Chapter One

Introduction

1 Introduction

In this section I shall provide a brief review of the zebrafish as a laboratory model organism, highlighting the features that make the zebrafish a highly advantageous system to study vertebrate development. I will also provide an overview of early zebrafish embryology and examine in some detail the molecular mechanisms and processes involved during development. I will pay particular attention to the development of the organiser and its derivative, the notochord. I will discuss knowledge derived from work on several model organisms, relating this to the development of the zebrafish. I will review the recent advances made in understanding processes involved in notochord differentiation, much of which stems from work on mutants generated as part of ENU mutagenesis screens (Development **123**, 1996). I will also discuss the structure and function of the notochord, emphasising its importance throughout development.

1.1 Overview of *Danio rerio*

1.1.1 The zebrafish as a model organism

The zebrafish (*Danio rerio*) has, in recent years, become a widely accepted model organism in the study of vertebrate developmental biology. The zebrafish exhibits many features that have helped make it a choice system for studying the processes of developmental biology. The zebrafish is a cheap and easy organism to

maintain, has a relatively short breeding cycle, taking only three months until it begins reproducing, a high fecundity, and produces relatively large (~0.7mm) translucent embryos that can be obtained throughout the year. The optical clarity of the embryo allows direct visualisation of individual cells and the cell movements that occur within the developing embryo. This visual accessibility, coupled with the short life cycle (a zebrafish embryo is fully formed and patterned by 5 days post fertilisation) and the external fertilisation of the zebrafish egg, makes studying the developmental processes of the zebrafish a relatively easy task. The short life cycle and high fecundity also make genetic studies a much easier venture.

As such, the zebrafish seemed the ideal organism on which to carry out the first vertebrate mutagenesis screen, similar to those carried out on *Drosophila* in the early 1980s (Nusslein-Volhard and Wieschaus, 1980). In 1996 the results of such a screen were published and an entire issue of Development (**123**; 1996) was dedicated to the characterisation and description of several hundred of the thousands of mutants isolated (Driever et al., 1996; Haffter et al., 1996). Thus, the zebrafish has a highly desirable and advantageous resource, a vast number of mutants with specific developmental defects. These mutants have been a source of intense study, helping to promote the zebrafish as a model of vertebrate development. The characterisation of these mutants and the molecular processes affected therein continue to reveal fascinating insights into the pathways involved during vertebrate development (Currie and Ingham, 1996; Holder and McMahon, 1996; Roush, 1996).

1.1.2 Brief summary of zebrafish embryology

In the first 72 hours following fertilisation, a zebrafish embryo develops from a single cell to free-swimming larvae with all its major axes and structures patterned. This 72 hour period can be separated into seven distinct phases: zygote, cleavage, blastula, gastrula, segmentation, pharyngula and hatching.

The zygote stage extends from fertilisation until the time of first cleavage and covers the first 40 minutes following fertilisation at 28ºC. During this time, the yolk cell, which consists of both yolk and cytoplasm, undergoes cytoplasmic streaming, where the cytoplasm separates from the yolk and segregates in the animal pole to form the blastodisc. This segregation continues beyond the zygote phase and into cleavage phase. After the first cleavage, the cells, known as blastomeres, undergo synchronous meroblastic cleavages every 15 minutes (Kimmel and Law, 1985). These six cleavages are confined to the animal pole and occur at regular orientations, resulting in a predictable pattern of blastomeres that is dependant on the number of cleavages that have occurred. The sixth cleavage is the first to occur in the horizontal plane, and results in a two-tiered arrangement of cells. This regular succession of cleavages continues until the tenth division, which correlates with the start of the mid-blastula transition (MBT) (Kane and Kimmel, 1993).

The MBT, which marks the beginning of zygotic transcription, occurs during the blastula period, which begins at the 128-cell stage (the 8^{th} cleavage), and continues until the start of gastrulation. During the early stages of the blastula period, cell divisions occur with some degree of synchrony, so that divisions can be seen as a wave that originates at the animal pole and then spans out to the marginal cells. It is only once the MBT occurs, at the 512-cell stage (the 10^{th} cleavage), that all synchronicity of division is lost (Kane and Kimmel, 1993). At cycle ten, cells can be divided into three distinct layers; the enveloping layer (EVL), which forms the

periderm, a layer that acts to surround and protect the developing embryo; the deep cell layer, which develops into the embryo proper and the yolk syncytial layer (YSL), which is thought to drive epiboly and to pattern the embryo at early stages. The YSL is formed when blastomeres of the marginal tier, which have remained cytoplasmically linked to the yolk cell, collapse, causing the release of their cytoplasm and nuclei into the adjoining yolk cells cytoplasm, generating the YSL. The nuclei then continue to undergo division after YSL formation, though after 3 divisions this ceases, with nuclei becoming enlarged, possibly indicating transcriptional activation (Kane et al., 1992), (Trinkaus, 1992).

As the embryo continues to develop, it eventually undergoes epiboly, which is the first major cell/morphogenetic movement of the developing embryo. Epiboly involves a coordinated movement of the cells of the blastoderm from their animal location towards the vegetal pole, so as to surround the yolk cell. The force necessary for this movement is generated through connections between the marginal cells of the EVL and the YSL, which is itself attached to force generating microtubules within the yolk cell. The gastrulation movements of involution, which marks the beginning of gastrulation at 50% epiboly when cells of the germ ring are subducted to form multiple layers, and of convergent extension, where cells stream to the dorsal side of the developing embryos, occur alongside epiboly movements (Solnica-Krezel et al., 1995). It is during epiboly that the hypoblast, a layer of cells residing between the epiblast and the yolk cell, is specified at the germ ring, the major axes of the embryo are also established and cells are first specified to distinct fates.

At approximately 50% epiboly, involution of marginal cells begins, forming the germ ring, which is visible as a thickening of the marginal region. This marks

the beginning of gastrulation, which acts to produce the three layered body plan of triploblastic organisms. There is a pause in epiboly shortly after 50% (approximately 20 minutes at 24ºC) at which point the embryo reaches shield stage, so named due to the formation of the dorsal organiser, called the embryonic shield in zebrafish. The shield marks the first obvious morphological identifier of the dorsal side and is the zebrafish equivalent to the node in mouse, Hensen's node in chick and Spemann's organiser in amphibians. Studies in zebrafish have established that transplantation of the shield to the ventral side of a host embryo is able to induce the formation of a complete secondary axis (Saude et al., 2000; Shih and Fraser, 1996). As epiboly continues, cells begin to converge on the dorsal region, the embryo extends along the Anterior-Posterior (AP) axis and the shield differentiates to form the axial mesoderm, which includes the notochord.

By approximately 10 hours post fertilisation, the embryo has reached tailbud stage. This stage marks the end of gastrulation, all major body axes are formed and the three germ layers are specified and organised. As the embryo progresses beyond tailbud, it begins the segmentation period, as first defined by formation of the somites. It is during during this stage that the embryo begins to elongate and tissues begin to differentiate. One of the earliest tissues to fully differentiate is the notochord.

Somitogenesis begins at the start of the segmentation period and represents one of this periods major events. Around 30 to 34 somites are formed, sequentially, in blocks along the AP axis from paraxial mesoderm. Somites form in pairs on either side of the notochord in the trunk and tail. The somites are blocks of undifferentiated mesenchyme surrounded by an epithelial layer and eventually differentiate into myotome and sclerotome, which will differentiate into segments of body muscle and

vertebral cartilage, respectively. The notochord plays a vital role in the choice between these two fates. The somites represent one of three segmental structures that form during this period; the other structures being rhombomeres, within the CNS, and the pharyngeal arches, which form the jaw and gills. The nervous system develops concurrently with somitogenesis, during which the neural plate undergoes an obvious thickening along the embryonic axis and the polster, a structure that will later form the hatching gland, develops at the anterior end. Analysis of neural markers makes it evident that, even at this early stage, a large degree of patterning has already taken place. By 24 hpf, the segmentation period is coming to an end, as characterised by a completion of somite formation, differentiation of blood and the first heartbeats. By this stage, the first fully differentiated structure, the notochord, has formed.

The final period before hatching occurs is known as the pharyngula period. Several structures necessary for the development into a free swimming and feeding larva are elaborated during this period, including, most obviously, the fins, jaws and gills. After two days, hatching of the developing embryo occurs. By approximately 4 days, all major organ systems have completed their extensive morphological movements. Hence, just 96 hours after fertilisation, the embryo has developed into a complex free-swimming fish. The events that occur during this time characterise the major challenges of developmental biology. The major aim of this thesis is to enhance the current understanding of the processes involved in the development of the notochord.

1.2 Early zebrafish Development

Recent work in the field of developmental biology has vastly increased our understanding of the stages and processes that occur during early vertebrate development. Advances in the field of molecular biology have made forward genetic studies increasingly plausible and the combination of these with classical embryological work and reverse genetic screens have revolutionised our understanding of the molecular and cellular processes involved during development. In the following sections the current understanding of the molecular processes involved in early development, specifically, the processes that are involved from fertilisation until gastrulation, will be discussed, including the mechanisms involved in the establishment of the three germ layers (ectoderm, endoderm and mesoderm), in forming the major body axes and in early patterning. It is during these early stages that the organiser is formed. The organiser constitutes a vital signalling centre that will eventually form the prechordal plate, hatching gland and, most relevantly to this thesis, the chordamesoderm, which itself differentiates to form the notochord.

Much of what is known about dorsal specification has been established through work on the systems involved in amphibian development. Such work has demonstrated that these amphibian systems are highly similar to those involved in the development of other vertebrate embryos. Thus, much of what is discussed concerning organiser will describe knowledge of *Xenopus* development, with comparisons and parallels being made to what has been established in the zebrafish.

1.2.1 Formation of the dorsal Organiser

Experiments published in 1924 by Spemann and Mangold identified the dorsal organiser, by virtue of its ability to induce a secondary axis when transplanted to the ventral side of a host embryo. The organiser functions through intercellular signals that act in several distinguishable roles. The organiser acts to dorsally pattern to the mesoderm, induces convergent-extension movements of the ectoderm and mesoderm and induces neurectoderm, providing signals to pattern the neurectoderm along the anterior-posterior axis (Harland and Gerhart, 1997). Transplantation studies have shown that structures equivalent to the amphibian Spemann organiser are present in the embryos representing the major vertebrate phyla. In teleost fish, such as zebrafish, the dorsal organiser is known as the embryonic shield (Saude et al., 2000; Shih and Fraser, 1996). In avians, the dorsal organiser is known as Hensen's node, and in mammals, the node (Beddington, 1994; Waddington, 1932). To easily understand the formation and specification of the organiser, it is both convenient and easy to divide organiser development into two processes; the determination of the dorsal side and the induction of mesoderm.

Dorsal specification in amphibians relies on the translocation of maternal, vegetally localised factors to the future dorsal side. By the first cleavage of an amphibian zygote, the vegetally localised dorsalising factors are segregated by a process known as cortical rotation (Gerhart et al., 1989; Vincent and Gerhart, 1987). However, recent work has suggested that cortical rotation may not be the only method of segregation. Initially, the observation that the vegetally localised dorsalising activity is broadly distributed during cortical rotation (Kageura, 1997; Sakai et al., 1996) and the observation that membrane bound organelles were able to

translocate faster than movement solely by cortical rotation would allow (Rowning et al., 1997). Further work, coupling dorsalising agents with GFP again demonstrated the rapid dorsally directed movement of the particles and demonstrated a link between the organisation of microtubules and the movement of the dorsalising factors (Miller et al., 1999; Weaver et al., 2003).

However the translocation of vegetally localised dorsal determinants occurs, the event itself acts to establish a group of vegetal cells, shown by Nieuwkoop to be capable of inducing a full secondary axis without itself contributing to the axial tissues and named, in honour of this fact, as the Nieuwkoop centre (Nieuwkoop, 1973). This group of cells then act as a signalling centre that induces the formation of organiser. However, neither the dorsal determinants, nor the Nieuwkoop centre signals are understood in precise detail. A clue to their identity was initially provided by the observation that the secreted signalling molecule, Wnt1, could induce a secondary axis in *X. laevis* when over expressed (McMahon and Moon, 1989). The details of Wingless/Wnt signalling in *Drosophila melanogaster*, in particular the protein armadillo, which was shown to play a key role in Wingless signal transduction, helped resolve the factors responsible for Wnt1's dorsalising ability (Peifer et al., 1991; Peifer and Wieschaus, 1990; Riggleman et al., 1989). The vertebrate homologue of armadillo, β-catenin, was known to be associated with the cell adhesion complexes of the Cadherin class and antibodies directed against β-catenin were found to result in axis duplication in *Xenopus* (McCrea et al., 1993). Over expression of β-catenin in either *X. laevis* or zebrafish was found to induce formation of a full secondary axis (Funayama et al., 1995; Kelly et al., 1995). Although the precise method of β-catenin's action in inducing organiser formation is not understood, it is clear that it is not β-catenin itself, as the vegetal cytoplasm of β-

catenin depleted embryos is still able to induce a secondary axis in host embryos (Marikawa and Elinson, 1999). However, recent evidence has provided likely candidates in the form of upstream factors involved in the stabilisation of β-catenin, such as GBP, *dishevelled* and GSK3 binding protein, which have been shown to be essential for dorsal specification (Miller et al., 1999; Weaver et al., 2003; Yost et al., 1998). In concert with transcription factors of the TCF/LEF family, β-catenin induces the expression of genes such as *siamois* and *twin* in *X. laevis,* which are thought to participate in organiser specification (Laurent et al., 1997; Lemaire et al., 1995; Moon and Kimelman, 1998; Nelson and Gumbiner, 1998).

Thus, cortical rotation in amphibia, which has been shown to be microtubule dependent, leads to the stabilisation and activation of β-catenin and the subsequent formation of a Nieuwkoop centre. The equivalent process in teleost fish is not clear, but does apparently culminate in the localised activation of β-catenin at the dorsal side. Direct manipulations of developing zebrafish embryos have been particularly helpful in defining the zebrafish equivalent of a Nieuwkoop centre. For example, in studies where the vegetal third of the yolk cell is removed within 20 minutes post-fertilisation, the embryo becomes completely ventralised (Ober and Schulte-Merker, 1999). Such embryos lack all dorsal mesoderm, neurectoderm and the most anterior 14-15 somites, indicating that a dorsal determinant, localised vegetally within the yolk, acts to specify the organiser. In other studies, disruption of microtubules within the early embryo has shown that an activity, located in the vegetal hemisphere and dependant on microtubule transport, is necessary for the formation of the shield and the construction of correct axes in the embryo (Jesuthasan and Stahle, 1997). Thus, although no obvious cortical rotation takes place in activated zebrafish eggs, a microtubule dependent process, possibly

analogous to that in *Xenopus*, is apparently required for the proper activation of β-catenin in the correct region.

The maternal mutant *ichabod* provides additional clues as to the nature of β-catenin localisation and activation. Mutant embryos are severely ventralised and closely resemble the ventralisation generated via removal of the vegetal yolk region. Embryos from a homozygous mutant mother can be rescued through injection of β-catenin (Kelly et al., 2000). Thus, it can be suggested that activation of β-catenin, specifically on the dorsal side by some unknown factor, possibly involving *ichabod,* produces organiser inducing activity, which may reside in the YSL, marginal blastomeres or both (Schneider et al., 1996). Indeed, recent work has provided evidence to suggest that the zebrafish functional equivalent of the Nieuwkoop centre is distributed between both the YSL and the dorsal marginal blastomeres. Injection of RNAse has shown that RNAs within the YSL are required for its ventrolateral and mesodermal inductive capabilities, as well as the proper expression of Nodal related genes in ventrolateral marginal blastomeres (Chen and Kimelman, 2000). However, it was also shown that YSL localised mRNAs are not essential for the induction of the dorsal mesoderm, suggesting that dorsal specification is due to the stabilisation of β-catenin in dorsal marginal blastomeres.

Dorsal activation of β-catenin in *X. laevis* is known to induce the expression of organiser specific homeodomain transcription factors, including *siamois* and *twin* (Laurent et al., 1997; Lemaire et al., 1995; Moon and Kimelman, 1998; Nelson and Gumbiner, 1998). The zebrafish gene *bozozok*/*dharma*/*niewkoid (boz)* encodes a homeodomain containing protein that is also regulated by β-catenin (Koos and Ho, 1998; Shimizu et al., 2000; Yamanaka et al., 1998). *boz* mutant embryos show a

complete lack of the axial mesendoderm tissues of notochord and prechordal plate (Fekany et al., 1999; Koos and Ho, 1999). Thus, though *boz* differs from *siamois* and *twin* in primary sequence, they appear to have similar roles in organiser specification and there are several lines of evidence that suggest a role for *boz* downstream of β-catenin in organiser specification (Ryu et al., 2001). Over expression of a mRNA encoding a constitutively active β-catenin is able to induce *boz* expression in wild type embryos and is also able to induce axis duplication in *boz* mutant embryos. However, this does not rescue the lack of axial mesendoderm phenotype. Contrasting this, over expression of a constitutively active type I activin receptor, Taram-A, in *boz* mutant embryos is able to induce both axis duplication and rescue the loss of axial mesendoderm (Fekany et al., 1999; Renucci et al., 1996). Finally, injection of *boz* mRNA is sufficient to rescue ventralised *ichabod* mutants (Kelly et al., 2000).

So, though it is clear that *boz* is involved in dorsal specification, there appear to be key organiser activities that do not involve *boz*. Severely affected *boz* mutant embryos have an incomplete organiser, failing to express dorsalising factors such as *chordin* and *dkk1*. These embryos also lack axial mesendoderm and show defects in anterior neural specification. Complete removal of the shield region can replicate this range of phenotypes, resulting in a loss of tissues derived from the shield region and in central nervous system (CNS) patterning defects (Saude et al., 2000). In spite of the CNS defect in both *boz* and shield ablated embryos, both the anterior-posterior (AP) and dorsal-ventral (DV) axes are specified properly, thus suggesting that *boz* is involved primarily in the specification of axial mesendoderm and that other factors are involved in specifying the organisers neural inducing and neurectodermal patterning activities.

Nieuwkoop demonstrated that a signal, which originates from the vegetal region of the embryo, is responsible for the induction of mesoderm in the cells located at the embryonic equator. This observation was exploited to identify secreted molecules that are able to act in the process of mesoderm formation. Indeed, such screens identified members of the fibroblast growth factor (FGF) family, and transforming growth factor β (TGFβ) superfamily as being able to induce mesoderm. Among these, Activin was demonstrated to be able to act as a morphogen, since it is able to induce varying mesoderm types that are dependent on the concentration of Activin (Asashima et al., 1990; Green and Smith, 1990; Smith et al., 1990). Furthermore, Activin is sufficient for the formation of dorsal mesoderm, i.e. organiser (Piepenburg et al., 2004; Smith et al., 1990). However, more recently Nodal related proteins have also been implicated as essential inducers, where both Nodals and Activin are known to operate though a common signal transduction mechanism (reviewed in (Schier and Shen, 2000)).

Loss of function studies in *X. laevis* have implicated VegT, a member of the T-box transcription factor family, in the control of initial Nodal related gene expression (Horb and Thomsen, 1997; Lustig et al., 1996; Stennard et al., 1996; Zhang and King, 1996). VegT is initially localised to the vegetal region and at the start of zygotic transcription activates zygotic signals that are vital to the correct patterning of the developing embryo. Indeed, in the absence of VegT activity there is a failure of Nodal-related growth factor expression (Kofron et al., 1999; Xanthos et al., 2001). It has also become apparent that the major targets of VegT are in fact the Nodals (Clements et al., 1999). Analysis of the promoter regions of *Xnr 1* (*Xenopus* nodal related 1) identified T-box binding sites, suggesting that VegT may act directly to up regulate the Nodals (Hyde and Old, 2000). Where there are six nodals in

Xenopus, three in zebrafish and one nodal in mouse. Although a zebrafish homologue of VegT has been identified, encoded by the *spadetail* locus, it is not expressed maternally and the phenotype resulting from loss of *spadetail* function does not produce the same range and severity as loss of VegT function in *X. laevis* (Griffin et al., 1998). However, recent work has identified the T-box protein Eomesodermin (Eom) as having an important role in organiser formation in zebrafish (Bruce et al., 2003). *Eom* is expressed in a manner resembling *VegT* expression in frog embryos, where *Eom* is expressed specifically on the dorsal side of the embryos shortly after the MBT. Removal of Eom function was noted to cause defects in organiser gene expression, with over-expression of *Eom* resulting in the formation of secondary axes. However, it was also noted that expression of zebrafish *Eom* was unable to rescue VegT depleted frog embryos, thus suggesting that though *Eom* is expressed maternally in zebrafish, much like VegT in *Xenopus*, they are not functionally equivalent leaving open the possibility that another, as yet uncharacterised T-box protein may be acting during early zebrafish development.

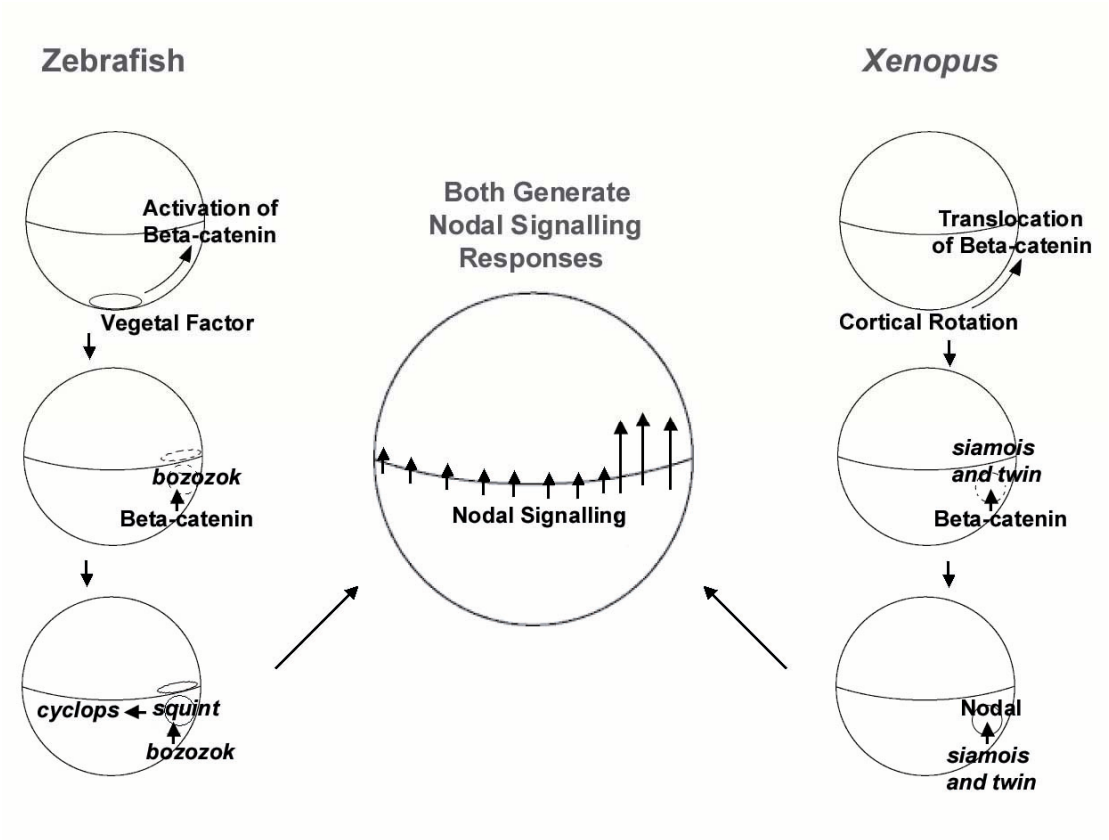
Genetic studies in mouse and zebrafish have demonstrated the essential nature of Nodals in mesoderm induction (Conlon et al., 1994; Feldman et al., 1998; Rebagliati et al., 1998; Sampath et al., 1998; Zhou et al., 1993). Two of the zebrafish nodal-related proteins, Squint and Cyclops, play essential though partially redundant roles in the specification of zebrafish mesendoderm. Double mutants for both Squint and Cyclops demonstrate a complete lack of endoderm and mesoderm, with the exception of a few somites in the tail (Feldman et al., 1998). This phenotype is replicated by maternal/zygotic (MZ) loss of the Nodal co-receptor One-eyed pinhead or the over expression of Nodal antagonists, such as Antivin/Lefty-1 (Gritsman et al., 1999; Thisse and Thisse, 1999). Fish embryos lacking *schmalspur* lack floorplate,

demonstrate reduced prechordal plate and have no medial mid or hindbrain (Brand et al., 1996). This mutation was identified in the zebrafish mutagenesis screen and encodes FoxH1, a transcription factor downstream of nodal signalling (Sirotkin et al., 2000). Despite the lack of mesoderm in Nodal mutants, embryos still possess a neuraxis with distinct anterior and posterior identities (Feldman et al., 2000; Feldman et al., 1998). Thus, at least two properties of organiser activity, neural induction and neural AP patterning, are present in the absence of the nodal derived organiser. However, ventralised embryos generated through removal of the vegetal yolk region lack not only the tissues absent in the Nodal mutants, but also the neurectoderm. Suggesting that other signals, possibly an FGF or another, as yet unidentified signal, acts to induce and pattern the neurectoderm (Reim and Brand, 2002; Streit et al., 2000).

The differentiation of mesoderm in response to nodals is complicated by the activity of mesoderm inducers of the bone morphogenic protein (BMPs) family. Several BMPs are able to induce a ventral/posterior type mesoderm (Fainsod et al., 1994; Hemmati-Brivanlou and Thomsen, 1995; Schmidt et al., 1995). In addition, over-expression of BMPs has been shown to prevent the formation of dorsal mesoderm (Schmidt et al., 1995). In light of this, considering several BMPs are expressed within the lateral/ventral margin, it is not unreasonable to assume that BMPs normally act as antagonists, favouring the formation of ventral/lateral mesoderm over dorsal mesoderm. Indeed, secreted inhibitors of BMPs, which include noggin, chordin and follistatin (Hemmati-Brivanlou et al., 1994; Piccolo et al., 1996; Zimmerman et al., 1996), are among the earliest dorsal-specific genes to be expressed.

In addition to members to the BMP family, Wnt signalling is also known to play an essential role in dorsal ventral patterning of the developing mesoderm (Christian et al., 1991; Christian et al., 1992). While activation of the canonical Wnt signalling pathway, involving β-catenin, will specify dorsal identity during cleavage stages, zygotic activation of the pathway will suppress organiser formation. For both the BMPs and Wnts and their antagonists, it is not clear if they have a definitive role in the establishment of organiser tissue, though they clearly are important in organiser function (Harland and Gerhart, 1997). Recent work in the zebrafish has helped define the method of Wnt8's repression of organiser formation. The transcriptional repressors Vox and Vent are direct transcriptional targets of BMP and Wnt8 signalling and embryos lacking both Vox and Vent have expanded organisers, similar to that observed in Wnt8 loss of function embryos, linking the action of Wnt8 in establishing the ventral side of the embryo directly with the up-regulation of Vox and Vent (Ramel and Lekven, 2004).

Thus, the earliest post-fertilisation events act to establish a gradient of activated, nuclear-localised β-catenin, the peak activity of which lies at the future dorsal side of the embryo. Independently of this, vegetal signals act to specify marginal, mesendodermal fates. The coincidence of high-levels of activated β-catenin with vegetally derived signals that are acting to specify mesendoderm, serves to specify the organiser as distinct from basic ventrolateral mesendoderm. At the dorsal side, high levels of Nodal activity are sufficient to specify dorsal organiser fate. In this situation Nodals are critical for mesoderm formation laterally and ventrally. Obvious comparisons can be made between this process in both *Xenopus* and Zebrafish (**Figure 1.1**).



Zebrafish:

Vegetal Factor → β-catenin → *bozozok* → Nodal

***Xenopus*:**

Cortical Rotation → β-catenin → *siamois* and *twin* → Nodal

Figure 1.1 Establishment of the dorsal-ventral axis in *Xenopus* and zebrafish.

A vegetally located factor in zebrafish and cortical rotation in *Xenopus* results in the activation of β-catenin specifically at the dorsal side in the region of YSL/Nieuwkoop centre formation. This results in the expression of *boz* in the zebrafish and *siamois* and *twin* in frog, both of which are thought to act to amplify the maternal signal, which results in the induction of nodal signalling. Nodal signalling then acts to pattern the developing mesoderm, which include the developing organiser.

1.2.2 Properties of the Dorsal Organiser

Organiser transplantation studies have revealed much about the structure and function of the organiser. Initially, work by Oppenheimer demonstrated that the *teleost* embryonic shield is the equivalent of the amphibian dorsal organiser (Oppenheimer, 1936). This has been confirmed in more recent zebrafish studies (Saude et al., 2000; Shih and Fraser, 1996). In these more recent studies, micro-dissection of organiser tissue demonstrated that the shield has separable head and trunk/tail organiser activities (Saude et al., 2000; Zoltewicz and Gerhart, 1997). In such studies, it was shown that the shield consists of a superficial epiblast layer and a deeper hypoblast layer sitting on the yolk cell, both covered with the tight-epithelial EVL. Donor tissue, dissected to enrich for deeper layer cells, was often able to induce second axes possessing anterior structures but completely lacking posterior structures, while superficial layer donor tissue was often found to induce axes consisting only of posterior structures. When the two layers are transplanted together a complete second axis was induced in the majority of experiments (Saude et al., 2000).

Expression patterns of dorsal-specific genes within the shield complement the experimental embryology. By the time the morphological shield is apparent, the expression of the homeobox genes *goosecoid* (*gsc*) and *floating head* (*flh*) is specifically restricted, since the expression of *gsc* and *flh* confined to the deep and superficial layers respectively. These regions are fated to develop into the prechordal plate and notochord (Gritsman et al., 2000; Stachel et al., 1993; Talbot et al., 1995). Prior to the formation of the embryonic shield the region fated to form prechordal plate resides close to the blastoderm margin, whereas the notochord progenitors are

situated further from the margin (Gritsman et al., 2000; Melby et al., 1996) (**Figure 1.2**). Studies on the induction of both *gsc* and *flh* in the organiser have shown that the differential activity of nodal is necessary for the correct patterning of the organiser (Gritsman et al., 2000). Over-expression of *sqt* and *cyc* induces *flh* at low doses and both *flh* and *gsc* at higher doses, demonstrating that Nodal signalling is vital for proper patterning of the organiser before gastrulation.

The defining properties of the organiser are understood primarily in the context of grafting experiments (reviewed in (Harland and Gerhart, 1997)). In such experiments, organiser tissue is capable of inducing neural development in tissue that would otherwise form non-neural ectoderm and patterns adjacent mesoderm to a dorsal fate. In searching for factors that have a role in organiser function, one successful approach has been to screen cDNA libraries to identify proteins able to induce dorsal structures in *Xenopus laevis*. Many genes identified in such a way have been found to be expressed within the organiser and have been demonstrated to have roles in the patterning activities of the organiser. Among the most abundant types of molecules identified in these screens are secreted antagonists of BMP or Wnt signalling, such as Noggin, Chordin and Follistatin, which antagonise BMP activity and prevent ventralisation. Such action promotes the development of dorsal mesoderm and neural fates (Hemmati-Brivanlou et al., 1994; Piccolo et al., 1996; Zimmerman et al., 1996). Similarly, several antagonists of Wnt signalling have suggested roles in the control of DV patterning of mesoderm and AP patterning of the ectoderm (Bradley et al., 2000; Kazanskaya et al., 2000). This growing list of molecules includes Dickkopf (Dkk1) and secreted forms of Frizzled receptors, FrzB, Crescent and Sizzled .

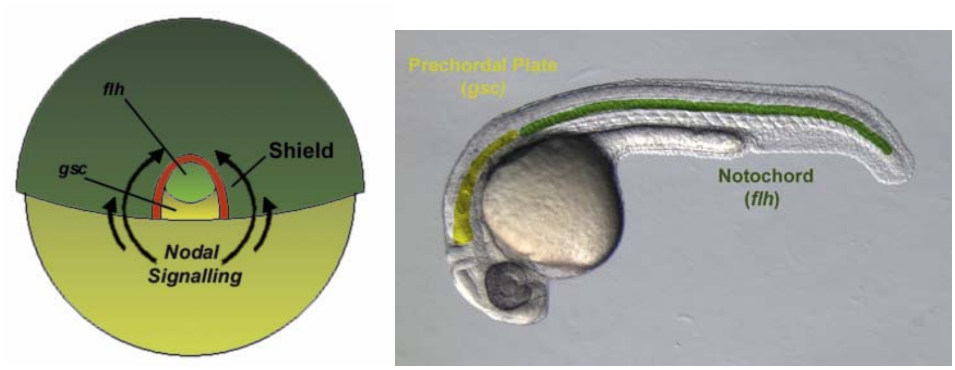


Figure 1.2 Patterning of anterior and posterior shield regions.

Left. Nodal signals pattern the organiser (shield) to form two distinct types of shield tissue, depending on the level of nodal signalling encountered. The highest levels of nodal signalling give rise to the deep, *gsc* expressing, domain, while lower levels of nodal give rise to the superficial, *flh* expressing, fated domain. These domains are fated to form the prechordal plate and the notochord respectively.

Right. In a 24-hour embryo, the prechordal plate and notochord are highlighted to show the fate of the shield regions. The deep (*gsc*) cells in yellow give rise to the prechordal plate and the superficial (*flh*) cells give rise the notochord

Genetic screens in zebrafish have also helped isolate several genes underlying the organiser's inductive capabilities. The mutants *swirl*/BMP2b, *snailhouse*/BMP7 and *somitabun*/Smad5 all encode components of the BMP signalling pathway and mutant embryos are substantially dorsalised (Hild et al., 1999; Kishimoto et al., 1997; Schmid et al., 2000). Recently, the zebrafish locus *ogon* has been found to encode Sizzled, which, similar to *Xenopus* Sizzled, was found not to inhibit Wnt8 activity but instead to modulate BMP signalling in a chordin dependent fashion, since Sizzled functions differently to Wnt inhibitors Dkk1 and Crescent and since Chordin was required for Sizzled dorsalisation (Collavin and Kirschner, 2003; Salic et al., 1997; Yabe et al., 2003). So, the model of organiser activity is one in which secreted factors that act to antagonise BMP and Wnt, establish a DV gradient within the mesoderm specifying different fates at different levels (De Robertis et al., 2001; Harland and Gerhart, 1997). While such a simple model is attractive, it does not fit several observations concerning the specification of, for example, blood, which is considered to be the most ventral mesodermal fate, though it in fact arises from nearly all regions of the mesoderm (Lane and Sheets, 2005). In addition, specification of what is considered to be the most dorsal mesoderm fate, trunk chordamesoderm, is relatively unaffected by increased or decreased levels of BMP signalling, as seen in the many zebrafish mutants that are defective in some component of BMP signalling. Thus, it appears that BMPs and zygotic Wnts act in a complicated and not yet fully understood mechanism, to pattern the established mesendoderm.

Direct ablation of organiser tissue has been achieved both genetically, as seen in *boz* mutant embryos, and surgically (Fekany et al., 1999; Saude et al., 2000; Shih and Fraser, 1996). In either case, despite the lack of organiser derived tissue,

embryos are able to develop with an essentially complete AP axis, i.e. there is a head, a spinal cord, a trunk and tail somites. Though some embryos have a partial lack of the most posterior tissue, it is clear that neural induction and patterning does occur and some somites are formed. Indicating that there is some patterning of the mesoderm. However, the removed organiser tissue is fully capable of patterning a complete secondary axis in host embryos. Thus, either the organiser, as defined by transplantation assays, is only transiently required to induce surrounding tissues, or alternatively, the organiser is a dynamic, possibly regenerative entity.

1.2.3 Specification of the three germ layers

An early process in all vertebrates is the specification of the three germ layers, where cells are specified as ectoderm, endoderm or mesoderm. It is during gastrulation that previously unspecificed cells are fated to form either the ectoderm, endoderm and mesoderm. The hypoblast, formed through the subduction of cells of the germ ring during epiboly, develops to form the endoderm and the mesoderm, with the overlying superficial layer forming the ectoderm. Work by Pieter Nieuwkoop in *Xenopus* established that a vegetal region in the egg was capable of inducing mesoderm cells in the overlying cells at the equator and that co-culture of animal cells, normally fated to become ectoderm, with this region could induce mesoderm specification (Gerhart, 1999; Nieuwkoop, 1973). As discussed previously, screens performed in the late 1980s demonstrated that Activin possesses morphogen activity (Green and Smith, 1990; Smith et al., 1990) and was shown to be sufficient for formation of organiser (Smith et al., 1990). Though it was later

established that the Nodals were the endogenous mesoderm inducers ((Jones et al., 1995), reviewed in (Kimelman and Griffin, 2000)).

VegT, discussed previously in the context of nodal signalling, is also intricately involved in the process of endoderm specification. Indeed, many endodermal genes, including *sox17, Gata5* and *Mixer*, as well as organiser specific genes are downstream of *VegT* (Xanthos et al., 2001). However, it is worth noting that TGF-β signalling is required for the proper expression of genes downstream of VegT and that a lack of VegT also results in a lack of mesoderm induction (Kofron et al., 1999). The specification of endodermal fates has also been closely linked to the specification of mesoderm. The double *sqt;cyc* and the MZ*oep* mutants that lack almost all mesoderm and also lack all endoderm (Feldman et al., 1998; Gritsman et al., 1999). Additionally, fate mapping and gene expression studies have shown that both mesoderm and endoderm arise from a bi-potent region near the vegetal margin of the developing blastoderm, termed the mesendoderm (Rodaway and Patient, 2001). However, it is not clear how mesodermal and endodermal cell fates are segregated, though it has been suggested that the timing and dose of nodal signalling are important (Aoki et al., 2002).

Further factors involved in the specification of endoderm have been characterised through analysis of endoderm mutants isolated from the zebrafish mutagenesis screen. The *casanova* locus has been shown to play an essential role in endoderm formation (Alexander et al., 1999) and has been shown by several groups to encode a novel member of the *sox* transcription factor gene family (Dickmeis et al., 2001; Kikuchi et al., 2001; Sakaguchi et al., 2001). This fits with the observation that the transcription factor Sox17 is necessary for endoderm formation in both mouse and frog (Hudson et al., 1997; Kanai-Azuma et al., 2002). The mutants

bonnie and clyde (*bon*) (Kikuchi et al., 2000), *faust* (Reiter et al., 2001), and *schmalspur* (*sur*) (Pogoda et al., 2000a), have also been shown to be defective in endoderm specification. These mutants were found to encode Mixer, a homeodomain protein, Gata5 and FoxH1 respectively (Kikuchi et al., 2000; Pogoda et al., 2000b; Reiter et al., 2001). Both FoxH1 and Mixer are required for facets of Nodal signalling in the induction of mesendoderm (Kunwar et al., 2003). The expression of *sur* is independent of Nodal, as is initial expression of *bon*, though Nodal signalling and Sur is required for its enhanced and maintained expression, suggesting, along with the observation that Smad2 associates with both FoxH1 and Mixer, a role for these factors as components of the Nodal-signalling pathway (Kunwar et al., 2003). Though the fact that complete loss of Nodal signalling results in a more severe phenotype than loss of both *sur* and *bon,* suggests that they do not compose the entire downstream pathway of Nodal-signalling. Over expression of Gata5 has been shown to lead to an expansion of endodermal cells and also induces the expression of endodermal genes in both *oep* and *bon* mutants. However, the induction of endodermal genes is less effective in *cas* mutants. Suggesting that Gata5 function downstream of *oep* and nodal, parallel to *bon* and upstream of *cas* (Reiter et al., 2001). It is perhaps unsurprising then, that homologues or both of these genes have been shown to be vital in endoderm formation in frogs (Henry and Melton, 1998; Weber et al., 2000).

1.3 Notochord

The derivative of the organiser is axial mesendoderm, which forms the hatching gland and prechordal plate in the anterior and the chordamesoderm in the

posterior. The chordamesoderm is fated to become the notochord, the defining structure of the phylum chordata. The notochord is a rod like structure, that forms early in development and serves two main roles in vertebrate development. First, as a mechanical structure, the notochord acts as the major embryonic skeletal element in lower vertebrates. Second, the notochord is essential for normal development of all vertebrates, providing signals that pattern adjacent tissues such as the gut, somites and spinal cord. Notochord development in zebrafish is relatively simple, as the notochord comprises a single cell type, surrounded by an extracellular sheath, that undergoes a characteristic series of differentiation events, marked by dramatic morphological changes. Our understanding of notochord differentiation has been significantly informed by studies of mutant zebrafish. Phenotypically, the notochord differentiation process can be broken into two discrete transitions. Firstly, the chordamesoderm is specified as a specialised mid-line mesoderm, and secondly there is a transition from chordamesoderm to notochord, which we term notochord differentiation.

1.3.1 Differentiation of the Notochord

After acting to establish the initial body pattern, the organiser differentiates and develops to form the axial mesoderm, which, in the posterior, develops into the notochord. There are two morphological features that mark the differentiation of the notochord. First, the cells of the chordamesoderm develop a thick basement membrane that forms of a sheath surrounding the notochord. Second, coupled to basement membrane formation, each cell acquires a large vacuole that acts to exert turgor pressure against the sheath. Failure to properly vacuolate leads to a

substantially shortened embryo that is easily seen in phenotypic mutagenesis screens. For this reason mutations affecting notochord differentiation are relatively easy to recognise (Odenthal et al., 1996; Stemple et al., 1996). Mutants have been identified that affect both the development of chordamesoderm and the differentiation of chordamesoderm to notochord. Analysis of these mutants has helped to reveal much concerning the processes involved in notochord development.

Identification of the mutant *flh* provided the first real insights into chordamesoderm specification. This mutation, isolated from the background of pet store zebrafish stocks, was found to encode the zebrafish homologue of the *Xenopus Xnot* gene (Talbot et al., 1995). These mutants fail to form a notochord but still form other mesoderm derivatives, such as prechordal plate and somites. In *flh* mutants, tissue that would normally form chordamesoderm instead forms somite and tissues dependent on notochord signalling, such as hypochord and floorplate, largely fail to form (Halpern et al., 1995). The gene *spadetail (spt)*, which encodes a T-box transcription factor homologous to *VegT*, is vital for embryo development and correct patterning of trunk somitic mesoderm (Griffin et al., 1998). Analysis of *flh/spt* double mutants has provided additional insight into the processes of chordamesoderm development. While *flh* mutants lack notochord, *flh*/*spt* double mutants possess trunk notochord. Thus, the *spt* mutation is able to suppress the *flh* phenotype, suggesting that *flh* acts in midline development to promote chordamesoderm and notochord fate by suppressing the induction of somatic fates in this region by *spt* (Amacher and Kimmel, 1998).

In *ntl* mutants, which lack a functional zebrafish homologue of the mouse T brachyury T-box transcription factor (Schulte-Merker et al., 1992; Schulte-Merker et al., 1994), the chordamesoderm develops normally but development arrests prior to

notochord differentiation. This contrasts *flh* mutants, in which chordamesoderm is converted to somitic mesoderm. However, the fate of chordamesoderm in *ntl* mutants is not clear. Some cells may die by apoptosis but others end up in the spinal cord and have been interpreted to form the medial floorplate, although some of these cells have been noted to express *ntl* mRNA at stages when ntl expression is normally extinguished (Stemple et al., 1996). There is also good evidence that *ntl* expression, like its counterpart in *Xenopus*, Xbra, is substantially controlled by FGF signalling (Cao et al., 2004; Griffin et al., 1998; Schulte-Merker and Smith, 1995). It is proposed in *Xenopus* that *Xbra* is involved in an indirect auto-regulatory feedback loop involving FGF. So it may be that FGF acts to maintain *ntl*, where FGF induces *ntl* expression (Cao et al., 2004; Griffin et al., 1998) and where *ntl* is able to function upstream of FGF (Casey et al., 1998; Griffin and Kimelman, 2003; Isaacs et al., 1994).

During normal development *ntl* is first expressed by marginal cells in the late blastulae and early gastrulae stages, then in internalised deep cells. Expression is then maintained only in chordamesoderm at later stages. Double mutant studies of *ntl*, *flh* and *cyc* have helped to establish the relationship between these genes in control of mid-line identities. Despite the dramatic loss of floorplate cells in *cyc* mutant embryos, double mutant *ntl/cyc* embryos display an apparent rescue of floorplate. Similarly, the majority of *ntl/flh* double mutants were found to resemble *ntl* single mutants demonstrating midline tissue not found in *flh* single mutants (Halpern et al., 1997). In the case of *ntl/flh* double mutants, since no marker of floorplate was used in the analysis, it is possible that undifferentiated chordamesoderm, which is persistently expressing early marker genes, has infiltrated the ventral neural tube. However, it is clear that midline tissue not present in *flh*

mutant embryos is present in the *ntl/flh* double mutants. While *ntl* single mutants suggest a role for *ntl* in notochord differentiation, the double mutant results show that *ntl* also has a role in chordamesoderm specification. So, considering that rescue of midline mesoderm also occurs in *spt*/*flh* double mutants and that *ntl*/*spt* double mutants have no trunk mesoderm, it appears as though ntl has some function partially overlapping with other T-box genes (Amacher et al., 2002; Amacher and Kimmel, 1998). One hypothesis is that *ntl*, *spt* and *flh* are controlling the choice between medial floorplate and chordamesoderm fate as seen with the *ntl*/*flh* double mutants, and between medial and lateral fate seen with the *spt*/*flh* double mutants and the three competing activities are balanced through feedback loops, possibly involving Nodal or FGF signalling, to ensure the appropriate amount of each tissue is specified (Griffin et al., 1995; Griffin and Kimelman, 2002; Schier et al., 1997).

Later in development, the notochord acts in the formation of vertebral bodies (centra). In zebrafish, the centra form through the secretion of bone matrix from the notochord, rather than the somites (Fleming et al., 2004; Fleming et al., 2001; Trout et al., 1982).

1.3.2 Patterning by the Notochord

The most studied signalling role of the notochord is in patterning of the neural tube. The neural tube develops distinct cell types at specific locations along its DV axis, and hence the notochord, situated just ventral to the neural tube, was considered a strong candidate for a source of patterning signals. Embryological work performed with chick demonstrated that the notochord is able to co-ordinate correct

neural tube formation, and that the absence of notochord results in abnormal formation of the neural tube (Smith and Schoenwolf, 1989; van Straaten et al., 1988). Ablation of both the notochord and the floorplate, which is itself dependent on notochord derived signals, prevents the differentiation of motor neurons and other ventral neuronal cell types in chicken as well as zebrafish (Saude et al., 2000; van Straaten and Hekking, 1991; Yamada et al., 1991). Further to this, grafting either the notochord or the floorplate to the dorsal midline of the neural tube is able to suppress dorsal neural tube fates and promote the ectopic formation of ventral neuronal cell types (Monsoro-Burq et al., 1995; Yamada et al., 1991). Similar studies have demonstrated that a diffusible signal, derived first from the notochord and then later from the floorplate, acts to pattern the neural tube (Yamada et al., 1993).

The diffusible signal involved in neural tube patterning has since been identified as Sonic hedgehog (Shh) (Echelard et al., 1993; Roelink et al., 1994). Zebrafish express three hedgehogs in the midline: *echidna hedgehog* in the chordamesoderm, *tiggywinkle hedgehog* in the floorplate and *sonic hedgehog* in both (Currie and Ingham, 1996; Ekker et al., 1995; Schauerte et al., 1998). Shh is essential for both correct patterning of the neural tube and formation of the floorplate (Ericson et al., 1996; Matise et al., 1998). It was observed, however, that ectopic Shh alone cannot induce formation of the floorplate (Patten and Placzek, 2002). However, explants of chick neural plate treated with a combination of Shh and Chordin, which is normally expressed by the notochord, develop floorplate, suggesting that the notochord produces Chordin to inhibit dorsally derived BMPs, generating a permissive environment to allow Shh to induce floorplate. The prevailing model suggests that the combination of Shh, produced ventrally, and BMPs, produced dorsally, establish opposing gradients that impart information

concerning DV position within the neural tube. *Shh* is initially expressed by notochord, then later by floorplate, with its expression being confined to the floorplate later in development.

Notochord derived hedgehog signals also have some role in both the muscle fibre type and the chevron shape that is characteristic of zebrafish somites. Normally, adaxial cells, which form immediately adjacent to the chordamesoderm and express *myoD*, will migrate to the outer surface of the developing muscle and differentiate to form slow twitch muscle fibres (Devoto et al., 1996). A few adaxial cells will eventually express Engrailed and form the muscle pioneer cells that define the horizontal myoseptum, imparting the chevron shape of the somite. When ligand activated hedgehog signalling is abolished, as in *slow-muscles-omitted* (*smu*) mutants, which lack the hedgehog signalling component Smoothened, slow twitch muscle fibres as well as the Engrailed-positive muscle pioneers fail to form (Barresi et al., 2000). Similarly, mutants lacking Shh (*sonic you)* or Gli2 (*you-too*), a transcription factor that mediates hedgehog signalling, also fail to form muscle pioneers and slow-twitch muscle fibres (Blagden et al., 1997; Karlstrom et al., 1999; Pownall et al., 1996; Xue and Xue, 1996). In notochord differentiation mutants, the somites form in an abnormal 'U' shape since the horizontal myosepta fails to form. Mutants also show compromised Engrailed expression, despite the persistent expression of midline hedgehogs in undifferentiated notochord (Odenthal et al., 1996; Stemple et al., 1996). This most likely results from a diminished capacity of the undifferentiated notochord to transmit the signal from the notochord to the forming somites (Parsons et al., 2002b).

The notochord has also been demonstrated to play a role in the development of the heart and vasculature. Removal of the anterior region of the notochord causes

an expansion of the expression domain of *Nkx2.5*, a marker for the region fated to become heart, suggesting a role for the notochord in defining the posterior limit of the heart field (Goldstein and Fishman, 1998). There is also a suggested role for the notochord in the formation of the major blood vessels of the trunk. In both *ntl* and *flh* mutants the dorsal aorta (DA) fails to form (Fouquet et al., 1997; Sumoy et al., 1997). The DA and posterior cardinal vein (PCV) form in a highly conserved fashion in vertebrates, with the DA forming just ventral to the notochord and the PCV forming dorsal to the trunk endoderm. Vascular endothelial growth factor (Vegf) is vital for the correct formation of these vessels and is thought to be sufficient for arterial specification. Over expression of Vegf in zebrafish embryos leads to ectopic expression of *ephrin-b2a*, an arterial marker, in tissue that would otherwise be venous (Lawson et al., 2002). Recent work has indicated a role for Shh in blood vessel formation. Mutants deficient in Shh were found to lack *ephrin-b2a* in the vasculature, though interestingly Vegf over-expression was sufficient to rescue arterial differentiation in the absence of Shh. In contrast, Vegf was unable to rescue arterial defects in notch signalling mutants. Taken together these data suggest a model of blood vessel formation in which Shh emitted from the notochord induces the expression of Vegf in the somites, with Vegf then acting in the DA in a Notch-signalling dependent fashion to induce proper arterial development.

The notochord also has as important role in the development of both the pancreas and the hypochord. By mechanically separating notochord from endoderm, expression of markers normally associated with pancreatic development are extinguished (Kim et al., 1997). Culture of presumptive pancreatic endoderm with the notochord induces expression of pancreatic markers, which are lacking when cultured without notochord. However, when notochord is cultured with other

endodermal tissue, pancreatic markers are not induced, suggesting that the notochord is able to induce pancreatic development only in preconditioned endoderm. The hypochord is a transient rod-like structure situated immediately ventral to the notochord and also expresses high levels *Vegf*, so may well be an important source of signals in the development of the vasculature (Cleaver and Krieg, 1998). Removal of the notochord during early neurulation stages results in a failure of hypochord formation, whereas removal of the notochord later in development does not (Cleaver et al., 2000). Thus notochord dependent hypochord induction is complete by late neurula stages. Chick transplantation studies in which notochord is grafted adjacent the endoderm have demonstrated that the ability of endoderm to form hypochord is restricted to the dorsal most region of endoderm. Moreover Notch signalling is essential for proper hypochord development (Latimer et al., 2002). Although specific roles have not been assigned, candidate notochord-derived signals controlling hypochord induction include Shh, Activin-βB and FGF2 (Hebrok et al., 1998). Finally, the notochord is vital in proper formation of the vertebral column. Removal of the notochord from both urodele and avian embryos at neural plate and 12-30 stage embryos respectively, results in a lack of proper vertebral column formation (Fleming et al., 2001).

In summary, the signalling activities of the notochord include patterning of ectoderm, specification of DV pattern in the neural tube, induction of somite, vascular and cardiac mesodermal tissues and patterning of the pancreas and hypochord endodermal tissues (see **Figure 1.3** for overview).

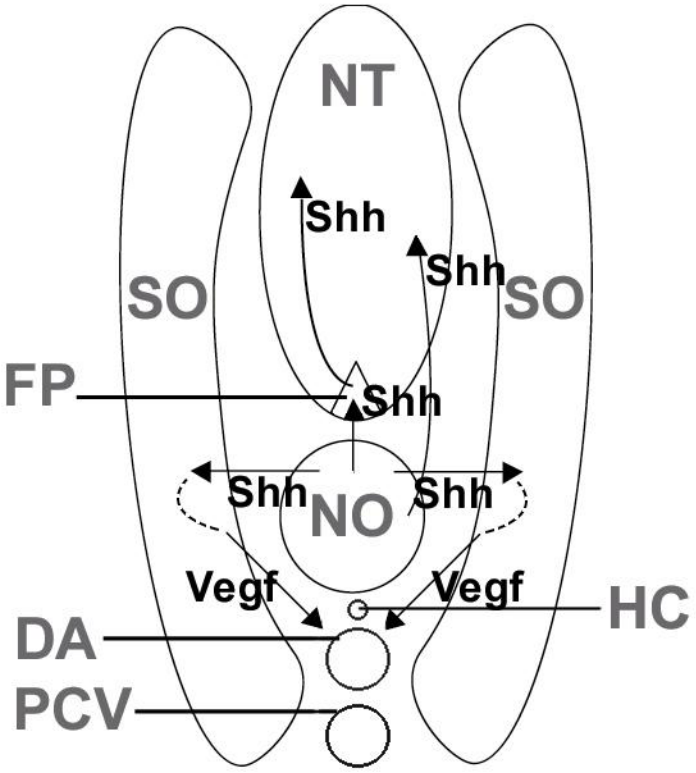


Figure 1.3 Patterning of surrounding tissues by the notochord.

Overview of the notochords role in patterning surrounding tissues; NT, neural tube; SO, somites; FP, floorplate; NO, notochord; HC, Hypochord; DA, dorsal aorta; PCV, pericardinal vein. Shh from the notochord induces floorplate and acts in early patterning of the neural tube, once floorplate is induced, shh is extinguished in the notochord and shh from the notochord patterns the neural tube along the D-V axis. Shh and Ehh (echidna hedgehog) are also involved in patterning the somites and shh signalling to the somites is able to induce vegf in the somites which then acts to pattern the dorsal aorta.

1.3.3 Mechanical Properties of the Notochord

The notochord plays a vital mechanical role in early embryos, especially for lower vertebrates where it acts as the major skeletal element, functioning in locomotion. The notochord consists of a stack of single cells, that each acquire a large vacuole surrounded by a thick sheath of basement membrane. This sheath serves as a physical boundary to limit and control the length and shape of the notochord. Turgor pressure, generated by vacuolation, is constrained by the fibrous sheath, which acts to strengthen and stiffen the notochord. In vitro experiments with *Xenopus* notochord demonstrate that notochord vacuoles will respond to environmental osmolarity, causing the notochord to lengthen and stiffen under physiological osmolarities and to become flaccid under conditions of higher osmolarity (Adams et al., 1990). The lengthening and stiffening of notochord is not observed at stages prior to sheath formation, suggesting a pathway where sheath formation must take place in order for vacuolation to proceed.

The cells of the notochord differentiate in an anterior to posterior wave, thus, the large change in cell volume of anterior cells acts to push more posterior cells caudally, causing the extension of the notochord. This extension is driven by inflation of the vacuoles constrained by the sheath, which stiffens the notochord, preventing buckling. Notochord cells are effectively "rolled" to the posterior, along the tube formed by the sheath, since strong mechanical connections, in the form of hemidesmsomes, between notochord cells and the sheath are not formed until notochord cells are mature (Coutinho, Parsons, Hirst and Stemple, unpublished observations).

The fibres of the sheath are arranged precisely and electron micrographs of transverse sections through the notochord indicate that fibres are arranged to run both parallel and perpendicular to the notochord (Parsons et al., 2002b). Studies of the precise fibre angle in the notochords of *Xenopus* embryos demonstrated that the average fibre angle within the sheath is 54°, an angle that allows the sheath to resist longitudinal and circumferential stress equally. Such as arrangement means that, provided the shape of the notochord is determined solely by inflation of the notochord cells, that the length/diameter ratio will always remain constant (Adams et al., 1990).

The structure of the notochord also functions to constrain in the type of tail movements an early embryo can make. If the notochord consisted only of a thick sheath filled with vacuolated cells it would be able to bend in any direction. However, the notochord is coupled to two other structures that mechanically serve as restraints. Dorsal to the notochord is the floorplate, which expresses many of the same extracellular proteins as the notochord, including α1 Collagen Type II (Yan et al., 1995). Ventral to the notochord is the hypochord, which also expresses similar proteins. These two structures serve as cables, running along the dosal and ventral side of the notochord, limiting its movement. Thus any force exerted on the notochord by surrounding muscle can only result in a left-right movement of the tail, due to the lateral positioning of the somites. Such motion is consistent with the requirements for forward locomotion in the early embryo.

Hence, cells of the notochord act, via vacuolation, to generate a sufficient force to support the embryo. Cells enlarge and exert pressure on the thick sheath of basement membrane that surrounds the notochord generating a hydrostatic force

similar to the turgor pressure of plant supportive networks. This inflation also acts to elongate the embryo, since an absence of inflation leads to a dramatic reduction along the AP axis. The inflation, which begins at the anterior end of the notochord and proceeds towards the posterior, effectively pushes posterior cells towards the posterior end as they expand, where these cells then expand and exert the same force on their neighbouring cells, resulting in a general extension of the embryos along the AP axis.

1.4 Notochord Mutants

In October of 1980 a paper published by C. Nusslein-Volhard and E. Wieschaus reported the first systematic search for genes involved in early development in *Drosphilia* (Nusslein-Volhard and Wieschaus, 1980). The genes identified in this systematic screen have revolutionised our understanding of animal development. Orthologous genes to those originally identified in *Drosphilia* have been uncovered in essentially every other species of animal and have helped demonstrate a remarkable conservation of developmental mechanisms throughout evolution.

Soon after this screen was published, G. Streisinger proposed that a similar screen could be performed with relative ease using the zebrafish (Streisinger et al., 1981). Just over a decade later Christianne Nusslein-Volhard and Wolfgang Driever initiated just such large-scale mutagenesis screens, for recessive-zygotic mutations in the zebrafish. Although much had been learned concerning metazoan development from the fly screen, unique developmental processes, including those involved in

development of the notochord and neural crest, could only be dissected through studies of vertebrate developmental genetics. The results of the zebrafish screen, performed in two parts, in Boston and in Tübingen, were published in 1996 and gained a great deal of publicity (Eisen, 1996)(Development **123**, 1996).

The loci identified by this screen have provided developmental biologists with an incredible resource with which to piece together the molecular mechanisms involved in early vertebrate development (Felsenfeld, 1996; Granato and Nusslein-Volhard, 1996; Holder and McMahon, 1996). The completion of this screen has prompted many groups to perform further smaller-scale screens, more focussed on specific processes (Patton and Zon, 2001).

The screen generated and isolated many mutations that resulted in notochord defects. From the Boston screen, 65 mutations corresponding to 29 complementation groups were identified with four loci identified in Tübingen (Odenthal et al., 1996; Stemple et al., 1996). These mutants demonstrate defects in notochord specification, differentiation, degeneration, maintenance and shape. Many of these mutants have a characteristic shortening of the body axis due to a lack of extension along the AP axis. Mutants were divided into several classes according to the type of observed phenotype. Two of these notochord mutants, *floating head* and *no tail*, had been isolated previously and are defective in chordamesoderm specification and differentiation into notochord respectively.

As well as *no tail*, eight other mutants were identified as defective in the differentiation of chordamesoderm to notochord, as demonstrated by the maintenance of early notochord markers such as *collagen type II* and *shh*. Of these eight mutants,seven were named after the seven dwarves *sneezy* (*sny*), *dopey* (*dop*),

happy (*hap*), *doc*, *bashful* (*bal*), *grumpy* (*gup*) and *sleepy* (*sly*), because of the stark reduction of the AP axis.

1.4.1 Dwarf Mutants

The "seven dwarves" have been grouped into three classes based on their phenotype and analysis of these mutants has revealed much about the process involved in the development and differentiation of notochord. Characterisation of these mutants has also revealed a startling similarity in the components affected in each class.

In the first class, *bal*, *gup* and *sly*, were all identified with a large number of alleles. These mutants are grouped together based on their shared lack of notochord differentiation, as demonstrated by maintained expression of early notochord markers and their shared brain defects (Odenthal et al., 1996; Stemple et al., 1996). In *bal, gup* and *sly* the brain has an irregular morphology, the hindbrain ventricle is enlarged and the axonal scaffold is disrupted (Schier et al., 1996). It is also noteworthy that all three of these mutants were also identified in a screen for retino-tectal path finding mutants (Karlstrom et al., 1996). The mutants *gup* and *sly* have the same overall morphological defects, with all *bal* alleles identified showing a much weaker phenotype than either *gup* or *sly*. The *bal* mutants fail to develop notochord in the anterior but demonstrate the same eye and brain defects as *gup* and *sly* and contain large amounts of apparently WT notochord caudally.

Cloning of *bal, gup* and *sly* identified the three mutants as the α1, β1 and γ1 laminin chains respectively (Parsons et al., 2002b; Pollard, 2002). These three specific laminin chains interact to form the Laminin-1 chain, an essential component of the notochord basement membrane sheath. Loss of laminin α1, β1 or γ1 leads to a dramatic reduction in the levels of Laminin-1 throughout the embryo, thus preventing the formation of the basement membrane surrounding the notochord. The lack of basement membrane results in a notochord differentiation defect. Loss of laminin β1 or γ1 results in a complete failure to form the notochord basement membrane. Loss of laminin α1 in *bal* mutants does not affect posterior notochord basement membrane and hence the posterior notochord is able to differentiate normally. Recent work has demonstrated that this posterior differentiation is due to the ability of laminin α4, which interacts with laminin β1 and γ1 to form Laminin-8, to act in concert with laminin α1 to form notochord basement membrane. One possibility is that laminin-α1 is acting to form basement membrane along the anterior notochord while laminin α4 contributes to the basement membrane in the posterior notochord (Pollard, 2002). Lack of both laminin α1 and α4 results in a phenotype comparable to that of *gup* and *sly*, where there is a complete lack of notochord differentiation. Hence, Laminin isoforms can act interchangeably in forming the basement membrane of the notochord.

In the second class of mutants, *dopey* (*dop*), *happy* (*hap*) and *sneezy* (*sny*) are grouped together based on their near identical phenotype. All three mutants show a similar failure in notochord differentiation, maintaining the expression of early markers, and have disrupted notochord sheath formation, similar to the *bal, gup* and *sly* mutants. However, *dop, hap* and *sny* also exhibit a marked reduction in pigmentation and show widespread degeneration by 48 hpf (Coutinho et al., 2004).

Recent work, including some performed as part of this thesis, has identified *dop, hap* and *sny* loci as coatomer (COP) β', β and α respectively ((Coutinho et al., 2004), this thesis). These are all subunits of the seven subunit COPI complex, which is involved in retrograde transport in the secretory pathway and maintenance of the composition of processing vesicles involved in secretion (Letourneur et al., 1994; Orci et al., 1997; Schmid, 1997). COPI vesicles are required for proper secretion, hence *dop, hap* and *sly* mutants h.ave a compromised secretory network, which results in defective notochord basement membrane sheath formation and defective notochord differentiation. The specific developmental phenotype observed in *dop, hap* and *sly* is reinforced by the observation that, although COPα is ubiquitously expressed, it is specifically up-regulated in chordamesoderm cells. This supports the observation that, as with the mutants *bal, gup* and *sly*, there is a link between formation of the basement membrane sheath and differentiation of the notochord. Perhaps most interestingly, work on the COPI mutants has demonstrated that expression of the COPα subunit may well be regulated in some way by the demand for secretion and COPI activity.

The final class of notochord differentiation mutations comprises *doc* and *ntl*, which fail to form fully differentiated notochord, leading to the persistent expression of some early markers and a failure in formation of the vacuoles. However, unlike the laminin and COPI mutants, *doc* and *ntl* mutants possess normal basement membrane. Transplantation experiments have shown that the notochord differentiation defect is cell-autonomous for both *ntl* and *doc* (Halpern et al., 1993; Odenthal et al., 1996). Of these two loci, *doc* has the most notochord-specific defect. Though *ntl* mutants fail to generate tails, in the trunk region they are phenotypically very similar to *doc* mutants. The *doc* mutants however, demonstrate defects only in

notochord differentiation, which in turn leads to defects in the patterning of surrounding tissues. This is the case in all the notochord mutants and is likely to be due to a lack of signalling from a properly differentiated notochord. A detailed understanding of the upstream factors controlling *doc* and *ntl* should elucidate the nature of the notochord differentiation signal and an understanding of their downstream effectors should reveal further insights into how differentiation is manifest. One of the primary aims of this thesis is to clone the *doc* locus and to determine how this gene product interacts with the known process of notochord development to control and coordinate differentiation. The presence of a normal basement membrane sheath surround the notochord in *doc* mutants suggests a possible role for *doc* in the final stages of notochord differentiation that take place only after the basement membrane has formed (see **Figure 1.4**).

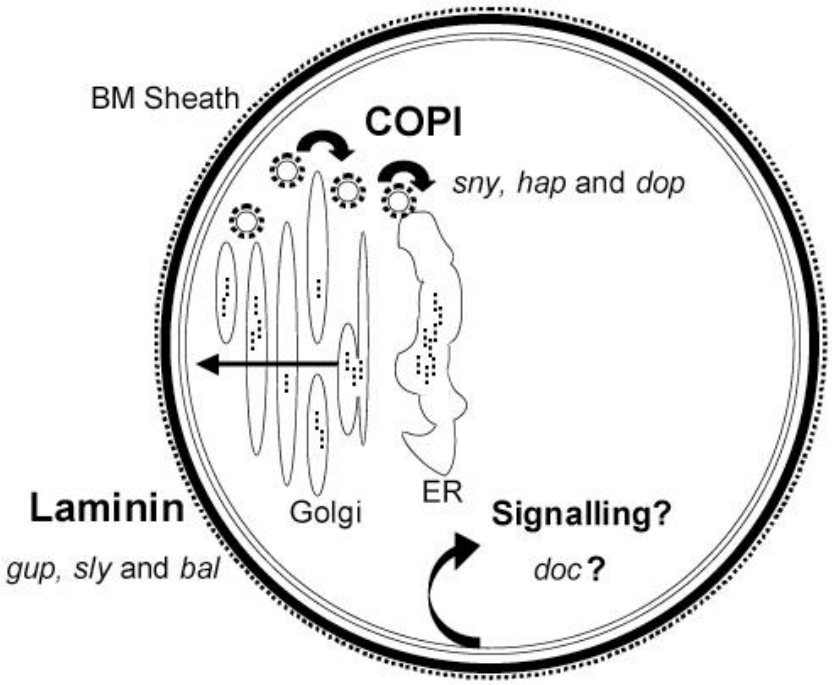


Figure 1.4 Roles of notochord dwarf mutants in notochord development.

The mutants *gup, sly* and *bal* encode the laminin chains β1, γ1 and α1 lack vital components of the notochord BM sheath. The mutants *sny, hap* and *dop* lack the COPI subunits α, β and β'and are defective in retrograde transport, causing defects in the secretory pathway. The mutant *doc* may well be defective in signalling important for notochord differentiation, possibly relating information about BM sheath formation back to notochord cells.

1.5 Notochord Sheath

The cells of multicellular organisms are surrounded and supported by the extracellular matrix (ECM), which can be essectially described as secreted molecules that are immobilised outside cells and can be broadly said to consist of three classes of molecules: collagens, non-collagenous glycoproteins and proteoglycans. The ECM is capable of affecting many cellular processes in an instructive manner (Adams and Watt, 1993) and during embryogenesis, populations of cells undergo many morphogenetic events that involve direct cell-ECM interactions (Zagris, 2001). Noteably, Epithelial cells form sheets and tubes, neural crest cells migrate large distances and cell-ECM interactions affecting cell migration have been characterised.

One important property of the ECM arises through the formation of a specialised type of matrix known as basement membrane (BM). Many proteins including fibronectin, collagen and laminin have been shown to make up this matrix, which is essential in early vertebrate development.

The BM can control many aspects of cell/tissue behaviour during development and following injury (Schwarzbauer, 1999). Investigations into the properties of BM have demonstrated that laminin, which is a major constituent of BM, is a mediator of ductal or tubular morphogenesis and differentiation (Edwards et al., 1998; Jiang et al., 1999; Schuger, 1997; Streuli et al., 1991; Thomas and Dziadek, 1994). BM also acts in the kidney glomerulus as an important component of the selective barrier that prevents passage of macromolecules from the blood into the urine and is known to have an important role in localisation of the synapse in the

neuromuscular junction both during embryogenesis and after injury (Carbonetto and Lindenbaum, 1995; Sanes and Lichtman, 1999).

1.5.1 Components of the Notochord Basement Membrane

The properties of the BM are a direct result of the properties of its component parts. BM consists primarily of laminin, which is cross-linked to type IV collagen by entactin or nidogen and includes proteoglycans such as aggrecan.

The laminins are a family of heterotrimeric glycoproteins and are one of the earliest extracellular matrix proteins secreted during development (reviewed in (Colognato and Yurchenco, 2000)). Three polypeptide chains, α, β and γ, make up the laminin heterotrimer complex. To date, five α, four β and three γ genes have so far been identified in mouse. Combinations of these proteins give rise to the multiple laminin isoforms, though there appears to be restrictions so that only a subset of all possible combinations are produced. Currently twelve isoforms have been reported. The Laminin heterotrimer is formed through interactions between the coiled-coil domains in the C-terminus, known as the long arm and the N-terminus of each chain contains, which contains globular domains, gives rise to the short arms. The globular domains within the N-termini of α chains are the major sites of interaction with cell-surface receptors such as integrins. As well as binding sites for collagen IV and nidogen, laminins are also able bind to each other and hence form large BM networks (Tunggal et al., 2000).

Laminin 1 (α1β1γ1) was identified in 1979 in extracts from the Englebreth-Holm-Swarm (EHS) murine tumour and teratocarcinoma cells (Timpl et al., 1979) and is the most well characterised of the Laminins. Laminin 1 appears to be the main laminin involved in early development and was the first to be completely sequenced and structurally analysed. Many of the domains responsible for the various ligand interactions were identified using purified Laminin 1. The roles of various Laminins *in vivo* have been better characterised through genetic studies of certain human diseases and targeted gene disruption in mice, reviewed in (Colognato and Yurchenco, 2000). Characterization of these phenotypes has revealed an unexpected diversity of function, demonstrating roles in processes as diverse as cell migration, differentiation, metabolism and polarity (Colognato and Yurchenco, 2000; Gustafsson and Fassler, 2000).

1.5.2 Formation of the Notochord Basement Membrane

Analysis of the notochord mutants has demonstrated the importance of BM formation in notochord development and has also provided much information on the processes involved in the formation of the basement membrane sheath, demonstrating what tissues are involved in the establishment of the notochord sheath. Transplantation studies in sheath mutants, where the shield from either a mutant or wild-type embryo is transplanted onto a wild-type or mutant host respectively, have shown much. In such studies a secondary notochord of the donor genotype is generated that is completely surrounded by tissues of the host genotype, allowing the origin of laminin components of the basement membrane to be dissected. Such

transplantation studies performed with the *bal, gup* and *sly* mutants have demonstrated that the laminin chains could be supplied either by the notochord or by non-notochordal tissues, since both transplantation of mutant shields onto wild-type hosts and wild-type shield onto mutant host leads to embryos that have proper notochord differentiation. Hence, the notochord basement membrane sheath can be supplied both autonomously and non-autonomously.

Although the Laminin rich layer of the notochord basement membrane can be supplied by either the notochord or the surrounding tissues examination of three other notochord differentiation mutants, *dop, hap* and *sny* demonstrated that this was not the case for the medial layer of the sheath. An absence of Laminin, which contributes greatly to the inner layer of the sheath, causes an absence of organised basement membrane sheath. However, in *dop, hap* and *sny*, the inner, Laminin rich layer, still forms but there is still an absence of organised notochord basement membrane. Transplantation experiments, to examine if the medial and outer layers were notochord autonomous or non-autonomous, established that the establishment of the medial and outer layers of the sheath are notochord autonomous. Shields transplanted from *sny* embryos onto wild-type hosts resulted in secondary notochords with disrupted sheaths lacking the medial layer, whereas wild-type shields transplanted into *sny* mutant hosts, generated secondary notochords with wild-type sheaths. So, it can be said that Copα, and most probably the entire COPI complex and secretory system, acts autonomously within the chordamesoderm/notochord to ensure formation of proper medial layer basement membrane formation and thus ensure notochord development.

1.6 Summary of Thesis Results

The results of this thesis are presented in five chapters, with the sixth chapter providing a discussion of results and an overview of the future perspectives raised by this work. In the first chapter, I describe the work performed to define the *doc* locus to a specific genomic location and identify candidate genes within this region. In the second chapter, the work undertaken to characterise which candidate is responsible for *doc* is described. In this chapter, a novel multiple WD40 domain protein is proposed as *doc* and evidence to support this is described. In chapter three, the work in characterising the *dop* and *hap* loci is discussed and evidence that they encode the COPI subunits COPβ' and COPβ is provided. Following that, the characterisation of the remaining COPI subunits is covered and evidence for an auto-regulatory mechanism in COPI subunit expression is discussed. The following chapter, chapter four, describes the work undertaken to uncover the regulatory mechanism involved in COPI expression. In the next chapter, a brief examination of the effect of COPI loss of function on ER and Golgi structure is reviewed. Following this, chapter seven describes the work undertaken to uncover the regulatory mechanism for COPI and the UPR is put forward as an essential regulatory mechanism required for proper development. The discussion describes the arguments based on the results of this thesis and discusses the future directions suggested by the work described herein.