Structure and function of the notochord: an essential organ for chordate development

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Summary

The notochord is the defining structure of the chordates, and has essential roles in vertebrate development. It serves as a source of midline signals that pattern surrounding tissues and as a major skeletal element of the developing embryo. Genetic and embryological studies over the past decade have informed us about the development and function of the notochord. In this review, I discuss the embryonic origin, signalling roles and ultimate fate of the notochord, with an emphasis on structural aspects of notochord biology.

Introduction

The notochord is an embryonic midline structure common to all members of the phylum Chordata (Fig. 1). In higher vertebrates, the notochord exists transiently and has at least two important functions. First, the notochord is positioned centrally in the embryo with respect to both the dorsal-ventral (DV) and left-right (LR) axes. Here, it produces secreted factors that signal to all surrounding tissues, providing position and fate information. In this role, the notochord is important for specifying ventral fates in the central nervous system, controlling aspects of LR asymmetry, inducing pancreatic fates, controlling the arterial versus venous identity of the major axial blood vessels and specifying a variety of cell types in forming somites (Christ et al., 2004; Danos and Yost, 1995; Fouquet et al., 1997; Goldstein and Fishman, 1998; Lohr et al., 1997; Munsterberg and Lassar, 1995; Pourquie et al., 1993; Yamada et al., 1993; Yamada et al., 1991). Second, the notochord plays an important structural role. As a tissue, it is most closely related to cartilage and is likely to represent a primitive form of cartilage. Accordingly, the notochord serves as the axial skeleton of the embryo until other elements, such as the vertebrae, form. In some vertebrate clades, such as the agnathans (lampreys), and in primitive fish, such as sturgeons, the notochord persists throughout life. In higher vertebrates, however, the notochord becomes ossified in regions of forming vertebrae and contributes to the centre of the intervertebral discs in a structure called the nucleus pulposis (Linsenmayer et al., 1986; Smits and Lefebvre, 2003; Swiderski and Solursh, 1992). For the ascidian (tunicate) invertebrate chordates, the notochord exists during embryonic and larval free-swimming stages, providing the axial structural support necessary for locomotion (Satoh, 2003). Similarly, for the cephalochordates, the notochord is essential for locomotion and persists throughout life (Holland et al., 2004). Thus, the notochord is essential for normal vertebrate development; but how does it arise?

Embryonic origins of the notochord
Organiser activities of notochord progenitors

In vertebrates, the notochord arises from the dorsal organiser. Originally identified by Spemann and Mangold in amphibians, the dorsal organiser is a region of a vertebrate gastrulae that, when transplanted into prospective lateral or ventral regions of a host embryo, induces the formation of a second embryonic axis, while only contributing to notochord and prechordal mesendoderm (Harland and Gerhart, 1997; Spemann and Mangold, 1924). In amphibians, this region is the dorsal lip of the blastopore. In other species, homologous structures have been found: the embryonic shield of teleost fish, Hensen's node in the chick and the node of mouse embryos all possess essentially the same activities as Spemann and Mangold's dorsal organiser (Beddington, 1994; Oppenheimer, 1936; Waddington, 1930). The functions and activities of the dorsal organiser are complex and have been discussed in detail elsewhere (Harland and Gerhart, 1997). For this discussion, it is useful to consider the relationship between specification of notochord fate and dorsal organiser activities. First, however, what are the distinct morphological stages that the dorsal mesendoderm progresses through on its way to becoming a mature notochord?

The first major transition is from dorsal organiser to chordamesoderm. During early gastrula stages, the chordamesoderm, which is the direct antecedent of the notochord, becomes morphologically and molecularly distinct from other mesoderm. Cellular rearrangements involving the mediolateral intercalation and convergence of cells towards the dorsal midline, force the chordamesoderm into an elongated stack of cells. Genetic screens in zebrafish have identified two loci, *floating head* (*flh*) and *bozozok* (*dharma* – Zebrafish Information Network), as being essential for this transition to occur (Amacher and Kimmel, 1998; Fekany et al., 1999; Solnica-Krezel et al., 1996; Talbot et al., 1995). As development proceeds, chordamesoderm cells acquire a thick extracellular sheath and a vacuole. Osmotic pressure within the vacuole acts
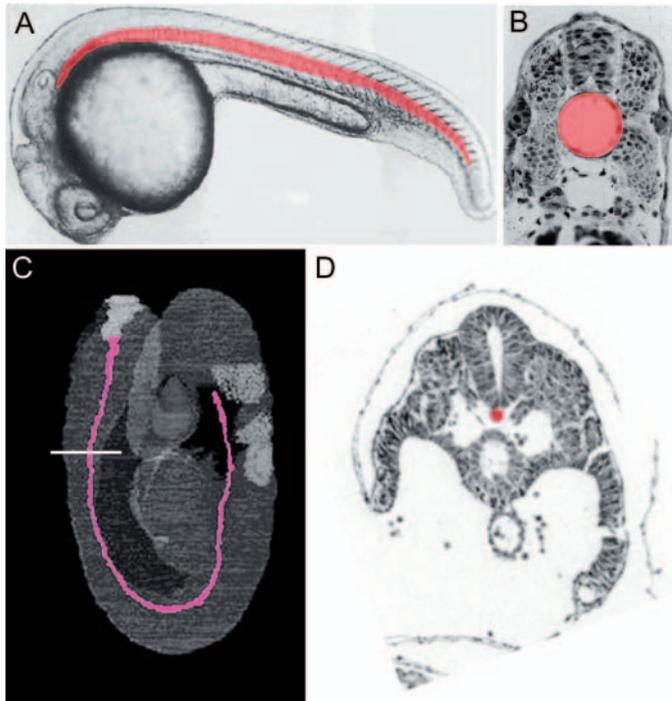
Fig. 1. A comparison of notochords. (A) At 24 hours post fertilisation (hpf), the zebrafish notochord (red) occupies a considerable volume of the embryo. (B) This is especially apparent in cross-section, where the area of the notochord is nearly equal to that of the neural tube. (C) By contrast, in an embryonic day (E) 9 mouse embryo, the notochord is proportionally smaller, (D) taking up a cross-sectional area of only a fraction of the neural tube. (A) Lateral view of live 24 hpf zebrafish embryo. (B) Trunk-level cross section of a 24 hpf zebrafish embryo. (C) Three dimensional reconstruction of an E9 mouse. White line indicates level of section shown in D, with notochord highlighted in red. (C,D) Adapted, with permission, from the Edinburgh Mouse Atlas Project (Baldock et al., 2001; Brune et al., 1999).

against the sheath, gives the notochord its characteristic rod-like appearance, and provides mechanical properties that are essential for the proper elongation of embryos and for the locomotion of invertebrate chordates and many vertebrate species (Adams et al., 1990; Koehl et al., 2000). This transition, from chordamesoderm to mature notochord, requires a host of loci that have been identified in zebrafish genetic screens (Odenthal et al., 1996; Stemple et al., 1996).

Studies of several early-acting zebrafish genes have been helpful in investigating the relationship between dorsal organiser activity and specification of notochord fate. It is clear that organiser activity, per se, is separable from the specification of notochord fate. For example, *bozozok* mutant embryos lack a morphologically distinct shield, and both *bozozok* and *floating head* mutant embryos fail to form a notochord (Fekany et al., 1999; Talbot et al., 1995). Yet in both types of mutant embryo, the embryonic axes form and embryos possess clear DV and anterior-posterior (AP) identities (Fekany et al., 1999; Talbot et al., 1995). Similarly, when organiser tissue is surgically ablated, the resulting embryos develop with clearly defined axes, but lack notochord and prechordal mesoderm, despite the fact that the ablated tissue can induce

the formation of a complete second axis in another host (Saude et al., 2000; Shih and Fraser, 1996). In addition, within the organiser region, there are separable prechordal versus notochordal fates that correlate with separable head-inducing, versus trunk/tail-inducing organiser activities. Expression of *flh* mRNA is a good prospective marker of notochord fate (Gritsman et al., 2000). In early gastrula zebrafish embryos, *flh* is expressed superficially within the organiser region. Simultaneously, another homeodomain-encoding gene, *goosecoid* (*gsc*) is expressed in deep organiser tissues. The distinct activities of deep versus superficial organiser have been measured in transplantation assays. In these experiments, deep tissue, which expresses *gsc,* was found to induce secondary axes that possess only head, whereas superficial *flh*-expressing tissue was found predominantly to produce secondary axes that possess only trunk and tail (Fig. 2) (Saude et al., 2000). The specification of notochord and prechordal fates are, however, controlled by the earlier acting processes – dorsal specification and mesoderm induction – that are also responsible for specifying dorsal organiser activities (De Robertis and Kuroda, 2004; Kimelman and Griffin, 2000; Sokol, 1999).

Mesoderm induction and dorsal specification

One key event during chordate early development is the induction of mesoderm. Defined today in terms of characteristically expressed genes that are known to correlate with future mesodermal fate, we now know many of the molecules involved in mesoderm induction, largely owing to classical embryological experiments carried out by Pieter Nieuwkoop (Boterenbrood and Nieuwkoop, 1973; Gerhart, 1999; Kimelman and Griffin, 2000). Using frog embryos, Nieuwkoop found that, when left on its own, the animal pole region, the animal cap, of a frog embryo would form only a ciliated epidermis; the yolky vegetal cells would form only endoderm. In combination, however, Nieuwkoop found that the vegetal-derived factors could convert animal cap cells to a mesoderm fate. In the past 15 years, this assay has led to the discovery of the signalling pathway that underlies mesoderm induction (Green and Smith, 1990; Schier and Shen, 2000; Smith et al., 1990). We now know that Nodal and Nodal-related proteins are secreted and serve to induce mesoderm formation. Importantly, the response of animal cap cells to Nodal is graded so that different levels of Nodal signalling lead to different mesodermal and axial mesendodermal fates. High levels of Nodal signalling specify the deep *gsc*-expressing cell fates, while lower levels specify *flh*-expressing prospective chordamesoderm (Gritsman et al., 2000).

Nodal ligands employ the TGFβ signalling pathway, including receptors that also serve as activin receptors. Nodal ligands, however, require a co-receptor of the EGF-CFC family. In zebrafish, the *one-eyed pinhead* (*oep*) locus encodes an EGF-CFC protein that is essential for Nodal signalling (Gritsman et al., 1999; Schier et al., 1997; Zhang et al., 1998). Initially characterised by zygotic loss-of-function, *oep* mutant embryos were found to be cyclopean but to possess a notochord (Schier et al., 1997). The *oep* gene product, however, is also supplied maternally, and mutants lacking both zygotic and maternal Oep lack all endoderm and mesoderm, apart from a small amount of muscle that forms in the tail (Gritsman et al., 1999). The type of mesoderm formed in response to inductive signals is dose dependent. This was first appreciated

in *Xenopus* animal cap experiments, where different concentrations of activin were found to elicit different mesodermal fates (Green et al., 1992; Green and Smith, 1990). More recently this dose dependence with respect to Nodal signalling was revealed when maternal-zygotic *oep* (MZ*oep*) mutant zebrafish embryos were compared with zygotic *oep* mutants (Gritsman et al., 2000). The major defect in zygotic *oep* mutants, in which low-level Nodal signalling still

occurs, is the lack of *gsc*-expressing prospective prechordal mesendoderm, which is the tissue that requires the highest level of Nodal signalling for proper specification. Indeed, this tissue, which only transiently expresses *gsc* in *oep* mutants, instead turns on *flh* expression and becomes chordamesoderm. The maternal supply of Oep protein is likely to be depleted with time in these embryos, suggesting that one difference between prechordal and notochord specification is that the prechordal tissue requires persistent Nodal signalling, while transient Nodal signalling is sufficient for chordamesoderm specification. Notochord specification is a stable property of the superficial dorsal organiser. This is most clearly indicated by the observation that when prospective chordamesoderm is transplanted anywhere into an equivalently staged host, the transplanted tissue will form notochord (Saude et al., 2000; Shih and Fraser, 1996).

The Nodal signalling pathway is required for specification of dorsal mesendodermal fates and for early mesoderm induction. It is not, however, required for dorsal specification or neural induction. Although endoderm and virtually all mesoderm fails to form in zebrafish MZ*oep* mutants and in mutants simultaneously lacking the Nodal ligands Squint and Cyclops, these embryos nevertheless posses DV and AP axes (Feldman et al., 2000; Feldman et al., 1998; Gritsman et al., 2000; Gritsman et al., 1999).

Relationship between notochord and cartilage

Consistent with its structural role in vertebrate development, the notochord shares many features with cartilage. It expresses many genes that are characteristic of cartilage, such as those that encode type II and type IX collagen, aggrecan, Sox9 and chondromodulin (Dietz et al., 1999; Domowicz et al., 1995; Ng et al., 1997; Sachdev et al., 2001; Zhao et al., 1997). There is, however, one clear difference between chondrogenesis and notochord formation. Chondrocytes normally secrete a highly hyrdrated extracellular matrix, which gives cartilage its main structural properties (Knudson and Knudson, 2001). By contrast, while notochord cells produce a thick basement membrane sheath, they retain hydrated materials in large vacuoles (Adams et al., 1990; Coutinho et al., 2004; Koehl et al., 2000; Parsons et al., 2002). These vacuoles allow notochord cells to exert pressure against the sheath walls, which gives the notochord its structural properties (Adams et al., 1990; Koehl et al., 2000). There may be a direct relationship between notochord and cartilage in which cartilage has evolved to secrete certain materials; by rerouting those materials to an internal vacuole, notochord cells have co-opted some of the essential structural properties of cartilage to their needs.
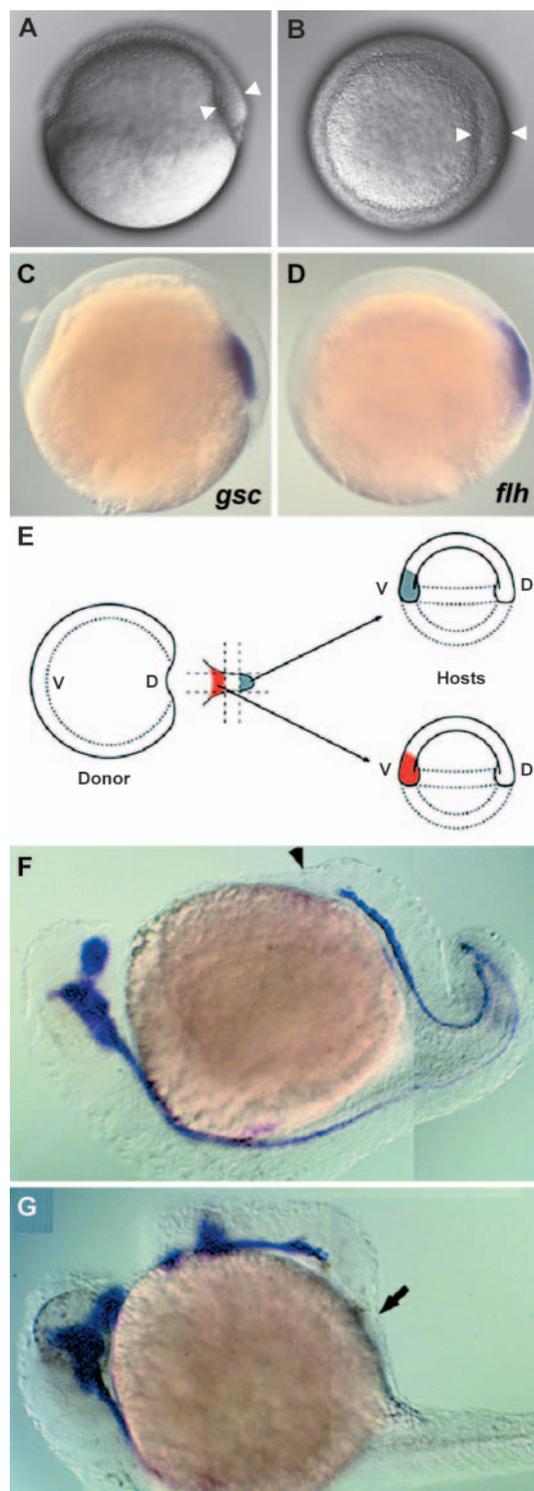

Fig. 2. Separable organiser activities. (A,B) The zebrafish dorsal organiser is the embryonic shield (between the arrowheads), and is shown in (A) lateral and (B) animal-pole views. (C) Deep organiser tissue expresses *gooscoid* (*gsc*), while (D) superficial tissue expresses *floating head* (*flh*) mRNA. (E) By separating deep (grey) and superficial (red) organiser tissues and grafting the individual pieces, superficial tissue was found to induce a secondary axis that possessed only trunk and tail (F), while deep tissue induced a secondary axis that possessed only a head (G). (F,G) Embryos are stained for *sonic hedgehog* expression by in situ hybridisation. Arrows indicate (F) anterior limit of secondary axis and (G) posterior limit of secondary axis. Adapted, with permission, from Saúde et al. (Saúde et al., 2000).

The ultimate fate of the notochord also emphasises the relatedness of notochord and cartilage. During endochondral bone formation, the type II collagen-rich extracellular matrix of cartilage is deposited with type X collagen, which signals the eventual replacement of cartilage by bone (Linsenmayer et al., 1986; Schmid et al., 1991; Solursh et al., 1986). Similarly, during the development of vertebrae, notochord that runs through the middle of each vertebra first expresses type X collagen and is then replaced by bone (Linsenmayer et al., 1986). Between the vertebrae, the notochord does not express type X collagen and is not replaced by bone, but becomes the centre of the intervertebral disc – the nucleus pulposis (Aszodi et al., 1998; Smits and Lefebvre, 2003). Thus, notochord can become ossified in a fashion similar to cartilage. Consistent with this view, in mutant mice that lack type II collagen, the notochord is not replaced by bone, presumably because the type II collagen network is required for proper deposition of type X collagen.

Evolutionary origins of the notochord

By definition, the notochord arose with the chordates; however, there are some possible hints to its origin in hemichordates. Hemichordates, along with echinoderms and chordates, constitute the three monophyletic phyla of the Deuterostomia (Jefferies, 1991; Peterson et al., 1999). Morphologically, several features of the hemichordates suggest a close relationship with the chordates. For example, in the head of hemichordates, there is a structure called the stomochord, which bears some structural resemblance to the notochord. There is, however, little direct evidence of a notochord in hemichordates. A more definitive answer is likely to come from an analysis of the expression of canonical notochord genes in hemichordates (Lowe et al., 2003), which might give a clear indication of the relationship between stomochord and notochord. An analysis of the expression of *brachyury*, a gene normally associated with notochord development, in the hemichordate *Ptychodera flava* revealed, however, that *brachyury* is never expressed in the stomochord (Peterson et al., 1999). As such, an analysis of cartilage-specific genes could also be helpful in elucidating the relationship between these structures.

In contrast to hemichordates, the ascidians have definitive notochords. A wide variety of ascidian species have been studied by developmental biologists, and much attention has been given to the notochord of ascidian larvae (Satoh, 2003). In one study, a comparison was drawn between two closely related ascidian species, *Molgula oculata* and *Molgula occulta*; the first sports a conventional ascidian tail, the other is tailless (Swalla and Jeffery, 1996). In hybrid crosses, the tail of the normally tailless species is restored and a gene called *Manx*, which encodes a zinc-finger protein, was identified by differential gene expression analysis between the two species. The disruption of *Manx* expression by antisense oligonucleotides in the tail-bearing species leads to tail loss (Swalla and Jeffery, 1996; Swalla et al., 1999). More recent screens have identified a host of notochord-specific genes and mutations that affect notochord development in ascidians (Di Gregorio and Levine, 2002; Jeffery, 2002; Moody et al., 1999; Nakatani et al., 1999; Satoh, 2003).

One process that is important to notochord development is convergent extension. A variety of studies in vertebrates have implicated the planar cell polarity (PCP) pathway in the control of convergence and extension during gastrulation. In zebrafish, for example, embryos in which PCP has been disrupted exhibit a widened chordamesoderm and a subsequent defect in tail notochord development (Hammerschmidt et al., 1996; Heisenberg et al., 2000; Jessen et al., 2002; Topczewski et al., 2001). A mutation that affects ascidian notochord development has been characterised by positional cloning and was found to encode a homologue of Prickle, which is a PCP component known to be involved in vertebrate convergent extension (Carreira-Barbosa et al., 2003; Di Jiang et al., 2005; Takeuchi et al., 2003; Veeman et al., 2003).

Roles of the notochord in vertebrate development
Patterning

The notochord has several well-established roles in patterning surrounding tissues. As these issues have been comprehensively reviewed elsewhere (Cleaver and Krieg, 2001; Dodd et al., 1998; Hogan and Bautch, 2004; Holland et al., 2004), I only mention some of the main roles here. Perhaps the best characterised is the role of the notochord in patterning the neural tube. A series of experiments involving both the transplantation and the removal of the notochord during development showed that the notochord can signal the formation of the floor plate, which is the ventral-most fate of the spinal cord (Placzek et al., 1991; van Straaten et al., 1989; Yamada et al., 1991). Among the signals secreted by the notochord are the Hedgehog (Hh) proteins. Sonic Hedgehog, in particular, induces a range of ventral spinal cord fates in a graded fashion while simultaneously suppressing the expression of characteristically dorsal genes (Placzek et al., 1993; Yamada et al., 1993; Yamada et al., 1991). Reinforcing and maintaining earlier developmental events, notochord signals are also involved in establishing LR asymmetry; the ablation of the notochord in *Xenopus* gastrulae results in randomisation of asymmetry (Danos and Yost, 1995; Lohr et al., 1997). In teleosts, notochord-derived Hh signals control the formation of the horizontal myoseptum, as well as specifying slow-twitch muscle fates (Barresi et al., 2000; Devoto et al., 1996). Notochord-derived signals are important for specifying the formation of the dorsal aorta (Cleaver et al., 2000; Fekany et al., 1999; Isogai et al., 2003; Lawson et al., 2002), as well as for normal specification of the cardiac field (Goldstein and Fishman, 1998). Finally, the notochord is important for the normal development of early endoderm and the pancreas (Cleaver and Krieg, 2001; Kim et al., 1997).

Structural

Although the patterning roles of the notochord are essential for normal vertebrate development, the notochord also has an essential structural role. The notochord is the main axial skeletal element of the chordate early embryo; without a fully differentiated notochord embryos fail to elongate (Odenthal et al., 1996; Saúde et al., 2000; Schulte-Merker et al., 1992; Schulte-Merker et al., 1994; Stemple et al., 1996; Talbot et al., 1995). For many species, this results in the inability to swim properly, to escape predation and to feed. Some understanding of this function of the notochord has been derived from studies of zebrafish mutations (Driever et al., 1996; Haffter et al., 1996). A number of loci involved in notochord formation were

identified in several large-scale systematic screens for mutations affecting zebrafish embryogenesis (Fig. 3) (Odenthal et al., 1996; Stemple et al., 1996). As I have discussed, a few of the loci identified in these screens have been found to be important for the formation of the chordamesoderm, highlighting important signalling roles of the notochord. Most loci, however, have been found to be profoundly important for structural aspects of notochord function.

To understand notochord structure, it is helpful to consider the notochord as part of a mechanical system required for locomotion (Fig. 4). Zebrafish embryos, for example, are able to flip their tails within 1 day of fertilisation, and hatch and swim within 3 days. By analogy, the notochord is like a fire hose, possessing a strong but flexible sheath that can resist high hydrostatic pressures. Consider then a situation in which the fire hose is filled with water balloons, each pushing against the other and against the sheath. In such an arrangement, the fire hose would be elongated and stiff, but able to bend in any direction. Finally, with cables running along the top and bottom of the inflated fire hose, the cables would resist any upward or downward bending of the hose, and any force acting on the fire hose would deflect it laterally. For the zebrafish embryo, the equivalent of the fire hose is the peri-notochordal basement membrane and the water balloons are the vacuolated notochord cells. Running along the top of the notochord is the floor plate and along the bottom is the hypochord. Consistent with this structural role, both the floor plate and the hypochord express a variety of cartilage proteins, such as type II collagen (Yan et al., 1995). In addition, mutations in genes such as *oep* and *cyclops* (*ndr2* – Zebrafish Information Network), which lead to substantial loss of floor plate, also produce embryos with a profound downward curvature (Hatta, 1992; Hatta et al., 1991; Schier et al., 1997). Thus, although not yet directly established, it is possible that the floor plate and hypochord act as cables on the respective dorsal and ventral sides of the notochord.

In zebrafish, the formation of the peri-notochordal basement membrane and vacuole are each affected by two phenotypic classes of mutations. One class, originally found to affect both notochord and brain development, comprises the *bashful*, *grumpy* and *sleepy* loci. These loci have recently been found to encode zebrafish laminin α1 (*bashful*), laminin β1 (*grumpy*) and laminin γ1 (*sleepy*), which are constituents of the basement membrane (Parsons et al., 2002) (S. M. Pollard, PhD thesis, University College London, 2002). The second class of mutations was originally found to affect notochord and melanophore development early in embryogenesis, leading eventually to catastrophic cell death throughout mutant embryos. This class comprises *sneezy*, *happy* and *dopey*, which encode, respectively, the α, β and γ chains of the coatomer complex, which is an important component of the secretory pathway and is essential for survival of all eukaryotic cells (Coutinho et al., 2004). The coatomer complex would normally be considered to perform a eukaryotic housekeeping function. It is surprising that loss of coatomer activity in zebrafish embryos would lead to a notochord phenotype. One explanation is that coatomer is maternally supplied, and that the forming notochord is among the first tissues in which secretory demand exceeds the maternal supply. This loss of secretory activity leads to the loss of notochord basement membrane and to a failure of vacuole formation. Together, the

zebrafish laminin and coatomer mutants highlight the importance of notochord structure to its function.

Laminins and notochord structure

Laminins are obligate heterotrimeric protein complexes that consist of an α, β and γ chain, and are integral to basement
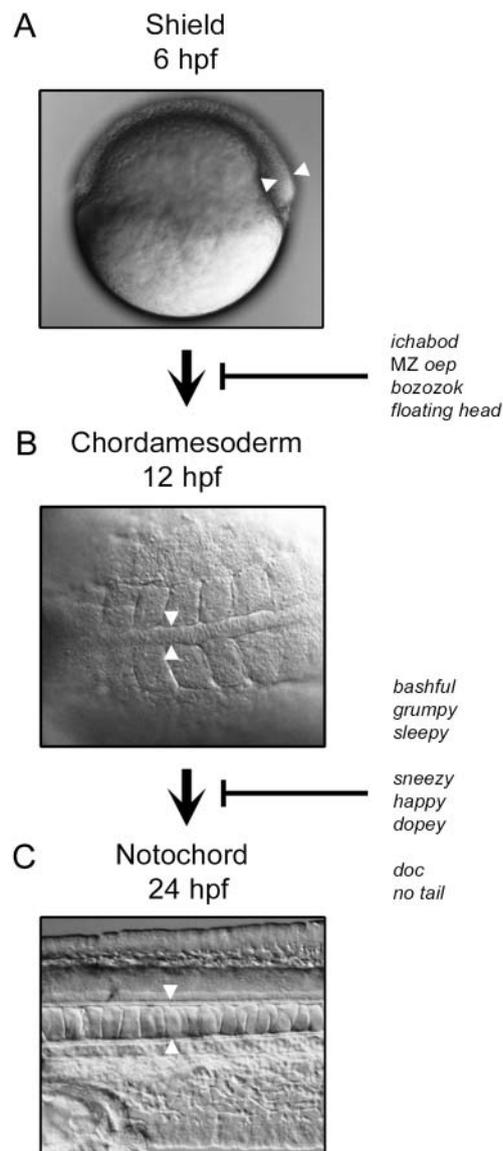

Fig. 3. Stages of notochord development affected by mutations. The transition from dorsal organiser to chordamesoderm is affected by mutations in several genes. At the top of the dorsal specification cascade are *ichabod* and β-catenin proteins (Kelly et al., 2000). In their absence, ventral types of mesoderm can form but not chordamesoderm. The *bozozok* and *floating head* loci each encode homeodomain-containing proteins that are necessary for chordamesoderm specification. In the complete absence of *oep*, as in the maternal-zygotic (Mz) mutant, mesendoderm is not specified, including the chordamesoderm. The laminins (*bashful*, *grumpy* and *sleepy*), the coatomers (*sneezy*, *happy* and *dopey*), brachyury (*no tail*) and one unknown locus, *doc*, are all required for proper notochord differentiation. Arrowheads indicate (A) chordamesoderm progenitor region of the shield, (B) the chordamesoderm and (C) the differentiated notochord.

Fig. 4. Structural aspects of the notochord. (A) A lateral view of a living zebrafish tail at 24 hpf, showing the main features of the notochord. Dorsal to the notochord is the floor plate, in the ventral-most part of the forming spinal cord. Ventral to the notochord is the hypochord. (B) A schematic diagram of lateral and cross-sections of the notochord, showing the floor plate and hypochord acting as cables running along the top and bottom of the notochord. (C) As well as the notochord, the floor plate and hypochord express type II collagen (Yan et al., 1995). cc, central canal; fp, floor plate; hy, hypochord; no, notochord; nt, neural tube.
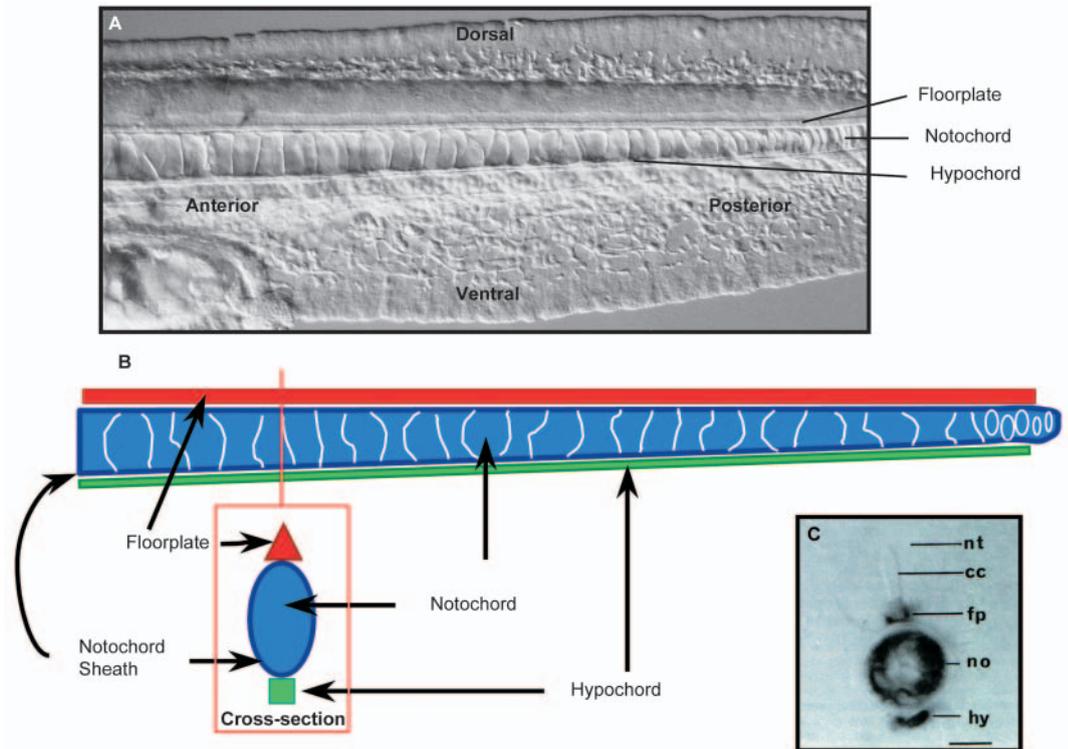
membranes. Basement membrane are ubiquitous structures with diverse roles, but they are usually associated with an epithelium. Typically in a basement membrane, laminin trimers polymerise to form a meshwork, which is crosslinked to a type IV collagen meshwork by nidogens (Colognato and Yurchenco, 2000; Iozzo, 1998; Timpl and Brown, 1996; Yurchenco and O'Rear, 1994). In addition, associated with basement membranes are heparin-sulphate proteoglycans (Iozzo, 1998). Basement membranes are important for defining boundaries between tissues, for signalling between cells and, in some situations, for providing a strong mechanical barrier (Timpl and Brown, 1996). The latter role is of particular importance in the kidney glomerulus, where the basement membrane serves as an integral part of the filtration apparatus while withstanding high hydrostatic pressures (Miner and Li, 2000). This is similar to the role that the peri-notochordal basement membrane plays.

Laminins are diverse, probably reflecting their diverse roles. In humans there are five α, four β and three γ laminin-encoding genes. Through various combinations of α, β and γ chains, as many as 12 different laminin isoforms have been identified (Colognato and Yurchenco, 2000). In zebrafish, thus far, most of the laminin genes can be found in genomic sequence, although only a few have been sequenced as full-length cDNA.

The positional cloning of the zebrafish *bashful*, *grumpy* and *sleepy* loci led to the identification of three laminin chains; α1, β1 and γ1 (Parsons et al., 2002) (S. M. Pollard, PhD thesis, University College London, 2002). The mutant phenotypes of *grumpy* and *sleepy* are indistinguishable, whereas the *bashful* phenotype is always weaker. During the development of *grumpy* and *sleepy* mutant embryos, the notochord phenotype first becomes apparent when vacuoles fail to form properly and when genes that are characteristically expressed by

chordamesoderm, such as *echidna hedgehog*, fail to be extinguished, as they would during normal notochord differentiation. Electron microscopic analysis of the peri-notochordal basement membrane shows that it is completely absent in these mutants, and antibody staining shows a complete lack of laminin 1 immunoreactivity in the most severely affected *grumpy* and *sleepy* mutant embryos. In the weaker *bashful* mutant embryos, the peri-notochordal basement membrane is missing only in the anterior affected regions of the notochord. In the posterior of these embryos, the notochord is normally differentiated and has laminin 1 immunoreactivity. The differences between *bashful* and *grumpy* or *sleepy* are due to a redundancy between laminin α chains. Disruption of laminin α4 expression in *bashful* mutant embryos, which lack laminin α1, leads to the severe notochord phenotype seen in *grumpy* and *sleepy* mutants, and a complete loss of laminin 1 immunoreactivity (S. M. Pollard, PhD thesis, University College London, 2002).

These four laminin chains – α1, α4, β1 and γ1 – participate in the formation of the peri-notochordal basement membrane, and the chordamesoderm expresses mRNA for each chain. But does the notochord alone account for the laminin in its basement membrane? This question has been addressed by an organiser graft assay. In these experiments, a wild-type dorsal organiser was grafted into the ventral region of a mutant host embryo, or vice-versa, and the resulting twinned embryos were analysed for morphological notochord differentiation. Surprisingly, for both *grumpy* and *sleepy* mutant tissues, both type of grafts led to the same result, a normally differentiated notochord (Parsons et al., 2002). An analysis of laminin immunoreactivity showed that when wild-type organiser is transplanted into mutant hosts, laminin 1 proteins surround the transplanted notochord. Thus, laminins can be
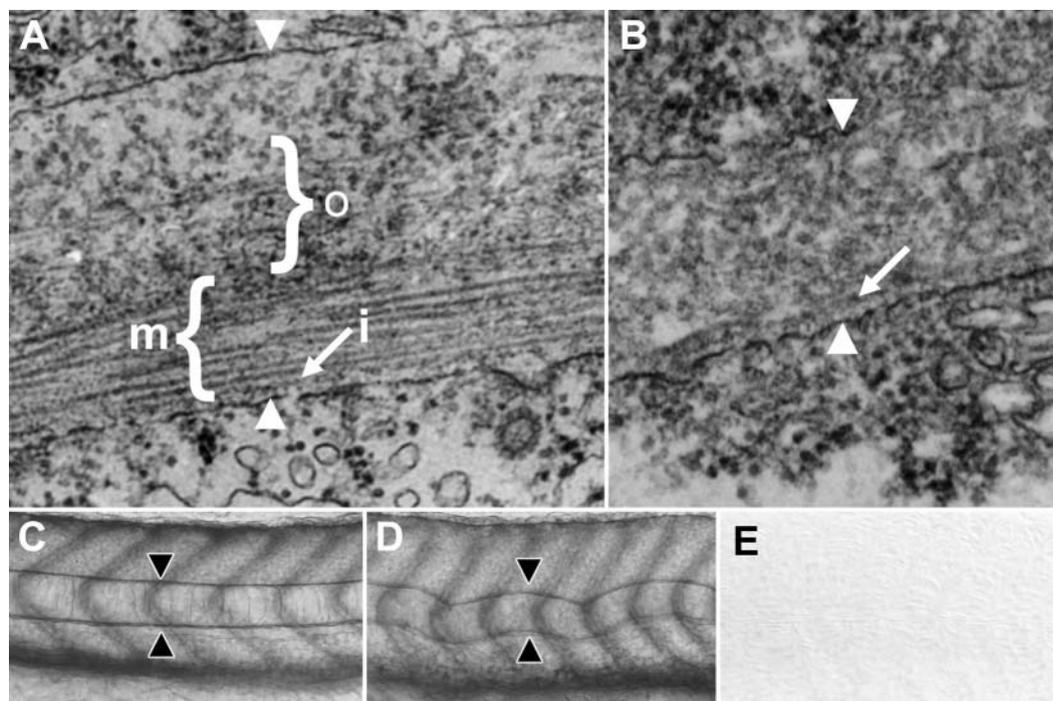
Fig. 5. Peri-notochordal basement membrane. (A) The tri-laminar structure of the peri-notochordal basement membrane (between the arrowheads) shown using electron microscopy, from the sheath of a wild-type notochord cell. i, inner layer; m, medial layer; o, outer layer. (B) This structure is disrupted in the sheath of a *dopey* mutant notochord cell, where the outer and medial layers are missing. The inner layer is likely to consist of laminin as (C) wild-type levels of laminin immunoreactivity are clearly seen in (D) *sneezy* mutant embryos, but are abolished in (E) *sleepy* mutants.

supplied either by extra notochordal sources or by the notochord itself.

There is some relationship between notochord differentiation and the presence of the basement membrane. This is likely to involve signalling from the basement membrane to chordamesoderm. The state of differentiation can be determined by analysis of gene expression. For example, *echidna hedgehog*, which is a zebrafish homologue of mammalian Indian hedgehog, is normally expressed in chordamesoderm, but when the notochord differentiates and vacuoles inflate, *echidna hedgehog* expression is extinguished (Currie and Ingham, 1996). In the mutants, however, *echidna hedgehog* is persistently expressed (Parsons et al., 2002). By contrast, when mutant notochord develops in a wild-type background, the notochord differentiates normally, suggesting that a signal residing in the basement membrane is received. One possibility is that laminin itself is the signal. There are many cell-surface laminin receptors, but disrupting several of these receptors fails to copy the laminin loss-of-function phenotype, although other phenotypes are apparent. Another possibility is that another signal residing in the basement membrane serves as a notochord differentiation cue. The presence of heparin-sulphate proteoglycans in basement membranes in general, and specifically in the notochord basement membranes, would provide the correct context for a variety of soluble growth factor proteins. The nature of the notochord differentiation signal, however, remains to be resolved.

These studies show that formation of a peri-notochordal basement membrane is essential not only to the structure of the notochord, but also for proper notochord differentiation. Indeed, loss of several other genes that are not basement membrane components, per se, but that affect formation of the peri-notochordal basement membrane, also affects the differentiation of the notochord.

Coatomers and notochord structure

Similar to the *sleepy* and *grumpy* laminin mutants, *sneezy*, *happy* and *dopey* mutants are also phenotypically indistinguishable (Coutinho et al., 2004). In these mutants, the notochord fails to differentiate, as measured both by the persistent expression of early marker genes, such as *echidna hedgehog*, and by the failure to form normally inflated vacuoles. As in the laminin mutants, the basement membrane fails to form properly. Unlike the laminin mutants, however, beginning at about 48 hours after fertilisation *sneezy*, *happy* and *dopey* mutants undergo widespread cell death by apoptosis and the embryos disintegrate. By positional cloning, we identified that *sneezy*, *happy* and *dopey* encode the zebrafish α, β and β' coatomer proteins (Coutinho et al., 2004).

Seven coatomer subunits, in combination with the small GTPase Arf1, make up the coat of COPI vesicles (Bannykh et al., 1998; Cosson and Letourneur, 1997; Lowe and Kreis, 1998; Nickel and Wieland, 1998). In eukaryotic cells, there are at least three vesicular coating systems. Clathrin-coated vesicles handle endocytosis and COPII-coated vesicles mediate the transport of secretory cargo from the endoplasmic reticulum to the Golgi. COPI-coated vesicles are thought to be largely responsible for the retrograde transport of enzymes within the Golgi that maintain its polarity, and for the machinery that transports proteins from the Golgi back to the rough endoplasmic reticulum (RER).

Electron microscopic analysis revealed two important features of notochord cells in zebrafish coatomer mutants (Coutinho et al., 2004). First, the secretory pathway is blocked, resulting in massive accumulations of materials within the RER, and disruption of the Golgi apparatus is evident. Second, the peri-notochordal basement membrane fails to form. Normally, the peri-notochordal basement membrane has a tri-laminar structure (Fig. 5). The layer closest to the plasma membrane is a single, darkly stained material with apparent

crosslinks to the underlying plasma membrane. Distal to this layer is a medial layer of fibres oriented in one direction around the notochord cell, and distal to this is an outer layer of fibres oriented in an orthogonal direction. In the coatomer mutants, the outer two layers are not present, but a distinct inner layer is often observed. Staining with laminin 1 antibody suggests that the inner layer largely comprises laminin. Using dorsal organiser grafts of mutant tissue into wild-type embryos, and vice-versa, the embryonic origin of the outer two layers of this basement membrane was investigated. Although similar grafting experiments with laminin mutants showed that laminin could be supplied either by the notochord itself or by extra-notochordal sources, grafts of coatomer mutant tissue show that both the RER and basement membrane phenotype are autonomous to the notochord. Thus, whereas the laminin-rich inner layer may be supplied by surrounding tissues, the outer layers of the peri-notochordal basement membrane are likely to be secreted by notochord cells themselves.

The notochord has two features, a thick basement membrane and a vacuole, both of which place unusual demand on the secretory pathway. Coatomer proteins are essential to all cells and both protein and coatomer mRNAs are supplied maternally. During wild-type development, mRNAs encoding coatomer proteins are expressed in chordamesoderm at a high level. Indeed, when observed by in situ hybridisation, coatomer mRNA expression appears to be chordamesoderm-specific and has a similar temporal and spatial pattern of expression to genes such as *flh*. The high-level of coatomer expression in the developing chordamesoderm is likely to be zygotic, and thus provides some explanation for how mutations in a cell-essential gene could lead to specific early developmental defects.

Conclusions

The notochord is an essential structure for the normal development of chordates. Although much attention has been focussed on its patterning roles, the notochord also has important structural roles. A more complete understanding of the mechanical basis of notochord structure is beginning to emerge from mutational analyses. Given the simplicity of the notochord as an embryonic organ, it is likely to be a useful model of organogenesis, especially for the integration of extracellular matrix formation with cellular differentiation.

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