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ZIC3 in heterotaxy

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Abstract

Mutation of ZIC3 causes X-linked heterotaxy, a syndrome in which the laterality of internal organs is disrupted. Analysis of model organisms and gene expression during early development suggests ZIC3-related heterotaxy occurs due to defects at the earliest stage of left-right axis formation. Although there are data to support abnormalities of the node and cilia as underlying causes, it is unclear at the molecular level why loss of ZIC3 function causes such these defects. ZIC3 has putative roles in a number of developmental signalling pathways that have distinct roles in establishing the left right axis. This complicates the understanding of the mechanistic basis of Zic3 in early development and left-right patterning. Here we summarize our current understanding of ZIC3 function and describe the potential role ZIC3 plays in important signalling pathways and their links to heterotaxy.

Keywords

left-right patterning; gastrulation; mutation; planar cell polarity; node; cilia

15.1 Introduction

ZIC3 was the first ZIC family member identified as a cause of a human developmental disorder. This finding was facilitated by the fact that ZIC3 is located on the X chromosome and therefore causes disease in an X-linked inheritance pattern in which males are affected. Since its initial identification as the cause of X-linked heterotaxy 20 years ago, there has been significant progress in understanding its role in human congenital anomalies, importance in left-right (LR) patterning, and interaction with multiple developmental signalling pathways. In this chapter, we review the current understanding of ZIC3 function and gaps that remain to be investigated.

15.2 Heterotaxy

The internal organs show asymmetry when comparing left and right sides of the body. Such asymmetry is identified in organ placement, with organs such as the heart, stomach,

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and spleen found on the left of the body, whereas the liver and gallbladder are located on the right. Insults to early development alter such asymmetry causing a spectrum of disorders from isolated organ abnormalities to complete reversal (termed *situs inversus*). These abnormalities include loss of normally asymmetric structures, improper symmetry or lateralization resulting in organ isomerism, and/or failure to regress symmetric embryonic structures (e.g. persistent left superior vena cava). Heterotaxy, or *situs ambiguus*, is a syndrome consisting of multiple such congenital anomalies without complete mirror image reversal.

'Classic' heterotaxy is defined as situs abnormalities in at least two organs or tissues. Historically, it has been further subdivided into a variety of subgroups based on spleen status (polysplenia or asplenia) or type of symmetry (right isomerism, left isomerism). However, the range of anatomic defects that occur in heterotaxy often defies such simplistic classification. For example, the heart defects identified in heterotaxy are tremendously variable ranging from simple septal defects (atrial septal defect, ventricular septal defect), atrioventricular canal defects, conotruncal anomalies (e.g. double outlet left ventricle, d-transposition of the great arteries), ventricular inversion and l-transposition of the great arteries; isomerism of the atria are sometimes present (right atrial isomerism, left atrial isomerism), with an effect on the conduction system; and anomalies of the vessels are also common (e.g. right aortic arch, interrupted inferior vena cava, anomalous pulmonary venous return). Heterotaxy has a population incidence of approximately 1/10,000, with a greater incidence in Asian (Kim et al. 2008) and African American (Correa-Villasenor et al. 1991) races, and a slightly higher prevalence in females (0.9 per 10,000 births) then males (0.7 per 10,000 births) (Lin et al. 2014). It accounts for 3% of all congenital heart defects (CHD) and of all cardiovascular malformation it is the most highly heritable (Oyen et al. 2009). An estimated 10% of heterotaxy patients have a close family history of CHDs. Although the genetic cause for heterotaxy in a family is not often identified, it is known from studying X-linked heterotaxy, caused by mutations in ZIC3, that the same mutation that causes heterotaxy in one patient can cause isolated CHD in another.

At the chromosome level, causes of heterotaxy include aneuploidy (trisomy 13 and trisomy 18), small chromosomal arrangements (inversions, unbalanced translocations) and copy number variants (CNVs; submicroscopic deletions or duplications) with CNVs estimated to explain 20–30% of heterotaxy cases (Fakhro et al. 2011; Rigler et al. 2015). The inheritance patterns of heterotaxy include sporadic, autosomal recessive, autosomal dominant and X-linked. Patients may have two causative loci (Bamford et al. 2000) or exhibit oligogenic inheritance which may partially explain the phenotypic diversity and variable penetrance within families. Additional investigation is required to better understand complex inheritance. Most pathogenic single gene mutations are dominantly inherited with reduced penetrance, but 6.3%−12.1% of patients with Primary Ciliary Dyskinesia have heterotaxy (Kennedy et al. 2007; Shapiro et al. 2014) which is primarily inherited in an autosomal recessive manner. Genes known to cause heterotaxy include *Nodal*, *ZIC3* (3% sporadic; >75% familial), *CFC1*, *CRELD1*, *FOXH1*, *SESN1*, *LEFTYA*, *GDF1*, *ACVR2B* and *NKX2.5* (Cowan et al. 2014; Sutherland and Ware 2009). Of these genes only ZIC3 has been assessed in large enough cohorts to give reliable estimates of the rate of mutation in heterotaxy patients; similar estimates in the other genes remain to be determined.

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15.3 Establishment of LR asymmetry

LR asymmetry initiates at a structure termed the embryonic node in mouse with similar structures found in zebrafish (Kupffer's vesicle), *Xenopus* (gastrocoel roof plate), chick (Hensen's node) and rabbit (posterior notochordal plate) (Blum et al. 2014). Formation of these structures requires several transcription factors (including *Brachyury*, *Noto*, *Foxa2* and *Zic3*) and several major signalling pathways. The node forms from epiblast cells in the anterior primitive streak at the midline of the embryo (Hamada and Tam 2014). The node cells are organised into columnar epithelium, which, in the case of mice, form a fluid filled cavity covered by the parietal yolk sac. These cells can be divided into two groups: the 200–300 pit cells in the central region of the node and the crown cells around the periphery of the node (Lee and Anderson 2008). Both groups of cells are monociliated, but only the cilia of the pit cells are motile and rotate clockwise when viewed from the 'apical aspect'. The motility or immobility of the cilia in these regions is reflective of the type of 9 + 0 cilia present; the motile central cilia express the dynein encoded by *Dnah11* whereas cilia at the periphery of the node are thought to be mechanosensory due to expression of the calcium canal *Pkd2* and *Pkd1l1* (Field et al. 2011; McGrath et al. 2003; Yoshiba et al. 2012).

Establishment of LR asymmetry involves integration of the two established embryonic axes (anterior/posterior and dorsal/ventral) to create a third. The node is positioned on the ventral surface of the embryo, with the cilia positioned towards the posterior side of the cells (Babu and Roy 2013). Because the cells are dome-shaped, the posterior localisation causes the cilia to tilt and thus the cilia rotary motion to vary in distance from the cell surface, the end result of which is a leftward flow of fluid across the node (Hamada and Tam 2014). The leftward flow has been shown to be critical for establishment of LR asymmetry as loss of the flow through loss of cilia motility or abnormal cilia structure results in randomisation of LR asymmetry (Babu and Roy 2013), whereas artificial reversal of cilia fluid flow results in activation of left signals on the right of the embryo (Nonaka et al. 2002). There are two main theories as to how the leftward flow creates the asymmetry. The first is that the leftward flow generates a gradient of left determinant particles or morphogens such as signalling ligands (Okada et al. 2005; Tanaka et al. 2005). The second theory is immotile cilia of the crown cells sense the flow and cause an influx in Ca^{2+} ions through the calcium signal Pkd2 (McGrath et al. 2003). The influx results in the activation of *Nodal* in the perinodal crown cells on the left.

However the flow functions to initiate LR asymmetry, the end result is to lower levels of Cerl2 (also called Dand5) on the left side of the node through increasing degradation of *Cerl2* mRNA (Nakamura et al. 2012). Since Cerl2 is a Nodal antagonist, this Cerl2 asymmetry results in an asymmetry in Nodal activity, with higher levels of Nodal activity in the left crown cells than in those right of the node. The increased expression of *Nodal* in the left crown cells is transient as Cerl2 protein is translocated between cells. Thus, the Cerl2 protein that continues to be produced in the crown cells to the right of the node will gradually move to those cells lacking *Cerl2* mRNA. However, the temporary change in Cerl2 levels is critical as loss/lack of *Cerl2* in mice results in bilateral or right-sided expression of *Nodal* in lateral plate mesoderm (LPM) (Marques et al. 2004). The 'Leftness' signal created by *Nodal* asymmetry is transferred/transmitted from the crown cells on the

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left node to activate *Nodal* in the left LPM. How the message is transferred is not completely understood, but it may be transmitted via crown cell secretion of Nodal-Gdf1 heterodimer. Nodal positively regulates itself in the LPM to enhance the signal, but also activates feedback inhibitors Lefty1/2 in the midline and left LPM to antagonise Nodal leading to restriction of the extent/duration of Nodal signalling. Lefty also acts to prevent transfer of left-sided signal across the midline to the right side. Expression of *Nodal* in the left LPM also upregulates the homeobox transcription factor *Pitx2*. The expression of *Pitx2* is more stable than *Nodal* and persists after loss of *Nodal* signal in the LPM, thereby acting as the major left determinator.

Situs inversus usually results from defects in 'signalling at the node', whereas heterotaxy can also be caused by early defects in node formation or later defects in transfer of signal to LPM and from the LPM to organ primordia. The latter two steps can also cause isolated CHD.

15.4 ZIC3

ZIC3 is part of the ZIC family and GLI superfamily which are defined by the presence of five highly conserved tandem C_2H_2 zinc fingers (Fig. 15.1b). ZIC proteins (named ZIC1–5) are distinguished from other superfamily members by an atypical first finger which contains 6–38 amino acids between the two cysteine residues rather than the normal 2–4. The atypical finger is highly conserved between ZIC1–3 and more divergent for ZIC4 and ZIC5. Functions of the zinc finger domain include interaction with DNA (Badis et al. 2009; Lim et al. 2010), interaction with proteins (Koyabu et al. 2001; Pourebrahim et al. 2011) and localisation to the nucleus, as both nuclear localization signals and a nuclear export signal is located within this domain (Bedard et al. 2007; Hatayama et al. 2008). Other domains conserved within the family include the ZF-NC (zinc finger N-terminally conserved) domain, a small (14–21 amino acids) region of unknown function highly conserved in vertebrate and arthropod ZICs except for zebrafish Zic6 (Aruga et al. 2006; Keller and Chitnis 2007). ZIC3 and most other ZIC proteins also contain the ZOC (Zic-odd paired conserved) domain which is a small conserved region (9–10 amino acids) found in a subset of the ZICs (ZIC1–3 in mammals) (Aruga et al. 2006). This region has been suggested to be necessary for certain protein-protein interactions, in particular with MDFI (also called I-mfa) (Mizugishi et al. 2004). ZIC3 also contains several low complexity domains, including polyalanine and polyhistidine tracts and has an alternatively spliced transcript (Fig. 15.1a) (Bedard et al. 2011).

Expression of *Zic3* during early development shows overlap with other family members' expression patterns but also demonstrates unique expression domains. *Zic3* expression is initially found in the embryonic and extraembryonic ectoderm prior to gastrulation (Elms et al. 2004). Expression is lost from the anterior portion of the ectoderm during gastrulation but is expanded to include some of the embryonic mesoderm. Expression of *Zic3* overlaps with *Zic2* and *Zic5* at this stage but has unique expression in the anterior of the gastrula in the prechordal plate and anterior definite endoderm as well as in the embryonic node at 7.75 dpc (Sutherland et al. 2013). By the early head fold stage, *Zic3* ectoderm expression becomes restricted to the neuroectoderm and as neurulation progresses, *Zic3* neuroectoderm

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expression becomes restricted to the dorsal regions that represent the future site of neural crest production and dorsal neural production. *Zic3* is also expressed in the presomitic population of the lateral mesoderm and at later stages of development is found in the dorsal spinal cord, dorsal cranial neural tube, the eye and the limb buds.

Of note, despite the cardiac defects seen in *Zic3* null mice, analysis by *in situ* hybridisation has shown a distinct lack of *Zic3* expression in the heart at any point in development (Nagai et al. 1997). In one publication, quantitative RT-PCR on cardiac tissue from E10.5 suggested low levels of *Zic3* transcripts were present in the heart (Zhu et al. 2007a), but attempts to confirm this expression using a highly sensitive *Zic3*-LacZ-BAC reporter line have also indicated a lack of *Zic3* in the heart (Sutherland et al. 2013). Furthermore, tissue specific deletion of *Zic3* in cardiac cells using several different cardiac specific Cre lines does not alter cardiac development or viability (Jiang et al. 2013; Sutherland et al. 2013), suggesting Zic3 does not have a role in these tissues.

15.5 *Zic3* mutant phenotype

Several null *Zic3* mouse lines have been generated, through interstitial deletion, targeted insertion or by spontaneous mutations during an ethylnitrosurea (ENU) mutagenesis screen (Ahmed et al. 2013; Bogani et al. 2004; Haaning et al. 2013; Jiang et al. 2013; Purandare et al. 2002; Sutherland et al. 2013). The phenotypes of these *Zic3* null mice indicate the role/function of Zic3 begins early in development, with a portion of null embryos failing to begin gastrulation. Of those that do gastrulate, another 40% show defects during gastrulation including excess mesoderm formation and a subset show axis duplication. Shortly after gastrulation, mouse embryos develop further phenotypes including caudal truncation, heart abnormalities, defects in somitogenesis and delayed growth. Zic3 continues to have roles throughout development, thus later stage *Zic3* nulls show a variety of phenotype including neural tube defects (both exencephaly and spina bifida), craniofacial defects, omphalocele, skeletal defects, limb defects, defects in folia and olfactory bulb patterning and microphthalmia.

Aside from these defects, the *Zic3* null mice show a number of phenotypes consistent with heterotaxy. The heart defects seen in *Zic3* null mice are varied, but include dextro-transposition of the great arteries, interrupted aortic arch, right aortic arch, atrial septal defect, ventricular septal defect and abnormal systemic venous connections (Purandare et al. 2002). Consistent with classic heterotaxy, mice with heart anomalies also have additional anomalies in the LR patterning of other organs including lung isomerisms, right sided stomach with reversed liver and hypoplastic spleen (Purandare et al. 2002). Quantification of the rate of these heart defects at d10.5-d12.5 has found that roughly 47% of *Zic3* null have defects in heart looping that can further divided into sinistral (leftward) looping (16.7%), ventral (forward) looping (23%) and no looping (6.7%) (Ware et al. 2006a). The *Zic3* mutant mice also show defects in expression of laterality genes during the establishment of the LR axis. Normally *Nodal* expression begins in the crown cells at the 0–2 somite stage and is maintained past the 6 somite stage. *Zic3* null and hypomorphic mice retain the initial expression of *Nodal*, but the expression is not maintained and is lost by the 4–6 somite stage in a subset of embryos. At the later somite stages, when *Nodal* and *Pitx2* are normally

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expressed in the left LPM, mutants show a variable expression of these genes which can be in the right LPM, left LPM, bilaterally or absent (Purandare et al. 2002; Sutherland et al. 2013). The role of Zic3 is conserved between species as loss of *Zic3* in Xenopus and zebrafish embryos causes similar phenotypes including delayed gastrulation and defects in left-right patterning including abnormal heart and gut looping (Cast et al. 2012).

Notably, gain of Zic3 function can also result in similar phenotypes in a number of species. Analysis of a mouse line showing increased expression (but not ectopic expression) of *Zic3* due to insertion of a Neo cassette found 6.9% showed dextrocardia at E14.5–15.5 and 7.9% at 4-weeks old, and there were also individual cases of left-sided gall bladder and asplenia (Zhu et al. 2007b). Overexpression of *Xenopus Zic3* mRNA in the right side of *Xenopus* embryos causes defects in the laterality of the heart and gut and induces expression of major left determinants *Pitx2* and *Xnr1* (Kitaguchi et al. 2000). By injecting *Xenopus* embryos with *Zic3* tagged with part of the human glucocorticoid receptor (*Zic3*-GR), which is only active in the presence of dexamethasone, the effect of *Zic3* overexpression on laterality was found to be restricted to the last blastula and early gastrula stage, with later induction of Zic3 activity not able to induce expression of *Pitx2* (Kitaguchi et al. 2000). Similarly, overexpression of human *ZIC3* in zebrafish by mRNA injection results in an altered position of the heart tube in 40% of embryos (Paulussen et al. 2016). In addition it has been found that in at least one mice line a truncated allele of Zic3 escapes nonsense mediated decay (Ahmed et al. 2013). The stability of such transcripts raises the possibility that Zic3 truncation may produce dominate negative proteins which potentially interfere with other members of the Zic family, although further analysis of the mouse line has suggested the mutation produces a loss of function allele.

Though *Zic3* has a wide expression pattern, the temporal and tissue specific requirement of *Zic3* expression for the development of heterotaxy has been delineated by use of two conditional mouse lines, both of which delete the first exon of *Zic3* in the presence of recombinase enzyme Cre to abolish its function (Jiang et al. 2013; Sutherland et al. 2013). Knockout of *Zic3* in the epiblast using a *Sox2-Cre* line results in the same phenotypes as *Zic3* nulls including gastrulation, neural tube, exencephaly and laterality defects (Jiang et al. 2013; Sutherland et al. 2013), although lethality was not as high as in *Zic3* nulls despite all adults displaying kinked tails consistent with loss of *Zic3* (Jiang et al. 2013). The tissue for which Zic3 activity is required for normal establishment of LR asymmetry can be further delimited to migrating streak and mesodermal lineage derivatives as knockout of *Zic3* within mesendodermal cells using *T-Cre* results in laterality defects including looping defects of the heart at 9.5 dpc (Jiang et al. 2013; Sutherland et al. 2013) as well abnormal expression of *Pitx2* and *Lefty* and dysmorphic nodes (Sutherland et al. 2013). Notably, knockout of *Zic3* in cardiac progenitors/tissue using five different Cre lines (*Nkx2.5*, *Mef2c*, *Wnt1*, β*MyHC* and *Mesp1*) did not reduce viability or cause laterality defects, indicating the heart malformations produced by loss of *Zic3* are not a result of loss of *Zic3* in cardiomyocytes, cardiac neural crest, or the anterior heart field (Jiang et al. 2013; Sutherland et al. 2013). Loss of *Zic3* in the node through use of a *Foxj1-Cre* or a nodal dependent enhancer (*NDE*)-*Cre* line also did not cause laterality defect (Sutherland et al. 2013), suggesting that Zic3 expression is required prior to node formation to ensure normal cardiac development.

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15.6 Interactions of ZIC3 in developmental signalling pathways in heterotaxy

The ZICs are multifunction proteins which are able to influence several of the major signalling pathways including hedgehog, Wnt, planar cell polarity (PCP) and transforming growth factor β signalling. Herein we summarize the potential role of ZIC3 in these pathways for the initiation of LR asymmetry.

15.7 Hedgehog signalling

Mammals have three paralogous Hedgehog genes: Sonic hedgehog (*Shh*) which is the most broadly expressed hedgehog gene and is involved in several stages of development including patterning the somites, neural tube and limb as well as in development of tooth, lung and gut; Indian hedgehog (*Ihh*) which is involved in bone differentiation and development of the mammalian gut (Ramalho-Santos et al. 2000; St-Jacques et al. 1999); and Desert hedgehog (*Dhh*) which is involved in gonad differentiation and development of the perineural sheath (Bitgood et al. 1996; Parmantier et al. 1999). In the absence of these three hedgehog ligands (Fig. 15.2a), the 12-span transmembrane receptors Patched-1 (Ptch1) and Patched-2 (Ptch2) accumulate in and around the cilium to repress activity of 7-span transmembrane protein Smoothened (Smo) which thus inhibits downstream hedgehog signalling (Rohatgi et al. 2007). The repression of Smo occurs through Ptch controlling the localization, stability and phosphorylation of Smo. For example, in the repressed state Smo is localized mainly to plasma and vesicle membranes (Milenkovic et al. 2009) as Ptch prevents localization to cilia. When Smo is repressed, Suppressor of Fused (Sufu) negatively regulates the pathway by binding the Gli family transcription factors and thereby retaining Gli proteins in the cytoplasm (Dunaeva et al. 2003; Humke et al. 2010; Kogerman et al. 1999). The sequestering of Gli proteins both prevents their nuclear transcriptional activation of target genes and causes Gli2 and Gli3 to be processed by the proteasome to produce repressor forms of these Gli proteins (Humke et al. 2010; Wang et al. 2000). There is also evidence that Sufu/Gli form a repressor complex in the nucleus to suppress Gli-induced gene expression. In contrast, when hedgehog signalling is active (Fig. 15.2b), the secreted hedgehog ligand binds Ptch1, causing Ptch to exit the cilium leading to derepression of Smo. The exact mechanism of Smo derepression is not clear, but involves trafficking of Smo to the cilia and phosphorylation of the cytoplasmic tail by Ckiα and Grk2 (Chen et al. 2011). Active Smo inhibits Pka and promotes transport of the Gli/Sufu complex to the tips of cilia where the proteins disassociate, after which the full length active form of the Gli proteins enter the nucleus and activate downstream hedgehog target genes (Tukachinsky et al. 2010).

Given the central role of cilia localisation in this pathway, normal cilia assembly and function are critical for activation/transduction of hedgehog signalling. For example, cilia intraflagellar transport (IFT) controls the bi-directional transport of protein complexes between the base and tip of cilia and is critical for the assembly and function of cilia. Several IFT proteins, including Ift172, Ift88, Kif3a and Dync2h1 have been shown in mouse genetic studies to be required for mouse hedgehog signalling, and have been placed in the middle of the pathway downstream of Smo and upstream the Gli proteins (Huangfu and Anderson

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2005; Huangfu et al. 2003; May et al. 2005). Disruption of basal body proteins also causes defects in cilia formation including abnormal or absent cilia. Mice with such basal body disruptions show both cilia defects and phenotypes consistent with loss of hedgehog, such as polydactyly (Bangs and Anderson 2017; Vierkotten et al. 2007; Weatherbee et al. 2009).

Several lines of evidence demonstrate hedgehog signalling has a role in establishment of LR asymmetry. In particular, *Smo*$^{-/-}$ mice show several phenotypes consistent with loss of LR asymmetry including failure of embryonic turning and loss of heart looping (Zhang et al. 2001). Although the failure of such *Smo*$^{-/-}$ embryos to survive past 9.5 dpc precludes analysis of later loss of LR asymmetry phenotypes, analysis of molecular markers also supports the concept that the establishment of the LR axis is disrupted in these mice, as *Lefty1* and *Lefty2* expression is lost whereas *Nodal* and *Pitx2* is lost only in the LPM. Interestingly, loss of individual hedgehog ligands does not seem to alter LR signalling in exactly the same way, as loss of Ihh does not affect development and *Shh*$^{-/-}$ mice show some phenotypes indicating initiation of the asymmetry has been affected, such as bilaterally unilobed lungs and abnormal cardiac looping. In addition, a small proportion of mice (10%) show reversal of cardiac looping (Meyers and Martin 1999; Tsukui et al. 1999), but do not show the same differences as Smo mutants at the molecular level. In particular, *Lefty2*, *Nodal* and *Pitx2* expression is not lost but instead the three genes are ectopically expressed in the right LPM. Instead, it takes loss of both *Ihh* and *Shh* to produce embryos indistinguishable from the *Smo* mutants. As both genes are weakly expressed in the node, this suggests redundancy in hedgehog signalling in regulating formation of the asymmetry (Zhang et al. 2001). Restoration of hedgehog signalling in the LPM of *Smo*$^{-/-}$ mutants by use of a LPM-enhancer driven Smo transgenic line is sufficient to re-establish expression of *Nodal*, *Lefty* and *Pitx2* and normal heart looping (Tsiairis and McMahon 2009). However *Lefty* expression at the midline is still absent, suggesting hedgehog may have multiple roles in regulating asymmetry formation.

There is evidence that Zic family members can repress hedgehog signalling in multiple species. For example loss of zic1 in zebrafish via MO injection produces reduced expression of *ptc1*, *shh* and the zebrafish hedgehog homolog tiggywinkle (*twhh*) (Maurus and Harris 2009), loss of Zic1 in mice causes reduced expression of *Ptch1*, *Gli1* and Shh downstream gene *Mycn* in the cerebellar vermis (Blank et al. 2011) and knockdown of *ZIC2* in HeLa cells using RNAi reduces expression of several hedgehog signalling downstream targets including *GLI1*, *PTCH1* and *CYCLIN* (Chan et al. 2011). Regulation of hedgehog most likely occurs through interaction of ZIC with the GLI proteins. ZIC1, ZIC2 and ZIC3 have all been found to interact with multiple GLI proteins via cell based immunoprecipitation assays (Koyabu et al. 2001; Zhu et al. 2008). These ZICs also show synergistic activation with the GLI family of various luciferase reporters in cell based mechanisms (Koyabu et al. 2001; Pan et al. 2011; Zhu et al. 2008). There are several potential mechanisms by which ZICs influence GLI activity, all of which involve interaction of ZIC and GLI proteins through their respective zinc finger domains. Through this interaction the ZICs have been found to increase the localization of GLIs to the nucleus (Koyabu et al. 2001), which should enhance GLI activity. ZIC3 has also been shown to alter the ratio of the repressor and activator forms of GLI3. *In vivo*, the loss of Zic3 alters the ratio of Gli3 activator: repressor

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in their shared limb bud expression domains, leading to the ability of *Zic3* null mice to rescue the polydactyly phenotype in *Gli3*$^{-/-}$ mice (Quinn et al. 2012).

Given these influences on hedgehog signalling, it could be predicted that *Zic3* null mice would have downregulated hedgehog signalling and thus would be similar to *Smo* mutants at the molecular level, with loss of *Pitx2* and *Nodal* in the LPM. As *Zic3* null mice retain expression of left markers such as *Pitx2* and *Nodal* in the LPM, it is unlikely hedgehog signalling is completely lost in such mutants.

15.8 Canonical Wnt Signalling

There are several signalling pathways regulated by secreted Wnt ligands. Canonical Wnt signalling differs from the other pathways in that it is regulated by controlling the amount of β-catenin that reaches the nucleus. β-catenin protein is constantly produced by cells and has important roles outside of the canonical pathway in cell-cell adhesion, with total loss of β-catenin resulting in adhesion defects and embryonic disintegration (Hierholzer and Kemler 2010). In the absence of Wnt ligands (Fig. 15.3a) the pathway is in the OFF state and the amount of β-catenin is strongly depleted by activity of the β-catenin cytoplasmic degradation complex. The degradation complex includes scaffolding proteins Axin and adenomatous polyposis coli (Apc) which promote the phosphorylation of β-catenin through action of casein kinase 1 (Ck1) and glycogen synthase kinase (Gsk3). The phosphorylation of β-catenin targets it for ubiquitin dependent degradation via ubiquitin ligase β-Trcp.

The pathway is switched into the ON state (Fig. 15.3b) by the extracellular presence of one of at least 19 secreted Wnt ligands (other Wnt ligands activate other pathways). The Wnt ligands interact with Frizzled (Fz) and Lrp5/6 co-receptors on the surface of cells to form a receptor complex that results in phosphorylation of the intracellular portion of Lrp5/6. The phosphorylated intracellular domain of Lrp5/6 acts as a docking site for Axin causing Axin to be relocated to the cell membrane in a process dependent on the Dishevelled (Dvl) proteins. Due to the loss of Axin, β-catenin is no longer degraded and thus accumulates in the cytoplasm and translocates into the nucleus. Once in the nucleus, β-catenin activates transcription of target genes through interaction with the T-Cell Factor (Tcf)/ Lymphoid Enhancer-binding Factor 1 (Lef1) family of transcription factors. In the absence of Wnt ligands Tcf/Lef transcription factors inhibit the transcription of the same target genes through interaction with Tle/Groucho family of co-repressors. Nuclear β-catenin displaces co-repressors and recruits co-activators including Bcl9, histone acetylation Cbp and p300 to interact with Tcf/Lef to activate target genes.

Multiple Wnt ligands may act during the initial establishment of LR asymmetry in functions that are not completely redundant. For example, *Wnt3a* is symmetrically expressed in the dorsal posterior node and its loss produces various LR defects including delayed bilateral expression of *Nodal*, *Lefty2* and *Pitx2* in the LPM, as well as randomization of heart looping, randomization of embryonic turning, abnormal stomach positioning and abnormal lung lobation (Nakaya et al. 2005). Wnt3a protein, on the other hand, shows an asymmetric expression in the node crown cells, where it is thought to promote degradation of Cerl2 (Kitajima et al. 2013; Nakamura et al. 2012). Initially *Cerl2* and *Wnt3a* are both expressed

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symmetrically in the crown cells but both inhibit expression of the other gene: Wnt3a increases degradation of *Cerl2* mRNA due to a Tcf binding site in the 3' untranslated region (UTR) whereas Cerl2 likely causes degradation of Wnt protein (Nakamura et al. 2012). The repression of both genes is balanced so that neither is completely lost. Once degradation of *Cerl2* is triggered by nodal flow, the initial loss of Cerl2 in the left crown cells allows accumulation of Wnt3a which in turn increases degradation of *Cerl2* to maintain the reduction in Cerl2 in the left crown cells after flow is lost (Kitajima et al. 2013; Nakamura et al. 2012). It should be noted that so far loss of Wnt signalling has not been found to affect cilia structure or basal body position in mice (Hashimoto et al. 2010; Nakamura et al. 2012; Nakaya et al. 2005), though in other species such as zebrafish Wnt signalling regulates ciliogenesis in the equivalent of the node (Caron et al. 2012; Lin and Xu 2009; Zhang et al. 2012; Zhu et al. 2015).

The first suggestion that the Zic family might influence Wnt signalling came from the observation that *Zic3* null mouse embryos showed posterior axis duplications (Ware et al. 2006b), a phenotype that had previously been associated with increased or ectopic Wnt signalling (Popperl et al. 1997; Zeng et al. 1997). How the ZIC family influenced the pathway was not initially clear since some findings, such as the loss of *Wnt3a* expression in *Zic3* null mice (Ware et al. 2006b) suggest the ZICs act upstream of Wnt, whereas other evidence, such as the finding the Wnt pathway activity is necessary for ZIC expression at the neural plate border and tectum in zebrafish (Garnett et al. 2012; Nyholm et al. 2007) placed the ZICs downstream of Wnt. Recently two papers confirmed ZICs act to repress canonical Wnt signalling using cell based TOPFLASH reporter assays and by showing that Zics could repress Wnt *in vivo* to rescue *Xenopus* axis duplication (Fujimi et al. 2012; Pourebrahim et al. 2011). Using co-immunoprecipitation both groups found that ZICs influence the pathway though interaction with the TCF/LEF transcription factors. The ZIC/TCF complex is able to interact with DNA, though ZIC DNA binding ability is not required for such interaction suggesting a co-factor role for the ZICs in inhibiting Wnt signalling (Pourebrahim et al. 2011).

Given that the ZICs inhibit Wnt signalling it might be expected that loss of *Zic3* in mice would upregulate the pathway and thus produce bilateral activation of *Nodal* in the crown cells. Such activation has not been reported. Given the complex interactions with this pathway, is it possible ZICs regulate Wnt in cell-type specific manners and thus produce a different effect depending on the developmental stage or tissue type.

15.9 PCP signalling

PCP regulates the alignment of cell polarity across a tissue plane through interaction of proteins at the membranes of adjacent cells. The pathway is considered part of non-canonical Wnt signalling and is best studied in *Drosophila* in which the core pathway proteins include Frizzled (Fz) receptor, Van Gogh (Vang) and Flamingo (Fmi) at the membrane and Dishevelled (Dsh), Prickle (Pk) and Diego (Dgo) in the cytoplasm. These proteins form two distinct complexes at the membrane on either side of a cell: Fz, Dsh and Dgo at one side and Vang and Pk at the opposite side, with atypical transmembrane cadherin Fmi found on both sides (Axelrod 2001; Bastock et al. 2003; Feiguin et al.

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2001; Shimada et al. 2001; Strutt 2001). The complexes encourage/enforce the separation of the two complexes by inhibiting one another intracellularly. For example, the Vang/Pk complex inhibits formation of Fz/Dsh by Pk directly binding Dsh to both affect Dsh stability and prevent Dsh from localising to the membrane (Tree et al. 2002). Dgo antagonises the inhibition of Dsh by competing with Pk for the same binding sites Dsh and thus stabiles the complex (Jenny et al. 2005). Outside of the cells the Fz-Fmi complex interacts with Vang-Fmi complex on neighbouring cell, forming homodimers between cells. As the extracellular portions of Fz and Vang can be deleted without altering the localisation of these proteins, it is thought that the Fmi forms a bridge between proteins (Chen et al. 2008). The interaction serves to stabilise the complexes and enforce the orientation of PCP signalling across neighbouring cells and thus across tissue planes.

The PCP pathway is largely conserved in mammals (Fig. 15.4), but several vertebrate-specific PCP factors have been identified, such as the Ror2 and Ryk co-receptors. These proteins interact with Fzd (the mammalian homolog of Fz) and Vangl2 to form a receptor complex for the Wnt ligand Wnt5a to trigger phosphorylation of Dvl (the mammalian homolog of Dsh) and Vangl2. In zebrafish the phosphorylation of Vangl2 correlates with increased Vangl2 activity (Gao et al. 2011), thus Wnt5a may act as an instructional signal regulating the initial asymmetry formation of the PCP complexes. Once the asymmetry is established PCP membrane interactions have various tissue specific downstream consequences, through manipulation of two distinct pathways. The first pathway is mediated by the Rho subfamily of small GTPases and the second pathway by Rac and Cdc42 proteins. Both pathways activate Jnk to regulate nuclear responses, though the Rho subfamily also acts independently of the nucleus to trigger cytoskeletal re-arrangements.

One of the roles of PCP signalling is positioning the basal body and thus the cilia within the cell. The major PCP signalling components are asymmetrically localized within node cells, with Dvl2 and Dvl3 localized to the posterior of node cells (Hashimoto et al. 2010) whereas Prickle2 and Vangl1 are localized to the anterior (Antic et al. 2010). This asymmetry is thought to be caused be a gradient of PCP activity across the node produced by the combination of expression of Wnt5a/Wnt5b ligands posterior of the node and the PCP inhibitors Sfrp1/Sfrp2/Sfrp5 anterior of node (Minegishi et al. 2017). The node cilia are initially positioned towards the center of cells, but PCP signalling gradually causes a posterior shift of basal body and cilia of the node cells (Hashimoto et al. 2010), which is needed to produce the leftward flow that activates downstream LR signalling events. Disrupting either of these complexes prevents the posterior shift of cilia, causing loss of flow. For example, in mice without either Vangl1 and/or Vangl2 the cilia remain centrally localized and develop various LR asymmetry defects including failure of heart looping and embryonic turning as well as lung isomerism and randomized *Nodal* and *Pitx2* expression in the LPM (Antic et al. 2010; Song et al. 2010). Loss of multiple *Dvl* genes or inhibition of the downstream PCP gene *Rac1* also causes a similarly impaired basal body shift (Hashimoto et al. 2010). It should be noted that scanning electron microscopy has indicated the cilia are structurally normal in all of these mutants (Antic et al. 2010; Hashimoto et al. 2010; Song et al. 2010). PCP signalling may also be involved earlier as embryos that lack *Rac1* in the epiblast show defects in the formation of the node (Migeotte et al. 2011). In

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these mutants, the pit cells of the node are present in several distinct clusters though the cilia are structurally normal.

There is some evidence that Zic3 regulates PCP signalling. For example, Zic3 null mice show tail kinks, a phenotype that is seen in Prickle1 hypomorphs (Liu et al. 2014) and in loss of function PCP alleles of LRP6 (Allache et al. 2014). *zic3* zebrafish and *Xenopus* morphants also show defects in convergence extension morphogenesis, a process regulated by PCP signalling (Cast et al. 2012). Several PCP related genes have also been identified as putative Zic3 targets in zebrafish by CHIP-seq (Winata et al. 2013). Additionally the nodes of *Zic3* null mice show node abnormalities (Fig. 15.5) (Sutherland et al. 2013) similar to those seen in the PCP effector gene *Rac1* (Migeotte et al. 2011).

15.10 TGFβ (Nodal) Signalling

Nodal is part of the Tgfβ superfamily of ligands which exist as homodimers or in rare cases heterodimers to bind Tgf beta type II receptors. Upon ligand binding the type II receptor (Fig. 15.6), a heterotetrameric complex is formed with type I receptor (Alk1–7), causing the type II receptor to phosphorylate the type I receptor. These initial steps of activation are similar for the other Tgfβ superfamily of ligands, including BMPs (Bmp2, Bmp4 and Bmp7), Tgf-β and Activins, but Nodal signalling is unique in that it also requires presence of EGF-CFC co-receptor (Cripto and Cryptic in mammals). Thus Nodal signalling has the potential to be inhibited without affecting the other TGFβ signalling through targeting the co-receptor. Formation of the ligand receptor complex triggers the type I receptor to phosphorylate the C-terminal serine residue of cytoplasmic receptor SMADs (R-SMADs). The R-Smad targeted for phosphorylation varies depending on which ligand activated the pathway, with Smad1, Smad5 and Smad8 phosphorylated in the case of BMP signalling and Smad2 and Smad3 phosphorylated in the case of Tgf-β, Activin and Nodal signalling. Phosphorylated R-Smad form homomeric and heteromeric complexes with Smad4 (also called co-Smad) and accumulates in the nucleus to regulate target genes. Smads alone activate transcription weakly and require additional tissue specific transcription factor or co-factors. In particular Smad2 does not directly bind DNA.

Nodal signalling is critical for the initiation of LR asymmetry both due to its activity in the crown cells of the node and the LPM. In addition, Nodal is required for formation of the primitive streak and thus the node. In *Zic3* null mice *Nodal* expression is initiated in the crown cells but not maintained and *Nodal* LPM expression is randomized, suggesting Zic3 regulates this pathway (Purandare et al. 2002). Partial loss of *Nodal* also increases the severity of *Zic3* phenotypes, which also suggests the genes act within the same pathway (Ware et al. 2006a). Zic3 is able to influence the pathway via a Nodal enhancer to activate expression in mice and *Xenopus* (Ware et al. 2006a), placing Zic3 upstream Nodal signalling. However, recently ZIC2 was shown to interact with both SMAD2 and SMAD3 (Houtmeyers et al. 2016). If the interaction is conserved within the ZIC family, ZIC3 may influence Nodal signalling at multiple points in the pathway.

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15.11 Lessons from human ZIC3 mutations

Loss of ZIC3 was originally identified as a potential cause of heterotaxy through mapping of a submicroscopic deletion of the X-chromosome in a case of X-linked familial heterotaxy (Casey et al. 1993; Ferrero et al. 1997; Gebbia et al. 1997). Other cases of ZIC3 deletion have since been found in heterotaxy patients, including patients in which the entire ZIC3 sequence has been lost (Chung et al. 2011) and in one case a male sporadic heterotaxy patient was found to have a deletion of only the third exon of ZIC3 (Cowan et al. 2016). There have also been at least two instances of translocations involving the X chromosome resulting in heterotaxy (Fritz et al. 2005; Tzschach et al. 2006). In both cases, the female patients were found to have undergone X-inactivation of their normal X chromosome, suggesting that the translocation altered the transcriptional regulation of the *ZIC3* gene and thus resulted in loss of function.

To date 38 unique variants altering the sequence of *ZIC3* have been identified in heterotaxy patients (Fig. 15.1b) (Chhin et al. 2007; Cowan et al. 2014; D'Alessandro et al. 2013a; D'Alessandro et al. 2013b; De Luca et al. 2010; El Malti et al. 2016; Gebbia et al. 1997; Ma et al. 2012; Megarbane et al. 2000; Paulussen et al. 2016; Ware et al. 2004; Wessels et al. 2010). The variants affecting the *ZIC3* sequence include 16 missense mutations of which 13 alter amino acids conserved in all of human, mouse, *Xenopus* and zebrafish. Other variants affecting ZIC3 include one splice site, nine frame shift and eight nonsense mutations, as well as one in frame deletion and three in frame duplications. In total, 21/38 (52.6%) of the mutations are in the ZFD and 78.9% in total disrupt this region. As this region is involved in DNA binding it is expected that such disruption of the ZFD would prevent transcriptional regulation by ZIC3. The in frame deletions and duplications do not affect this region, but rather occur within or immediately adjacent to the Alanine tract, probably due to the repeated triplicate in this region. Whether the expansion of the Alanine tract is pathogenic is currently not clear. Although an alternatively spliced 4th exon of *ZIC3* has been identified, no mutations have been found within the exon. Together these mutations explain 75% of familial X-linked heterotaxy but less than 5% of sporadic cases (Cowan et al. 2014; Ware et al. 2004).

There are several pieces of evidence that suggest ZIC3 is intolerant to genetic variation and thus that it is important for human development. In particular ZIC3 is very highly conserved across organisms, particularly between zinc fingers 2–5. In addition, there are few polymorphisms in *ZIC3* in different races/ethnicities in humans and the introns are much more highly conserved than typically seen.

From studying familial heterotaxy it appears that the effect of ZIC3 mutation is variable. ZIC3 mutations appear to be highly penetrant, but at least one male with a ZIC3 mutation is known to be unaffected. Phenotypic variability is common within families with ZIC3 mutations. There is often a range of heart defects identified and other situs anomalies can be variable. Both asplenia and polysplenia have been identified as a result of ZIC3 mutations, as well as left isomerism and right isomerism (Chhin et al. 2007; Cowan et al. 2014; Cowan et al. 2016; D'Alessandro et al. 2013a; El Malti et al. 2016; Ma et al. 2012; Paulussen et al. 2016; Ware et al. 2004). This suggests that mutations in ZIC3 result in randomization of LR

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patterning. In general, male hemizygous patients show more severe phenotypes then female heterozygous patients carrying the same mutation. Nevertheless, female carriers are often found to have phenotypes, albeit at lower rates than males. The phenotypes in these female patients in some cases is attributed to skewed X-inactivation (Chhin et al. 2007).

Animal models suggest a role for ZIC3 in gastrulation, neural tube closure, and other congenital anomalies. Large cohorts of patients with neural tube defects have not been screened for ZIC3 mutations, but a smaller screen of 352 patients failed to identify any causative variants in ZIC3 (Klootwijk et al. 2004). Expansion of the Alanine tract in ZIC3 has been reported in association with VACTERL (Wessels et al. 2010), a multiple congenital anomaly association likely resulting from multiple distinct causes that affect gastrulation. VACTERL syndrome is defined as the co-occurrence of three or more vertebral anomalies (V), anal atresia (A), cardiovascular malformations (C), tracheoesophageal (T), renal anomalies (R) and limb anomalies (L). It remains to be determined whether ZIC3 mutations contribute significantly to isolated CHD or other isolated birth defects.

15.12 Conclusion

ZIC3 is a critical regulator of early development, particularly in establishment of the LR axis. Research over the past 20 years has shown ZIC3 to be a multifunctional protein with significant roles in several of the major signalling pathways, but it remains to be definitively determined which roles lead to heterotaxy when ZIC3 function is compromised.

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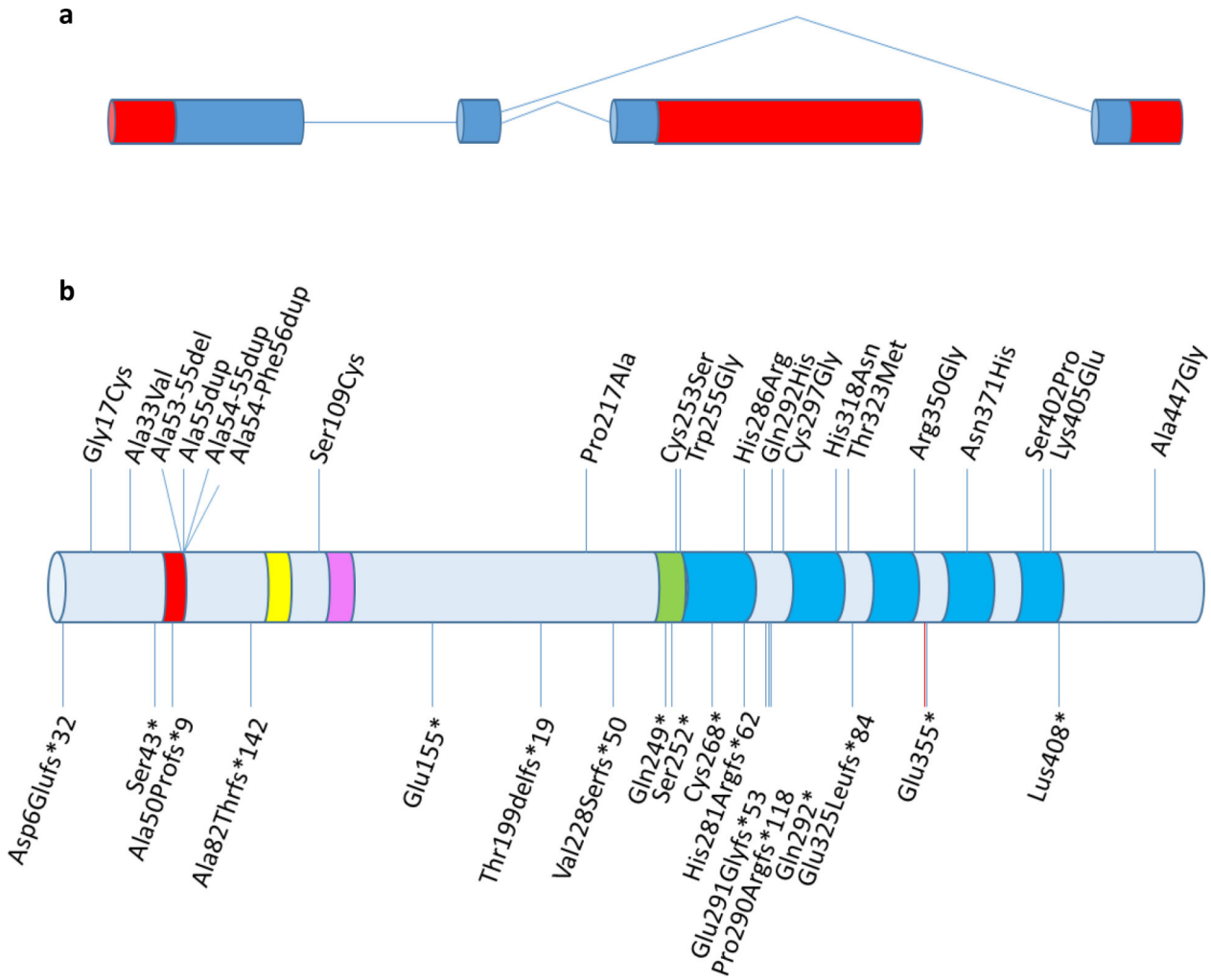

Fig. 15.1. The genomic arrangement and protein domains of ZIC3.

(a) ZIC3 consists of four exons, shown here in blue, with alternative splicing of exons 3 and 4 creating two isoforms. Untranslated regions are shown in red. (b) ZIC3 mutations. Missense mutations, in-frame expansions and in-frame deletions that have been identified in heterotaxy patients are shown above the protein whereas nonsense and frame shift mutations are shown below. Red = polyalanine tract; yellow = polyhistidine tract; pink = ZOC (Zic-odd paired conserved) domain; green = ZF-NC (zinc finger N-terminally conserved); Blue = zinc fingers

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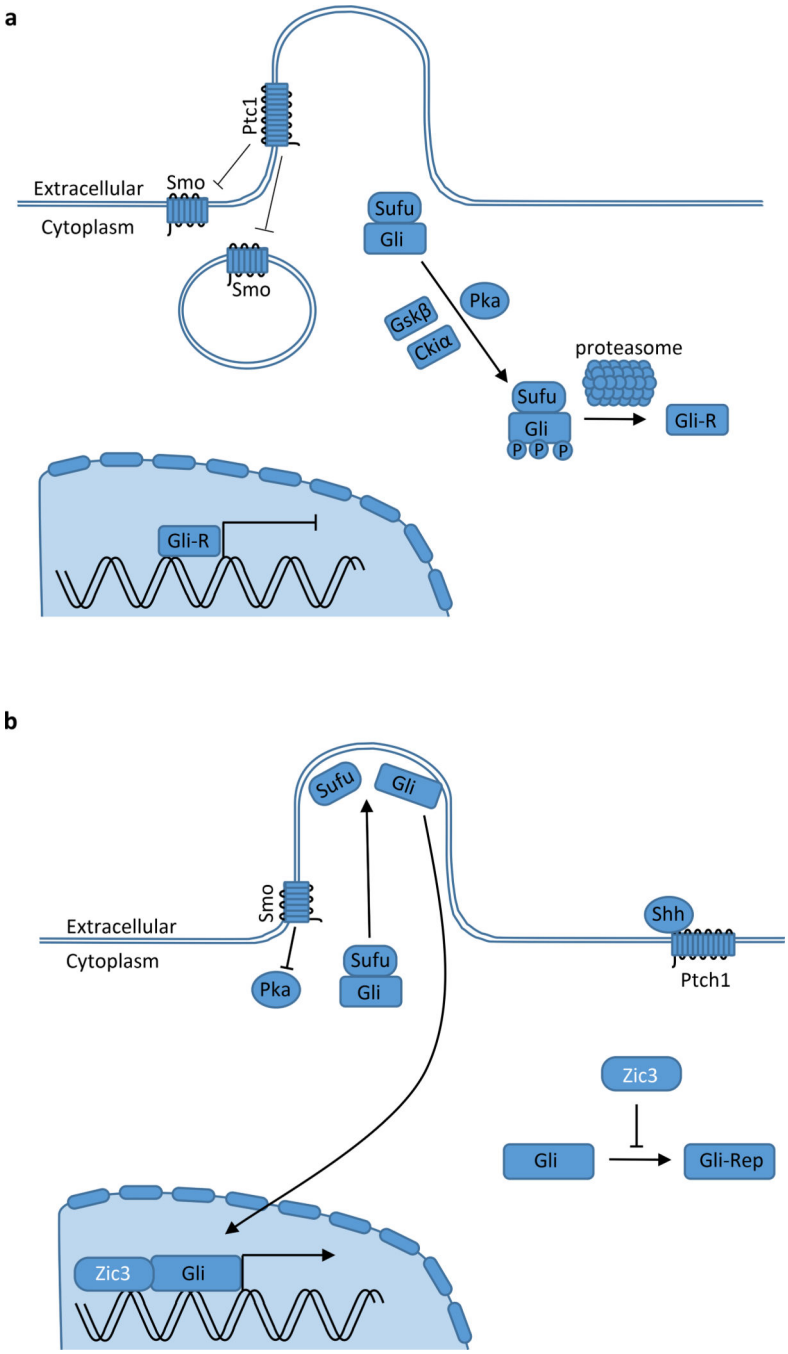

Fig. 15.2. Hedgehog signalling.

(a) In the absence of hedgehog ligands Ptch1 accumulates in and around the cilia where it inhibits Smo, partly by preventing it from entering the cilia. When Smo is repressed, Sufu binds Gli thus preventing Gli from entering the nucleus. The Sufu-Gli interaction also causes phosphorylation of Gli by the kinases Pka, Gskβ and Ckiα which in turn causes partial degradation by the proteasome resulting in the truncated Gli-repressor forms. (b) In response to binding of Shh ligands, Ptch exits the cilium which causes derepression of Smo. Sufu and Gli still interact, but active Smo inhibits Pka and promotes transport of the Sufu/Gli

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complex to tip of the cilia where the proteins disassociate. The full length activate form of Gli then moves into the nucleus to activate hedgehog target genes. Zic3 directly interacts with Gli proteins and potentially increases Gli localisation to the nucleus, but has also have been found to influence the ratio of activator and repressor forms of Gli.

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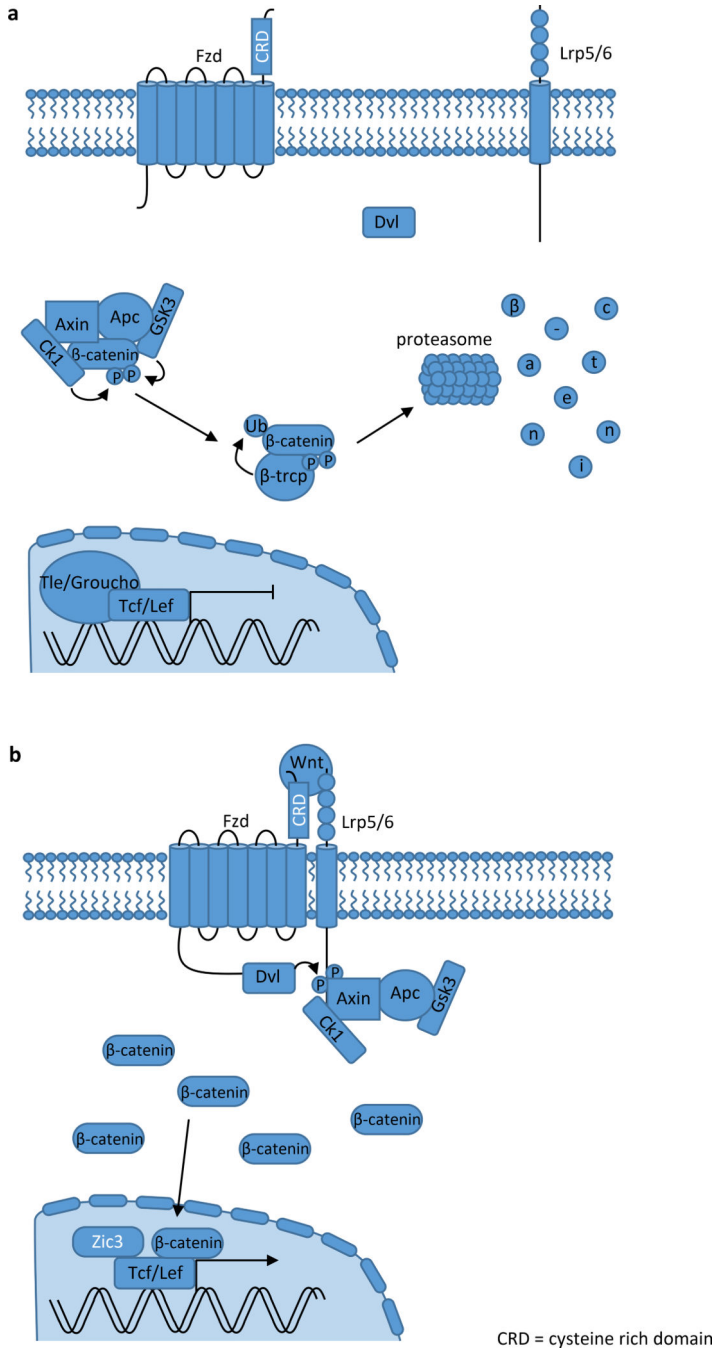

Fig. 15.3. Canonical Wnt Signalling.

(a) In the absence of Wnt ligands, β-catenin is bound by the β-catenin destruction complex consisting of Axin and APC which promotes phosphorylation of β-catenin by the kinases CK1 and Gsk3. The phosphorylation of β-catenin targets it for ubiquitination via the ligase β-Trcp and subsequent degradation. Due to the constant degradation of β-catenin it fails to enter the nucleus and the transcription factors TCF and LEF inhibit transcription of Wnt targets due to interaction with the TLE/Groucho family of co-repressors. Zic3 also interacts with TCF/LEF to promote repression of Wnt targets without directly interacting

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with DNA. (b) Wnt ligands interact with the extracellular portion of the Fzd receptor and LRP5/6 co-receptor to cause the phosphorylation of the intracellular portion of LRP5/6 thus producing an Axin docking site. Axin and the β-catenin complex is relocated to the cell membrane in a process dependent on the Dvl proteins. β-catenin is thus no longer degraded and accumulates in the nucleus where it interacts with TCF/LEF to displace Groucho and recruit co-activators.

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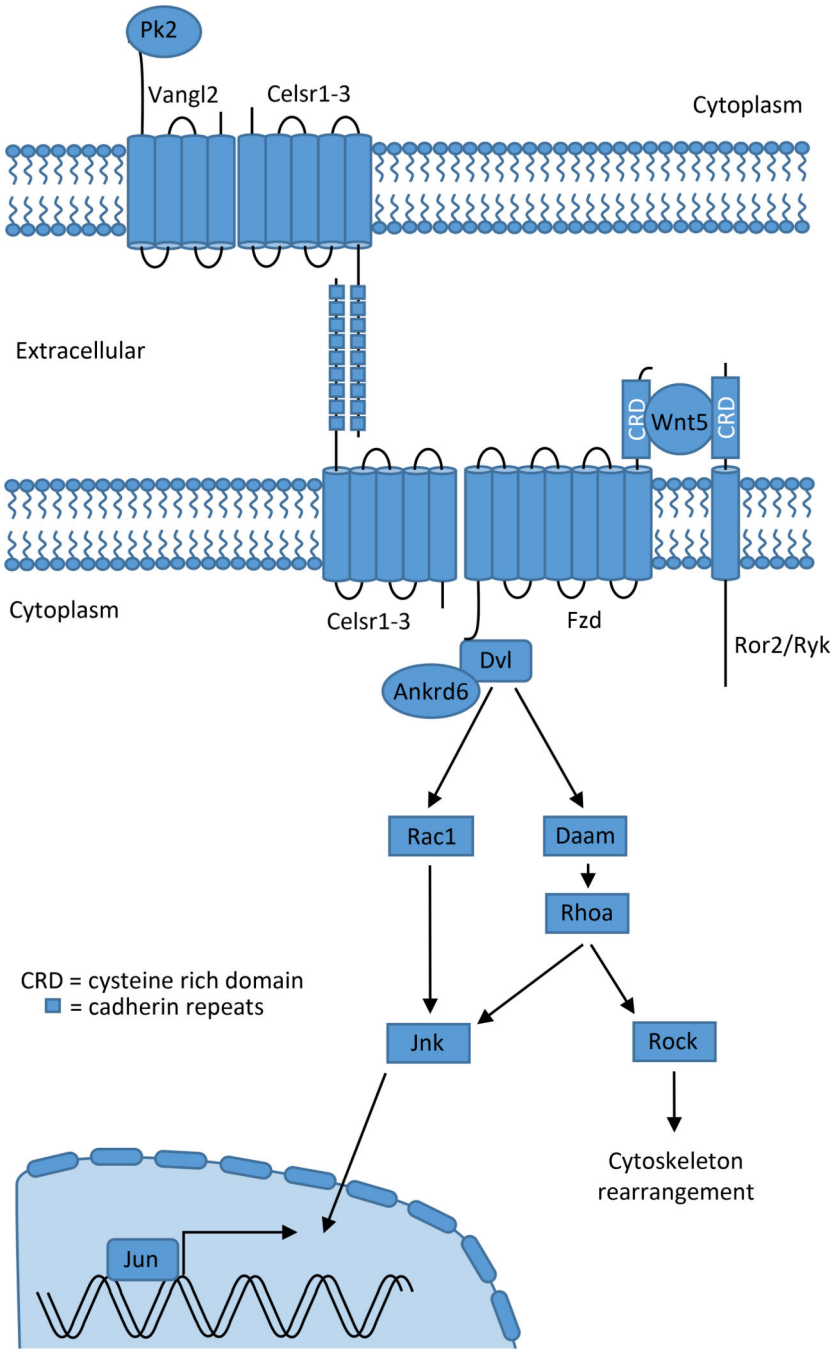

Fig. 15.4. PCP signalling.

Two complexes form at the membrane on either side of a cell, Fzd/Dvl at one end of the cell and Vangl2/Pk2 at the other side of the cell. Both complexes interact with the cadherin Celsr1–3, which enables the two complexes to interact extracellularly and form homodimers between cells. Mammalian PCP signalling also involves interaction of the Fzd/Dvl complex with the co-receptors Ror2/Ryk which enables Wnt5a to potentially act as an instructional signal. In response to Wnt ligand Dvl activates Rac1 and Daam to activate transcription of target genes via JNK/Jun and cytoskeletal rearrangement via Rock.

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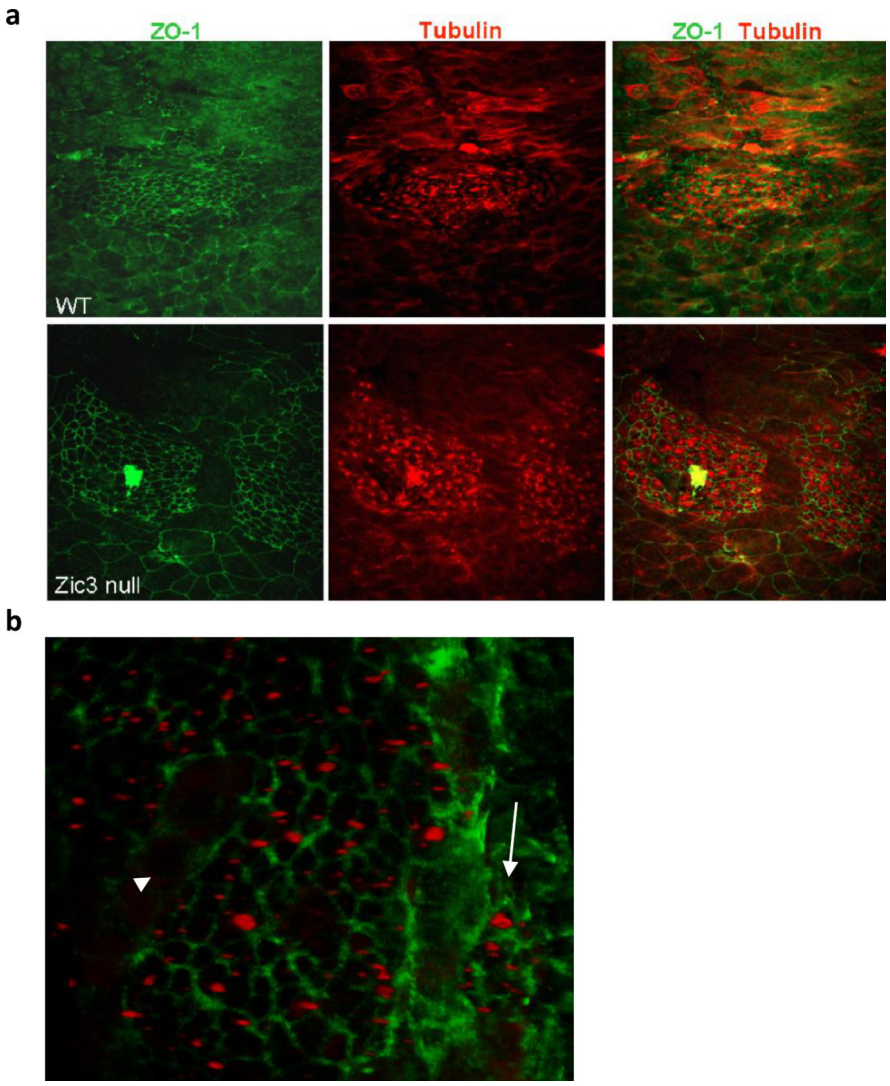

Fig. 15.5. Immunofluorescent image of a Zic3 mutant mouse node.

(a) ZO1immunohistochemistry (*green*) marks the cell borders whereas acetylated tubulin (*red*) marks the cilia. (b) A higher magnification view of the node in which phalloidin staining (green) marks the cell borders whereas gamma tubulin (red) marks the basal bodies at the base of cilia. Node cells normally cluster in a tear drop shape, but the Zic3 mutants often show ectopic foci of node cells or lack of continuity of the node ultrastructure. *Arrow* indicates a second smaller group of ciliated node cells physically separated from the other node cells. *Arrowhead* indicates a region of cells in the middle of the node which are larger and have no cilia and thus are likely endoderm cells.

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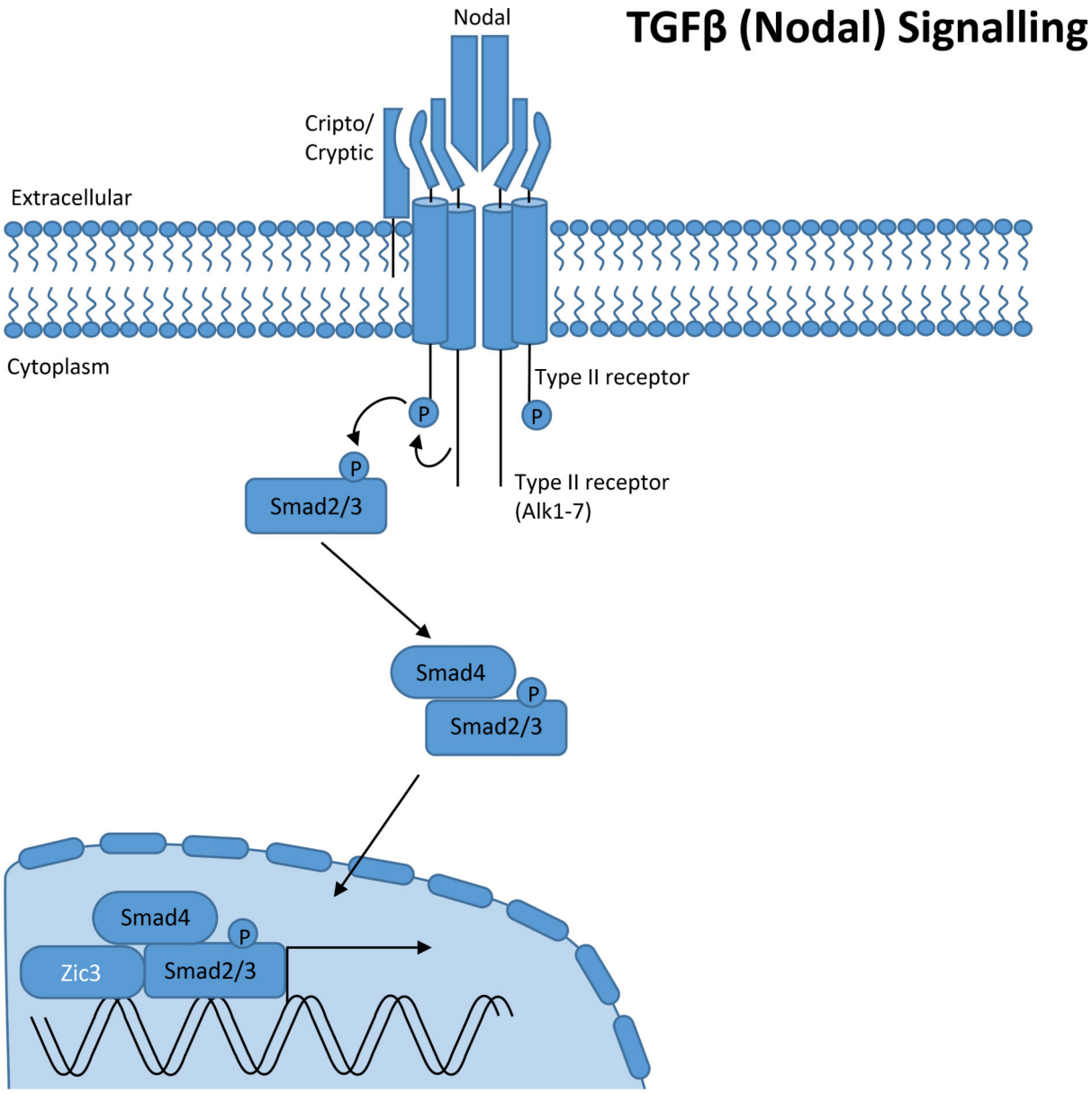

Fig. 15.6. TGFβ signalling.

TGFβ ligands interact with a heterotetrameric complex consisting of type I and type II receptors. In the case of Nodal, ligand binding also involves the co-receptor Cripto/Cryptic. The presence of Nodal causes the type II receptor to phosphorylate the type I receptor which in turn causes phosphorylation of cytoplasmic receptors Smad2 and Smad3. Phosphorylated Smad2/3 complexes with Smad4 and accumulates in the nucleus to activate target genes. Activation also requires tissue specific transcription factors and co-factors which potentially include Zic3.