are some differences between these mutants.

Deficiency for another Shh pathway negative regulator, PKA, results in increased Shh pathway activity and failure of cranial neurulation, further supporting the mechanistic link between these events. The compound mutants for two PKA isoforms, *Prkaca*^{-/-}; *Prkacb*^{+/-} results in reduced PKA activity, and a proportion of these embryos display exencephaly (Huang et al, 2002). PKA mutants have additional spinal defects (see below). A *Gli2* allele *Gli2*^{P1-4/P1-4} that cannot be phosphorylated by PKA and so is not processed normally, exhibits a slight expansion of ventral markers, and dorsal shift of Pax7 (Pan et al, 2009); this patterning disruption is even milder than observed in *Tulp3* mutants. Notably, these mutants exhibit midbrain exencephaly (Pan et al, 2009). Ectopic expression of Shh itself is also known to disrupt neural tube closure (Echelard et al, 1993).

The tetratricopeptide repeat domain 21B *Ttc21b* (*Thm1*) mutants exhibit reduced velocity of transport of proteins out of the cilia, and expansion of ventral markers within the neural tube indicating increased Shh pathway activity (Tran et al, 2008). These mutants were first identified from a plethora of developmental anomalies including a low frequency of exencephaly and spina bifida (Herron et al, 2002). *Fkbp8* mutants demonstrate expansion of ventral markers within the spinal cord (Bulgakov et al, 2004; Cho et al, 2008; Wong et al, 2008) and some exhibit exencephaly (Shirane et al, 2008). Kinesin family member 7 (*Kif7*)

mutants exhibit a moderate activation of the Shh pathway within the neural tube. Ventral marker domains for V3 progenitors (Nkx2.2) and motoneuron progenitors (Olig2 or HB9) are expanded, as is *Ptch1* expression, although Shh itself is not altered (Liem, Jr. et al, 2009; Endoh-Yamagami et al, 2009; Cheung et al, 2009). A subset of these mutants exhibits exencephaly.

Further correlative evidence linking increased Shh activity with failure of cranial neurulation comes from mutants not formally associated with the Shh pathway. Leucine zipper protein 1 *Luzp1*^{-/-} embryos exhibit exencephaly, and show ectopic expression of Shh in the dorsolateral neural folds of the forebrain and (less obviously) hindbrain (Hsu et al, 2008). Folate receptor 1 (*Folr1*, previously *Folbp1*) mutant embryos have exencephaly, if rescued from lethality by folic acid administration, and show an increased and expanded domain of Shh expression and reduction of dorsal markers in the midbrain (Tang and Finnell, 2003).

Hedgehog interacting protein (Hhip) was identified from screens to detect Shh-binding proteins (Chuang and McMahon, 1999). Over-expression studies show diminution of Ihh signalling, suggesting that Hhip attenuates Hh. Indeed, loss of Hhip results in up-regulation of Hh signalling in the mouse, with disruption of lung and skeletal morphogenesis (Chuang et al, 2003). Loss of Hhip exacerbates the defects of mutants with reduced *Ptch1* activity, so that compound mutants have exencephaly (Jeong and McMahon, 2005).

Although insufficient data are available to make a definitive judgement, there are indications that the degree of disruption of DV patterning (and therefore degree of ectopic Shh pathway activation) correlates with the prevalence of exencephaly. The incidence of exencephaly is higher in *Tulp3* null mutants (77%) than in *Tulp3*^{hhkr} hypomorphs (37%) (Ikeda et al, 2001; Patterson et al, 2009) and evidence from double mutant studies suggests that the Shh pathway is not as highly activated in *Tulp3*^{hhkr} as it is in the *Tulp3* targeted allele (Patterson et al, 2009; Norman et al, 2009). Consistent with this idea, *Sufu* and *Ptch1* mutants exhibit close to complete penetrance of exencephaly and also have complete ventralization of the neural tube (Goodrich et al, 1997; Caspary et al, 2002) (Cooper et al, 2005; Svard et al, 2006), while PKA deficient embryos exhibit exencephaly in 25% of *Prkaca*^{-/-};*Prkacb*^{+/-} mutants (Huang et al, 2002). *Kif7* mutants exhibit only a mild ventralization of the neural tube, and demonstrate exencephaly in 9% of homozygotes (Endoh-Yamagami et al, 2009). It is well known that the incidence of exencephaly is influenced by genetic background so it will be important, in future, to make comparisons of exencephaly frequency between such mutants when bred onto matched backgrounds.

Evidence from mutation or over-expression of nine different genes clearly indicates that increased activation of the Shh pathway causes exencephaly. What about spinal neurulation? Many of the mutants with increased Shh activity also exhibit spina bifida but, on examination, there are two distinct defects. A proportion of *Ptch1*, *Sufu*, *Rab23* and *Tulp3* mutants exhibit open spina bifida, as the result of failure of spinal neural tube closure (Gunther et al, 1994; Milenkovic et al, 1999; Ikeda et al, 2001; Patterson et al, 2009; Norman et al, 2009; Cameron et al, 2009). This clearly suggests that increased Shh activity can inhibit spinal neurulation, as well as cranial neurulation. In addition, *PKA* and *Fkbp8* mutants (and a subset of *Tulp3* mutants) exhibit spina bifida, but with a closed neural tube (Huang et al, 2002; Wong et al, 2008; Patterson et al, 2009). In these cases, neural tube closure occurs, but development of the vertebral arches is defective. Thus, these mutants exhibit a phenotype of “splayed vertebrae” (also called spina bifida occulta). This defect probably does not arise from faulty neural tube closure, although it may be secondary to abnormalities within the neural tube (see below).

What is the cause of the neurulation defects, following pathway activation, at the cellular level?

Several tissue or cellular defects may contribute to failure of cranial or spinal neurulation, following increased activation of the Shh pathway. Perhaps the best defined cellular mechanism involves the stereotypical pattern of neural plate bending in the mouse embryo, which has previously been demonstrated to be regulated by Shh signalling. The neural tube exhibits three “modes” of closure along the spinal cord (Figure 3A) (Shum and Copp, 1996). In upper spinal regions, the neural tube bends solely at the ventral midline, termed the median hinge point (MHP), the forerunner of the floor plate. At mid spinal levels, the neural tube bends both at the MHP and also at paired dorso-lateral hinge points (DLHPs), whereas in the caudal spinal region, the closing neural tube exhibits only DLHPs, and no MHP is formed. In the cranial region, the neural folds undergo a complex morphological switch from convex to concave and, thus, also require considerable bending of the dorsolateral region of the neural folds. Recent studies have demonstrated that DLHP formation is obligatory for closure of the mid and low spinal neural tube (Ybot-Gonzalez et al, 2002; Ybot-Gonzalez et al, 2007). Moreover, there is a correlation between reduced Shh expression in the notochord and increased formation of DLHPs, within the posterior neuropore of embryos of different ages. Local release of Shh-N peptide from implanted beads inhibits DLHP formation on the operated side, an effect that appears to be mediated via inhibition of the BMP antagonist Noggin (Ybot-Gonzalez et al, 2002; Ybot-Gonzalez et al, 2007). Hence, Shh is a physiological inhibitor of dorsolateral bending, supporting the idea that a genetically-induced increase in Shh signalling may prevent neural tube closure by repressing the formation of DLHPs (Figure 3B).

This hypothesis is supported by evidence from the *Tulp3* mutant. We have documented delay or failure of cranial and spinal neurulation, in the *Tulp3^{hhkr}* mutant (Patterson et al, 2009). Histological sections through the spinal region demonstrate reduced dorsolateral hinge points. It is striking, however, that both *Tulp3* and *Rab23* mutants exhibit a failure of spinal neural tube closure at a level caudal to the region where DV patterning is most severely disrupted. Indeed, failure of DLHPs in the *Zic2^{kumba}* mutant prevents neural tube closure even at mid spinal levels (Ybot-Gonzalez et al, 2007). It is possible that a partial suppression of DLHP formation in *Rab23* and *Tulp3* mutants results in NTDs only at low spinal levels, since at this axial level no MHP forms and neural tube closure is entirely reliant on DLHPs. At mid spinal levels, the MHP and presence of albeit reduced DLHPs appears sufficient for closure. Alternatively, other cellular mechanisms may be involved. Of relevance here is the finding that ectopic Shh expression in the chick embryo has early effects on cell-cell and cell-matrix adhesion that, while critical for neural plate bending, precede and are distinct from alterations in DV patterning (Fournier-Thibault et al, 2009). Hence, it seems likely that the effects of over-activation of the Shh pathway may inhibit neural tube closure by mechanisms independent of DV spinal cord patterning, which can be seen as a later developmental result of increased Shh signalling.

One unresolved paradox is why defects in spinal neural tube closure are not observed in all mutants with increased Shh pathway activation. For example, *Fkbp8* mutants and PKA deficient embryos exhibit increased pathway activation to a similar extent as that observed in *Rab23* and *Tulp3* mutants. Yet, in both cases, spinal neural tube closure occurs normally. One idea is that these mutants may have a regionally localised effect, such that Shh activity is not substantially altered in the caudal region of the spinal cord, where DLHP-dependent closure occurs. While different genes are well known to have regionally localised effects, this speculation remains to be experimentally verified.

Other causes of NTDs in mutants with increased Shh pathway activity must also be considered. Cranial neurulation is a complex morphological process and a number of

different cellular events are involved (Copp et al, 2003; Copp, 2005). These include balanced regulation of neuroepithelial cell proliferation, differentiation and apoptosis, contraction of apical microfilaments and emigration of neural crest cells. While there are no reports of altered cytoskeletal changes in Shh pathway mutants, nor of neural crest cell defects, there is some evidence of disruption of cell proliferation and apoptosis in many of these Shh-pathway mutants. Indeed, Shh pathway activity is known to be involved in influencing the balance between proliferation and differentiation, or apoptosis, in many tissues. Thus, part of the effect of increased Shh activity on neural tube closure could involve disruption of cell number within the neuroepithelium.

Elevated levels of apoptosis are observed in the cranial neuroepithelium of several mutants with increased Shh activity. These include the *Tulp3* targeted mutant allele, with increased apoptosis in the ventral hindbrain (Ikeda et al, 2001), *Sufu* mutants (Svard et al, 2006), *Luzp1*^{-/-} embryos (Hsu et al, 2008) and *Folr1*^{-/-} embryos (Tang and Finnell, 2003; Tang et al, 2005). In contrast, the *Tulp3*^{hhkr} allele exhibits no difference in apoptosis rates, compared with wild-type (Patterson et al, 2009) while *Rab23*^{ppb} mutants show no increase in cell death at E10.5 (Gunther et al, 1994).

Increased proliferation is observed in the cranial neuroepithelium in the *Tulp3*^{hhkr} mutant, but this is only a small (though statistically significant) increase, making its biological importance unclear (Patterson et al, 2009). Differentiation may also be disrupted by the *Tulp3* targeted allele, since the number of β III-tubulin-positive cells is reduced in the hindbrain (Ikeda et al, 2001). *Ptch1* mutant embryos are larger than normal, with apparent overgrowth of the cranial neuroepithelium, while *Ptch1* heterozygous mice develop tumours (Goodrich et al, 1997; Milenkovic et al, 1999). Conversely *Sufu* mutants are smaller than littermates (Cooper et al, 2005) and have reduced proliferation in the neuroepithelium (Svard et al, 2006), although some heterozygous *Sufu* mice also exhibit skin lesions suggestive of cellular over-proliferation (Svard et al, 2006). *Folr1* mutants also demonstrate reduced cell proliferation (Tang et al, 2005).

Expression of many of the genes is not restricted to the neuroepithelium. *Tulp3* is expressed ubiquitously during neurulation (Ikeda et al, 2001), and *Sufu* is also widely expressed (Svard et al, 2006). *Fkbp8* is expressed ubiquitously (Bulgakov et al, 2004) and so could be acting in other tissues. *Luzp1* is detected in both the neuroepithelium and mesenchyme in the cranial region at the time of neurulation (Hsu et al, 2008). *Ptch1* is expressed in the ventral somites as well as regions of the head (Ding et al, 1998), and *Rab23* is expressed in the branchial arches, eyes, somites and limb mesoderm (Eggenchwiler et al, 2001). Therefore, regulation of activities outside the neuroepithelium must also be considered. Indeed, expansion of the cranial mesenchyme is an important process for cranial neurulation (Copp et al, 2003). Detailed studies on the mechanisms involved in cranial closure in these mutants are required to fully understand the cause of the exencephalic phenotype.

Another consideration is that, although these mutants are defined as having increased activation of the Shh pathway, this is based on analysis of patterning within the spinal cord. Patterning in the head has largely not been assessed. While it is reasonable to assume that Shh pathway activity is likely to be up-regulated also in the cranial region, experimental analysis is required to verify this. In particular, concerns are raised since some mutants exhibit regional differences in the extent of pathway misregulation. For instance, *Fkbp8* and *Tulp3* mutants exhibit expanded expression of ventral markers in the caudal spinal cord, yet patterning is normal in more anterior regions of the spinal cord. Therefore it is formally possible that some of these proteins may have different functional roles in cranial and spinal regions of the embryonic axis.

How does Shh pathway activation result in the splayed vertebrae phenotype?

A subset of mutants with increased Shh pathway activation exhibit spina bifida, with splayed vertebrae but a closed neural tube. There is evidence that this defect may be secondary to a defect in the neural tube.

The phenotype of “splayed vertebrae” is observed in mutants for *Fkbp8* (Wong et al, 2008; Shirane et al, 2008), *PKA* (Huang et al, 2002) and a subset of *Tulp3^{hhkr}* mutants (Patterson et al, 2009) (Figure 4). In all cases, the neural tube has a markedly abnormal morphology, prior to vertebral formation, exhibiting a “triangular” shape with narrowed basal and expanded alar domains. The neuroepithelium is thin, and the lumen is expanded. Indeed, between the embryonic stages of E10.5 and about E13.5, the neural tube appears to bulge along the dorsal midline. This defect is seen in the thoraco-lumbar region of *Fkbp8* and *Tulp3* mutants, and corresponds closely to the region of abnormal vertebrae seen at later stages. Since the vertebrae form by fusion of the neural arches at the dorsal midline, the abnormal neural tube morphology may mechanically inhibit formation of closed vertebral arches. An interesting recent finding is that Shh signalling promotes proliferation of cells in the hindbrain choroid plexus (Huang et al, 2009). Since the choroid plexus is responsible for cerebrospinal fluid production, it is tempting to speculate that mutants with increased Shh signalling could possess an enlarged choroid plexus. This in turn could lead to increased production of cerebrospinal fluid, which might contribute to the swelling of the neural tube lumen. This hypothesis remains to be tested experimentally.

As well as mechanical inhibition, there may be a role for disrupted signalling from the abnormally patterned neural tube. The vertebral column develops from the somites, and it is well known that somite differentiation requires signalling from the surrounding structures, including the notochord and neural tube (Chiang et al, 1996). The dorsal part of the vertebral neural arch, the spinous process, forms from the dorsomedial portion of the sclerotome, termed the dorsal mesenchyme (Christ et al, 2007). Interestingly, while Shh signalling seems to act to maintain the sclerotome, formation of the dorsal mesenchyme is regulated by BMP signals from the roof plate and surface ectoderm. Thus, reduction of these dorsal signals, following neural tube ventralization, would be expected to have a negative effect on formation of the dorsal vertebral structures. Indeed, grafting a notochord dorsal to the neural tube inhibits formation of the spinous process, and causes failure of closure of the vertebrae (Monsoro-Burq et al, 1994). Thus, reduced dorsal signals from the ventralized neural tube may contribute to the defect of spina bifida.

A third consideration is a primary defect within the sclerotome itself. Expression of Gli2 and Gli3 within the developing somites is required to mediate the Shh response and allow correct somitic patterning (Buttitta et al 2003), indicating an endogenous requirement for the Shh pathway in this tissue. Since *Tulp3* and *Fkbp8* are expressed ubiquitously (Ikeda et al, 2001; Bulgakov et al, 2004), they may play a role within the somites during development of the vertebrae. However, *Fkbp8* mutants have no detectable defect in the somites, exhibit no evidence for enhanced Shh signalling in non-neural tissues, and mutation of *Fkbp8* does not restore sclerotomal fate in *Shh* mutants (Bulgakov et al, 2004). Pax7 expression in the somites appears normal in *Tulp3^{hhkr}* mutants, arguing against a role in somite development (Patterson et al, 2009). PKA-deficient pups have occult spina bifida in thoracic and lumbar regions, whereby the vertebral arches have failed to fuse at the dorsal midline, yet all other components of the vertebrae are present with regular ossification. Thus, these data suggest the absence of a somite-specific role for *Tulp3*, *Fkbp8* and *PKA* (Huang et al, 2002). However, the *Rab23* mutant shows similar malformations of the spinal cord, with a protruding, enlarged central canal (Gunther et al, 1994) and marked dysmorphology of the axial skeleton and this has been attributed to defective somite development (Sporle and Schughart, 1998). Since there is evidence that Gli2 and Gli3 function as both activators and

repressors in the developing somites (Buttitta et al, 2003), the influence on somitic development may vary in different mutants, depending on the differential effects on the Gli proteins. Clearly, tissue-specific gene knockouts are required to fully understand the cause of the vertebral abnormalities in these mutants.

CILIAL ABNORMALITIES AND THE RELATIONSHIP TO NTDS

Disruption of the cilia can result in a variety of effects, depending on the precise genetic and functional abnormality. As mentioned above, disruption of retrograde intraflagellar transport in the *Ttc21b* (*Thm1*) mutants results in increased Shh pathway activity in the neural tube with expansion of ventral markers in the spinal cord (Tran et al, 2008). Disruption of ciliary structure in ADP-ribosylation factor-like 13B (*Arl13b*) mutants results in expansion of intermediate marker domains, indicating a constitutive moderate level of Shh signalling within the neural tube (Caspary et al, 2007). Mutants for the kinesin motor protein *Kif7* also exhibit a moderate activation of the Shh pathway within the neural tube (Liem, Jr. et al, 2009; Endoh-Yamagami et al, 2009; Cheung et al, 2009). In these three mutants, a proportion of the homozygous embryos exhibit exencephaly, reflecting the phenotypic defects following Shh pathway activation observed in other mutants.

In contrast, a number of other mutants that disrupt ciliogenesis or ciliary function have reduced Shh pathway activity within the spinal cord, characterised by loss or reduction of ventral markers. These include mutants for many of the intraflagellar transport proteins, *Ift52*, *Ift57*, *Ift88*, *Ift122* and *Ift172*, as well as mutants for the retrograde motor subunit, dynein cytoplasmic 2 heavy chain 1 (*Dync2h1*) (Murcia et al, 2000; Huangfu et al, 2003; Haycraft et al, 2005; May et al, 2005; Liu et al, 2005; Huangfu and Anderson, 2005; Houde et al, 2006; Haycraft et al, 2007; Cortellino et al, 2009; Gorivodsky et al, 2009). Additional genes whose disruption results in abnormal cilia and loss or reduction of ventral markers in the neural tube include retinitis pigmentosa GTPase regulator interacting protein 1 like 1 (*Rpgrip1l*), dynein cytoplasmic 2 light intermediate chain 1 (*Dync2li1*), C2 calcium-dependent domain containing 3 (*C2cd3*), Meckel syndrome type 1 (*Mks1*), *Fuzzy*, *Inturned* and *D630037F22Rik* (*bromi*) (Rana et al, 2004; Vierkotten et al, 2007; Hoover et al, 2008; Gray et al, 2009; Heydeck et al, 2009; Weatherbee et al, 2009; Zeng et al, 2010; Ko et al, 2010). Thus, the neural tube patterning defect observed in these mutants is the opposite of that observed in *Ttc21b*, *Kif7* and *Arl13b* mutants, with reduced (rather than increased) Shh pathway activity. Surprisingly, however, many of these mutants also exhibit exencephaly (Table 3).

Mutants for the *Scl/Tal1* interrupting locus (*Stil*, formerly *Sil*) protein (Izraeli et al, 2001) also have reduced Shh signalling, as determined by the absence of Shh and *Foxa2* expression from the neural tube, and greatly reduced expression of *Ptch1* and *Gli1*. *Stil* mutants exhibit an HPE-like defect, with lack of midline separation at the anterior end of the cranial neural folds, and additionally have a delay or failure of neural tube closure (Izraeli et al, 1999). Interestingly, *Stil* mutants have left-right patterning defects, with randomized heart looping and bilateral expression of *Nodal* and *Pitx2*, characteristic of mutants with a disruption of nodal cilia. Although to our knowledge, cilia have not been examined in *Stil* mutants, it is possible that the defects are a consequence of impaired ciliary function.

How does a defect in the cilia result in exencephaly?

The differential effect of ciliary abnormalities – that is, gain or loss of hedgehog signalling – depends on the precise functional defect in each mutant and also varies in different tissues. The role of the cilium is complex and is not yet fully understood. The cilia are required for the normal processing of full length Gli proteins to form the Gli transcriptional activators, and also for the proteolytic cleavage of Gli to form the Gli repressors. Recent data also

reveal that Gli flux through the cilium is important for normal pathway activation. The downstream consequence of mutations disrupting ciliogenesis or ciliary function hinges on the precise biological effects on Gli protein stability or processing and the resultant levels of Gli activators and repressors. The variable consequences in different tissues is likely owing to the differential importance of the activators and repressors. However, it is also possible that specific mutations differentially affect Gli2 and Gli3 or, indeed, that ciliary mutations have other as yet undefined molecular effects in the cell.

A disturbance in the balance of Gli activators and repressors, within the cranial neuroepithelium, may be causative for the exencephalic phenotype observed in many of the ciliary mutants. Indeed, in almost all of the ciliary mutants in which Gli3 processing has been examined, homozygous embryos exhibit reduced processing (Table 4). This may be apparent either as substantially increased levels of full length Gli3 (Gli3FL), or as substantially decreased levels of cleaved Gli3 (the repressor form; Gli3R), relative to wild-type embryos. The net result is the same: an increase in the ratio of Gli3FL to Gli3R in mutant embryos. We hypothesise that reduced Gli3R is the fundamental cause of the failure of neural tube closure. Indeed, mutants for the *Gli3* gene itself exhibit failure of cranial neurulation (Hui and Joyner, 1993). Genetic ablation of *Gli2* from *Ptch1* mutants prevents the ventralization of the spinal cord seen in *Ptch1* mutants, yet the double mutants still have exencephaly, consistent with an effect on cranial closure mediated through the loss of Gli3R (Bai et al, 2002). In addition, Ski interacts physically with Gli3, functioning both as a co-repressor with Gli3R and inhibitor of Gli3FL (Dai et al, 2002). Consistent with this model, *Ski* mutants exhibit exencephaly (Berk et al, 1997).

Since Shh stimulation inhibits proteolytic cleavage of Gli3, increased Shh activity in the cranial region would be expected to lead to a reduction in Gli3R level. It is intriguing to speculate that the increase in Shh pathway activation seen in other mutants (e.g. *Ptch1*, *Sufu*, *Tulp3*; Table 2), may also disrupt neurulation through an effect on the balance of Gli activators and repressors in the brain. Increased Shh activity would be expected to inhibit processing of Gli3, resulting in an increased Gli3FL:Gli3R ratio. Indeed, reduced Gli3R has been reported for *Rab23* mutants (Eggenschwiler et al, 2006), and for *Sufu* mutants (Kise et al, 2009). In contrast, *Tulp3* mutations do not appear to alter Gli3 levels (Patterson et al, 2009; Norman et al, 2009), although these analyses used either whole embryos or limb buds, and may therefore obscure a difference localised within the cranial neuroepithelium.

The evidence supporting the “Gli3R hypothesis” is only correlative, and there are data that do not appear to fit with this model. *Ar113b* mutants exhibit exencephaly, have reduced expression of ventral-most domains within the spinal neural tube, and exhibit abnormal (short) cilia with a structural defect in the arrangement of microtubules in the axoneme (Caspary et al, 2007). However, *Ar113b* mutants exhibit no detectable difference in Gli3FL:R ratio, compared with wild-type embryos. In this mutant, unlike many of the other IFT mutants, there is an expansion of the domain of neural tube markers such as HB9, revealing constitutive Shh activity at an intermediate level. It has been proposed that the Gli activators may be formed inappropriately, or not tethered in the cilia (Caspary et al, 2007), and this may be sufficient to cause an imbalance of Gli activator and repressor activity. A further apparent challenge to the Gli3FL:Gli3R imbalance hypothesis comes from mice that carry a small deletion within the Gli3 locus (*Gli3*^{Δ68}), in which there is reduced formation of Gli3R (Wang et al, 2007). Although loss of Gli3R is predicted to recapitulate the exencephalic phenotype, as observed in the ciliary mutants, no NTDs have been reported for *Gli3*^{Δ68} mutants. The *Gli3*^{Δ68} deletion lowers the Gli3R level by 50%, and causes only a slight increase in Gli3FL, compared with wild-type. Moreover, the limbs show only one or two extra digits in *Gli3*^{Δ68/Δ68} mutants, compared with three or four extra digits in *Gli3*^{-/-}

mutants. Thus, the absence of exencephaly in *Gli3*^{A68} may result from a relatively smaller effect on Gli3 balance than in the IFT mutants.

Again, it is important to note that neural tube patterning has been examined only in the spinal cord of most mutants, whereas the NTDs affect predominantly the cranial neural tube. It will be important to examine the precise abnormalities occurring in the cranial region, particularly since disruption of ciliogenesis or ciliary function results in tissue-specific effects. For instance, complete loss of *Kif3a* results in embryonic lethality at around E9.5, with loss of ventral marker domains in the neural tube but no reported defects in neural tube closure (Takeda et al, 1999; Huangfu et al, 2003). However, conditional ablation of *Kif3a* in cranial neural crest cells reveals a gain of hedgehog signalling (Brugmann et al, 2010). The *Kif3a* mutant neural crest cells exhibit increased response to Shh ligand, resulting in increased *Gli1* and reduced *Gli3* expression. The mutant neural crest cells undergo increased proliferation, leading to increased width of the craniofacial midline, a phenotype in marked contrast to the reduced craniofacial midline phenotype and holoprosencephaly observed in *Shh* mutants. The differential effects of loss of cilia in different tissues may be determined by the relative importance of particular Gli proteins in these tissues. Gli3 is the predominant Gli factor in craniofacial development, and loss of Gli3 results in increased proliferation. Several IFT mutants exhibit preaxial polydactyly, a “gain of hedgehog signalling” phenotype, owing to reduction in Gli3R (Huangfu and Anderson, 2005).

What about other pathways?

Cilia are gradually being revealed to have important roles in other signalling systems, not just the Hedgehog pathway (Berbari et al, 2009). Therefore, the possibility arises that the ciliary mutants may have NTDs as the result of misregulation of other signalling events. *Fuzzy* and *Inturned* were originally identified (in *Drosophila*) through effects on the planar cell polarity (PCP) pathway (Lee and Adler, 2002), and there is some evidence to support a role for cilia in function of the mammalian PCP pathway (Ross et al, 2005; Jones et al, 2008). Disruption of core components of the PCP pathway also leads to NTDs, although the types of NTDs that characterise PCP mutants differ from those seen in Hh pathway mutants. Specifically, loss of function of the core PCP genes such as *Vangl2*, *Celsr1* and *Dishevelled*, results in the severe NTD craniorachischisis in which the neural tube is open along most of the body axis, owing to failure of initiation of neural tube formation, at Closure 1 (Murdoch et al, 2001; Curtin et al, 2003; Wang et al, 2006a). This phenotype is distinct from the exencephaly and spina bifida seen in the ciliary mutants with abnormal Shh pathway activity, arguing against a commonality. However, partial disruption of PCP pathway activity can result in spina bifida, as seen in some *Vangl2* heterozygous mutants (Copp et al, 1994), or in compound heterozygous mutants between *Ptk7* and *Vangl2* (Lu et al, 2004), while exencephaly is observed in a proportion of compound mutants between *Vangl2* and Cordon bleu (*Cobl*) (Carroll et al, 2003) or between *Vangl2* and *Bbs4* (Ross et al, 2005). Thus it is not possible on phenotype alone to fully exclude an involvement in the PCP pathway.

Cilia have also been proposed to be required for canonical Wnt signalling (Berbari et al, 2009), although other evidence argues against this idea (Ocbina et al, 2009). Wnt signalling is important for regulation of DV patterning in the neural tube, since Wnt and BMP signals from the roof plate and surface ectoderm are important for regulation of dorsal neuronal identity. Indeed, increasing evidence suggests Wnt signalling is important in regulating ventral identity also, through antagonistic action on the Shh pathway through Gli3 (Ulloa and Briscoe, 2007; Yu et al, 2008; Alvarez-Medina et al, 2008; Ulloa and Marti, 2010). Stabilized β -catenin promotes dorsal and inhibits ventral cell identities in the spinal cord while, conversely, genetic ablation of β -catenin from the ventral spinal cord leads to expansion of ventral cell fates (Yu et al, 2008). Disruption of some molecules involved in canonical Wnt signalling also leads to NTDs, including *Axin1* (Zeng et al, 1997) and *Lrp6*

(Pinson et al, 2000). Disruption of Frizzled or Dishevelled proteins also yields NTDs (Hamblet et al, 2002; Wang et al, 2006b; Etheridge et al, 2008), although in these mutants both canonical and non-canonical Wnt pathways are affected.

Disruption of IFT proteins can also affect the Nodal signalling pathway. Notably, *Ift172* null embryos exhibit exencephaly, accompanied by holoprosencephaly, reduced forebrain size and abnormal anterior-posterior patterning of the developing brain (Gorivodsky et al, 2009), and this may also influence cranial neural tube closure. Early anterior defects include failure to maintain expression of markers (*Wnt1*, *Pax2*, *Gbx2*) in the isthmic organizer, a signalling centre at the midbrain-hindbrain boundary involved in growth and patterning, likely owing to an early disruption of *Fgf8* expression. Nodal expression is greatly reduced in the epiblast at E7.0 and in the node at E7.5, prior to the onset of Shh expression, indicating Shh-independent effects. *Ift172* mutants demonstrate abnormalities in the anterior mesendoderm, a structure involved in patterning the prospective fore and midbrain and regulating forebrain growth, likely as a consequence of the node defect (Gorivodsky et al, 2009). Clearly, the complex interplay between Shh and Wnt, PCP, Nodal and perhaps other signalling pathways must be considered in deciphering the precise functional effects following disruption of primary cilia.

Human SHH pathway mutations and ciliopathies

Searches of Online Mendelian Inheritance in Man (OMIM; www.ncbi.nlm.nih.gov/omim) reveal that, although the majority of SHH pathway homologues are not yet associated with a human disease, polymorphisms in some of these genes are linked to a common set of disorders (Table 5). Similar to the mouse, mutations of *SHH* are associated with holoprosencephaly (HPE), in both familial and sporadic cases - for review, see (Dubourg et al, 2007). Loss of function mutations of *DISP1* are also associated with HPE-like features (Roessler et al, 2009), as are mutations in *GLI2* or *PTCH1* (Ming et al, 2002; Roessler et al, 2003). The *PTCH1* mutations are predicted to enhance the action of PTCH1 in repression of the SHH pathway. Formation of tumours is seemingly a common affliction in patients with SHH pathway gene disruption (Villavicencio et al, 2000; Lupi, 2007). Basal cell carcinomas are found in some *SMO* patients, while disruption of *PTCH1* predisposes to basal cell carcinoma or Basal Cell Nevus Syndrome. Polymorphisms in *SUFU* are associated with medulloblastoma, while *GLI1* mutations are found in gliomas. These associations are perhaps not surprising, given the link between Shh signalling and regulation of cell proliferation and apoptosis.

Interestingly, mutation of *RAB23* is found in patients with Carpenter Syndrome (Jenkins et al, 2007), a disorder that includes craniosynostosis, polysyndactyly, obesity and cardiac defects (OMIM #201000). This is the only example of a Hedgehog signalling molecule affecting cranial suture biogenesis, perhaps indicating a specific functional role for *RAB23*.

Mutations in *MKS1* lead to either Meckel Gruber or Bardet-Biedel Syndrome, while disruption of *RPGRIP1L* causes Meckel Gruber or Joubert Syndrome (Delous et al, 2007). Mutations in *ARL13B* can also lead to a form of Joubert syndrome (Cantagrel et al, 2008). Meckel-Gruber Syndrome (MKS, OMIM #249000), Bardet-Biedl Syndrome (BBS, OMIM #209900), and Joubert's syndrome (JBTS, OMIM #213300) are some of the diseases attributed to defective ciliary function, termed the ciliopathies (Cardenas-Rodriguez and Badano, 2009). Ciliary dysfunction is also considered as the cause of Short Rib-Polydactyly Syndrome (SRPS Type 3, OMIM #263510) and Asphyxiating Thoracic Dystrophy 3 (ATD3 #613091), disorders caused by disruption of *DYNC2H1*.

It is striking that, although one fetus with *RPGRIP1L* mutation displayed anencephaly (Delous et al, 2007), the human genes are largely not associated with any form of NTD. On

the other hand, the congenital malformation encephalocele is a characteristic feature of Meckel-Gruber Syndrome (MKS) (Chen, 2007), and is sometimes observed in patients with Joubert's syndrome (Joubert et al, 1999), but not in the other ciliopathies. Encephalocele is occasionally reported in mouse mutants for ciliary genes: for example, some *Fuzzy* mutants exhibit encephalocele (Gray et al, 2009; Heydeck et al, 2009). However, encephalocele represents a malformation of the skull vault following closure of the neural tube and, like the splayed vertebral defect as discussed above, encephalocele is not a primary anomaly of neural tube closure. It could, however, arise as a secondary consequence of abnormal signalling from an inappropriately patterned neural tube. Other CNS abnormalities are sometimes observed in human ciliopathies, such as sensorineural deafness in Alstrom syndrome (ALMS #203800) (Marshall et al, 1997) and mental retardation with cerebral or cerebellar malformations in some types of Orofaciodigital Syndrome (OFD1 #31120, OFD4 #258860, OFD6 #277170) (Towfighi et al, 1985; Adès et al, 1994; Doss et al, 1998). Although mutation of mouse *Bbs* genes results in a low frequency of exencephaly (Ross et al, 2005; Eichers et al, 2006), to date none of the human ciliopathies have been associated with defects in closure of the neural tube.

CONCLUSIONS

A wealth of genetic mutants exist that affect Hedgehog signalling in mice, either directly or via effects on ciliary function. Of these, a number disturb neural tube closure in the brain, spine or both, leading to NTDs. The mutants that have direct effects on the Shh pathway fall into two distinct groups: those that diminish signalling cause holoprosencephaly and cyclopia, but not NTDs, whereas those that activate signalling produce NTDs in almost all cases. A third group of mutants, principally those that affect Shh signalling indirectly via effects on ciliary function, have rather variable consequences for neural tube closure. Further studies, specifically targeted at an understanding of the level of activation of Hedgehog signalling in the vicinity of the closing neural tube, will be required to fully evaluate these mutants. At the developmental level, Shh signalling inhibits dorsolateral bending of the neural plate in the spinal (and perhaps also cranial) regions, a morphogenetic event that is essential for closure. It remains to be determined whether this effect of Shh signalling is mediated via DV gene expression patterning, or through earlier effects on cell-cell and cell-matrix adhesion that may underlie neural plate bending. While these studies have not yet provided insight into the etiology of human NTDs, there is known to be a strong genetic component to these defects and it will be interesting to determine in future whether dysregulation of the Shh pathway may be implicated as a risk factor in some cases of human anencephaly or spina bifida.

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Figure 1.

Schematic diagram of the Shh signalling pathway

1. Shh is released from cells, following processing to N-fragment; release requires Disp1.
2. Shh binds avidly to several transmembrane proteins, including Gas1, Boc and Cdon; these proteins are thought to enhance presentation of Shh to the key receptor protein, Ptch1.
3. Ptch1 acts to inhibit Smo, by preventing translocation of Smo into the cilium.
4. Upon binding of Shh, the inhibitory action of Ptch1 on Smo is repressed, allowing activation of Smo.
5. Smo is phosphorylated by GRK2, and physically interacts with β -arrestin2, triggering internalisation in clathrin-coated pits.
6. A complex is formed between activated Smo, β -arrestin2 and Kif3a; activated Smo relocates to the tip of the cilium.
7. This causes relocation of the Gli proteins from cytoplasmic microtubule-associated punctae to the distal cilial tip.
8. Formation of cilia and transport of proteins into (anterograde) and out of (retrograde) involves a number of IFT proteins, including Complex B, Complex A, kinesin motors and cytoplasmic dynein motors. Sufu interacts with full length Gli proteins, at the tip of the cilia.
9. Full length Gli2 (and to a lesser extent Gli3) undergoes "activation" to become a transcriptional activator. The precise molecular details are poorly understood, but a key step is the dissociation of full length Gli proteins from Sufu, an event that is inhibited by PKA and stimulated by Kif3a. Other activation events include phosphorylation by ULK3, translocation into the nucleus and destabilization. Activation of Gli2 is potentiated by MIM/BEG4.

BEG4 and inhibited by Sufu and by recruitment of SAP18 and the histone deacetylation machinery (HDAC).

10. Gli3 (and to a lesser extent Gli2) undergoes cleavage to form a repressor; this involves sequential phosphorylation by PKA then GSK3 β and CK1, binding of β -TrCP and proteolytic processing.

11. Gli proteins can also be targeted for degradation; Spop promotes ubiquitination by Cullin E3 Ubiquitin ligase and degradation through the 26S-proteasome. Sufu stabilises Gli proteins by inhibiting Spop.

12. A number of proteins are required for ciliogenesis (Mks1, Fuz, Intu, C2cd3, Rpgrip11) and are required to form both Gli activator and repressor proteins. Arl13b and Tulp3 inhibit formation of Gli activator, but do not affect Gli repressor; Fkbp8 also results in reduced Gli activator; Rab23 inhibits Gli activator and promotes Gli repressor formation.

13. Transmembrane protein Hhip binds Shh ligand and represses pathway activity.

Figure 2.

Neural tube closure and neural tube defects

(A) Schematic diagram of the multi-site process of mammalian neural tube closure, and the defects (boxed) arising as a consequence of failure of neurulation. Modified from (Copp et al, 2003).

(B) Fetus at E17.5 with craniorachischisis as a result of loss of *Vangl2* function; this is the most severe form of NTD in which the neural tube is open from the midbrain throughout the hindbrain and spinal cord (between arrowheads).

(C-E) E12.5 fetuses demonstrating normal neural tube closure (C), the NTDs exencephaly (ex) and spina bifida (sp bif) in a *Tulp3^{hhkr}* homozygous mutant (D), and a *Shh* null embryo with a proboscis (prob), a feature of holoprosencephaly (E).

A Normal neurulation

B Effect of increased Shh pathway activity within the neural plate

Figure 3.

Morphology of spinal neurulation and the effect of increasing Shh pathway activity, at different axial levels.

(A) During normal spinal neurulation, different axial levels exhibit different modes of closure, regulated by the strength of Shh signalling. These modes are characterised by the presence of either; a median hinge point (MHP) only (Mode 1), MHP and paired dorsolateral hinge points (DLHPs; Mode 2), or DLHPs only (Mode 3). Shh signalling inhibits DLHP formation, and the intensity of Shh signalling decreases along the spine. Shading within the neural tube depicts Ptch1 expression.

(B) Increasing Shh pathway activity in the neural tube (observed as increased Ptch1 expression) leads to neural tube defects in the low spinal region. Increased Shh pathway

activity suppresses formation of DLHPs; this has no effect on Mode 1, but reduces DLHPs at mid spine levels, converting closure from Mode 2 to Mode 1-like. At low spinal levels, where a MHP does not form, suppression of DLHPs inhibits closure and leads to NTDs.

Figure 4.

Phenotype of splayed vertebrae in the *Tulp3^{hhkr}* mutant

(**A,B**) Dorsal views of E18.5 wild-type fetus (**A**) and *Tulp3^{hhkr}* homozygous mutant fetus (**B**) demonstrating a spina bifida phenotype, with splayed vertebrae (at axial levels between arrowheads). The neural tube was closed in both fetuses. (**C,D**) Dorsal views of skeletal preparations of wild-type (**C**) and *Tulp3^{hhkr/hhkr}* (**D**) E18.5 fetuses. The bony (red) vertebral pedicles are clearly visible in the mutant (**D**), as a result of the splayed phenotype. In **A-D**, rostral is to the left and caudal to the right. (**E,F**) E13.5 wild-type embryo demonstrating normal morphology (**E**), while *Tulp3^{hhkr/hhkr}* embryo (**F**) shows marked swelling and bulging of the neural tube along the back of the embryo, particularly in the thoraco-lumbar region (between the arrowheads in **F**). (**G,H**) Histological sections through the lumbar region of E13.5 embryos demonstrating closed neural tube in both wild-type (**G**) and *Tulp3^{hhkr}* mutants (**H**), but grossly abnormal neural tube morphology in the *Tulp3^{hhkr}* mutant.

Table 1
Mutants with loss of activation of Shh signalling

Gene ¹	Alleles	NT patterning ²	NTD	Other defects ³	References
<i>Adrbk1</i> (<i>Grk2</i>)	Targeted null	ND	No	Heart defects; ventricular hypoplasia, thin myocardium	(Jaber et al, 1996)
<i>Cdk11b</i> (<i>Cdc211</i>)	Targeted null	ND	ND, die early	Inhibits Sufu; null embryos die early from cell cycle arrest	(Li et al, 2004)
<i>Cdon</i> (<i>Cdo</i>)	Targeted null	Reduction of floor plate	No	Craniofacial defects, HPE, eye defects	(Cole and Krauss, 2003; Tenzen et al, 2006; Zhang et al, 2006; Zhang et al, 2009)
<i>Disp1</i>	Targeted null; ENU mutant, icbins (<i>icb</i>)	Loss of ventral markers, Pax6 & Pax7 throughout	No	Cyclopia and HPE; arrest ~E9-9.5; small; LR defects, random embryonic turning, abnormal heart looping; enlarged pericardial sac,	(Caspary et al, 2002; Ma et al, 2002; Kawakami et al, 2002)
<i>Gas1</i>	Targeted null	Reduction of ventral markers	No	Craniofacial defects; hypodactyly (absence of digit 2); decreased Gli3FL:Gli3R ratio	(Seppala et al, 2007; Allen et al, 2007; Martinelli and Fan, 2007)
<i>Gas1</i> ; <i>Cdon</i>	Digenic	Reduction of ventral markers	No	HPE	(Allen et al, 2007)
<i>Gli1</i>	Targeted null	No phenotype	No	No phenotype	(Park et al, 2000; Bai et al, 2002)
<i>Gli1</i> ; <i>Gli2</i>	Digenic	Reduced ventral markers	No	Postaxial nubbin; lung hypoplasia; undescended testes	(Park et al, 2000; Bai et al, 2002)
<i>Gli2</i>	<i>Gli2^{zfd/zfd}</i>	Reduced ventral markers	No	Craniofacial defects, cleft palate	(Mo et al, 1997; Matisse et al, 1998; Ding et al, 1998; Bai et al, 2002)
<i>Ptch1</i>	Overexpression under control of Metallothionein promoter	ND		Reduced number of digits; extra ribs; incomplete neural arch formation; reduced size	(Milenkovic et al, 1999)
<i>Ptch1</i>	Overexpression under control of Nestin enhancer	Mild dorsalization; expansion of dorsal markers further ventrally, but maintenance of ventral markers	In some embryos, open region of NT seen forebrain, hindbrain or along spine	NB: not clear if this is a failure to close or a re-opening event; some have defect resembling holoprosencephaly	(Goodrich et al, 1999)
<i>Shh</i>	Targeted null	Loss of ventral markers	No	Cyclopia and HPE; short limbs with a single digit	(Chiang et al, 1996)
<i>Smo</i>	Targeted null; ENU mutant, bent body (<i>bnb</i>)	Loss of ventral markers, Pax6 throughout	No	Cyclopia and HPE; arrest ~E9-9.5; small; LR defects, random embryonic turning, abnormal heart looping; enlarged pericardial sac	(Kasarskis et al, 1998; Zhang et al, 2001; Caspary et al, 2002; Wijgerde et al, 2002)
<i>Tctn1</i> (<i>Tectonic</i>)	Gene trap	Loss of ventral markers	No	HPE; die before E16.5	(Reiter and Skarnes, 2006)

¹Gene names as in MGI, March 2010; former names in brackets.

²ND, not determined;

³HPE, holoprosencephaly; LR, left-right.

Table 2
Mutants with increased activation of Shh signalling

Gene ¹	Alleles	NT patterning ²	NTD	Other defects ³	References
<i>Fkbp8</i> (<i>Fkbp38</i>)	Gene trap; targeted null	Expansion of ventral markers, reduction of dorsal markers.	Exencephaly (low frequency)	Splayed vertebrae, lumbar and thoracic region	(Bulgakov et al, 2004; Cho et al, 2008; Wong et al, 2008; Shirane et al, 2008)
<i>Gli2</i>	<i>Gli2</i> ^{P1-4/P1-4} ; not phosphorylated by PKA so not processed	Slight expansion of ventral markers, and dorsal shift of Pax7	Exencephaly	Preaxial polydactyly	(Pan et al, 2009)
<i>Gli3</i>	Spontaneous, <i>Xt1/Xt1</i> <i>Pdn/Pdn</i>	Subtle defect, expansion of intermediate markers into more dorsal domains	Exencephaly	Shortened nasal process, microphthalmia, polydactyly	(Hui and Joyner, 1993; Persson et al, 2002; Maekawa et al, 2005; Ohta et al, 2006)
<i>Kit7</i>	Targeted null; ENU mutant <i>matariiki</i> , <i>maki</i>	Shh reduced in notochord and floor plate but other ventral markers expanded,	Exencephaly	Preaxial polydactyly; microphthalmia.	(Liem, Jr. et al, 2009; Endoh-Yamagami et al, 2009; Cheung et al, 2009)
<i>Prkaca</i> , <i>Prkacb</i> (<i>PKA</i>)	Digenic, Ca ²⁺ /Cβ1 ^{-/-} and Ca ²⁺ /Cβ1 ^{+/-}	Expansion of ventral markers; only posterior to forelimb bud	Exencephaly	Splayed vertebrae	(Huang et al, 2002)
<i>Ptc</i> ^{-/-} ; <i>Gli2</i> ^{zfd/zfd}	Digenic	Ventral markers reduced, similar to <i>Gli2</i> mutant, but motoneurons also expand dorsally (unlike in <i>Gli2</i> mutant)	Exencephaly		(Bai et al, 2002)
<i>Ptch1</i>	Targeted null; conditional null	Severe expansion of ventral markers and reduction of dorsal markers, most severe anteriorly	NT fails to close	Most die ~E9.5, heart defects; 1% polydactyly	(Goodrich et al, 1997; Caspary et al, 2002; Ellis et al, 2003)
<i>Ptch1</i>	Knock-out partially rescued with MT- <i>Ptch1</i> transgene	Expansion of Nkx2.2, Isl1/2, Pax6; Shh looks normal; loss of Pax7	Exencephaly and severe spina bifida	Polydactyly; rarely ectopic ventral digit (DV defect)	(Milenkovic et al, 1999)
<i>Ptch1:Hhip</i>	Digenic	Expansion of ventral markers	Exencephaly (100%) or spina bifida	Loss or reduction in ligand dependent antagonism of Shh signalling	(Jeong and McMahon, 2005)
<i>Rab23</i>	Spontaneous mutant <i>Openbrain</i> , <i>Opb</i> , ENU mutant, <i>Openbrain2</i> , <i>Opb2</i>	Expansion of ventral markers, dorsal shift of dorsal markers, loss of roof plate	Exencephaly, spina bifida	Polydactyly, abnormal or absent eyes	(Gunther et al, 1994; Kasarskis et al, 1998; Eggenschwiler et al, 2001)
<i>Sufu</i>	Targeted null	Ventral markers expressed over entire DV extent; Pax6 and Pax7 virtually absent.	Exencephaly, or failure to close neural tube	Die ~ E9.5; LR defects, abnormal heart looping; abnormal node shape	(Cooper et al, 2005; Svard et al, 2006)
<i>Tic21b</i> (<i>Thm1</i>)	ENU mutant <i>alien</i> , <i>aln</i>	Increased ventral markers	Exencephaly, spina bifida (low frequency)	Preaxial polydactyly, split and fused ribs, cortical layering defects; cilia have abnormal, bulbous tip and reduced retrograde transport	(Herron et al, 2002; Tran et al, 2008)
<i>Tulp3</i>	Targeted null; ENU mutant <i>hitchhiker</i> , <i>hhkr</i>	Expansion of ventral markers, reduction of dorsal markers posterior to forelimb bud only	Exencephaly and spina bifida	Preaxial polydactyly; splayed vertebrae; rib defects.	(Ikeda et al, 2001; Patterson et al, 2009; Norman et al, 2009; Cameron et al, 2009)

¹ Gene names as in MGI, March 2010; former names in brackets.

² DV, dorso-ventral;

³ DV, dorso-ventral; LR, left-right

Table 3
Mutations with reduced Shh signalling and reduced or abnormal cilia

Gene ¹	Alleles	NT patterning ²	NTD	Other defects ³	References
<i>Arl13b</i>	ENU mutation, <i>hennin</i> , <i>hnn</i>	Loss of Shh, expansion of HB9 dorsally and ventrally; expanded domain of intermediate level activity	Exencephaly and spina bifida	Structural defect in cilia; maybe Gli activators formed inappropriately or not tethered in cilia, Gli3R still formed	(Caspary et al, 2007)
<i>C2cd3</i>	Gene-trap; ENU mutant <i>hearty</i> , <i>hty</i>	Loss or reduction of ventral markers,	Exencephaly	Severe polydactyly; random heart looping; loss of cilia	(Hoover et al, 2008)
<i>D630037F22Rik</i>	ENU mutation, <i>bromi</i>	Loss of Shh, FoxA2; Nkx2.2 reduced and seen over ventral midline; HB9 expanded dorsally and ventrally; Pax6 expanded ventrally	Exencephaly	Poorly developed eyes, preaxial polydactyly; cilia bulbous or spherical; abnormal axonemal shape, abnormal relationship between axoneme and ciliary membrane	(Ko et al, 2010)
<i>Dync2h1</i> (<i>Dnchc2</i>)	ENU mutants, <i>ling-ling</i> , <i>lln</i> , <i>Q397STOP</i> and <i>W2502R</i>	Loss of ventral markers, Shh, Nkx2.2; MN markers expressed in caudal but not rostral spinal cord	Exencephaly in <i>lln</i>	Polydactyly; LR defect, randomised heart looping; nodal cilia short and bloated	(May et al, 2005; Huangfu and Anderson, 2005)
<i>Dync2li1</i>	Targeted null		Exencephaly	Die ~E11.5; arrest of or randomisation in embryonic turning and heart looping; forebrain truncation; cilia short or absent; ballooning of pericardial sac;	(Rana et al, 2004)
<i>Fuz</i> (<i>Fuzzy</i>)	Gene-trap, loss of function	Reduction of ventral markers, Shh, Foxa2, Nkx2.2 reduced no. of cells but dorsal limit shifted dorsally, Isl1 extends further dorsally, Pax6 shifted dorsally	Exencephaly	Short cilia; heart defects; craniofacial defects; curly tails; polydactyly; hypoplastic lungs; fewer cilia; encephalocele	(Gray et al, 2009; Heydeck et al, 2009)
<i>IFT122[&Med1]</i>	Targeted null	Loss of Shh, expansion of MNR2 dorsally and ventrally: expanded domain of intermediate level activity	Exencephaly	LR defects, loss of nodal cilia, severely disrupted limb development, ectopic Shh expression in anterior limb bud	(Cortellino et al, 2009)
<i>IFT172</i>	Targeted null; ENU mutant <i>wimple</i> , <i>wim</i>	Loss of ventral markers, floor plate, V3 and MN markers not expressed	Exencephaly	Craniofacial defects; HPE; randomised heart looping; absent or truncated cilia; defects in cranial anterior-posterior patterning	(Huangfu et al, 2003; Huangfu and Anderson, 2005)
<i>IFT52</i>	Gene-trap hypomorph	Loss of floor plate, reduced Nkx2.2 ventral markers	Exencephaly	Craniofacial defects: LR defects; polydactyly; reduced Ptc1 in limbs	(Liu et al, 2005)
<i>IFT57</i> (<i>hippi</i>)	Targeted null	Loss of ventral markers Foxa2, Shh, Isl1; ventral expansion of Pax6; no FP	Exencephaly	Disrupted turning, LR defect; polydactyly; loss of node cilia	(Houde et al, 2006)

Gene ¹	Alleles	NT patterning ²	NTD	Other defects ³	References
<i>IFT88(Tg737, polaris, orpk)</i>	Targeted null; ENU mutant, <i>flexo</i>	Loss of floor plate, loss or reduction in Nkx2.2; Pax6 throughout DV axis	Exencephaly	Polydactyly; defects in endochondral bone formation; absent or short cilia; reduced Gli3 processing; LR defects; double mutant with Shh suggests loss of both GliA and GliR function	(Murcia et al, 2000; Huangfu et al, 2003; Haycraft et al, 2005; Liu et al, 2005; Haycraft et al, 2007)
<i>Intu (Inturned)</i>	Targeted null	Posterior spinal cord: loss of Shh and Foxa2; Nkx2.2 in midline; Isl1 domain expanded ventrally; dorsal patterning normal. Anterior spinal cord less affected.	Exencephaly	Fewer and shorter cilia in node; no cilia in limb bud cell cultures; severe polydactyly; smaller eyes; reduced telencephalon, expanded dorsal diencephalon; limb bud disrupted AP patterning, Ptch1 and Gli1 reduced	(Zeng et al, 2010)
<i>Kif3a</i>	Targeted null	Loss of ventral markers, floor plate and V3 markers not expressed	No	Die around E9.5; fail embryonic turning; defect in heart looping; nodes lack cilia; LR defects; failure to form posterior region	(Takeda et al, 1999; Huangfu et al, 2003)
<i>Mks1</i>	ENU mutant <i>kerouac, krc</i>	Disrupted Shh signalling; HB9 and Nkx6.1 +ve cells found more dorsally than in wt, suggests broader domain of low-level Shh signalling; reduction in FP and V3, ventral expansion of MN, reduced high-level signalling; similar (milder than) <i>Ar1.3b</i> mutant	Exencephaly in 28%; splitting of supraoccipital bone in 80% at late term, probably corresponds to the occipital meningo-encephalo-coele (protrusion of brain through occipital bone) seen in 85% MKS cases.	Defective formation of cilia, reduced numbers of cilia; preaxial polydactyly; liver, cystic kidneys; defects in skull; lung hypoplasia, rib cage, long bones; LR asymmetry disruption	(Weatherbee et al, 2009)
<i>Rpgrip11 (Fantom)</i>	Targeted null	Reduction of ventral markers Shh, Nkx2.2, MNR2	Exencephaly at E10.5, but not reported at E18.5	LR defects, heterotaxia; preaxial polydactyly; craniofacial defects; cells can still form cilia but with "slight change in architecture"	(Vierkotten et al, 2007)
<i>Sil (Sil)</i>	Targeted null	Lack of ventral FoxA2, reduced Ptch1, Gli1	Delay or failure of NT closure	Die ~ E10.5, developmentally delayed; HPE defect, lack of anterior midline separation; LR defect, randomized heart looping, bilateral nodal, lefty2, Pitx2 expression	(Izraeli et al, 1999; Izraeli et al, 2001)

¹Gene names as in MGI, March 2010; former names in brackets.

²DV, dorso-ventral; FP, floor plate; MN, motoneurons

³AP, anterior-posterior; HPE, holoprosencephaly; LR, left-right;

Table 4
Effect on Gli processing

Mutant	Change to Gli3FL:Gli3R ratio	References
<i>Arl13b</i>	Not altered	(Caspary et al, 2007)
<i>C2cd3</i>	Increased	(Hoover et al, 2008)
<i>D630037F22Rik</i>	Increased	(Ko et al, 2010)
<i>Dync2h1</i>	Increased	(May et al, 2005; Huangfu and Anderson, 2005)
<i>Fuz</i>	Increased	(Gray et al, 2009; Heydeck et al, 2009)
<i>Ifi122</i>	Increased	(Cortellino et al, 2009)
<i>Ifi172</i>	Increased	(Huangfu et al, 2003; Huangfu and Anderson, 2005)
<i>Ifi52</i>	Increased	(Liu et al, 2005)
<i>Ifi88</i>	Increased	(Liu et al, 2005; Jia et al, 2009) (Haycraft et al, 2005; Haycraft et al, 2007) and Murcia 2000(Huangfu et al, 2003)
<i>Intu</i>	Increased	(Zeng et al, 2010)
<i>Kif3a</i>	Increased	(Huangfu and Anderson, 2005; Chen et al, 2009)
<i>Kif7</i>	Increased	(Liem, Jr. et al, 2009; Endoh-Yamagami et al, 2009; Cheung et al, 2009)
<i>Ptch1</i>	Increased	(Huangfu and Anderson, 2005; Jia et al, 2009; Chen et al, 2009)
<i>Rab23</i>	Increased	(Eggenchwiler et al, 2006)
<i>Rpgrip11</i>	Increased	(Vierkotten et al, 2007)
<i>Sufu</i>	Increased; Also, decreased Gli2, Gli3FL	(Jia et al, 2009; Chen et al, 2009; Kise et al, 2009)
<i>Ttc21b</i>	Increased	(Tran et al, 2008)
<i>Tulp3</i>	Not altered	(Patterson et al, 2009; Norman et al, 2009)