Specific Roles of Laminins During Vertebrate Embryogenesis

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Abstract

The notochord is critical for the normal development of vertebrate embryos. Zebrafish mutants, bashful (bal), grumpy (gup) and sleepy (sly) were identified in mutagenesis screens and have defects in notochord differentiation. By positional cloning I identified lamal, encoding Laminin α1, as a candidate for the bal gene. Analysis of mRNA expression, antisense knockdown and sequencing of the mutant lamal, confirmed that mutations in this gene are responsible for the bal phenotype. A similar approach was taken to identify the gup gene. Meiotic mapping identified a region containing lamb1, encoding Laminin β1. Cloning and sequencing of lamb1 from gup^{m189} mutants revealed a non-sense mutation. These results identify a role for Laminin α1 and Laminin β1 in notochord differentiation. Concurrent work in our laboratory revealed that the third mutant, sly, encodes the Laminin γ1 chain (Parsons et al., 2002). Thus, the laminin 1 isoform, a heterotrimer comprising the α1β1γ1 chains, is necessary for notochord differentiation.

bal mutants differ from gup and sly, as failure of notochord differentiation is not as extensive, occurring only in anterior regions. We hypothesised this was due to a redundant role of another α chain. Characterisation of lama4 and lama5, encoding Laminin α4 and α5 chains, respectively, showed that both control aspects of notochord differentiation. Furthermore, lama5 has a functionally redundant role with lamal during CNS development. Characterisation of lama2 identifies a role for Laminin α2 chain in muscle function, consistent with loss-of-function phenotypes in human and mouse. Hence, I demonstrate different roles for specific laminin chains during zebrafish development, confirming and extending results from studies in other vertebrate systems. Finally, I studied integrin α6, integrin β4, and integrin-linked kinase (ILK), to determine their role during development. The results suggest these genes control cell movements during gastrulation, rather than notochord differentiation.
To Mum and Dad, thank you for everything.
Man with all his noble qualities, still bears in his bodily frame the indelible stamp of his lowly origin.

*Charles Darwin 1809-82 (The Descent of Man)*

Happy is he who gets to know the reasons for things.

*Virgil (70-19 BCE)*
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<td>AP</td>
<td>Antero-posterior</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>BAC</td>
<td>Bacterial artificial chromosome</td>
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<tr>
<td>bal</td>
<td>bashful</td>
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<tr>
<td>BCIP</td>
<td>X-phosphate/5-Bromo-4-chloro-3-indol-1-phosphate</td>
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<td>BM</td>
<td>Basement membrane</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<tr>
<td>CTP</td>
<td>Cytidine 5'-triphosphate</td>
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<tr>
<td>DAB</td>
<td>Diaminobenzidine</td>
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<tr>
<td>DEPC</td>
<td>Diethylpyrocarbonate</td>
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<tr>
<td>DIG</td>
<td>Digoxigenin</td>
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<tr>
<td>DTT</td>
<td>Dithiothritol</td>
</tr>
<tr>
<td>DV</td>
<td>Dorso-ventral</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene-diamine-tetra-acetate</td>
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<tr>
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<tr>
<td>GTP</td>
<td>Guanidine 5'-triphosphate</td>
</tr>
<tr>
<td>gup</td>
<td>grumpy</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>hpf</td>
<td>Hours post-fertilisation</td>
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<tr>
<td>ILK</td>
<td>Integrin-linked kinase</td>
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<tr>
<td>IPTG</td>
<td>Isopropylthio-β-D-galactosidase</td>
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<tr>
<td>LG</td>
<td>Linkage group</td>
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<tr>
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<td>Definition</td>
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<tr>
<td>MO</td>
<td>Antisense morpholino oligonucleotide</td>
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<tr>
<td>NBT</td>
<td>4-Nitro blue tetrazolium chloride</td>
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<td>OD</td>
<td>Optical density</td>
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<tr>
<td>PAC</td>
<td>P1 artificial chromosome</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>Rapid amplification of cDNA ends</td>
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<tr>
<td>RAPD</td>
<td>Random amplified polymorphic DNA</td>
</tr>
<tr>
<td>RH</td>
<td>Radiation hybrid</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>sly</td>
<td>sleepy</td>
</tr>
<tr>
<td>SSLP</td>
<td>Simple sequence length polymorphism</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris, acetate, EDTA</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris, borate, EDTA</td>
</tr>
<tr>
<td>TE</td>
<td>Tris EDTA</td>
</tr>
<tr>
<td>TTP</td>
<td>Thymidine 5'-triphosphate</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl Transferase-mediated dUTP nick end labelling</td>
</tr>
<tr>
<td>UTP</td>
<td>Uridine 5'-triphosphate</td>
</tr>
<tr>
<td>X-Gal</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-galactosidase</td>
</tr>
<tr>
<td>YAC</td>
<td>Yeast artificial chromosome</td>
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<tr>
<td>YSL</td>
<td>Yolk Syncytial layer</td>
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During this general introduction I initially discuss the reasons why the zebrafish has become a major model system for the study of developmental biology. I give an overview of zebrafish embryology, and then look in more detail at the molecular mechanisms of processes that have been well characterised. In particular, I concentrate on development of axial mesendoderm and notochord differentiation, which is the focus of my investigation. I then discuss the use of mutant screens in the study of developmental biology and how this approach has been used to identify many zebrafish developmental mutants. Those mutants with notochord differentiation defects are the basis of this study and are discussed in detail. The results from this thesis show a vital role for laminin genes in the development of the notochord and therefore I review the literature concerning the extracellular matrix, and its role during early embryogenesis.

1.1 Zebrafish as a model system

1.1.1 The Zebrafish

Over the past decade the zebrafish (Danio rerio) has become a popular choice of model organism for the study of vertebrate developmental biology. There now exists a large zebrafish research community with many resources available. The zebrafish has many features that make it an excellent system for studies in developmental biology,
including: low maintenance costs, a short breeding cycle, high fecundity, and translucent embryos that can be obtained all year round in large numbers. The optical clarity permits direct visualisation of individual cells and cell movements within the developing embryo, and the short life cycle allows genetic studies to be carried out routinely.

Each of these attributes made the zebrafish an ideal system in which to perform vertebrate mutagenesis screens akin to those carried out for Drosophila (Nusslein-Volhard and Wieschaus, 1980). Such screens were performed for zebrafish and were published in 1996 as a single volume of the journal Development (Driever et al., 1996; Haffter et al., 1996). Thus, a vast number of zebrafish mutants with specific developmental defects are the subject of intense study in many labs worldwide. The molecular characterisation of these mutants promises new insights into vertebrate development (Currie, 1996; Eisen, 1996; Holder and McMahon, 1996; Roush, 1996).

1.1.2 Overview of zebrafish embryology

Zebrafish development during the first three days following fertilisation has been described in terms of seven broad periods: zygote, cleavage, blastula, gastrula, segmentation, pharyngula, and hatching periods (Kimmel et al., 1995). I will briefly describe each of these.

The zebrafish egg contains intermixed cytoplasm and yolk. Following fertilisation the zygote undergoes cytoplasmic streaming where the cytoplasm separates and moves to the animal pole. This yolk-free zone is called the blastodisc. As is the case for all vertebrate zygotes the initial stages of zebrafish development involve synchronous meroblastic cleavage divisions. These begin 40 minutes after fertilisation and occur at 15-minute intervals (Kimmel and Law, 1985). Unlike amphibian embryos, which undergo holoblastic cleavage, zebrafish cleavages occur only at the animal pole within the blastodisc and leave cytoplasmic bridges between early cleaving cells and the yolk (Kimmel and Law, 1985). The sixth cleavage is the first to occur in the horizontal plane, and results in two tiers of cells. Regular cleavages continue until the tenth division, which correlates with the start of the mid-blastula transition (MBT) (Kane and Kimmel, 1993).
MBT begins at the 1024-cell stage and is the point at which zygotic transcription commences. At this stage in development three cell lineages can be described: the yolk syncitial layer (YSL), enveloping layer (EVL), and deep cell layer. The deep cell layer forms the embryo proper. The YSL is formed following a collapse of the marginal nuclei into the yolk cell forming a syncytium. The EVL is fated to become the periderm, a protective layer surrounding the embryo. During this period there is an increase in cell cycle length and commencement of zygotic transcription. Later, cell motility begins through radial intercalations and subsequently epiboly (Solnica-Krezel et al., 1995; Warga and Kimmel, 1990). These events are also characteristic of MBT in other metazoans. In *Xenopus laevis*, for example, MBT is triggered through an unknown mechanism that monitors the nucleo-cytoplasmic ratio (Newport and Kirschner, 1982).

Epiboly is the first major gastrulation cell movement. It involves a thinning and spread of the blastoderm from its position on top of the yolk vegetally so as to cover the yolk. It has been likened to pulling a ski cap over one's head. The blastoderm is pulled down over the embryo through strong attachments between the marginal cells of the EVL and the YSL, which in turn is attached to force-generated microtubules within the yolk cell (Warga and Kimmel, 1990). Other gastrulation movements such as, involution, and convergent extension occur simultaneously with epiboly (Solnica-Krezel et al., 1995). During this period the mesendodermal germ layer (hypoblast) is specified and the primary axes of the embryo are defined (see section 1.2).

Gastrulation is perhaps the single most important event in the development of an organism. The extensive morphogenetic processes that occur are incredibly complex, and it is during this stage that cells are first specified to particular fates. Use of vital dyes for fate mapping has been particularly successful in studies of zebrafish due to the optical clarity of the embryo (Kimmel et al., 1995; Kimmel and Law, 1985). This type of analysis has shown that in broad outline the fate map of the zebrafish is topologically similar to other vertebrates (Figure 1-1).

At about 50% epiboly (5.25 hpf) the germ ring begins to form through involution of cells, forming a clearly visible thickening of the marginal region. Approximately 20 mins later an important stage is reached, known as shield stage. This is a landmark event as it represents the first clear morphological identification of the dorsal side of the embryo. There is a temporary pause in epiboly as the shield forms through convergence.
of cells toward the dorsal side. The zebrafish shield is the gastrula dorsal organiser and is equivalent to Spemann's organiser (frog), Hensen's node (chicken) and the node (mouse). Transplantation of the zebrafish shield to the ventral side of a host embryo induces a complete second axis (Saude et al., 2000; Shih and Fraser, 1996). Deep cells of the shield are termed hypoblast, while those more superficial are known as epiblast. Hypoblast gives rise to mesendoderm derivatives and the epiblast gives rise to ectodermal derivatives such as epidermis and the nervous system. As cells continue to converge on the dorsal region, the embryo extends along the AP axis. The shield tissue itself differentiates into axial tissues, including the notochord (Saude et al., 2000).

At the completion of gastrulation, just 10 hours after fertilisation the embryo has established the main body axes, specified and organised the three germ layers and has reached a stage known as tailbud. The subsequent period of development is known as the segmentation period (10-24 hpf). During this period the tailbud becomes more prominent, the embryo elongates and tissues begin to differentiate. The notochord is one of the first tissues to differentiate fully and I discuss in detail the embryological and molecular nature of its development in later sections.

Somitogenesis occurs soon after the tailbud stage and represents one of the major events during the segmentation period. Blocks of somites arise sequentially in an anterior to posterior direction from the paraxial mesoderm either side of the notochord in the trunk and tail. Each somite represents a block of undifferentiated mesenchyme surrounded by an epithelial layer. Between 30 and 34 somites form in the zebrafish that differentiate into myotome and sclerotome, giving rise to the body-muscle segments and vertebral cartilage respectively. The choice between these two fates is influenced by the notochord (see section 1.3). Two other segmental structures form during this period; the rhombomeres, within the CNS, and the pharyngeal arches, which give rise to the jaw and gills. The notochord has no obvious morphological segmentation, but has been shown to have regional identity at the molecular level (Prince et al., 1998).

The development of the nervous system is concurrent with somitogenesis. The neural plate thickens substantially along the embryonic axis and at the anterior end a structure known as the polster (fated to become hatching gland) forms due to accumulation of anterior-most axial mesendoderm. Even at this early stage in the segmentation period it
is clear from analysis of neural marker genes that a large degree of patterning has already taken place.

Space constraints prevent a detailed description of the many other processes that occur during this period, such as development of the sensory placodes, pronephric duct, neural crest and brain. The end of this period (24 hpf) is characterised by a completion of somite formation, differentiation of blood cells, and commencement of the heartbeat. At this point in development the first fully differentiated organ, the notochord, has formed.

The final period prior to hatching is known as the pharyngula period. A number of body structures that are required for the transformation into a freely swimming and feeding larva are elaborated during this period. Most conspicuous are the forming fins, jaws and gills. At about 4 days all of the major organ systems have completed their extensive morphological movements and hatching occurs. Thus, just 96 hours after the embryo consisted of a single cell it has been converted into a complex free-swimming fish. Understanding all of the events that occur during these 4 days is the major challenge of developmental biology. The major aim of this thesis is to further understand the development of the notochord.
Figure 1-1  Fate map of the zebrafish late cleavage stage embryo. (A) The fate map of zebrafish was found to be very similar in character to that of *Xenopus laevis*. In both frog and zebrafish the endoderm forms from cells in the vegetal region, with mesoderm tissue forming above this (Wolpert, 2002). (B) Stages of zebrafish development from each period of development during the first 5 days post-fertilisation.
1.2 Early vertebrate development

The advances made in our understanding of vertebrate development over the last decade have been truly astonishing. The advent of recombinant DNA technology has revolutionised the field of developmental biology and we are now in the process of uncovering the molecular and cellular mechanisms underlying major developmental processes, such as patterning, morphogenesis and differentiation. The following sections briefly describe aspects of vertebrate development that have been well studied at the molecular level. I will describe events that occur from fertilisation through to the gastrula period, specifically, the establishment of the germ layers and body axes, and early patterning. It is these processes that result in specification of chordamesoderm. I mainly discuss studies of *Xenopus laevis*, which have provided the foundations of our current understanding, and compare the equivalent events in zebrafish.

Two major questions have occupied vertebrate developmental biologist for many years. First, how are the three germ layers (ectoderm, mesoderm and endoderm) established? This represents one of the first major events in the development of a vertebrate embryo. Second, what processes generate pattern in the early embryo? The last 20 years have seen rapid progress in our understanding of each of these processes at the molecular level and recent work in zebrafish has confirmed and extended these findings (Kimelman and Griffin, 2000; Schier, 2001).

1.2.1 Establishing the germ layers and induction of the organiser

All vertebrates undergo an early specification event whereby signals lead to a specification of cells as ectoderm, mesoderm or endoderm. Formation of these germ layers has been extremely well studied in amphibians, with landmark experiments being performed in the 1970’s by Pieter Nieuwkoop (Gerhart, 1999; Nieuwkoop, 1973). These studies suggested the presence of a signal, emanating from the vegetal region of the embryo that is capable of inducing mesoderm in the overlying cells in the equator. Animal pole tissue normally fated to become ectoderm could be converted to a mesoderm fate through co-culture with this vegetal region (Nieuwkoop, 1973). Attempts to identify this mesoderm-inducing signal were successfully made during the late-1980s and the TGF-β protein activin, and FGF were identified as good candidates...
Chapter 1  General Introduction

(Kimelman et al., 1988; Slack et al., 1990; Smith et al., 1990). Subsequent work has shown that neither of these two factors is the endogenous mesoderm inducer. In fact, in frog, members of the nodal family (also a TGF-β family member), are expressed in a manner spatially and temporally consistent with a mesoderm inducer, and are sufficient for mesoderm induction (Jones et al., 1995), reviewed in (Kimelman and Griffin, 2000).

Recent studies of zebrafish mutants have supported the frog studies and helped confirm the role nodal plays (Schier and Shen, 2000; Stemple, 2001). Double mutant zebrafish for cyclops and squint, both of which encode nodals, lack all endoderm and head/trunk mesoderm (Feldman et al., 1998). The mesoderm is still present in the tail suggesting the presence of another uncharacterised mesoderm inducing factor. Further, positional cloning of the zebrafish gene, one-eyed pinhead (oep) identified a novel EGF-CFC type protein that has been shown to act as a co-receptor for nodals (Zhang et al., 1998). A mouse homologue of this protein has been identified and is termed Cripto (Shen et al., 1997). A secreted TGF-β protein known as Lefty/antivin antagonises nodal signalling, possibly through action at the level of the nodal receptor (Meno et al., 1999; Thisse and Thisse, 1999). Another mutant of the nodal pathway is schmalspur, whose phenotype includes: lack of floorplate, reduced prechordal plate and no medial mid- and hindbrain (Brand et al., 1996). This mutation was identified in the zebrafish screen and encodes FAST1, a transcription factor downstream of nodal signalling (Sirotkin et al., 2000).

Thus, several mutants have been identified that lie at various point along the nodal signalling pathway. These support the results from other vertebrate model organisms and reveal that nodal signalling is required for mesoderm induction (Table 1-1). A direct target of the nodal signalling pathway is the transcription factor Xbra (a homologue of mouse Brachyury) (Smith et al., 1991). In zebrafish the mutant no tail was shown to encode an Xbra homologue (Schulte-Merker et al., 1994). Its role in notochord development is discussed in later sections.

What signal controls the expression of nodals? Several lines of evidence implicate a member of the T-box family of transcription factors known as VegT (Horb and Thomsen, 1997; Lustig et al., 1996; Stennard et al., 1996; Zhang and King, 1996). VegT is localised to the vegetal region and at the start of zygotic transcription activates zygotic signals that induce endoderm. Antisense knockdown of maternal supplies of VegT mRNA in Xenopus laevis results in a loss of capacity both to form endoderm and
to release mesoderm-inducing signals (Zhang et al., 1998). It appears that the major targets of VegT are in fact the nodals (Clements et al., 1999). In frog there have been six nodals identified \((Xnr1-6)\) (Whitman, 2001). Studies of the promoter regions of \(Xnr1\), identified T-box binding sites, and so VegT may act directly to turn-on nodals (Hyde and Old, 2000). The zebrafish homologue of VegT is spadetail, but it is not expressed maternally (Griffin et al., 1998). A T-box gene with an analogous role to VegT has yet to be identified in zebrafish.

Specification of the endoderm appears to be intimately linked to mesoderm specification. Nodal signalling can induce endoderm formation in fish and maternal zygotic oep mutants completely lack endoderm, resembling the squint/cyclops double mutants (Feldman et al., 1998; Gritsman et al., 1999). Gene expression and fate mapping studies both suggest that mesoderm and endoderm arise from a bipotential layer of cells near to the vegetal margin of the blastoderm. This region is termed the mesendoderm (Rodaway and Patient, 2001). It is not clear how mesoderm and endodermal cell fates are segregated within the mesendoderm, but it has been suggested that the timing and dose of nodal signalling are important factors (Aoki et al., 2002).

Several mutants that lack endoderm were identified in the large-scale mutagenesis screen. casanova plays an essential non-autonomous role in endoderm formation (Alexander and Stainier, 1999) and was recently identified by three groups as a member of the Sox gene family (Dickmeis et al., 2001; Kikuchi et al., 2001; Sakaguchi et al., 2001). Sox17 is necessary in mouse and frog for endoderm specification (Hudson et al., 1997; Kanai-Azuma et al., 2002). bonnie and clyde and faust mutants also have defects in endoderm specification and have been identified as a genes encoding a Mix-like homeodomain protein and Gata5, respectively (Kikuchi et al., 2000; Reiter et al., 1999). This is perhaps unsurprising given that homologues of both these genes are known from studies in frog to be important for endoderm specification (Henry and Melton, 1998; Weber et al., 2000). How these factors act in relation to one another and to the nodal pathway is not clear, though a detailed characterisation of these mutants should shed light on some of the outstanding questions.
1.2.2 The gastrula organiser and DV patterning

A landmark paper in the field of embryology was published in 1924 by Spemann and Mangold, in which a series of transplantation studies using salamander embryos are described (Spemann and Mangold, 1924). A remarkable discovery was that transplantation of a specific region of an embryo (dorsal gastrula) to ventral regions of a host is capable of inducing a complete secondary axis. The donor tissue gave rise to axial mesendoderm derivatives, while the nervous system and somites were derived from host cells (Spemann and Mangold, 1924). This region of tissue is now termed the gastrula dorsal organiser, and an equivalent structure with similar capacities has been identified in chick, fish and mouse (Beddington, 1994; Saude et al., 2000; Shih and Fraser, 1996; Waddington, 1932). The formation of the organiser is particularly relevant with regard to this thesis, as specification of axial mesoderm and induction of the organiser represent parts of the same problem.

Studies of the various gastrula organisers suggest at least three major roles of the organiser tissue: secretion of molecules capable of altering fates of neighbouring cells; self-differentiation into axial structures; and regulation of morphogenesis during gastrulation, reviewed in (Harland and Gerhart, 1997). Recent studies of the gastrula organiser have suggested there may be heterogeneity within the organiser, with separate cell populations responsible for induction of the head and trunk. This was particularly well illustrated in zebrafish, where goosecoid or floating head expressing cells (marking hypoblast and epiblast, respectively) were independently transplanted to the ventral side of host embryos. This led to induction of head or trunk, respectively (Saude et al., 2000). Surgical removal of the organiser (shield) in zebrafish results in embryos lacking all axial mesoderm derivatives (hatching gland, prechordal plate, hypochord, chordamesoderm and floorplate) (Saude et al., 2000).

Induction of the gastrula organiser has been intensively studied over the last decade. As with germ layer specification, the current model is based upon much earlier embryological work performed using amphibian embryos, by Pieter Nieuwkoop. He showed that transplantation of dorsal-vegetal blastomeres could induce a full secondary axis without contributing to axial tissues (Nieuwkoop, 1973). It was postulated that a signalling centre, now termed the Nieuwkoop centre, exists in the early blastula that can induce organiser tissue. The dorsal YSL is thought to be a functionally equivalent
signalling centre in zebrafish (Koos and Ho, 1998). Induction of the Nieuwkoop centre is mediated through cortical rotation (from a vegetal to dorsal region) of a modifier signal that results in the translocation of β-catenin to the nucleus on the dorsal side (Gerhart et al., 1989; Vincent and Gerhart, 1986). Overexpression of β-catenin in both *Xenopus laevis* and zebrafish is capable of inducing a secondary axis (Funayama et al., 1995; Kelly et al., 1995). β-catenin binds to transcription factors of the TCF/LEF family and then induces organiser specific genes such as *siamois* in the frog (Lemaire et al., 1995; Nelson and Gumbiner, 1998). *bozozok* seems to play an analogous role in zebrafish and is discussed in more detail in the context of notochord formation in the next section (Kodjabachian and Lemaire, 1998). Embryological studies in zebrafish support this model of organiser induction as surgical removal of the vegetal region of the yolk results in retention of yolk mesoderm inducing potential, but loss of the capability to induce ectopic organiser gene expression upon transplantation (Mizuno et al., 1999). A summary of these early events in frog development is given in Figure 1-2.

Once formed, cells of the organiser signal to adjacent ectoderm and mesoderm to induce neural and dorsal mesoderm cell fates respectively. Many genes have been identified that are specifically expressed within organiser tissue that play a role in these inductive processes (Harland and Gerhart, 1997). The discovery of these genes, and characterisation of the roles they play provides a foundation of our understanding of the major patterning events through gastrulation. The current model is that the organiser secretes antagonists of BMP proteins, such as, chordin, noggin and follistatin, which promote dorsal mesoderm and neural fates in neighbouring tissues (Hemmati-Brivanlou et al., 1994; Piccolo et al., 1996; Zimmerman et al., 1996). Thus, induction of neural tissue occurs through inhibition of the ventral specification pathway (BMP signalling), rather than direct induction of neural fates. This has led to the neural default model, reviewed in (Weinstein and Hemmati-Brivanlou, 1997).

Noggin, chordin and follistatin induce neural tissue that has an anterior character, and so it was proposed that the mechanism of AP patterning of the nervous system may involve a second ‘transforming’ signal that specifies posterior fates. Wnts and FGFs were both shown to be capable of posteriorising neural tissue, and moreover, antagonists of the Wnt pathway are expressed in prechordal plate, reviewed in (Gamse and Sive, 2000; Wodarz and Nusse, 1998). Thus, it is thought restriction of the Wnt antagonists results
in adjacent cells acquiring anterior fates through inhibition of the posteriorising Wnt signals (Niehrs, 1999). Many regulatory and potentiating molecules for each of the signalling pathways that operate during specification of cell fates have been identified (e.g. Arkadia, Cerberus, FrzB, Tolloid and Twisted gastrulation). We are still some way from a complete understanding of how these fit together to generate the complexity of pattern and form within the embryo.

In summary, specification of the endoderm in *Xenopus* occurs through a maternally localised transcription factor that in turn controls the expression of secreted mesoderm inducing molecules. Concurrent with this a dorsal modifier activates the nuclear accumulation of \( \beta \)-catenin that in turn initiates organiser specific gene expression, such as antagonists of Wnt and BMP signalling that pattern the embryo. There is considerable evidence that this program of events, which establishes the vertebrate body plan, is remarkably conserved at the molecular level (Fraser and Harland, 2000; Whitman, 2001; Wolpert, 2002).
Figure 1-2  Summary of the molecular mechanisms operating in frog during the establishment of the germ layers and dorso-ventral axis. Maternal transcription factors such as VegT and Vg-1 are responsible for endoderm specification in the ventral part of the blastula. They in turn switch on secreted members of the nodal family, which are capable of mesoderm induction. Concurrent with this, translocation of a dorsal modifying signal results in the formation of the Nieuwkoop centre, that in turn switches on organiser gene expression, such as BMP and Wnt antagonists (Wolpert, 2002).
Table 1-1  Summary of zebrafish specification and patterning mutants. The majority of these mutants were identified during the large-scale mutagenesis screens (see section 1.4). The similarities between the genes they encode and those from other species, highlights the evolutionary conservation of developmental mechanisms. Adapted from (Schier, 2001).
1.3 The notochord

The most anterior portion of the axial mesendoderm differentiates to form the hatching gland and prechordal plate. Prechordal plate has been shown to be a signalling centre involved in patterning of the ventral CNS (Macdonald et al., 1995). Posterior to the prechordal plate lies a thin stripe of axial mesendoderm called the chordamesoderm, which is fated to become notochord. The notochord is common to members of the phylum chordata and is a rod-like structure formed early in vertebrate embryonic development. It serves both as the major skeletal element of the embryo and as a source of signals required to pattern the surrounding tissues.

The zebrafish notochord is an ideal system in which to study cell differentiation, as it is a simple organ comprising only one cell type. The cells differentiate early in development acquiring a large central vacuole that inflates to occupy the majority of the cell. A peri-notochordal basement membrane (BM) surrounds the notochord in zebrafish and serves to restrain the swelling cells. The resulting turgour pressure gives the notochord its distinctive morphology and mechanical strength (Kimmel et al., 1995). Such a BM has also been described in the chick, monkey and human embryos (Camon et al., 1990; Jerome and Hendrickx, 1988; Shinohara and Tanaka, 1988). Later in development the notochord degenerates and becomes integrated into structures of the adult spinal column. Within the forming vertebrae notochord cells are replaced by bone and between vertebrae they become part of the tissue at the centre of the intervertebral discs, known as the nucleus pulposus (Fleming et al., 2001; Trout et al., 1982).

1.3.1 Specification of chordamesoderm

The first insights into the molecular events underlying zebrafish chordamesoderm specification came from studies of the mutant floating head (flh). flh is an embryonic lethal mutation that was isolated from maintained zebrafish stocks (Talbot et al., 1995). flh mutants do not form a notochord, as determined by morphology and analysis with the notochord specific antibody MZ15. Prechordal plate and other mesoderm derivatives, such as somites, are still present, as seen by expression of goosecoid and α-tropomyosin, respectively. In fact, chordamesoderm is re-specified to somitic mesoderm, as seen by abnormal co-expression of MyoD and no tail (Halpren et al., 2001; Trout et al., 1982).
1995). Tissues induced by the notochord are also disrupted, such as the dorsal aorta, hypochord, and floorplate.

The *flh* gene was cloned and found to be the homologue of the *Xenopus* homeobox gene *Xnot* (Talbot et al., 1995). Overexpression of *Xnot* in frog embryos results in production of excess notochord tissue (Gont et al., 1996). Transcription of the zebrafish gene *flh* is first detected around the entire circumference of the late blastula, and is restricted to the dorsal side of the embryo within the embryonic shield by the onset of gastrulation (6 hpf). As somitogenesis proceeds, *flh* expression within the notochord is extinguished in an anterior to posterior direction. Understanding the events upstream and downstream of this transcription factor should identify the molecular basis of notochord formation.

*spadetail (spt)* is a mutation in a T-box transcription factor homologous to *VegT*, and the mutant phenotype suggests *spt* is a crucial component of the pathway leading to specification of trunk mesoderm (Griffin et al., 1998). Results from analysis of *spt/flh* double mutants suggest that repression of *spt* function by *flh* is critical to promote notochord fate and prevent midline muscle development, as these double mutant embryo have a greatly restored midline compared to *flh* mutants (Amacher and Kimmel, 1998).

### 1.3.2 Differentiation of the notochord

Notochord differentiates in an anterior to posterior direction. The differentiation of the mature zebrafish notochord can be considered in terms of three major processes, which occur subsequent to chordamesoderm specification. Firstly, a chordamesoderm cell must acquire its distinctive notochord morphology, consisting of a large central vacuole and peri-notochordal BM *(Figure 1-3)*. Secondly, at the molecular level, gene expression must be altered such that expression of early signalling molecules (e.g. Shh) is extinguished in chordamesoderm and notochord differentiation genes are turned on. Finally, apoptosis must be prevented, i.e. cell survival mechanisms have to operate. The relationship between these processes is unclear, though there is evidence suggesting that they are independent events. For example, in both *cyclops* and *one-eyed pinhead* mutants the notochord vacuolates normally, but collagen type II is maintained (Strahle et al., 1997). Conversely, the zebrafish notochord mutant *doc*, (see next section) extinguishes *sonic hedgehog* expression but fails to inflate the vacuole (Stemple et al.,
1996). It is therefore possible that independent mechanisms exist to control each aspect of notochord differentiation.

*no tail (ntl)* was identified as the homologue of the mouse *T* gene (*Xbra* in frog) and is implicated in the differentiation of notochord (Schulte-Merker et al., 1994). *ntl* differs from *flh* in that the cells at the midline that would normally differentiate into notochord are still present in mutants and are not trans-fated to somite tissue. *ntl* expression is seen within all cells of the germ ring early on, and then only maintained in cells involuting dorsally within the shield. Analysis of *ntl* expression in *flh* mutants and vice versa, showed that each is required for the expression of the other. Importantly *ntl/flh* double mutants do not have additive defects and look very similar to single *ntl* mutants. Therefore, it has been proposed that *ntl* acts upstream of *flh* to promote a notochord fate (Halpern et al., 1997).

Morphogenesis is one aspect of axial mesoderm development that is distinct from cell specification or differentiation, but is intimately linked to both. Morphogenesis involves the formation of distinct tissues through cell migration, cell-cell interactions, and establishment of boundaries. It is clear that members of Eph receptor and ephrin families are required for this, as disruption of these in zebrafish results in abnormal morphogenesis of axial mesoderm (Chan et al., 2001). Further, previous studies with dominant negative forms of EphA3 and ephrin-A5 suggested the Eph-ephrin interaction is necessary for convergence movements during gastrulation in zebrafish (Oates et al., 1999). Whether this family of proteins also have instructive roles during the transition from chordamesoderm to notochord is not known. Cell adhesion molecules such as paraxial-protocadherin (PAPC), and components of the planar polarity pathway (e.g. Wnt-11) are also involved in these morphogenetic events, and may well act in parallel with signalling systems affecting the notochord differentiation (Heisenberg et al., 2000; Yamamoto et al., 1998).
Figure 1-3  Timing of vacuole formation in zebrafish notochord. The large central vacuole of each cell within the notochord (n), begins to inflate at ~18 hpf (A) in the anterior regions and is fully formed throughout the notochord by 28 hpf (C). Adjacent to the notochord are the spinal cord (c), floor plate (f), hypochord (h) and aorta (a). The notochord affects the development of each of these tissues. Adapted from (Kimmel et al., 1995). Scale bars 50µm.
1.3.3 Signalling roles of the notochord

Within the early embryo the notochord is ideally placed to perform its extensive signalling functions (Figure 1-4). The notochord is involved in specification of the floor plate, DV patterning of the neural tube, patterning of somites and establishment of left-right asymmetry (see below). Notochord not only patterns ectodermally and mesodermally derived tissues, but also the adjacent endoderm in an AP manner, reviewed in (Cleaver and Krieg, 2001).

Floor plate is the name given to the cells located within the ventral region of the neural tube. The floor plate controls axon guidance through secretion of netrin, (Colamarino and Tessier-Lavigne, 1995). Some controversy surrounding the specification of this cell fate has emerged recently following studies of zebrafish mutants. Two alternative explanations have been put forward. The first suggests that Shh secreted from the notochord induces floor plate, and is largely based upon mouse knockout phenotypes and grafting experiments in chick (Placzek et al., 2000). A second, more recent hypothesis is that induction of the floor plate occurs much earlier, within the gastrula organiser, and that notochord merely maintains its differentiated state (Le Douarin and Halpern, 2000). The evidence supporting this second hypothesis comes from studies of zebrafish mutants. no tail and flh mutants lack a notochord but still develop floor plate (Halpern et al., 1997; Schulte-Merker et al., 1994; Strahle et al., 1996). In addition fate-mapping studies indicate a common progenitor for medial floor plate and notochord (Gritsman et al., 2000). Conversely, cyclops and one-eyed pinhead mutants have a notochord, but the floor plate does not form (Rebagliati et al., 1998; Sampath et al., 1998; Strahle et al., 1997; Zhang et al., 1998). One observation that may resolve the issue is that zebrafish floor plate consists of two cell populations, a medial floor plate cell and two lateral floor plate cells, which differ in their requirement for Shh (Odenthal et al., 2000). Alternatively, the mechanism of induction may simply differ between amniotes and teleosts.

DV patterning of the neural tube via the notochord is not just restricted to induction/maintenance of the floor plate cell type. The notochord, in conjunction with the floor plate is also responsible for the induction of interneurons, motoneurons and oligodendrocytes in the spinal cord, via secretion of Shh (Lee and Jessell, 1999).
Distinct concentration thresholds give rise to a homeodomain protein code that specifies particular cell types (Briscoe and Ericson, 1999; Ericson et al., 1995).

Notochord is also necessary for correct patterning of the somites (Stickney et al., 2000). In amniotes, the ventral half of the somite gives rise to sclerotome, which contains the precursors of the axial skeleton and ribs, while the dorsal region forms the dermamyotome. Lateral dermamyotome is fated to become limb and ventral body musculature and dermis (hypaxial muscle), while the medial cells form back and intercostal muscles (epaxial). The induction and maintenance of sclerotome requires signals from the notochord (Dockter, 2000).

In zebrafish, two types of muscle precursors, medial and lateral, were identified by fate mapping studies (Devoto et al., 1996). The medial population are termed adaxial cells and will migrate laterally to form the slow muscle. The role of the notochord in the induction of slow muscle was investigated following characterisation of the zebrafish mutants, floating head (flh), no tail (ntl) and bozozok (boz). These all show a variable degree of deficiencies in slow muscle formation, muscle pioneers and horizontal myosepta, which result from abnormalities in notochord development (Blagden et al., 1997; Halpern et al., 1993; Stemple et al., 1996; Talbot et al., 1995). In flh and boz mutant embryos, the notochord precursors do not form, while in ntl mutant embryos notochord precursors are present, but do not differentiate. Improper hedgehog signalling from the notochord is responsible for these phenotypes. In zebrafish there are three different hedgehog genes expressed within the axial mesoderm. Shh and echidna hedgehog (ehh) are both expressed within the notochord (Currie and Ingham, 1996), while tiggy-winkle hedgehog (tghh) is expressed in the floorplate (Ekker et al., 1995). The functions of these genes are thought to have some degree of redundancy. In WT embryos, overexpression of each hedgehog cRNA can lead to expansion of slow muscle and reduction of fast muscle, and possibly sclerotome. In particular, the combination of shh and ehh overexpression results in the increase of the number of muscle pioneer cells at each somite, i.e., normal slow muscle territories (Currie and Ingham, 1996).

Endoderm is patterned along both the AP and DV axes (Cleaver and Krieg, 2001). Such patterning establishes the domains where the organ primordia form along the gut tube. Notochord is known to express in an AP restricted manner four zebrafish hox genes: hoxb1, hoxb5, hoxc6 and hoxc8 (Prince et al., 1998). Given that the vertebrate Hox code
confers regional identity along the AP axis, it is possible that this restricted expression in the notochord is reflected in molecular differences that may alter signalling capacities along its length.

The major endodermally derived organs include: the respiratory system, liver, thymus and thyroid, and digestive system (oesophagus, stomach, pancreas, intestines and colon). A role for the notochord in the formation of each of these organ systems has been documented (Barlow, 2001; Kim et al., 1997; Korzh et al., 2001). Evidence for patterning of endoderm by notochord came from heterologous recombination experiments, performed in chicken, mouse and rat, where mesoderm and endoderm from different AP levels were recombined (Fukamachi et al., 1979; Fukamachi and Takayama, 1980; Yasugi, 1994). Furthermore, clinical cases reveal that abnormal notochord development occurs in conjunction with gastrointestinal defects (Kiristioglu et al., 1998). In molecular details, we are still very far from understanding how endoderm is patterned, and shared signalling molecules may function differently within different species. For example, studies of zebrafish pancreas development, through analysis of the marker pdx-1 seem to contradict work performed in amniotes, which suggested shh was a negative regulator of pancreas specification (Hebrok et al., 2000; Roy et al., 2001).

The major axial vessels (dorsal aorta and axial vein) form in close proximity to the notochord and endoderm. In flh and ntl mutants the dorsal aorta fails to form, indicating a necessary role for notochord in this process (Weinstein, 1999). Moreover, genetic mosaic experiments and analysis of flk-1 expression (a vascular progenitor marker) suggest that there may be a signal from the notochord that is essential for guiding the migrating angioblasts to the midline (Fouquet et al., 1997). Left-right asymmetry is also affected by loss or removal of the notochord and many of the midline zebrafish mutants also have defects in left-right asymmetry. The best studies of left-right determination have been performed using chick (Tamura et al., 1999). Although the mechanism maintaining left-right asymmetry is not fully understood interactions of midline cells are clearly essential (Schier and Shen, 2000; Yost, 1998).
Figure 1-4  Signalling of the notochord in zebrafish embryos. Schematised view of a transverse section through: (A) an early somite stage embryo, and (B) late somite stage embryo. The notochord (blue) lies ventral to the neural tube (green), and its position reflects its important role as a signalling centre, involved in the patterning of somites (pink), neural tube, and underlying endoderm. Adapted from (Gilbert and Rainio, 2000).
1.4 Mutant screens

In October 1980 a seminal paper was published in which C. Nusslein-Volhard and E. Wieschaus reported the first systematic search for genes involved in early development (Nusslein-Volhard and Wieschaus, 1980). The mutants generated from this approach have revolutionized our understanding of developmental biology. Homologous genes to those originally isolated in fly have been uncovered in many diverse organisms, and an unexpected conservation of developmental mechanisms throughout the animal world has been revealed.

Soon after the fly screen was published G. Streisinger proposed that vertebrate development could also be approached in this way using the zebrafish (Streisinger et al., 1981). Just over a decade later Christianne Nusslein-Volhard and Wolfgang Driever initiated large-scale mutagenesis screens for recessive-zygotic mutations in the zebrafish. Although much had been learned concerning metazoan development from the fly screen, uniquely vertebrate tissues such as notochord and neural crest, could only be understood through studies of vertebrate developmental genetics. The results of the zebrafish screen (in fact two screens, one in Boston and one in Tübingen) were published in 1996 and gained a great deal of publicity (Eisen, 1996).

Although not carried out to saturation, >400 complementation groups were identified that have been classified according to phenotype. Among these are genes affecting well-studied processes such as DV patterning, neural specification and somitogenesis as well as less well-understood processes such as organogenesis, axonal pathfinding, and locomotion behaviour. These mutants provide developmental biologists with an incredible resource with which to piece together the molecular mechanisms of early vertebrate development (Felsenfeld, 1996; Granato and Nusslein-Volhard, 1996; Holder and McMahon, 1996). The completion of these large zebrafish screens prompted the undertaking of many other smaller-scale screens more focussed on specific processes, that make use of fluorescent reporters or inventive behavioural assays (Patton and Zon, 2001).
1.4.1 Notochord mutants

The screen revealed many mutations that result in embryos with notochord defects. In all, 65 mutations corresponding to 29 complementation groups were identified in the Boston screen with four others identified in Tübingen (Odenthal et al., 1996; Stemple et al., 1996). Defects of notochord specification, differentiation, degeneration, maintenance and shape, were found. Many of these mutants have a characteristic shortened body axis due to lack of extension along the AP axis. Mutants were divided into several classes according the type of defect observed, providing a phenotypic pathway. Two of these notochord mutants, *floating head* and *no tail*, had been isolated previously and are defective in chordamesoderm specification and differentiation respectively (see section 1.3). *bozozok* (*boz*) mutants were the only other mutant identified that failed to specify chordamesoderm. Severe *boz* mutants lack all axial mesoderm, have no chordamesoderm or floorplate, and show cyclopia (Stemple et al., 1996). *boz* was identified as the homeobox gene *Dharma/Nieuwkoid* and is necessary for formation of the organiser, acting downstream of β-catenin in an analogous manner to *siamois* in frog (Fekany et al., 1999).

As well as *no tail*, 8 other genetic loci were characterised as having defects in notochord differentiation, based on maintenance of early notochord markers such as *collagen type II*, *shh*, and *no tail*. These were: *sneezy* (*sny*), *dopey* (*dop*), *happy* (*hap*), *mikry* (*mik*), *doc*, *bashful* (*bal*), *grumpy* (*gup*) and *sleepy* (*sly*). In addition to the failure of notochord differentiation, *bal*, *gup* and *sly* also have brain defects, where the brain has an irregular morphology, the hindbrain ventricle is enlarged and the axonal scaffold is disrupted (Schier et al., 1996). These three mutants were also identified in a screen for retinotectal pathfinding mutants (Karlstrom et al., 1996). Accordingly, they were grouped together in the same phenotypic class (Figure 1-5). All *bal* alleles identified show a much weaker phenotype than either *gup* or *sly*, in that they contain large amounts of apparently WT notochord caudally. Further characterisation of *bal*, *gup* and *sly*, is the major focus of this thesis. Identifying the genes disrupted in *bal*, *gup* and *sly*, should be highly informative, as shared phenotypes are commonly due to disruptions within same biochemical pathway. Furthermore, we will learn whether the brain and notochord phenotypes are directly linked, or more likely represent dual functions of a similar pathway.
Figure 1-5 Notochord Mutants. (A) Phenotypic pathway of notochord development. bozozok and floating head have defects in chordamesoderm specification. no tail is the homologue of mouse gene brachyury (Xbra in frog). dopey, happy and sneezy were recently identified as components of the coatmer complex (P. Coutinho, PhD thesis). bal, gup and sly, also lack a terminally differentiated notochord but in conjunction have CNS defects including axon pathfinding errors. doc is the only mutant that has a specific defect within notochord differentiation. doc has not been characterised at the molecular level. (B) Morphology of bal, gup and sly mutants at 48 hpf. Notice the weaker nature of the notochord defect in bal compared to gup and sly; only the anterior notochord fails to mature (arrow head).
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**Specification mutants:**
- bozozok
- floating head

**Differentiation mutants:**
- no tail
- doc
- dopey
- sneezy
- happy
- bashful
- grumpy
- sleepy

+ general degeneration
+ CNS defects

A

B

bashful (bal)  grumpy (gup)  sleepy (sly)

WT
The results presented in this thesis identify an essential role for the peri-notochordal basement membrane during formation of the notochord. Therefore, in the next section I discuss the extracellular matrix and its role during embryonic development.

1.5 Extracellular matrix

The extracellular matrix (ECM) fills the space between the cells in a tissue and is defined as essentially all secreted molecules that are immobilised outside cells (Kreis and Vale, 1999). It comprises a multitude of proteins and polysaccharides that have vital roles during embryogenesis and later in the adult. Three broad classes of molecules are typically found: collagens, non-collagenous glycoproteins and proteoglycans. The variety of forms that these ECM molecules can assemble into is vast, and this provides a repertoire of materials for constructing the organism. For example, connective tissues contain massive amounts of matrix molecules, secreted by fibroblasts, which provide the major architectural support for the adult body. The mechanical integrity, rigidity, and elasticity of organs such as skin, vasculature, tendons, lungs, bone and teeth all vary due to differences in the matrix composition.

A realisation over the past decade has been that the ECM fulfils a more active role than was previously believed, and is capable of affecting many cellular processes in an instructive manner (Adams and Watt, 1993). A detailed discussion of ECM in the adult is beyond the scope of this thesis, and so I concentrate on the major types of ECM molecules present in the early vertebrate embryo.

1.5.1 Extracellular matrix during vertebrate development

During embryogenesis populations of cells undergo many morphogenetic events that involve cell-ECM interactions (Zagris, 2001). Epithelial cells form sheets and tubes, neural crest cells migrate large distances and a range of other morphogenetic processes occur. ECM can also affect cell differentiation and can regulate the activity of secreted patterning molecules. Cell-ECM interactions that affect cell migration have been well-characterised, though an equally important property of the ECM arises when it forms a specialised type of matrix known as basement membrane (BM). ECM proteins such as fibronectin, collagen and laminin have all been shown to be major classes of molecule important during early vertebrate development. Recently proteoglycans have received
interest due to their regulatory effects on various signalling molecules (Gustafsson and Fassler, 2000; Perrimon and Bernfield, 2001). I discuss each of these major protein families and some of their well-characterised roles during development.

1.5.2 Fibronectins

Fibronectins are large disulphide-linked dimers encoded by a single gene. There are many forms of fibronectin formed through alternative splicing and the resulting proteins are modular in design with many functionally different domains linked by regions of flexible polypeptide. The best-characterised module is known as the type III fibronectin repeat, which is a domain common to many vertebrate proteins (Campbell and Spitzfaden, 1994). The type III domain interacts with integrins through the RGD peptide, and this interaction (in conjunction with a functional actin cytoskeleton) promotes the formation of the fibrillar form of fibronectin that attaches to the cell surface (Darribere et al., 1990). Fibronectin is present at sites of cell migration such as during gastrulation, neural crest cell migration, and migration of primordial germ cells. It has been shown to be necessary for gastrulation movements in salamander and frog. These studies showed that these cell movements could be inhibited through injection of either antibodies to fibronectin and integrins, or RGD peptides (Boucaut et al., 1984; Smith et al., 1990). Similar studies carried out in the chick prevented migration of heart precursors to the midline (Icardo et al., 1992). Gene targeting of the fibronectin locus in mouse results in mesodermal defects, including lack of somites, and cardiovascular defects, though gastrulation seems unaffected, somewhat contradicting the earlier studies (George et al., 1993). Cell attachment during these migrations is not the sole function of fibronectin, as it can also be restricted to specific regions of the embryo, thereby acting as a path to guide cells to their final destination. This is particular obvious from studies of fibronectin in neural crest cell migration (Perris and Perissinotto, 2000).

1.5.3 Collagens

Collagens are a family of fibrous proteins that can account for up to 25% of the total protein mass of the adult in mammals. Mutations within collagen genes are responsible for a variety of genetic disorders such as Alport syndrome, chondrodysplasias, and some
forms of osteoporosis (Prockop and Kivirikko, 1995). There are 25 known collagen α chains, encoded by separate genes that form in restricted combinations over 20 different isoforms. These have tissue specific expression and unique roles but their specific roles during development are not well understood.

Collagens are a major component of BM, of which the network-forming type IV \([\alpha 1(IV)\alpha 2(IV)]\) is best characterised. Humans contain six type IV α chains while fly and worm each have two type IV collagens. Type VII collagen forms also forms networks that can give rise to anchoring filaments associated with BM, particularly within the skin. Other isoforms are more flexible than the fibrillar collagens and form an insoluble mesh to which other components bind. Collagen type II, \([\alpha 1(II)]_3\), is a noteworthy member, as it is known to be an early marker of zebrafish chordamesoderm (Yan et al., 1995). The targeted deletion of type II collagen α1 results in chondrodysplasia and invertebral disc defects; these mice also fail to dismantle the notochord (Aszodi et al., 1998).

1.5.4 Laminins

Laminins are a family of heterotrimeric glycoproteins that are one of the earliest extracellular matrix proteins secreted during development, and are major components of basement membrane, reviewed in (Colognato and Yurchenco, 2000). Three polypeptide chains, α, β and γ make up the laminin heterotrimer complex, and there are multiple forms of each of these. To date five α, four β and three γ genes have been identified (Table 1-2). Various combinations of these give rise to the multiple laminin isoforms and, as with the collagens, there appear to be restrictions such that only a subset of all possible combinations is produced. Currently twelve isoforms have been reported (Table 1-3). The nomenclature for these various isoforms was revised in 1994, and obscure names such as EHS-laminin, merosin, and s-laminin, are replaced with a simpler Arabic numeral system, based on the order in which they were discovered, i.e. laminin-2, laminin-3 etc. (Burgeson et al., 1994).

Laminins are extremely large proteins (~850kD) that have a cruciform appearance when viewed using rotary shadow electron microscopy. Coiled-coil domains in the C-terminus form what is termed the long arm and hold the three chains together. The N-
terminus of each chain contains globular domains that give rise to the short arms (Figure 1-6). The globular domains within the C-termini of \( \alpha \) chains are the major sites of interaction with cell-surface receptors such as integrins and dystroglycan (see chapter 6). As well as containing binding sites for collagen IV and nidogen, laminins can also bind to one another and can thus form networks (Tunggal et al., 2000). This polymerisation occurs spontaneously \textit{in vitro} and is calcium-dependant (Yurchenco et al., 1992). Initial polymerisation of the laminin network \textit{in vivo} is thought to occur via mass action resulting from high local concentrations around the tissue following secretion.

The biochemistry of laminin 1 (\( \alpha_1\beta_1\gamma_1 \)) is the best understood. Laminin 1 was identified in 1979 in extracts from the Englebreth-Holm-Swarm (EHS) murine tumour and teratocarcinoma cells (Chung et al., 1979; Timpl et al., 1979). 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. Laminin 1 has also been implicated in axon guidance and has been shown to convert the attractive role of netrin to repulsion (Hopker et al., 1999).

Some of the roles of laminins \textit{in vivo} have been determined 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, and they can affect processes as diverse as cell migration, differentiation, metabolism and polarity (Colognato and Yurchenco, 2000; Gustafsson and Fassler, 2000).

The laminin \( \gamma_1 \) chain is present in 10 of the 12 known laminin isoforms, suggesting a pivotal role for this particular chain. Disruption of the \textit{lamc1} gene, encoding laminin \( \gamma_1 \), therefore affects the majority of known laminin isoforms, and the associated BMs. Homozygous mutant mice die as a result of failure to form Reichert’s membrane, which is required for proper implantation (Smyth et al., 1998; Smyth et al., 1999). The targeted disruption of either \textit{lamal} or \textit{lamb1}, has not been reported, though as the laminin 1 isoform (\( \alpha_1\beta_1\gamma_1 \)) is thought to be the major laminin of Reichert’s membrane one might expect them to have the same pre-implantation lethal phenotype as \textit{lamc1} (Amenta et
al., 1983; Hogan et al., 1980). Due to this early developmental requirement for laminin 1 in mice it has been difficult to address the later roles of isoforms containing one or more of α1, β1 and γ1 chains. In addition some laminin chains may be functionally redundant, making loss-of-function phenotypes difficult to interpret.

Disruption of the gene encoding mouse laminin β2 resulted in mutants with abnormal motor nerve terminals, revealing a role for the laminin β2 chain in formation of the neuromuscular junction (Noakes et al., 1995). The laminin α2 and γ1 chains are partners for laminin β2 at the neuromuscular junctions but elsewhere in the developing skeletal muscle and peripheral nerve they associate with β1 (laminin 2). The importance of α2-laminins in the muscle and peripheral nervous system is underlined by the fact that half of all congenital muscular dystrophies have mutations in this gene (Helbling-Leclerc et al., 1995; Xu et al., 1994). CNS defects are also reported in patients with defective α2 chains likely resulting from dramatic reduction of myelin in the brain. Functions of laminin chains in the nervous system have been reported in fly, frog and worm, where laminins have been known for many years to affect axon guidance (Liesi, 1990).

Laminin α3 chain is expressed in the skin and other epithelia and is present in laminin 5 (α3β3γ2), laminin 6 (α3β1γ1) and laminin 7 (α3β2γ1). When deleted in mice it results in neonatal lethality due to severe epithelial defects, most notably severe skin blistering phenotype from a lack of hemidesmosomes (Ryan et al., 1999). This type of phenotype was also observed in human patients with disruptions of genes encoding laminin β3 and γ2, suggesting a role for these chains in skin function and integrity (Pulkkinen et al., 1994; Pulkkinen et al., 1995). This represents one of the few cases where a specific loss-of-function phenotype for a laminin isoform has been uncovered, as the α3β3γ2 chain combination is unique to laminin 5.

Recently, laminin α4, which is present in laminin 8 and laminin 9 isoforms, has been disrupted in mice and was shown to have defects in micro-vessel formation. Mice presented with hemorrhages during the embryonic and neonatal period. Targeted disruption of laminin α5 also results in an early embryonic lethality. Homozygous mutants show exencephaly, syndactyly, placentopathy and defective glomerulogenesis (Miner et al., 1998). Laminin α5 chain is present in laminin 10 and 11.
Both *Drosophila* and *C. elegans* have laminin gene homologues, though only two different alpha subunits have been detected in each of these species, reviewed in (Brown, 2000). LamA (fly) and epi-1 (worm) have greatest similarity to laminin α5. *Drosophila wing blister* (wb) and *C. elegans lam-3* genes are equally similar to laminin α1 and α2. Drosophila wb mutants display a striking axon guidance defect as well as a wing blistering phenotype due to an adhesive failure during wing formation (Martin et al., 1999). Laminin A plays an important role during oogenesis and is essential for proper axis formation suggesting that the ancestral alpha gene was more similar to alpha 5 than to alpha 1-4 (Henchcliffe et al., 1993; Miner et al., 1995).

### 1.5.5 Basement membrane

One particular class of laminin-containing ECM, known as BM, is particularly prevalent during early vertebrate development and provide a compartmentalisation role by grouping together of cells into distinct tissues and the separation of these tissues from one another. It is principally composed of type IV collagen, laminin, nidogen, and several proteoglycans. BM is always found adjacent to or surrounding epithelial cells and the cells that rest upon are thought to secrete its principal components. Additional ECM molecules more loosely associated with the sheet-like BM can be supplied by underlying connective tissue (Slack, 2001).

BM can control many aspects of cell/tissue behaviour during development and following injury (Schwarzbauer, 1999). Investigations of lung, breast and pancreas have shown that laminin, 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; Streuli et al., 1995; Thomas and Dziadek, 1994). A more specific role is performed in kidney glomerulus where the BM acts as an important component of the selective barrier that prevents passage of macromolecules from the blood into the urine (Figure 1-6). BM has also been intensively studied in the neuromuscular junction where it is known to have an important role in localisation of the synapse both during embryogenesis and after injury (Carbonetto and Lindenbaum, 1995; Sanes and Lichtman, 1999).

The basic composition of BM has been known for over a decade and is well accepted. laminin and collagen form independent networks by self-polymerisation, which are
cross-linked to one another via nidogen. BM often has multiple roles, fulfilling not only architectural requirements, but also an instructive function through signalling pathways mediated by specific cell-surface receptors. Multiple genes encoding distinct laminin and collagen chains, as well as numerous types of cell-surface receptors and regulatory proteases have been characterised over the last few years. Thus, the composition, signal transduction, and processing of BM is much more complicated than first believed and differences in BM composition will give rise to tissue specific functions.

1.5.6 Proteoglycans

Proteoglycans are a group of extracellular matrix molecule that are grouped together because of their shared post-translational modification by attachment of glycosaminoglycans (GAGs). There are four types of GAG that differ in the type of disaccharide repeat they possess: hyaluronan, chondroitin sulphate/dermatan sulphate, keratan sulphate, and heparan sulphate. It is the GAG itself that is thought to dominate the functional properties of each type of proteoglycan (Kreis and Vale, 1999).

During embryonic development many intracellular signalling molecules are used to generate pattern formation and specify tissues to various fates. Many of these are regulated to some extent by proteoglycans associated with BM (Timpl, 1994), which can act to stabilize them against mis-folding and proteolysis (Perrimon and Bernfield, 2001). For example, the proteoglycan Perlecan is present in the majority of BMs and is known to be a low-affinity receptor for bFGF (Aviezer et al., 1994). Targeted disruption of the perlecan gene results in exencephaly and chondrodysplasia a phenotype very similar to FGFR3 mutants (Arikawa-Hirasawa et al., 1999). Also, in mouse the proliferative activity of shh on cerebellar granule cells is regulated through binding to heparan sulphate proteoglycans (HSPs) (Rubin et al., 2002).
Figure 1-6  **Structure of laminin and the roles of BM.** (A) A schematic view of the laminin 1 heterotrimer. Many of the ligand interaction sites that have been mapped are illustrated. The laminin α1 chain (green) and is larger than the β1 (red) or γ1 (blue) chains. All of the α chains contain a C-terminal series of globular domains that interact with laminin receptors. The short arm of each of the three chains is the region of self-polymerisation, adapted from (Colognato and Yurchenco, 2000). (B) Laminin heterotrimers associate with one another to form a sheet-like matrix. This laminin polymer also interacts with the collagen network via nidogen, adapted from (Alberts, 1994). Proteoglycans, such as perlecan, are often associated with BM. (C) BM is associated with epithelial tissues, and has specialised roles in tissues such as muscle and kidney, adapted from (Lodish, 1999).
<table>
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Table 1-2  **Identified Laminin chains.** Adapted from (Kreis and Vale, 1999).
Table 1-3   Chain composition of the twelve known laminin isoforms. (A) Laminin 1 was previously known as EHS laminin; laminin 2 as merosin; and laminin 3 as s-laminin. The γ1 chain is present in all but two isoforms, and this has been suggested to indicate an essential role of this chain in laminin function or assembly. (B) Schematic view of the structural organisation of the various laminin isoforms. Adapted from, (Colognato and Yurchenco, 2000).
1.6 Summary of thesis results

The results of this investigation are described in four chapters. In chapter 3, I introduce the method of positional cloning and then present the results of such an analysis for bal, for which I have identified the laminin α1 chain as a candidate. In chapter 4, I characterize the zebrafish lamal gene, including identification of the mutation responsible for the bal phenotype. Chapter 5 contains the results from the positional cloning of the related notochord mutant, gup, along with some characterization of the gene lamb1 (encoding laminin β1). I also present the analysis of other laminin α chains (lama2, lama4 and lama5) and make conclusions concerning roles for particular laminin isoforms during zebrafish development. In chapter 6, I provide evidence suggesting that some members of the integrin family and a downstream component, integrin-linked kinase (ILK) are not involved in differentiation of the notochord per se, but instead function control cell movements during gastrulation. Finally, the general discussion provides some speculations and future directions that this work might take.
Chapter 2

Materials and methods
Chapter 2

Materials and methods

2.1 Embryo collection

Zebrafish (Danio rerio) embryos were raised at 28°C in embryo water (red sea salt 0.03 g/l, methylene blue 2 mg/l) or in 0.3X Danieau solution. 1X Danieau solution is 58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO₄, 0.6 mM Ca(NO₃)₂, 5 mM HEPES, pH 7.6). Approximate stages are given in hours-post-fertilisation (hpf) at 28°C according to the morphological criteria provided in (Kimmel et al., 1995).
2.2 Embryo labelling and microsurgery

For shield transplantation experiments, chorions of donor embryos were removed by 4 minutes incubation in 0.5 mg/ml pronase (Sigma) in 0.3X Danieau solution followed by several washes in 0.3X Danieau solution. Donor embryos were then transferred into ramps made of 2% agarose in 0.3X Danieau solution covered with 0.3X Danieau solution. Donor embryos were labelled at the 1-4 cell stage by micro-injection into the yolk cell with rhodamine dextran (Molecular Probes) in 0.2 M KCl.

Transplantation pipettes were pulled from 1 mm borosilicate glass capillaries (World Precision Instruments, 1B100-4) and cut with a diamond pencil to an inner diameter of approximately 200 µm. A sharp inner edge is optimal and the plane of the cut was orthogonal to the long axis of the pipette. The pipettes were initially filled with medium and then loaded into a pipette holder filled with mineral oil. The pipette holder (World Precision Instruments, 5430-10), carried by a 3-axis micro-manipulator (Narishige, MN-153), was connected, via a continuous column of mineral oil, to a 50 µl Hamilton syringe driven by a micrometer controlled syringe pump (Stoelting, 51218).

Microsurgery was performed at 19-21°C in 1X Danieau solution containing 5% penicillin/streptomycin (Gibco-BRL, 15140-114). The chorion of host embryos was removed with watchmaker’s forceps shortly before transplantation. Donor and host embryos were loaded into transplantation wells that had been pre-formed with an acrylic mould in 2% agarose/1X Danieau solution. Transplantation wells were 1.0 mm deep by 1.0 mm wide, with the bottom surface of the well sloping from the back wall of the well approximately 1.3 mm to the surface of the agarose.

To remove shield tissue, donor embryos were oriented such that the shield faced the pipette tip and the transplantation pipette was placed over the shield. Shield tissue was gently drawn in and out of the pipette generally 3 or 4 times until the yolk cell and shield tissue became separated. To transplant the shield tissue, hosts were oriented so that the site of transplantation was 180° from the host shield. With the donor shield in the pipette, the tip of the pipette was placed onto the host embryo, at the margin, and a piece of ventral tissue was removed and discarded. The donor shield was pushed to the tip of the pipette, which was placed over the hole in the host embryo. The donor shield was gently expelled into the host embryo. Typically, the enveloping layer (EVL) of the
host inflated a little, making space for the donor tissue. For acceptable transplants, the donor shield tissue became trapped under the EVL either directly beneath or immediately adjacent to the hole. Transplanted embryos were left in the transplantation well for about 10 minutes to recover then transferred to 0.3X Danieau with 5% penicillin/streptomycin on 2% agarose/0.3X Danieau for overnight culture.

2.3 Culture of embryonic shields

The embryonic shield was cultured in complete medium (CM) comprising L15 medium + 10% fetal calf serum (Gibco) and 1mM HEPES pH 7.0 + 1% penicillin streptomycin solution (Gibco). Shields were cultured on the Permanox Chamber Slide System® (Lab-Tek). Each well was pre-treated as follows. 200µl of a 0.1mg/ml solution of poly-D-lysine (Sigma) was placed in each well and immediately removed. The wells were then air-dried for 2hours and washed twice with ddH2O. Finally, 200µl of 250µg/ml fibronectin solution (Biomedical technologies) was added to each well for 2mins and then removed, and replaced with CM.

The same procedure as described in 2.2 was used to remove the morphological shield with the exception that all embryos were dechorionated manually with watchmakers forceps. Typically, 5 shields were removed at a time, and then transferred using a P20 pipette in minimal volume of 1xDanieau, to a pre-treated well and allowed to settle and attach for 30mins, before adding 250µl of CM to just cover the shields. The chamber slide was then incubated overnight in a humidified incubator at 28°C. The following morning each well was flooded with 300µl CM.

2.4 General molecular biology techniques

2.4.1 Small scale preparation of DNA

The Qiagen Spin miniprep kit was used for all small scale plasmid preparations (Qiagen). From a 5ml overnight culture of bacteria in selective LB medium, 1.5 ml was transferred to a 1.5 ml microcentrifuge tube and spun for 20 seconds. The supernatant was removed completely and the pellet resuspended in 250 µl of Resuspension Buffer (Qiagen; 10 mM EDTA, 50 mM Tris.HCl pH 8.0, 100µg/ml RNase). 250 µl of Lysis Buffer (Qiagen; 0.2 M NaOH, 1% SDS) was added, mixed and left for 5 minutes at
room temperature to allow alkaline lysis of the cells. Lysis solution was then neutralised by adding 350 μl of ice-cold Neutralisation Buffer (Qiagen; 3M KOAc pH 5.5) and mixed carefully by inverting the tube a few times, followed by 10 minutes incubation on ice. The tube was spun for 15 minutes at room temperature and the supernatant was transferred into a fresh microcentrifuge tube and washed with 750μl of 100% EtOH. DNA was then eluted from the membrane using 50μl of sterile water.

DNA and RNA were quantified by spectrophotometry at 260 nm (an OD of 1 equates to 50 μg/ml double stranded DNA, 35 μg/ml single stranded DNA and 40 μg/ml RNA). The ratio between the readings at 260 nm and 280 nm provided an estimate of the purity of the nucleic acid preparation (pure preparations of DNA and RNA should have \( \frac{OD_{260}}{OD_{280}} \) values of 1.8 and 2.0, respectively).

2.4.2 Gel extraction of DNA

For the extraction of DNA from agarose gels, the QIAquick Gel Extraction Kit (Qiagen) was used according to manufacturers protocol. Samples were eluted in 30μl of water and 4μl of this was used for TOPO cloning or standard ligation reactions.

2.4.3 Phenol/Chloroform extraction

To remove proteins from nucleic acid solutions, a mixture of phenol:chloroform:isoamyl-alcohol (25:24:1 volume ration) was added in a 1:1 volume ratio to the DNA solution and vortexed for 1 minute. After a 5 minutes centrifugation, the upper (aqueous) layer was transferred into a new microcentrifuge tube and extracted with an equal volume of chloroform.

2.4.4 Ethanol Precipitation

Ethanol precipitation was carried out by adding 3 M NaOAc pH 5.5 (to a final concentration of 0.3 M) and 2.5 volumes of 100% ethanol to the DNA solution that was then left on dry ice for approximately 20 minutes. 1 μl of 10 mg/ml glycogen was often used as a carrier to visualise the pellet at the bottom of the tube. Centrifugation at 20 000 x g for 5 to 20 minutes was performed and the DNA pellet was then washed in 70% ethanol, dried and re-suspended in TE or distilled water.
2.4.5 TOPO cloning

The cloning of PCR products was performed using the TOPO TA Cloning® kit (Invitrogen). The cloning reaction was performed according to the following conditions: 4 µl fresh PCR product or 4µl of gel purified product, 1µl of 1.2M NaCl solution and 0.5 µl pCR®-TOPO® vector. These were mixed gently and incubated for 5 minutes at room temperature.

2.4.6 Transformation of chemically competent bacteria

Transformation of the ligated vector was performed using chemically competent TOP10 cells (Invitrogen). Briefly, 2µl of TOPO ligation mix was added to 25µl cells and incubated on ice for 30mins. Cells were then heat shocked for 30s at 42°C, then immediately transferred to ice, incubated for 2mins before adding 250µl SOC added. Cells were then incubated at 37°C for 1hr and an aliquot of 10 to 200 µl from each transformation was spread onto a selective agar plate (100 mg/ml of ampicillin) and incubated overnight at 37°C. 40 µl of X-Gal (20 mg/ml in dimethylformimide) and 40 µl of IPTG (200 mg/ml) were used per plate for selection.

2.4.7 Transformation of electrocompetent cells

Up to 100 ng of DNA was added to 20 µl of TOP10 electrocompetent cells (Invitrogen) cells that had been thawed on ice, then immediately transferred to a pre-cooled 0.1 cm electroporation chamber. Cells were electroshocked under 1.8 kV, 25 µF and 200 Ω. 1 ml of SOC medium was immediately added, the mixture was transferred to a plastic tube and incubated with shaking at 37 °C for 1 hour. Cells were plated as described for chemical transformation.

2.4.8 Restriction digestions

Restriction enzyme digests were performed at the recommended temperature for approximately 2hrs using commercially supplied restriction enzymes and buffers (Boehringer Mannheim, Promega, New England Biolabs). The enzyme component of the reaction never comprised more than 10% of the reaction volume. For enzyme
digests using more than one restriction enzyme, the buffer suggested by the manufacturer was used.

2.4.9 DNA Sequencing

DNA sequencing was performed using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit, according to the manufacturers instructions, in an ABI 377 automatic sequencer.

2.4.10 Bioinformatics

All PCR primers were designed using the program primer3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3 www.cgi). The RZPD site was used extensively to obtained cDNA clones, identified through EST searches of GenBank. All manipulations of DNA sequence was performed with Sequencher and DNAnstar software, with BLAST site from NCBI used for sequence analysis. Clustal was used for sequence alignments. The Sanger centre zebrafish genome project was used for identification of genomic fragments containing exons of interest (http://www.sanger.ac.uk/Projects/D_reio/).

2.5 Preparation of genomic DNA from Adult Fish

Whole tail fin from adult fish was dissected and placed in 0.5ml of extraction buffer (0.5% SDS, 0.1 M EDTA pH.8.0, 10mM Tris pH.8, 100 µg/ml Proteinase K) for 5 hours at 55°C. Phenol chloroform extraction and ethanol precipitation were carried out, and the final pellet was resuspended in 30µl TE. A 1:100 dilution of this was used PCR template.

2.6 Preparation of genomic DNA from embryos

The digestion of each single embryo was done by incubating in 100 µl of extraction buffer (0.5% SDS, 0.1 M EDTA pH.8.0, 10mM Tris pH.8, 100 µg/ml Proteinase K) for 5 hours at 55°C. The DNA was purified using Multiscreen-GV, sterile column plates (0.22 µm hydrophilic, low protein binding, Durapore Membranes) and recovered in 100µl of low TE (10mM Tris pH.8, 0.1mM EDTA).
2.7 Oligonucleotide labeling

When testing for SSLP markers we always labelled both oligonucleotides. For 30 reactions, we mixed 0.5\(\mu\)l 10x T4 polynucleotide kinase buffer, 2.9 \(\mu\)l of 10\(\mu\)M oligonucleotide, 0.1 \(\mu\)l T4 polynucleotide kinase (10 U/\(\mu\)l) and 1.5 \(\mu\)l \(\gamma^{32}\)P dATP, 6000Ci/mmole, 10mCi/\(\mu\)l. This reaction was then incubated for 30 minutes at 37°C.

2.8 Polymerase Chain Reaction (PCR)

2.8.1 SSLP mapping

All PCR reactions were performed in 96-well plates with a final reaction volume of either 10 \(\mu\)l or 20 \(\mu\)l. Purified DNA from mutant embryos was diluted 10 times in low TE before being used. Adult fish DNA was used in a concentration of about 10-50 ng/\(\mu\)l. For a 20 \(\mu\)l reaction we used 14.3 \(\mu\)l PCR mix, 0.15 \(\mu\)l of each labelled oligonucleotide, 0.2 \(\mu\)l of Taq polymerase (5u/\(\mu\)l) and 5 \(\mu\)l of DNA template. The PCR mix was made by adding 122 \(\mu\)l of 10x Taq buffer, 9.8 \(\mu\)l of 25 \(\mu\)M dNTPs, 740.8 \(\mu\)l of distilled water to final volume of 872.6 \(\mu\)l. Each reaction mixture was overlaid with 1 drop of mineral oil. The PCR conditions were as follows:

- 94°C for 3 minutes
- 35 cycles:
  - 92°C for 1 min
  - 58°C-60°C for 1 min
  - 72°C for 1 min and 30s
- 72°C for 7 minutes

This PCR program was also used for all RH mapping and for physical mapping of SSLP markers onto YAC, PAC and BAC templates. AmpliTaq Gold (Perkin Elmer) was used in all cases.
2.8.2 Long range PCR

Cloning of the laminin α1 cDNA required long range PCR. This was performed using the eLongase kit (GibcoBRL), following manufacturers guidelines. Optimal yield was achieved with 2mM MgCl₂. The PCR conditions were as follows:

94°C for 3 minutes
40 cycles:
  92°C for 1 min
  55°C for 1 min
  72°C for 8 mins
72°C for 7 minutes

2.9 RFLP analysis of genomic DNA from embryos

The bal⁴¹⁹⁰ allele is a mutation that results in the loss of a FokI restriction site. Primers were used to PCR amplify a genomic region surrounding this locus (TTCTGCGCTGACATCTGTTG and CACACAGTGCTGTTTTCCTCA). These were used in a 20µl reaction with SSLP PCR program described previously. 5µl of genomic DNA was used from a 1:10 dilution of DNA prepared as section 2.6. 30 cycles were performed and then 3U of FokI was added directly to each sample. Digests were run on a 2% agarose gel.

2.10 Polyacrylamide Gel Electrophoresis

For each gel, 80 ml of gel mix was added to 600 µl of 10% ammonium persulfate and mixed. 1L of gel mix consisted of 240ml of Ultrapure SEQUAGEL Concentrate, 660 ml of Ultrapure SEQUAGEL Diluent and 100 ml of gel mix buffer. 1L of gel mix buffer consisted of 500 g of Urea, 108 g Tris base, 53 g of boric acid, 40 ml of EDTA, 10 ml of N, N', N'-tetramethyl-ethylenediamine (TEMED) and distilled water up to 1 litre. After pouring, the gel was left to polymerise for at least 30 minutes. To each PCR product was added the same volume of loading buffer (50 ml: 75 µg Bromophenol Blue, 75 µg Xylene Cyanol, 1ml EDTA (0.5 M, pH 8.0) up to 1 ml, Formamide up to 50 ml). The mixture was denatured at 94°C for 5 minutes and then 4 µl of each sample were loaded onto the gel using multi-channel pipettes (Hamilton). The gels were run
for 90-150 minutes, depending on the size for PCR product, in 0.5x TBE at room temperature and 85 W with constant power. Finally, the gels were transferred onto Whatman filter paper, covered with plastic wrap and exposed overnight to X-ray films in cassettes at -80°C.

2.11 YAC libraries

2.11.1 Isolation of yeast genomic DNA

A PCR-based zebrafish library from Research Genetics was used during positional cloning. Yeast clones containing a specified YAC (Research Genetics) were streaked on YPD agar plates and incubated for 2 days at 30°C. From the plates, single clones were picked with a sterile loop and used to inoculate 30 ml YPD medium cultures. The yeast cultures were left to grow until saturation at 30°C, when they were collected by centrifugation for 2 minutes. Subsequently, supernatant was discarded and the pellet was re-suspended in 0.5 ml of distilled water. The suspension was transferred to a 1.5 ml and centrifuged for 5 seconds. The resulting supernatant was decanted and the cells were re-suspended in the residual liquid by vortexing. The cells lysis was performed by addition of 0.2 ml of 2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl pH8 and 1mM Na$_2$EDTA. To this mixture, 0.2 ml of phenol:chloroform:isoamyl alcohol (25:24:1) and 0.3 g of acid-washed glass beads (0.45-0.5 mm beads (Sigma) soaked in nitric acid and washed in distilled water) were added. After vortexing for 4 minutes, 0.2 ml of TE, pH8 was added. The lysate was then centrifuged for 5 minutes after which the aqueous layer was transferred to a fresh tube. 1 ml of 100% ethanol was added. The sample was mixed by inversion, centrifuged for 2 minutes and after decanting the supernatant, the pellet was re-suspended in 0.4 ml of TE plus 3 µl of a 10 mg/ml solution of RNAse A (in 50 mM potassium acetate pH 5.5, boiled for 10 minutes). After incubation for 5 minutes at 37°C, the DNA was precipitated with 10 µl of 4M ammonium acetate plus 1 ml of 100% ethanol. The DNA samples were centrifuged for 2 minutes and the DNA pellet (± 20 µg DNA) was resuspended in 50µl of Low TE. 1:100 dilution of this stock was used in PCR genotyping reactions.
2.11.2 Recovery of YAC ends

YAC ends were rescued through digestion followed by re-circularisation. 5 μl of yeast DNA (± 2 μg) was digested with BamHI (or SpeI), for 5 hours at 37°C. The restriction digests were set up according to the following conditions: 2 μl BamHI (SpeI), 10 μl buffer B (H), 5 μl yeast DNA and 83 μl deionised water. The digestion products were extracted with phenol:chloroform:isoamyl alcohol (25:24:1) and ethanol precipitated. The samples were then centrifuged for 15 minutes at 12000 x g at 4°C. The resulting supernatants were decanted and the pellets were re-suspended in 25 μl Low TE. YAC ends can form plasmids by self-ligation. The ligation reaction was set according to the following conditions: 25 μl restriction products, 10 μl ligase buffer, 5 μl ligase (400 units/μl New England Biolabs) and 60 μl deionised water. The mixture was incubated overnight at 16°C. The next day, ligation products were ethanol precipitated and centrifuged at 12000 x g for 15 minutes at 4°C. The pellet was re-suspended in deionised water and electroporated into electrocompetent E.coli.

2.12 PAC and BAC libraries

A zebrafish BAC library (‘Down-to-the-well’) was obtained from Genome Systems, Inc. (St. Louis). It is a PCR based library housed in 192 microtitre plates. The PCR protocol was the same as that given earlier for the meiotic mapping. The library is arrayed into 13 microtiter dishes. Initially 19 upper pools are screened to restrict the search to 10 possible plates. Screening of these 10 plate pools then identifies the plate. Finally, the specific clone is identified by performing 40 PCR reactions on ‘down-to-the-well’. A zebrafish PAC library was obtained from RZPD (Amemiya et al., 1999).

2.12.1 Isolation of PAC and BAC DNA

This procedure was based upon the Qiagen Maxi-prep kit. Bacterial clones were streaked on selective LB agar plates (10μg/ml kanamycin for PACs; 50μg/ml chloramphenicol for BACs) and incubated overnight at 37°C. From the plates, single clones were picked up with a sterile loop and used to inoculate 10 ml selective LB medium cultures. These 10 ml cultures were then used to inoculate 250 ml selective LB medium cultures, containing the appropriate antibiotic, which were left to grow overnight at 37°C. The final cultures were split into two tubes and centrifuged at 3500 x
g for 30 minutes. After decanting the resulting supernatant, the pellets were re-
suspended in 10 ml buffer 1 with Rnase, through shaking for 30 minutes at 37°C. To
lyse the cells, 10 ml of buffer 2 was added to each tube. The mixture was then mixed
gently and incubated for 5 minutes at room temperature and then neutralised by addition
of 10 ml of buffer 3. The samples were mixed gently again and placed overnight at 4°C.
The following morning, samples were centrifuged at 21000 x g for 30 minutes. During
this period, the elution buffer QF was heated to 65°C and the Qiagen Tip 500 were
equilibrated with 10 ml of buffer QBT. At this point, supernatants were recombined,
poured into a cartridge filter and strained through the filter into the column. Each
supernatant passed through the column twice, before the column was washed with 30 ml
of buffer QC, 4 times. DNA was then eluted using 2 times 10 ml of heated elution
buffer QF. This was followed by isopropanol precipitation. The samples were split
again and 0.7 ml isopropanol was added to each one. After mixing gently, they were
placed at -20°C for at least 4 hours and centrifuged at 15000 x g for 30 minutes. The
supernatants were discarded and the pellets washed with cold 70% ethanol, by
centrifugation for 2 minutes. The ethanol was decanted and the pellets left to air dry for
20 minutes. Pellets were re-suspended in 200 μl of deionised water, overnight.

### 2.13 Radiation hybrid panels

96 PCR reactions were performed using the LN54 panel (Hukriede et al., 1999). 20μl
total reaction using 0.2μl of AmpliTaq Gold (Perkin Elmer), 2μl of each primer
(10μM), and 4μl PCR buffer (5X). The SSLP PCR program was used (see previous
section). Analysis of the results and the calculation of linkage and LOD scores was

### 2.14 RT-PCR

For total RNA extraction, 20 embryos were pooled and homogenized in 0.5ml of
TRIzol reagent (GibcoBRL) and left at room temperature for 5mins. 100μl of
chloroform was then added and samples were mixed by hand for 15s and then
centrifuged at 13000rpm for 10mins at 4°C. The aqueous phase was transferred to a
fresh tube and RNA was precipitated with isopropyl alcohol, and then centrifuged for
20mins. The pellet was then washed with 70% ethanol, re-suspended in DEPC treated
water and the concentration of RNA determined. First strand cDNA was synthesized using superscript reverse transcriptase (GibcoBRL) as manufacturers instructions. Briefly, 1μg of total RNA was used in a 20μl reaction with either random hexamer or gene specific primers.

2.15 Rapid amplification of cDNA ends (RACE)

polyA+ RNA was isolated using dynal beads (Dynal), according to manufacturers instructions. 5'- and 3'-RACE PCRs were performed using cDNAs synthesized by the Marathon cDNA amplification kit (Clontech). Gene specific primers (GSP) were designed with a $T_m > 65^\circ C$, and were between 23-28nt, with 50-70% GC content (as suggested (Clontech). RACE reactions were always performed by nested PCR. Primers AP1 primer (Clontech) and gene specific primer 1 (GSP1) were used for 15 cycles in first round. This was then diluted 1 in 100 and used as template for the second round with the primers AP2 and GSP2 for 25 cycles. Touchdown PCR program was used in all cases. The standard PCR program used for all these was as follows:

94°C for 3 minutes
5 cycles:
  94°C for 30s
  72°C for 4 mins*
5 cycles:
  94°C for 30s
  70°C for 4 mins*
25 cycles:
  94°C for 20s
  68°C for 4 mins*

*(extension time in min was varied according to the expected length of the cDNA; e.g. 1kb = 2mins, 2kb = 4min etc.).
2.16 Whole-mount in situ hybridization

Whole-mount in situ hybridizations were performed essentially as described by Thisse (Thisse et al., 1993). Embryos fixed with 4% paraformaldehyde/PBS at 4°C were dehydrated with methanol at -20°C and rehydrated by soaking for 5 minutes each in 75% methanol/PBT (1x PBS + 0.1% Tween 20); 50% methanol/PBT; 25% methanol/PBT and then 4 times 5 minutes in 100% PBT. All embryos >24 hpf were digested with proteinase K (10 µg/ml) for 5 minutes and then refixed in 4% paraformaldehyde for 20 minutes at room temperature and then washed in PBT 5 times 5 minutes. They were then transferred to hybridisation buffer (50% formamide, 5X SSC (pH7.0), 500 µg/ml type VI torula yeast RNA, 50 µg/ml heparin, 0.1% Tween 20, 9 mM citric acid to pH 6.0-6.5) for 2-5 hours at 70°C (prehybridisation). The hybridisation buffer (Hyb) was then replaced with the mixture containing 150 ng of DIG-labelled RNA probe in 200 µl of preheated hybridisation solution and the embryos were incubated at 70°C overnight. Washes were performed at the hybridisation temperature with preheated solutions for 15 minutes each with 75% Hyb/2X SSC; 50% Hyb/2X SSC; 25% Hyb/2X SSC; 100% SSC and finally 2 times 30 minutes in 0.2X SSC. A series of washes were performed at room temperature for 10 minutes each in 75% 0.2X SSC/PBT; 50% 0.2X SSC/PBT; 25% 0.2X SSC/PBT and 100% PBT. Embryos were blocked in 2 mg/ml BSA, 2% goat serum in PBT for several hours and then incubated with alkaline-phosphatase (AP)-conjugated anti-DIG Fab fragments diluted 1:5000 in 2 mg/ml BSA, 2% goat serum in PBT at 4°C overnight with agitation. After washing at least 8 times for 15 minutes with PBT, the embryos were rinsed 3 times 5 minutes in NTMT reaction buffer (0.1 M Tris-HCl pH9.5; 50 mM MgCl₂; 0.1 M NaCl; 0.1% Tween 20). Detection was performed using NBT/BCIP (112.5 µl of 100 mg/ml NBT in 70% dimethylformamide and 175 µl of 100 mg/ml BCIP in 70% of dimethylformamide 1 added to 50 ml of NTMT). After stopping the reaction with 100% PBS (pH 5.5), the embryos were refixed in 4% paraformaldehyde/PBS. Embryos were cleared with 20% glycerol/80% PBS, 50% glycerol/50% PBS and stored at 4°C in 80% glycerol/20%PBS.
2.17 Whole-mount immunocytochemistry

Embryos were fixed in 4% paraformaldehyde, rehydrated in a series of methanol dilutions and then digested with proteinase K for 5 mins (10μg/ml), if >24 hpf. The embryos were blocked in 2 mg/ml BSA, 2% goat serum in PBTr (PBS + 0.1% Triton-X 100) for 2-3 hours and then incubated with the primary laminin 1 antibody (Sigma L-9393)(1:300), in blocking solution, at 4°C overnight, with agitation. After washing at least 8 times for 15 minutes with PBTr, the embryos were blocked again and incubated at 4°C overnight with the secondary antibody (1:300). The secondary antibody was washed at least 8 times 15 minutes. A secondary antibody conjugated to HRP were used and detected using DAB supplemented with NiCl₂.

2.18 Morpholino injection

Morpholino oligonucleotides (MOs) were obtained from Gene Tools, LLC. MOs work through an RNase-H independent process that blocks translational initiation. Thus, all MOs were arbitrarily designed to bind to the 5' UTR or sequences flanking and including the initiating methionine. The selected sequences were based on design parameters according to the manufacturer's recommendations, namely 21–25mer antisense oligonucleotides of 50% G/C and A/T content with no predicted internal hairpins. Four consecutive G nucleotides were also avoided. In addition, each design sequence was tested for representation elsewhere in the genome. Control MO was that suggested by Gene Tools, LLC.

Lyophilised MO was re-suspended in 63 μl of deionised water to obtain a final concentration of ± 4.7 nM/μl. Prior to microinjection, the MOs were diluted using MO buffer to titrate the dose (5 mM Hepes pH7.2, 0.2 M KCl and 2.5 mg/ml phenol red). A volume of 1.4nl was injected through the chorion of one-cell stage embryos to deliver 3ng of MO. E.g A 1:16 dilution of this stock solution equates to ~3ng injected per embryo.

2.19 Electron microscopy

All electron microscopy was performed by Elizabeth Hirst (Electron Microscopy lab, NIMR). Whole zebrafish embryos were de-chorionated manually and fixed overnight.
with 2% glutaraldehyde, 2% paraformaldehyde in 0.1M sodium cacodylate buffer, pH 7.2 (SCB). They were washed for 10 minutes in SCB and postfixed for 1 hour in 1% osmium tetroxide, SCB. They were washed again with SCB and stained en bloc with 1% aqueous uranyl acetate for 1 hour. The samples were then dehydrated through a graded ethanol series, followed by 2 changes of propylene oxide over 20 minutes and embedded in Epon resin (Agar Scientific). 50nm ultra thin sections were cut and mounted on pioloform coated slot grids and stained with 1% aqueous uranyl acetate for 15 minutes followed by Reynold's lead citrate for 7 minutes. Sections were visualised in a Jeol 1200 EX electron microscope.

2.20 Photomicrography

Nomarski and live fluorescence images were obtained using a Leica compound microscope fitted with a Princeton Instruments, MicroMax cooled-CCD camera. MetaMorph image processing software was used to acquire images and overlay fluorescence and Nomarski images. Whole-mount in situ hybridisation images were obtained using a Zeiss Axiophot microscope fitted with a Kodak DCS420 digital camera.
<table>
<thead>
<tr>
<th>Solution</th>
<th>Formulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1X PBS</td>
<td>137 mM NaCl, 2.7 mM KCl, 4.3 mM Na&lt;sub&gt;2&lt;/sub&gt;HPO&lt;sub&gt;4&lt;/sub&gt;.7H&lt;sub&gt;2&lt;/sub&gt;O, 1.4 mM KH&lt;sub&gt;2&lt;/sub&gt;PO&lt;sub&gt;4&lt;/sub&gt;</td>
</tr>
<tr>
<td>1X PBT</td>
<td>1X PBS, 0.1% Tween 20</td>
</tr>
<tr>
<td>1X TAE</td>
<td>40 mM Tris.Acetate, 2 mM Na&lt;sub&gt;2&lt;/sub&gt;EDTA.2H&lt;sub&gt;2&lt;/sub&gt;O (pH 8.5)</td>
</tr>
<tr>
<td>1X TE</td>
<td>1 mM EDTA, 10 mM Tris.HCl pH 8.0</td>
</tr>
<tr>
<td>20X SSC</td>
<td>3 M NaCl, 0.3 M Na&lt;sub&gt;3&lt;/sub&gt;citrate.2H&lt;sub&gt;2&lt;/sub&gt;O, adjust pH to 7.0 with 1 M HCl</td>
</tr>
<tr>
<td>Sequencing loading buffer</td>
<td>Formamide: 25 mM EDTA pH8 + blue dextran (50 mg/ml) (5:1)</td>
</tr>
<tr>
<td>Polyacrylamide gel electrophoresis load buffer</td>
<td>98% formamide, 0.025% bromophenol blue, 0.025% xylene cyanol, 10 mM EDTA pH 8</td>
</tr>
<tr>
<td>Agarose gel electrophoresis gel loading buffer (6X)</td>
<td>6X TAE, 50% v/v glycerol, 0.25% w/v bromophenol blue</td>
</tr>
</tbody>
</table>

Table 2-1  Formulation of frequently used solutions.
<table>
<thead>
<tr>
<th>Protein</th>
<th>Origin</th>
<th>Enzyme</th>
<th>Pol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laminin β1</td>
<td>(Parsons et al., 2002)</td>
<td>BamHI</td>
<td>T7</td>
</tr>
<tr>
<td>Laminin β4</td>
<td>RZPD clone AA495063 (fragment sub-cloned into Bluescript)</td>
<td>Xba</td>
<td>T7</td>
</tr>
<tr>
<td>Echidna hedgehog</td>
<td>(Currie and Ingham, 1996)</td>
<td>EcoRI</td>
<td>SP6</td>
</tr>
<tr>
<td>Laminin α1</td>
<td>RACE product (this thesis)</td>
<td>BamHI</td>
<td>T7</td>
</tr>
<tr>
<td>Laminin γ1</td>
<td>(Parsons et al., 2002)</td>
<td>Xba1</td>
<td>T7</td>
</tr>
<tr>
<td>Laminin α2</td>
<td>RACE product (this thesis)</td>
<td>EcoRV</td>
<td>SP6</td>
</tr>
<tr>
<td>Laminin α4</td>
<td>RACE product (this thesis)</td>
<td>Xba</td>
<td>SP6</td>
</tr>
<tr>
<td>Laminin α5</td>
<td>RZPD clone, AA545720</td>
<td>EcoRI</td>
<td>SP6</td>
</tr>
<tr>
<td>Laminin α5</td>
<td>RACE product (this thesis)</td>
<td>EcoRV</td>
<td>SP6</td>
</tr>
<tr>
<td>ILK</td>
<td>RZPD clone, AI883753 (fragment sub-cloned into pCR 2.1)</td>
<td>NotI</td>
<td>SP6</td>
</tr>
<tr>
<td>Integrin β4</td>
<td>RZPD clone, F0934Q8</td>
<td>EcoRI</td>
<td>SP6</td>
</tr>
<tr>
<td>Integrin α6</td>
<td>RZPD clone, D0317Q8</td>
<td>EcoRI</td>
<td>SP6</td>
</tr>
<tr>
<td>Fli-1</td>
<td>(Fouquet et al., 1997)</td>
<td>Xba</td>
<td>T3</td>
</tr>
</tbody>
</table>

**Table 2-2**  **In situ hybridisation probes.** (RZPD) Resource Centre/Primary Database, Germany. (Pol) RNA polymerase used for in vitro transcription.
<table>
<thead>
<tr>
<th>Targeted mRNA</th>
<th>Sequence of injected MO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laminin α1</td>
<td>ATCTCCATCATCGCTCAAACTAAAG</td>
</tr>
<tr>
<td>Laminin β1</td>
<td>TATTTCCAGTTTTCTTTCTTCAGCGG</td>
</tr>
<tr>
<td>Laminin β4</td>
<td>CTAGACGGAGACAGCATAACTG</td>
</tr>
<tr>
<td>Laminin α2</td>
<td>GCCACTAAACTCCGCGTGTCCATGT</td>
</tr>
<tr>
<td>Laminin α4</td>
<td>GCCATGATCCCCCTGCAAAACTT</td>
</tr>
<tr>
<td>Laminin α5</td>
<td>CTCGTCCTGATGGTCCCCTCGCCAT</td>
</tr>
<tr>
<td>Integrin α6</td>
<td>ATTGCCCTGAAATGATGCTGTTGTA</td>
</tr>
<tr>
<td>Integrin-linked kinase</td>
<td>ATGTTCATCCATTTCGGCACCCCTCTC</td>
</tr>
<tr>
<td>Standard control</td>
<td>CCTCTTACCTCAGTTACATTATA</td>
</tr>
</tbody>
</table>

**Table 2-3**  Morpholino oligonucleotides (MOs) used during this thesis. Each MO was designed around the translation start site following the recommendations of Gene Tools.
Chapter 3

Positional cloning of bashful
Chapter 3

Positional cloning of *bashful*

3.1 Introduction
This chapter introduces the techniques required to identify a mutant gene on the basis of its position in the genome and provides some examples where this approach has been used successfully in the zebrafish. The results of the positional cloning of *bashful* are then presented.

3.1.1 Identifying genes disrupted in zebrafish mutants
Two main approaches, the candidate gene approach or positional cloning, are used when attempting to clone mutant genes. The candidate approach has been used successfully for a number of zebrafish mutants and uncovers the gene by deduction, based on functional information previously obtained. Two good examples of this approach are the zebrafish mutants *no tail* and *floating head* (see 1.3), which were found to be homologues of the *Xenopus* genes *Xbra* and *Xnot* respectively (Schulte-Merker et al., 1994; Talbot et al., 1995).

In cases where there is no obvious candidate, and also for the isolation of novel genes, cloning of the zebrafish mutant gene by its position in the genome is possible (Talbot and Schier, 1999). This approach was initially developed as a method for discovering human disease genes (Collins, 1992). Proof of principle was established in 1986 with
the identification of the gene for the inherited human disorder, chronic granulomatous disease (Royer-Pokora et al., 1986). Since then the genes disrupted in many inherited human diseases have been uncovered based solely on their chromosomal location, notably: Duchenne muscular dystrophy, cystic fibrosis, fragile X syndrome and breast cancer (Fu et al., 1991; Kerem et al., 1989; Miki et al., 1994; Monaco et al., 1986; Riordan et al., 1989; Rommens et al., 1989; Verkerk et al., 1991). The technique is now termed positional cloning and improvements in the method have resulted from work carried out as part of the human genome-sequencing project.

Positional cloning has been used to successfully identify many zebrafish genes including, novel genes (Zhang et al., 1998), genes important as models for human disease (Zon, 1999), and genes highlighting the evolutionary conservation of developmental mechanisms (Kikuchi et al., 2001) (Table 3-1). The process of positional cloning is labour intensive and requires a large number of genetic tools. Since the publication of the large-scale zebrafish screens in 1996 progress in developing these tools for zebrafish has been remarkable. Today there are simple sequence length polymorphisms (SSLP) and radiation hybrid (RH) maps, large-insert genomic libraries and over 100000 published expressed sequence tags (ESTs), reviewed (Beier, 1998). In addition, the zebrafish genome sequencing effort has generated >4Gb of whole genome shotgun sequence that can be readily searched. Finally, the function of genes expressed early in development can be easily tested using antisense morpholino oligonucleotides (MOs). Taken together these tools make positional cloning a straightforward means to characterise genes identified by mutation (Fishman, 1999; Talbot and Schier, 1999).
Table 3-1  Zebrafish genes identified by positional cloning. one-eyed pinhead was the first zebrafish gene identified by positional cloning, and is an important part of the nodal signalling pathway. sauternes provides a model for the human disease congenital sideroblastic anaemia, whilst weissherbst may be responsible for mammalian disorders involving iron deficiency/overload. A deficiency of UROD causes hepatoerythropoietic porphyria (HEP) in humans. casanova was identified as a Sox17-like protein, and provides a good example of the evolutionary conservation of developmental mechanisms.
3.1.2 Overview of positional cloning methodology in zebrafish

There are several steps involved in positional cloning of a zebrafish gene (Figure 3-1). Firstly, one needs to establish linkage to a particular chromosome. Gynogenic half-tetrad diploids can be produced by early pressure and used to identify the linkage group on which the mutation lies, and also provide a distance from the centromere (Johnson et al., 1995). An alternative to this is to use bulk segregant analysis (BSA), whereby polymorphic markers that are linked to a mutation can be identified by looking for the segregation of markers between pools of WT and mutant embryo genomic DNA (Beier, 1998). Once linkage has been determined one carries out genetic mapping to find closely linked markers either side of the mutation. The zebrafish represents an excellent system in which to perform meiotic mapping as a large number of meioses can be analysed. This increases the likelihood of identifying useful crossovers and enables the mutation to be mapped finely. Crossing two genetically distinct strains of fish (one carrying the mutation and the other a mapping strain) generates hybrid fish (Figure 3-2). Using previously mapped genetic markers (see section 3.1.3), one can perform meiotic mapping and identify a closely linked marker, or ideally two close markers that flank the mutation. This involves genotyping many hundreds of mutant fish, in search of recombination events that give an indication of the distance and order of markers. A candidate interval is thus defined which is narrowed further through identification of new markers and further mapping. If the closest genetic marker is still far from the mutation then chromosome walking is required in order to identify new markers. One aims to genetically map the mutation between two markers, each of which is located on the same PAC or BAC (see 3.1.4), i.e. the mutation and possibly the whole gene physically isolated within a single clone. This typically requires analysis of around 4000 meioses. In zebrafish 1cM corresponds to a physical distance of 660kb (Postlethwait et al., 1994). Candidate genes can then be identified through either complete sequencing of the clone, or through hybridisation of the BAC/PAC with cDNA libraries. Once candidate genes have been identified there are three well-established techniques that can be used to test and prioritise them: phenotype rescue, through over-expression of mRNA or DNA (Yan et al., 1998); analysis of expression patterns, by in situ hybridisation; and phenocopy by targeted knockdown using MOs. Once a good
candidate has emerged from these types of study then one can proceed to cloning and sequencing of WT and mutant cDNA in search of the mutation.

MOs are short oligonucleotides that contain a morpholino sugar within the backbone rather than ribose. Thus, they are resistant to degradation and are capable of hybridising with endogenous nucleic acids. Though only very recently developed as reagents for gene knock-down in developmental biology studies they have proven particularly effective in zebrafish and have very rapidly become a powerful tool (Heasman, 2002; Heasman et al., 2000). The phenotypes of no tail, chordino, one-eyed pinhead, nacre and sparse have all been successfully phenocopied using MOs (Nasevicius and Ekker, 2000). MOs can be designed to prevent translation through hybridisation to the endogenous mRNA and therefore reduce protein levels. They are stable (operating via steric blocking), cheap, and extremely effective. Furthermore, they can enter the nucleus, and so can be designed to interfere with pre-mRNA splicing events (Draper et al., 2001). Injection of a MO against a particular candidate gene can provide strong evidence that it represents the mutant gene through recapitulation of the mutant phenotype.

3.1.3 Genetic maps

Identification of a polymorphic marker that is closely linked to the mutation through genetic mapping is a critical step in any positional cloning project. If such a marker can be found then this greatly reduces the amount of work involved later in chromosome walks and candidate identification. Thus, availability of dense genetic maps is vital. For zebrafish, the first genetic map was based on random amplified polymorphic DNA (RAPD) (Postlethwait et al., 1994) and identified 401 loci, which was increased to 652 by 1996 (Johnson et al., 1996). However, routine genetic mapping using RAPD analysis has many drawbacks and SSLPs have become the preferred type of marker for genetic mapping (Beier, 1998). One can identify PCR primers that flank short microsatellite repeats, typically the dinucleotide cytosine-adenine (CA), that are interspersed throughout the genome and are variable in repeat number. A map based on this type of marker was published for the zebrafish in 1996 and characterised 102 SSLPs. Since then further markers have been added and the total number now exceeds 2000 providing a marker on average every 1.2cM (Shimoda et al., 1999). One drawback of SSLP maps is
that a high proportion of markers will not be polymorphic, and accordingly are not useful for meiotic mapping. Thus, each marker must initially be tested to determine whether it is polymorphic on the mapping line. Once a close marker is found one can commence a chromosome walk using large-insert constructs.

3.1.4 Chromosome walking and large insert libraries

Once the position of the mutant locus has been roughly determined through genetic analysis, one has an estimate of the distance of the mutation from the marker. If the marker is close enough (usually <0.5cM) then it becomes feasible to perform a chromosome walk. Such walks became possible following the development of libraries of constructs holding large inserts (YACs, BACs and PACs). YACs are able to hold extremely large fragments of DNA (Murray and Szostak, 1983). In zebrafish YAC libraries have been made that have insert sizes of ~240kb (Amemiya et al., 1999). They were constructed through ligation of vector arms that contain all the sequence necessary for replication within yeast to the ends of genomic fragments. Such large fragments are especially useful for initial ‘walks’ if there is not an existing close marker, as large distances can be covered with few steps. BACs and the related P1 artificial chromosome (PAC) are based on F-factor replicon and P1 bacteriophage replicon, respectively. They carry much smaller inserts than YACs (usually ~100kb) but have several advantages. As large bacterial plasmids they can be handled with similar techniques as standard plasmids (with modifications taking into account their low copy number). Obtaining sequence data from these clones is therefore fairly trivial. Both BAC and PAC libraries have been created for the zebrafish (Amemiya et al., 1999; Amemiya and Zon, 1999).

A chromosome walk from a particular marker involves three main steps. Firstly, a YAC, BAC, or PAC clone is identified within the library through PCR or filter hybridisation. Secondly, the ends are rescued and sequenced (PAC and BAC clones can be sequenced directly). Thirdly, PCR primers are designed based on the end sequences and are used to re-screen the library for new clones. This process is repeated and results in a series of overlapping clones, each new clone being further along the chromosome in a particular direction. One can orientate the walk and determine when the mutant locus has been reached by using the end sequences for either genetic mapping (using polymorphisms) or physical mapping by use of radiation hybrid (RH) panels.
Figure 3-1  Summary of positional cloning of mutant zebrafish genes.
Figure 3-2  Generation of heterozygous bal carriers. WT WIK/WIK zebrafish were crossed with heterozygous bal carriers with the AB genetic background (P0). This produces an F1 generation that includes fish that are heterozygous for bal and contain 50% of their genome from the WIK background (half of the fish will also be WIK/AB, but will not carry the mutation). The genomic DNA of the mutant progeny of these fish (F2) will be of AB character around the mutant locus. Analysing markers that are polymorphic between the AB and WIK strains allows the mutant locus to be mapped, as closer markers are less likely to have recombined. Adapted from www.eb.tuebingen.mpg.de.
3.1.4 Radiation hybrid panels

RH panels were developed in the early 1990's and provide a means of constructing high-resolution, contiguous physical maps of chromosomes (Cox et al., 1990). The technique involves generation of hybrid cell lines that contain random genomic fragments from the species of interest. The fragments are generated through x-ray fragmentation of chromosomes and then hybrid cells are made by fusion of these irradiated cells with those from another species. The DNA from hybrid clonal cell lines is extracted and then tested by PCR for retention of chromosomal fragments. Statistical comparison of the set of positive samples with that of other mapped markers, identifies the position of the locus, and relative distances between markers (i.e. the pattern of positive samples for any two markers will be more similar if they closely linked). An important advantage of RH panels is that polymorphic markers are not needed and therefore any genomic sequence can be mapped. Two RH panels have been generated for the zebrafish, called LN54 and T51, and use of these provide a complementary approach to recombination mapping (Ekker et al., 1999; Geisler et al., 1999; Hukriede et al., 1999).

3.1.5 ESTs and zebrafish genes

The expressed sequence tag (EST) was developed as a method for the rapid identification of novel genes and comparison of gene expression between different cell types (Adams et al., 1991). cDNA libraries are generated from developmental stages or adult tissues, and partial sequences are obtained from the ends of each clone. The sequences obtained are then tested for homology with known genes in the database. This approach was first taken by J. C. Venter and colleagues and has been crucial for the large-scale identification of expressed genes (Adams et al., 1992; Adams et al., 1991). Importantly, one can assign the EST to a particular locus by mapping, and those that map to regions previously shown to be associated with disease become candidates. For example, a genetic disorder known as CADASIL that results in stroke and dementia provides an early example of this strategy, identifying Notch3 as the disrupted gene (Joutel et al., 1996).
EST databases have been established for the zebrafish and are continually being updated (Gates et al., 1999; Gong, 1999). As more zebrafish transcripts are mapped then the positional candidate approach will become the predominant method for identifying mutant genes, where low-resolution genetic mapping will be sufficient to identify a large number of candidate EST clones around the mutant locus. This approach sidesteps some of the laborious positional cloning tasks, such as chromosome walking. The availability of a complete zebrafish genome sequence will clearly take the candidate approach a step further by not only revealing mapped ESTs, but all of the genes around the mutant locus (Butler, 2000). Advances in both mouse and human genomics also facilitates the cloning of zebrafish mutant genes, as regions of synteny between these species can be useful in identifying candidate genes. An example of this approach was the identification of Gli2 as the you-too gene (Karlstrom et al., 1999).
3.2 Further characterization of *bashful*

3.2.1 Expression of *echidna hedgehog* mRNA in *bal*, *gup* and *sly*

In the initial characterisation of the zebrafish notochord differentiation mutants the expression of several early chordamesoderm markers such as *sonic hedgehog*, *no tail*, *engrailed* and *collagen type II* was shown to be maintained (Stemple et al., 1996). I extended this analysis by testing *bal*, and the related mutants *gup* and *sly* with the chordamesoderm specific gene *echidna hedgehog* (*ehh*) (Currie and Ingham, 1996). Both *gup* and *sly* mutants show maintenance of expression throughout the length of the notochord at 24 hpf (Figure 3-3). These results are consistent with previous marker analysis and suggest that the cells of the chordamesoderm fail to differentiate. By contrast, however, *bal* embryos do extinguish *ehh* expression within anterior and trunk regions, and resemble WT embryos. This is somewhat paradoxical as it is the anterior regions of the *bal* notochord that are morphologically mutant due to lack of vacuole inflation. This suggests that during notochord differentiation gene expression and vacuolation are controlled independently.

3.2.2 Shield transplants

Recent work in our laboratory involved the development of a technique for transplantation of the zebrafish embryonic shield (Saude et al., 2000). The technique is also a useful method for performing genetic mosaic experiments, and was used to show that *silberblick/wnt-11* acts cell non-autonomously (Heisenberg et al., 2000). I have used the same approach in order to determine whether *bal* acts notochord autonomously or non autonomously. Donor embryos were injected with a rhodamine-dextran tracer. Once embryos reach shield stage the morphological shield was removed, and transplanted into the ventral sided of an unlabelled host embryo of the same stage. This gave rise to a complete second axis, with all axial mesoderm derived solely from the donor. Remarkably, donor embryos recover following shield removal and complete development normally. Thus, the transplanted tissue can be unambiguously scored as WT or mutant by monitoring the phenotype of the recovered donor. The results are shown in Figure 3-4, and show convincingly that the mutation acts cell non-autonomously. A mutant shield transplanted into a WT host environment is rescued.
Figure 3-3  Characterisation of ehh expression in bal, gup and sly. When compared to a WT sibling (A), the anterior region of the bal notochord has an abnormal morphology (B). However, like WT embryos, expression is extinguished anteriorly by 24 hpf (B). This is in contrast to the two other notochord differentiation mutants, gup and sly, where expression of ehh is maintained throughout the entire notochord (C and D).
Figure 3-4  Shield transplantation shows that *bal* acts cell non-autonomously. Donor *bal* embryos were labelled at the one-cell stage by injection of rhodamine-dextran (B). The labelled shield was removed and transplanted to the ventral side of an unlabelled host WT embryo (A). This results in formation of a secondary axis (C), containing axial mesoderm solely of mutant character (D). Mutant notochord is rescued (i.e. has WT morphology) by surrounding WT tissue (white arrow), indicating that the *bal* mutation acts non-autonomously.
3.3 Initial genetic mapping of *bashful*

3.3.1 Identification of polymorphic SSLP markers

Bulk segregant analysis had previously been carried out for *bal* and identified five potential linkage groups (Stefan Neuhaus, PhD thesis). Subsequently, a telomeric region of linkage group 24 near marker Z6923 was identified as the location of *bal* (M. Parsons, pers. comm.). No obvious candidates had been mapped to this locus (Figure 3-5), and so a positional cloning project was undertaken. Each of twenty-one SSLP markers within the region was tested on WT, mutant, and grandparent (WIK) embryos, in order to identify size polymorphisms (i.e. markers that could be used for genetic mapping). An example of this is shown for the marker Z8456 in Figure 3-6. To increase the number of useful polymorphic markers, initial mapping was carried out on two different backgrounds with two different mutant alleles of *bal*. Hybrid lines were generated for *bal*<sup>m190</sup> and *bal*<sup>nc45</sup> (AB and Tübingen backgrounds, respectively).

For *bal*<sup>m190</sup>, 10 of the 21 markers tested were polymorphic. *bal*<sup>nc45</sup> had 8 potentially useful markers, two of which were polymorphic at only one WIK allele. Three of these markers (Z8209, Z8327 and Z8456) were polymorphic on both mapping crosses and were used for the initial genetic mapping (Table 3-1).
Figure 3-5  Initial genetic mapping of bal. (A) Zebrafish SSLP map around the bal locus on LG 24 (http://zebrafish.mgh.harvard.edu). bal was known to lie close to Z6923 (boxed). (B) RH map of bal region from the T51 Goodfellow panel. (C) RH map of bal region from LN54 Ekker panel. None of the ESTs that mapped to this region represented obvious candidates for bal.
Figure 3-6  Identifying polymorphic SSLP markers. In this example Z8456 was tested by PCR on genomic DNA from twelve different mutants (odd lanes) and WTs (even lanes) from a cross between two heterozygous fish, \((baf^{m190}/\text{WIK})\). DNA from the grandfather (\text{WIK/\text{WIK}}) was also tested. Each mutant sample has the genotype AB/AB, while WT fish can be either AB/\text{WIK} or \text{WIK/\text{WIK}}. The results reveal two \text{WIK} alleles of different size, both of which are smaller than the AB allele. Therefore, this marker has a size polymorphism on our mapping fish and can be used to identify recombination events. This procedure was repeated for each of the SSLPs in the region on two different mapping lines and is summarised in Table 3-1.
<table>
<thead>
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<th>SSLP</th>
<th>Size polymorphism?</th>
<th>( \text{bal}^{\text{m190}} )</th>
<th>( \text{bal}^{\text{c45}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z6923</td>
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<td>Yes</td>
<td>No</td>
</tr>
<tr>
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<td>No</td>
<td>No</td>
</tr>
<tr>
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<td>No</td>
<td>Yes *</td>
<td>Yes</td>
</tr>
<tr>
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</tr>
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</tr>
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</tr>
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</tr>
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</tr>
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</tr>
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<td>Yes *</td>
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</tr>
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</tr>
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</tr>
<tr>
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</tr>
<tr>
<td>Z26694</td>
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**Table 3-1** Polymorphic SSLPs. The 21 markers tested had previously been mapped to the distal region of LG24 and were known to lie close to the \( \text{bal} \) locus. Each of these was tested for size polymorphisms on two mapping lines to determine their usefulness for meiotic mapping. Seven of these were not polymorphic on either line and could not be mapped genetically. Two markers were polymorphic at only one of two WIK alleles (*).
3.3.2 Initial mapping of SSLPs

Several hundred mutant fish were initially tested for recombination events, using both mapping lines with the marker Z8456. This marker gave a reliable PCR product and was polymorphic on both hybrid lines. Early results indicated a similar genetic distance for this marker on both mapping crosses, 1cM ($bal^{am545}$) and 0.7cM ($bal^{am190}$). Those fish recombinant for Z8456 were subsequently tested using the other polymorphic markers. None of these appeared to be on the telomere side, although six mapped closer to $bal$ than Z8456 (Figure 3-7). None of the recombinants identified were recombinant with Z7895 and so all of the fish were tested with this marker to establish whether it was on the other side of the mutation. No recombinants were found in 742 meioses and so Z7895 seemed to lie extremely close to the mutation, though on which side could not be established without analysis of more meioses.

For successful positional cloning, one needs genetic markers on both sides of the mutation to define a candidate interval. Once markers have been identified on either side of the mutation then one can rule out false-positives, and a more accurate genetic distance can be obtained. A particular problem with the initial mapping of $bal$ was that the locus is extremely close to the telomere and so identification of a marker on the telomere side was difficult as recombination events there are rare. As YAC clones are extremely large, and the markers I tested were all close to the mutant locus, it was reasonable to assume that walking over $bal$ with YAC clones identified with these markers would enable me to obtain genomic sequence telomeric to the mutation. The end sequences of these YACs could then be used to identify genetic marker on the telomere side of the $bal$ locus.
Figure 3-7 Preliminary genetic map of $bat^{m190}$ and $bat^{ac45}$. Two maps are shown, one for the $bat^{m190}$ allele and the other for $bat^{ac45}$. The genetic map is based on only a few hundred meioses and is therefore of low resolution. Also, the distances are likely to be an over-estimate due to the lack of a marker telomeric to $bal$ that would enable identification of false-positives. Z8456 was polymorphic on both lines and was initially tested on a few hundred meioses. Other markers were then tested on $bat^{m190}$ and Z7895 was found to be closest to the mutant locus.
3.4 Physical mapping of bashful

3.4.1 Construction of a YAC contig

YAC clones were identified using the markers shown previously to be closest to bal, (Z7895). This marker hit YACs: 165B10, 5E7 and 70F1 from the YAC library (see methods). These YACs were obtained and used to physically map all twenty-one SSLP markers by PCR. This confirmed the clones were ‘real’ and also physically mapped Z3038 and Z26694 (two of the non-polymorphic markers that could not be mapped genetically). The results are summarized in Figure 3-8.

In order to chromosome walk over the bal locus towards the telomere, the direction of the walk had to be determined. This was achieved by walking away from 165B10 and 5E7 in both directions and comparing YACs identified with Z7292, which had been mapped genetically as further away from bal and closer to the centromere (Figure 3-9).

Z7292 was present on YAC 158A11. The same YAC was identified using Z26694, which was the marker previously assigned to 165B10. Thus, the direction of the walk was established, as 165B10 is closer to the centromere than 5E7. This meant that 5E7 could be used to walk further towards the telomere by identifying YACs with Z3038. A new YAC, 89A10, was identified in this way. Once again all of the SSLP markers were mapped physically onto the YACs enabling a more accurate contig to be generated (Figure 3-9).
Figure 3-8 YAC clones identified using Z7895. Z7895 (red) had previously mapped very close to bal. A further twenty SSLP markers in the region were tested on these three clones by PCR. Two of these, Z26694 and Z3038, were found to lie on 165B10 and 5E7/70F1, respectively.
Figure 3-9 Establishing of the direction of the chromosome walk. Z7895 (red) was used to identify YAC clones. By identifying new YACs with Z26694 and Z3038 (green) we could walk away from *bal*. Z26694 hits the same YAC as Z7292 (blue) that had been previously mapped genetically as 0.3cM from *bal* on the centromere side. Therefore, one knows the direction of the telomere and accordingly the direction to pursue new markers for *bal*. Following the isolation of YAC clone DNA I was able to physically map more of the SSLPs in the region by PCR.
3.4.2 YAC end–rescue

The ends from four of these YAC clones were rescued and sequenced, in an attempt to identify a telomeric polymorphic marker. For each successfully rescued end, sequences were placed on the RH panel to ensure the YAC clone was not chimaeric, i.e. mapped to LG24. Four ends were rescued successfully and were linked to LG24 (Table 3-2). Two of the eight ends were found to be chimaeric and two of the ends could not be rescued. PCR primers were generated from each of these four end sequences and then tested on all of the YACs in the contig. This established the order and degree of overlap between adjacent clones (Figure 3-10).

A dinucleotide repeat (cytosine-adenine) was identified in the end sequence of 5E7 (T7 end) and primers were designed that flanked this sequence to test for size polymorphisms on our mapping lines. The marker was polymorphic between the WIK and AB backgrounds (bal\textsuperscript{m190} mapping cross) and was used for further high-resolution genetic mapping. Thus, I identified an SSLP that is useful as a genetic marker, and lies telomeric to bal. It is referred to hereon as 5T7 (YAC 5E7, T7 end). 5T7 was not polymorphic between WIK and Tü (bal\textsuperscript{ec45} mapping cross).
Table 3-2 Summary of YAC end rescue. Four different YACs were obtained and plasmid end-rescue was performed to obtain end sequence (ends are designated either T3 or T7 based on the sequencing primer used). Sequences from each rescued end were mapped using the RH panel to establish linkage. Four of the ends were ‘real’ and could be used as a source of sequence for identification of a genetic marker.
Figure 3-10  Rescued YAC ends and the establishment of a contig. Four ends were successfully rescued (red). Two of the ends were chimaeric (C) and two ends were unclonable (X). Each end was tested by PCR on all the YACs in the contig to establish the degree of overlap. 5E7 contains a CA repeat in the T7 end (5T7) that was used for further genetic mapping (see next section).
3.5 Identification of a candidate gene

3.5.1 High resolution genetic mapping

Following the identification of 5T7 as a polymorphic marker on the telomere side, a more accurate and higher resolution genetic map was obtained for bal<sup>m190</sup>. 5T7 was tested on 1054 fish and thirty-one recombinants were identified, confirming that the marker is on the telomere side at a distance of ~1.5cM from bal. To determine the position of Z7895, which was previously determined to be close to bal, the thirty-one recombinants were tested with this marker. Three were found to be recombinant. Therefore, Z7895 is a very close marker (~0.1cM), on the telomere side of bal (Figure 3-11).

1324 fish were tested using the marker Z7292, as this gave a good PCR product and was previously mapped on the centromere side 0.3cM from bal. Nine recombinants were identified. Four of these nine recombinants were also recombinant for Z7895 and 5T7. This indicates that these fish had been wrongly identified as mutants and are, in fact, WT fish that appear as false-positives*. Thus, the true number of recombinants for Z7292 is five, representing a distance of ~0.2cM.

From this higher resolution genetic map, and with false-positive samples excluded, I had determined that bal<sup>m190</sup> lies within a genetic interval of ~0.3cM between Z7292 and Z7895. This corresponds to a physical distance of ~200kb (Figure 3-11). At this point, with many markers genetically and physically mapped, I decided to attempt to cover the interval with BAC and PAC clones.

Further recombination mapping was also carried out with the second mapping allele (bal<sup>ace45</sup>). In total, 1004 fish were tested with Z8456, and a distance of ~0.75cM obtained (15 recombinants). As was shown for bal<sup>m190</sup>, this figure probably overestimates the true distance, as false-positives need to be excluded. Nevertheless based on this data the two mutations would appear to be 0.05cM apart (~33kb).

* bal mutants were difficult to score conclusively as their phenotype was suppressed on the WIK background.
Figure 3-11 High-resolution genetic mapping of $bal^{m190}$. Following the identification of the marker 5T7, further genetic mapping was carried out (>2000 meioses) using markers either side of $bal$. This defined an interval of 0.3cM, between Z7292 and Z7895, in which the $bal$ gene lies.
3.5.2 Construction of a BAC and PAC contig

As I knew that \textit{bal} was likely to be a large gene (many alleles were identified in the screen) my approach to finding candidates within the \(~200\text{kb}\) interval was to sequence as many BAC and PAC ends within this region as possible, rather than performing more extensive genetic mapping. By doing so one can generate a large amount of genomic sequence within the interval. E.g. 20 BAC and PAC ends of \(~500\text{bp}\) would give \(10\text{kb}\) of sequence information. This would likely contain some exon sequence from the \textit{bal} gene.

The following SSLP markers were used to screen the BAC and PAC libraries: Z7292, Z26694, Z7895, and Z23038. PCR primers generated from YAG end sequences T7, T3, 89T7 were also used (Appendix A2). Each of these markers identified many clones, and those clones positive for more than one marker reveal the order and degree of overlap between clones. Based on this, as well as the genetic map, I constructed a contig of BAC and PAC clones (Figure 3-12). No BAC or PAC clone could be identified with the markers T7 and Z7292 and so there appears to be ‘holes’ in both libraries around this region.

3.5.3 Sequencing of BAC and PAC ends

DNA was prepared for all of the BACs and PACs identified and used for end sequencing in order to identify candidate genes. The end of BAC 143J17 contained 141bp with 68\% identity at the amino acid level to the mouse \textit{lama1} gene\(^*\). This encodes the laminin \(\alpha1\) chain, which is an excellent candidate for \textit{bal}. No other candidates emerged from this sequencing. A summary of the whole positional cloning procedure for \textit{bal}\(^{m190}\), incorporating all the genetic data and the YAC and BAC contigs is given in Figure 3-13.

\(^*\) The predicted protein of this 141bp sequence is:

\[
\text{NVMGSHCDLCKQGFYNLQASNPGE} \text{C} \text{F} \text{G} \text{V} \text{D} \text{V} \text{C} \text{E} \text{S} \text{T} \text{W} \text{F} \text{S} \text{S} \text{S} \text{V}
\]
Figure 3-12  Contig of BAC and PAC clones. Clones were identified using SSLP and YAC end markers. There appears to be a ‘hole’ in both BAC (blue) and PAC (green) libraries around Z26694, as no clones could be found in this region. Therefore a contiguous span of clones across the candidate interval could not be achieved. The end sequencing of BAC 143J17 revealed sequence with high homology to mouse *lama1*, which was an extremely good candidate for the *bal* gene.
Figure 3-13  Summary of the positional cloning strategy used to identify \( \text{bal}^{m190} \). Following initial mapping, YAC clones (blue) were isolated and end-rescued. The end of YAC 5E7 contained a CA repeat that was used as a SSLP marker on the telomeric side of the mutant locus (5T7). Further meiotic mapping was performed and restricted the \( \text{bal}^{m190} \) locus to an interval of 0.3cM (orange). BAC clones (green) were identified that lay within this region and sequenced. The end of BAC 143J17 contains part of the gene encoding laminin \( \alpha1 \), which is an excellent candidate for \( \text{bal}^{m190} \).
Positional cloning of bastful

X - YAC end not rescued
C - chimeric YAC end

Laminin α1 (lama1)

Chapter 3

Meiotic mapping
3.6 Discussion

*bal* was shown to act cell non-autonomously in the shield transplant experiments, which suggested that *bal* encodes a secreted or transmembrane protein. This result also suggests that there is no inherent defect that would prevent these chordamesoderm cells from differentiating; that is, they have the potential to be rescued if the correct environment is provided. The maintenance of the early chordamesoderm marker *echidna hedgehog* was expected in *gup* and *sly* given the previously reported characterisation of the mutants. However, the result for *bal*, in which *ehh* expression is extinguished, is surprising, and suggests that the product of the *bal* locus might control the vacuole inflation process independently of gene expression. There are therefore differences between *bal* compared to *gup* and *sly*. Cloning of the genes responsible for each mutant may suggest a hypothesis for the differences.

One of the major technical problems with the positional cloning of *bal* was the weak nature of the phenotype on the mapping background (WIK) that inevitably resulted in mutant mis-scoring. Consequently, false-positive recombinants were found that resulted in an overestimate of the genetic distance. This was only resolved once a marker was found on the telomere side of the *bal* locus. The end of the YAC 5E7 provided such a marker. The meiotic mapping of *bal* was made simpler by the large number of SSLP markers in the region, allowing the construction of a YAC and PAC/BAC contig, and eliminating the need for a laborious ‘walk’ along the chromosome.

There are a number of criteria that make *lamal* gene an excellent candidate for *bal*. Firstly, laminin genes are extremely large, which fits well with the observation that 26 alleles of *bal* were identified in the screens; the most number of alleles reported for any mutant to date. Secondly, laminins have been studied intensively in other organisms and are known to be important for axon guidance and cell differentiation (Brown, 2000; Crisera et al., 2000; Pires Neto et al., 1999) which is consistent with the CNS defects in *bal* (Karlstrom et al., 1996). Finally, a peri-notochordal BM is present in the mature notochord of zebrafish. Loss of laminin might be predicted to disrupt this BM and hence organisation of the matrix surrounding the chordamesoderm. One might expect this to interfere with the turgour pressure necessary to create to vacuolated and rigid
notochord. Laminin can also control gene expression through signalling via their receptors, such as integrins and dystroglycan (see Chapter 6).

In summary, a positional candidate approach has been used to identify a good candidate for the gene disrupted in bal. An initial genetic map identified several SSLP markers close to the bal locus. These were used to identify YAC clones, and the end of one of these clones contained an SSLP (5T7). This was used as a genetic marker and was found to lie on the telomere side of bal. More detailed mapping narrowed the region containing bal to an interval of ~200kb. The end of BAC 143J17 contained part of the gene encoding laminin α1 (lama1). This is an excellent candidate for bal and was investigated further.

3.7 Summary

- bal notochord extinguishes expression of the early chordamesoderm marker ehh whereas gup and sly do not.

- bal chordamesoderm cells can be rescued by adjacent WT tissue.

- Low resolution genetic mapping identified Z7895 as a marker very close to the bal<sup>m190</sup> locus.

- Chromosome walking and further genetic mapping restricted the bal<sup>m190</sup> locus to an interval of 0.3cM (between Z7895 and Z7292).

- A BAC clone within this region contains part of the lama1 gene.

- The gene encoding laminin α1 is a good candidate for bal.
Chapter 4

Characterisation of zebrafish Laminin α1
Chapter 4

Characterisation of zebrafish Laminin α1

4.1 Introduction

In the previous chapter I showed that the gene encoding the laminin α1 chain is a strong candidate for bal. In this chapter I extend this finding and confirm that mutations in lama1 are responsible for the bal phenotype. In addition, I analyse the expression of the laminin α1 mRNA through development. Finally, using MO injections I disrupt expression of laminin α1 and show this phenocopies the bal mutation.

4.2 Cloning of the full-length laminin α1 cDNA

A search of the Genbank database revealed no zebrafish cDNA sequences (including ESTs) with significant homology to laminin α1. Thus, three approaches were simultaneous taken to clone the full length lama1 cDNA: screening of a shield stage phage cDNA library; 5' RACE; and a search for laminin α1 exon sequence within the zebrafish genome sequencing project, sequence repository. All of the PCR primers used for the cloning of the zebrafish laminin α1 cDNA are given in appendix A3, and a summary of the cloning strategy is given in Figure 4-1.
4.2.1 Rapid amplification of cDNA ends (RACE)

Initially 5’ RACE was performed from the exon sequence identified in BAC 143J17. Two rounds were required to obtain the start of translation and 5’UTR. Obtaining the 5’ end served two immediate purposes. Firstly, I could design a MO against the translation start site, and secondly, the cloned fragment could be used as a template to generate a probe to screen cDNA libraries. Identification of the 23 amino acid signal peptide and its predicted cleavage site, was performed using Signal P (Nielsen et al., 1997), and is illustrated in Figure 4-1B. In order to ensure that the cDNA obtained by RACE was real (i.e. part of the same gene as the BAC exon) I performed RH mapping using primers based on the 5’ RACE product. As expected these mapped the 5’ end of the lamal cDNA to the telomere of LG24 (i.e. the bal locus).

4.2.2 cDNA libraries

A shield stage cDNA phage library (Gift from M. Rebagliiti) and a cDNA plasmid library (RZPD), were screened using a probe prepared from the first 5’ RACE product. Sequencing of the positive phagemids identified several clones with laminin α1 sequence. However, no sequences were identified further 3’ to that already obtained by 5’ RACE. No positive clones were obtained from the plasmid library.

4.2.3 Identification and mapping of lamal exons

A PCR approach was taken in order to identify the remaining 3’ part of the laminin α1 cDNA. A search was made for sequences encoding Laminin α1 from the Sanger Centre zebrafish genome-sequencing project (http://www.sanger.ac.uk/Projects/D_rerio/). A TBLASTN search of existing whole genome shotgun sequence using the mouse Laminin α1 amino acid sequence as a query, revealed many traces with high homology to mouse Laminin α1. A comparison of these exon sequences with the human LAMA2 gene identified the sequences as homologues of exons 22, 23, 26, 44, 60 and 63 (Zhang et al., 1996). Each of these genomic fragments was physically mapped by using the RH panel. Exon 26 and 60 were shown be linked to the bal locus, and PCR primers were designed for use in long range PCR and 3’ RACE to obtain the remaining portion of the cDNA. The complete cDNA of 9686bp length was obtained.
Figure 4-1  Cloning of the laminin α1 cDNA. (A) Cloning of Laminin α1 cDNA was performed using: 5’ RACE, 3’ RACE and long range PCR. Italic numbers represent the plasmid clone number (S. Pollard stocks). PCR primers used to generate F1 → F5 are given in appendix A3 (pg192) (B) A predicted signal peptide of 23aa for Laminin α1 chain was identified using SignalP. The cleavage site located between A_{23} and Q_{24} (→) (http://www.cbs.dtu.dk/services/SignalP/).
4.2.4 Zebrafish Laminin α1 predicted protein

The 9686bp full-length zebrafish laminin α1 cDNA contains an open reading frame encoding a predicted protein of 3075aa (336kD) (Figure 4-2). An alignment of the full-length amino acid sequence was made against human and mouse laminin alpha chain genes. The greatest homology was with the laminin α1 encoding genes (% identity values of 50.0 and 49.9, for human and mouse, respectively). The phylogenetic tree shows that as expected the mouse and human genes diverged much more recently than the zebrafish homologue (Figure 4-3). The distinction between the α3/α4/α5 and the α1/α2 subgroup is evident. This reflects the evolutionary origin of two distinct forms of laminin and is reflected in invertebrates, which have one gene, of each type (see Introduction). The analysis of protein domains within zebrafish Laminin α1 by SMART analysis reveals the same domain organisation as mouse and human homologues (Schultz et al., 1998). Each contain one Laminin N-terminal domain, seventeen EGF repeats, two LamB domains and five C-terminal LamG domains (Figure 4-4).
Figure 4-2  Zebrafish predicted Laminin α1 protein. The cloning was performed by PCR as follows: exon sequence from BAC 143J17 (red) was initially used for the design of primers for 5' RACE (blue). Two of the exon sequences (obtained from the Sanger centre database) are shown (green). PCR primers based on these and the BAC exon were used to produce two long-range PCR products (purple). The 3' region and UTR were obtained by 3' RACE (orange), from the 3' Sanger centre exon.
### Figure 4-3 Alignment of zebrafish Laminin α1 with vertebrate homologues.

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#### B

![Dendrogram](image)
Figure 4-4  SMART analysis of Laminin α1. LamNT = N-terminal domain of laminins and laminin-related protein such as Unc-6/ netrins. EGFLam = Laminin-type epidermal growth factor-like domain. LamB = Laminin B domain (also known as domain IV). An extracellular module of unknown function that is found in a number of different proteins (e.g. heparan sulphate proteoglycan). LamG = A homology domain first described in the long arm globular domain of laminin. Similar sequences also occurs in a large number of extracellular proteins. A general function of laminin-G repeats is not known, but in laminin it seems to bind to heparin. Low complexity sequence (purple); Transmembrane segments (blue); Coiled-coil (green).
4.3 Identification of a mutation in \( \textit{bal}^{m190} \textit{lama1} \)

4.3.1 Mutation in \( \textit{bal}^{m190} \)

Conclusive evidence that \( \textit{bal} \) encodes Laminin \( \alpha1 \) comes through identification of a mutation within the gene. I therefore attempted to clone and sequence the \( \textit{lama1} \) cDNA from \( \textit{bal}^{m190} \) and \( \textit{bat}^{nc45} \) mutants. This was a time-consuming task given the large size of the cDNA (~9.7kb). It was expected that as the \( \textit{bal}^{m190} \) and \( \textit{bat}^{nc45} \) have the strongest phenotype of all the \( \textit{bal} \) alleles, the mutation would give rise to a completely non-functional protein, most likely an effective protein null. Thus, I initially cloned and sequenced the 5' region of mutant cDNA. Three overlapping fragments of ~800bp were cloned from \( \textit{bal}^{m190} \) and \( \textit{bal}^{nc45} \) 24hr cDNA libraries (representing the 5' 1.6kb).

Surprisingly, among these fragments I did not identify any lesions/substitutions that would clearly affect the function of the protein. I therefore proceeded to clone and sequence the remaining 3' region of the \( \textit{bal}^{m190} \) cDNA. This was performed using the PCR primers from the initial cloning of the WT cDNA. Two long-range PCR products were cloned and sequenced, representing sequences from exon10-26 (~2.4kb) and 26-63 (~4.5kb), respectively. Sequencing primers were the same as those used for PCR of the WT F3 and F4 fragments (appendix A3 and Figure 4-1).

A number of polymorphisms were identified. However, the actual mutation responsible for the \( \textit{bal}^{m190} \) phenotype was found at amino acid position 890, where a premature stop codon (TGA) was identified (Figure 4-5). In the WT cDNA this position is a glycine coded by GGA. Thus, a substitution from a guanine \( \rightarrow \) thymine results in a truncated protein of 889 residues (full-length is 3075). This result was seen for two different cDNA clones, and verified by sequencing the same region in \( \textit{bal}^{m190} \) genomic DNA. The second allele (\( \textit{bat}^{nc45} \)) was not characterised any further and the mutation remains unidentified.

4.3.2 RFLP mapping of \( \textit{bal}^{m190} \)

The change from G \( \rightarrow \) T results in loss of the recognition site of the restriction enzyme FokI. This property was used to generate a restriction fragment length polymorphic marker (RFLP). Fortunately, genomic sequence around the mutant locus became available from the zebrafish genome-sequencing project. Primers flanking the mutation
were obtained that generate a 338bp PCR product, which upon digestion with Fok1, results in two fragments of 220bp and 118bp. This RFLP was used to validate identification of the mutation. The PCR product from bal^m190 mutant embryos is not cut with Fok1, while both WT (WIK) and bal^ac45 PCR products are digested (Figure 4-6). Additionally, such an RFLP provided an ideal marker for genetic mapping, as there should be no recombination events over this locus if it represents the true bal^m190 mutation. Testing the recombinants (previously identified with Z7292 and Z7895) using the RFLP markers shows that all are mutant, i.e. did not cut with Fok1 (0 recombinants, representing >4000 meioses).
Figure 4-5 Mutations in lamal are responsible for the bal phenotype. (A) Sequencing of the mutant locus within the genomic DNA of bal^{m190} mutant fish compared to WT grandparent confirms the base substitution (G → T) previously identified in the cDNA. (B) The mutation leads to a premature stop codon at position 890 prior to the start of the coiled-coil domain.
Figure 4-6  \( \text{bal}^{m190} \) contains an RFLP that can be used for mapping. (A) RFLP products run on a 2% agarose gel. Two separate \( \text{bal}^{m190} \) mutants were tested (lanes 1 and 2) and contain no Fok1 site (338bp band). Positive controls were grandparent DNA (WIK/WIK) and a second allele (\( \text{bal}^{abc45} \)) (lanes 3, 4 and 5), both of which were digested to 220bp and 134bp fragments (though digests were only partial as uncut product remains). (B) Using this RFLP genetic marker no recombination events over this locus could be detected (>4000 meioses).
4.4 Expression of zebrafish *lama1* mRNA

Following the cloning of the zebrafish laminin α1 full-length cDNA, I studied the expression of the mRNA to further elucidate its role during embryogenesis. The results support the notion that *lama1* is involved in notochord differentiation and aspects of CNS development. The probe used was generated from the 5' region of the cDNA (Table 2-2). The results of the *in situ* hybridisation are shown in Figure 4-7.

*Lama1* does not seem to be expressed maternally, as no expression could be detected in early cleavage stages. Expression is first evident at around 70% epiboly throughout the hypoblast. During somitogenesis (5 somite stage) expression is detected throughout the developing brain and somites. More caudally, in more immature paraxial mesoderm, expression is seen in cells most closely associated with the notochord, known as adaxial cells. Interestingly, there is no detection of *lama1* mRNA in the chordamesoderm; however, expression is detected in the chordamesoderm of the tailbud. Later, at the 12-somite stage, expression within the somites is restricted to the adaxial cells, and there continues to be widespread expression within the developing eye and brain. During late somitogenesis and at 24 hpf, somitic expression appears to be down-regulated, though expression remains in the tailbud and surrounding tissue. Strong expression is detected throughout the hypochord, underlying the notochord, as well as in the eye and brain.

4.5 Antisense knockdown of *lama1*

Use of MOs to disrupt translation has proven to be valuable for testing of candidate genes identified during positional cloning projects. As further confirmation that the *bal* locus encodes Laminin α1, I designed a MO against the *lama1* start of translation site (based on the sequence obtained from 5' RACE). Injection of 3ng of this into embryos at the 1 cell stage resulted in a remarkable phenocopy of *bal*. Morphologically these morphants resemble *bal* in many ways including, an obvious shortening of the AP axis, an enlarged hindbrain, eye defects, and anterior notochord defects. As is the case in *bal* mutants the posterior notochord has a WT morphology (Figure 4-8).
Figure 4-7  Expression of lamal during early zebrafish development. There are no maternal transcripts detected at the 2-cell stage by whole-mount in situ hybridisation (A). Expression is first detected at 70% epiboly (E) and by 5-somite stage is present in the forming brain and eyes (J), somites (K), and strongly in cells adjacent to the chordamesoderm in more posterior regions (L). By 24 hpf expression remains strong in the head (P). Within the trunk and tail only hypochord (arrow head) and tailbud notochord retain expression (Q).
Chapter 4 Characterisation of laminin α1

Figure 4-8 Injection of a lamal MO recapitulates the bal^m190 phenotype. (A, B) Morphology of control MO-injected embryo at 48 hpf. (C, D) Morphology of lamal MO-injected embryo at 48 hpf. (E) uninjected control at 48 hpf. lamal morphants strongly resemble bal^m190 mutants.
4.6 Discussion

Following the identification of laminin α1 as a candidate for bal, I have cloned the full-length bal\textsuperscript{m190} cDNA. It is 9686bp and has ~50% sequence identity with mouse and human homologues. Ultimate proof that the bal phenotype arises because of non-functional laminin α1 protein, came with identification of the mutation in the bal\textsuperscript{m190} cDNA. The G → T substitution results in a premature stop codon, and consequently a truncated protein. The truncated protein expected from the bal\textsuperscript{m190} allele would not be capable of forming a heterotrimer with the laminin β and γ chains, due to lack of the coiled-coil region. There is a possibility, however, that it may be able to act as a dominant negative as the N-terminal region contains the domains responsible for polymerisation of laminins.

The spatial and temporal expression of the lamal RNA correlates with the bal phenotype, and is consistent with a role for Laminin α1 in notochord development. Expression of lamal is not seen within the chordamesoderm tissue itself, but rather in the adjacent adaxial cells. One interesting question arising from this observation concerns BM assembly. The expression pattern would be consistent with either assembly of laminin heterotrimers extracellularly, with each chain being supplied by different tissues. Alternatively, adaxial cells that surround the notochord may produce a mature laminin heterotrimer.

As expected, the pattern of expression within the brain is widespread, fitting well with the bal phenotype (gross morphological defects and axon guidance defects throughout brain). The developmental expression of both laminin α1 and β1 has been studied in early chick embryos (Zagris et al., 2000). Expression of both chains is strong within the chordamesoderm during neurula stages, but this is extinguished by 10-somite stage (HH10-11). Later both chains are expressed in the brain, neural crest, neural tube and kidney.

Rescue of the mutant phenotype and overexpression studies, by injection of WT laminin α1 cDNA, was not practical due to the large size of the message. The reciprocal experiment, however, of reducing protein levels with antisense lamal MOs generated
phenotypes identical to bal mutants, providing further evidence that the bal encodes laminin α1.

In parallel to the characterisation of bal, our laboratory had also found that the related sleepy locus encodes zebrafish laminin γ1 (Parsons et al., 2002). As discussed in the introduction, laminin γ1 is present in all but two of the twelve known laminin isoforms whereas the α1 and γ1 chains are present in only laminin 1 (α1β1γ1) and laminin 3 (α1β2γ1) (Table 1-3). Hence, one or both of these isoforms might be required for notochord differentiation. The results for the positional cloning of gup and characterisation of other laminin α chains, are described in the next chapter. This data suggests that it is the laminin 1 isoform that is necessary.

To summarise, the results presented in this section have characterised zebrafish laminin α1, and provide compelling evidence that the defects seen in the mutant bal are due to mutations in the lamal gene.

4.7 Summary

- The zebrafish lamal gene encodes a 3075 aa protein with 50% identity to the mouse Laminin α1.
- cDNA encoding Laminin α1 from balm190 contains a G → T substitution resulting in a premature stop codon at position 890.
- lamal mRNA is expressed during early embryogenesis adjacent to the chordamesoderm and throughout the CNS.
- Injection of a MO against lamal cDNA phenocopies bal.
- The related mutant sly encodes the laminin γ1 chain (laml).
- A laminin trimer containing α1 and γ1 chains is necessary for notochord differentiation.
Chapter 5

Laminin isoforms during embryogenesis
Chapter 5

Laminin isoforms during embryogenesis

5.1 Introduction

The grumpy (gup) mutant has a very similar phenotype to both bal (lamal) and sly (lamcl). It has a failure of notochord differentiation and general morphological defects within the CNS. This chapter describes the identification of the gup gene through a positional candidate approach. The shared phenotypes of bal, gup and sly suggested they would most probably be part of a similar biochemical complex or pathway. gup is shown in this chapter to be due to mutation in the gene encoding the laminin β1 chain (lamb1). Later parts of this chapter present the analysis of how the three laminin mutants, bal/lamal, gup/lamb1 and sly/lamcl, relate to one another and what the roles of various laminin isoforms are during early zebrafish development, with emphasis on those necessary for the differentiation of the notochord.
5.2 Mapping of grumpy

5.2.1 Genetic mapping

M. Parsons identified a genomic marker (cat/gca) 1.3cM from \( gup^{m189} \) using the amplified fragment length polymorphism (AFLP) method (Vos et al., 1995). By RH mapping he placed this marker to within 21cR of \( myoD \) on chromosome 25 (Parsons et al., 2002). I used primers flanking a polymorphic region of the \( myoD \) 3'UTR to perform recombination mapping, which showed that the \( myoD \) locus lies \(-0.4cM \) from \( gup \).

5.2.2 EST map

By monitoring publicly accessible RH maps we noted an EST that was both linked to \( myoD \), and homologous to a gene encoding a laminin \( \beta \) chain. The clone corresponding to this EST (accession number AA495063) was obtained and sequenced. It was found to be homologous to human \( LAMB4 \) (D. Stemple, pers comm). Although the human \( LAMB4 \) gene has been identified from genomic sequence, no laminin isoform containing this chain has been described. We obtained the complete \( lamb4 \) cDNA sequence by screening additional cDNA libraries and by 5' RACE. Furthermore, with database searches we discovered a zebrafish \( Iambi \) homologue (accession number AI641157), which we mapped to within 20cR of \( myoD \) using the LN54 RH panel. Using this clone and 5' RACE we determined the complete \( Iambi \) cDNA sequence. Thus, two genes encoding laminin \( \beta \) chains are located near the \( gup \) locus (Figure 5-1).

5.3 Expression of \( Iambi \) and \( lamb4 \)

As both \( Iambi \) and \( lamb4 \) genes were good candidates I looked by in situ hybridisation to determine whether they were expressed in a manner consistent with a role in notochord and brain development. I generated an in situ probe for \( lamb4 \) by sub-cloning an 824bp fragment into bluescript vector. \( lamb4 \) is expressed maternally and shows widespread expression at shield stage and 80% epiboly. By 20-somite stage expression is strong within the brain and eyes, though little expression is seen in the trunk or tail (Figure 5-2A). A \( Iambi \) probe was generated by M. Parsons. The results show that \( Iambi \) is also expressed maternally, while later expression is seen throughout embryo, with noticeably higher levels present within the chordamesoderm by tailbud.
stage. High levels of expression remain in the hypochord, fin-folds, tailbud and gut at 24 hpf.

These results do not exclude either gene from having a role in notochord differentiation, though the more restricted expression within the chordamesoderm of lamb1 suggests that this is more likely to be the gup gene.

5.4 **Antisense knockdown of lamb1 and lamb4**

I obtained MOs targeted against lamb1 and lamb4 mRNA in order to establish whether these would be capable of recapitulating the gup phenotype. Embryos injected with either the lamb4 MO or control MO, were phenotypically WT. By contrast, embryos injected with lamb1 MO displayed a morphological phenotype identical to that of gup mutants, strongly suggesting that lamb1 is defective in gup (Figure 5-2C). The lamb1 morphants show a shortened AP axis as well as defects with in the brain and eyes consistent with the gup mutant phenotype. Importantly, vacuoles within morphant notochord fail to inflate, and echidna hedgehog is persistently expressed in lamb1 but not lamb4 morphants (Figure 5-2C). These results strongly suggest that gup encodes laminin β1 and not laminin β4.

5.5 **Mutation in grumpy^m189**

As a final verification that gup corresponds to the lamb1 gene, cDNA and genomic DNA from gup mutant embryos were cloned and sequenced. A premature stop codon at amino acid 544 was identified (Parsons et al., 2002).
Figure 5-1  RH map of the *gup* locus. Genetic mapping had shown that the *gup* locus lies 0.4cM from *myod* on LG25. RH mapping of *lamb1* and *lamb4* reveals that both are located near this region. *lamb1* and *lamb4* are therefore both good candidates for *gup*.
Figure 5-2 mRNA expression and morphant phenotypes for lamb1 and lamb4. (A) lamb4 is expressed widely during early stages, but does not show any specific expression dorsally. (B) The expression of lamb1 becomes stronger within the chordamesoderm by tailbud stage. (C) Injection of a MO against lamb1 phenocopies gup, at the morphological level and molecular level (maintenance of ehh). A MO against lamb4 does not show any obvious phenotype.
5.6 Laminin immunocytochemistry

5.6.1 Laminin protein in WT embryos

To study expression of laminin 1 protein we obtained rabbit anti-mouse laminin 1 antibody (Sigma). This antibody is polyclonal and so will not only reveal laminin 1, but presumably other isoforms containing α1, β1 or γ1 chains. Hence, potentially nearly every known laminin isoform should be revealed if present during development.

The results for various stages of development are shown in Figure 5-3. Laminin immunoreactivity is clearly present at early stages prior to the start of zygotic expression. The fact that there is such strong maternal laminin protein is surprising given that lamal is not maternally expressed. It is likely that the antibody is reacting with β1 and γ1, which may be associated with another α chain during this stage. Strong immunoreactivity is seen in a more restricted manner by 70% epiboly around the chordamesoderm, coinciding with the first expression at around (70% epiboly) of the lamal mRNA. Later, at somite stages intense staining is seen surrounding the forming somites. At 24 hpf laminin protein is localised around the notochord, inter-somitic boundaries and throughout the brain and eyes. There is also strong expression within the fin folds, gut, tailbud and surrounding the yolk extension. Thus, laminin isoforms are present in a very wide range of tissues in the early embryo, including regions consistent with the defects seen in bal, gup and sly mutants.
Figure 5-3  **Laminin immunocytochemistry in WT embryos.** A polyclonal antibody against laminin 1 was used to analyse protein expression during early zebrafish development. Maternal immunoreactivity is seen (A), and by tailbud stages there is laminin localisation around the chordamesoderm (arrow, C) consistent with a role for laminins in the progression of this tissue into notochord. By 24 hpf strong staining is seen within the eyes, otic vesicle, brain, notochord, yolk sac membranes and fin folds (G and H).
5.6.2 Laminin immunoreactivity in bal, gup, and sly

Laminin polyclonal antibody was used to assess levels of expression in mutant embryos (Figure 5-4). sly embryos show a rather remarkable lack of immunoreactivity compared to control WT siblings and this seems likely to be due to a loss/degradation of other α and β chains that associate with laminin γ1 in the early embryo. Severely reduced staining is also seen in gup mutants, though some disorganised staining persists within the tailbud at 24 hpf. This may either be due to un-degraded α1 or γ1, chains or other isoforms containing the α1 and γ1 chains.

By contrast, bal mutants show WT levels of immunoreactivity in the posterior notochord, that most probably represents a functional laminin trimer, as this correlates precisely with the anterior boundary at which notochord cells become mutant. Other tissues such as yolk sac membranes, fin fold, tailbud and brain also show immunoreactivity in bal mutants. One possible explanation for the laminin immunoreactivity seen in bal, as well as its weaker phenotype, is that another laminin α1 gene with a more restricted expression domain than bal/lamal is present in zebrafish (i.e. a putative lamal(b)). A priori, this is a strong possibility given the ancestral genome duplication of teleost fish (Force et al., 1999; Taylor et al., 2001). An extensive search of the zebrafish genomic sequence database did not, however, reveal any genomic sequence encoding a second laminin α1. An alternative explanation for the weaker phenotype is that other α chains are used during notochord differentiation. Loss of gup/lamb1 or sly/lamcl affects many more isoforms of laminin than α1. Therefore another laminin isoform containing laminin α2, α3, α4 or α5 may be necessary for the notochord development. Both gup and sly have complete loss of notochord differentiation, suggesting that, for notochord differentiation the relevant isoform must contain both β1 and γ1 chains. Four other isoforms have been previously identified that associate with these two chains: laminin 2 (α2β1γ1), laminin 5 (α3β1γ1), laminin 8 (α4β1γ1) and laminin 10 (α5β1γ1) Table 5-1. It was therefore reasonable to hypothesise that one or more of these isoforms are present during development in a manner partially redundant to laminin 1.
Figure 5-4  Laminin immunocytochemistry in bal, gup and sly mutants. These three mutants have been shown to encode the laminin α1, β1 and γ1 chains respectively. Laminin immunoreactivity in WT embryos at 24 hpf is shown (A and B). There is very little remaining immunoreactivity within both gup and sly mutants (E-H). This is consistent with these chains being components of most of the known laminin isoforms. bal mutants, however, retain strong levels of immunoreactivity within the brain and lens of the eye (C), as well as surrounding the notochord (black arrow, D); with the exception of anterior regions that have mutant morphology (white arrow, D). The notochord expression in bal posterior notochord might be due to the presence of another Laminin α chains, functionally redundant with Laminin α1.
Laminin isoforms during embryogenesis

Table 5-1  Laminin α1, β1 and γ1 chains are present in all but one of the twelve known laminin isoforms. The β1 and γ1 chains have a widespread distribution in mouse embryonic and adult tissues that is reflected in their abundance in many isoforms. The Laminin α1 chain on the other hand has a more restricted expression and is associated with only two of the known isoforms. Five isoforms contain both β1 and γ1 chains (laminin 1, 2, 4, 8, and 10).
5.7 Expression of *lama1*, *lamb1* and *lamc1* mRNA in laminin mutants

Given the knowledge that *bal*, *gup* and *sly* encode three laminin chains collectively known to be components of all but one laminin isoform, and the observation that disruption of *gup/lamb1* and *sly/lamc1* each lead to a large loss of laminin 1 immunoreactivity, it became important to test for any regulatory interactions between these three chains. Although many biochemical investigations of laminins have been made, studies of laminin chain regulation at the genetic level have not been reported. I therefore analysed the expression, by in situ hybridisation, of laminin α1, β1 and γ1 chains in *bal/lama1*, *gup/lamb1* and *sly/lamc1* mutants.

The results of this analysis are shown in Figure 5-5. It is clear that *lama1* is expressed normally in *bal* embryos and likewise *lamb1* is seen in *gup*. In *sly*, however, there is a lack of *lamc1* expression (also seen in a second allele of *sly* and is consistent with nonsense mediated mRNA decay, since morpholino mediated disruption of laminin γ1 does not affect *lamc1* mRNA levels) (Parsons et al., 2002). I found that *lama1* expression is severely reduced in the *gup* and *sly* embryos, suggesting that *lama1* mRNA expression may be controlled at the transcriptional level through some feedback mechanism involving β1 and γ1 chains (Figure 5-5).
Figure 5-5  Expression of *lama1, lamb1* and *lmc1* in notochord mutants. Each of the notochord mutants, *bal, gup* and *sly*, was tested by whole-mount in situ hybridisation for expression of mRNA each of *lama1, lamb1* and *lmc1*. 
5.8 Characterisation of zebrafish lama2, lama4 and lama5

In the previous section I noted laminin immunoreactivity in the posterior region of bal notochords that may be due to a redundancy with another laminin α chain. Therefore, I decided to investigate some of the other known chains, through mRNA expression, and MO injection.

5.8.1 Cloning and RH mapping

There are five known genes encoding laminin α chains in mouse (lama1–lama5) (Colognato and Yurchenco, 2000). It was decided to clone lama2, lama4 and lama5 cDNAs, as genomic fragments for each of these were present in the genomic sequence database. I used 5' RACE from homologous genomic sequences and then mapped each cDNA fragment using the RH panel (Figure 5-6) (appendix A 7). The mapping of these genes was needed to confirm the identity of exon fragments, and is also useful to identify any of these genes as candidates for mapped mutants. LAMA2 and LAMA4 are syntenic in the human genome. This is also true in zebrafish as lama2 and lama4 are linked on LG20. The lama5 gene maps to LG23. No previously mapped mutants were present at either of these two loci. I did not investigate the lama3 as no sequences with homology to mouse could be identified.

5.8.2 Expression of lama2 and lama5 mRNA

A lama2 antisense probe was generated using a cDNA fragment generated by 5' RACE. Expression is seen specifically within the developing somites and this is consistent with the muscular dystrophy seen in humans and mice that lack this chain. Earlier stages were not analysed. An antisense in situ probe (1.1kb) against lama5 was generated using a cDNA clone obtained from the RZPD database as template. Lama5 is expressed maternally and by tailbud stage strong zygotic staining is seen within the axial mesoderm. At somite stages there is specific staining within the dorsal midbrain (tectum), within the fin folds and in the membranes surrounding the yolk sac extension. Laminin α5 chain is present in two of the heterotrimer combinations that have been characterised to date, laminin 10 (α5β1γ1) and laminin 11 (α5β2γ1). The strong expression within the chordamesoderm suggests that either of these isoforms may be present within the peri-notochordal BM.
Figure 5-6  Location of laminin genes in zebrafish and human genomes. (A) Location of cloned zebrafish laminin genes. (B) Chromosomal location of the 12 human laminin chains. The synteny seen in zebrafish between lamb1 and lamb4 is conserved in the human genome, as is that between lama2 and lama4. I mapped zebrafish lama5 to LG23.
Figure 5-7  Expression of *lama2* mRNA at 24 hpf. *lama2* mRNA is expressed specifically within the somites at 24 hpf (A). A higher power view (C and D) show that higher levels of expression are seen laterally within the slow muscle.
Figure 5-8 Expression of *lama5* during zebrafish development. Maternal transcripts of *lama5* are present at 64-cell stage (A). By tailbud stage strong staining is observed specifically within axial mesoderm (C and D, lateral and dorsal views). By 15-somite stage, expression remains strong within the chordamesoderm and prechordal plate (E and F, lateral and dorsal views), and lower levels of expression are also seen within the epidermis and fin folds. By 20-somite stage expression within the chordamesoderm is extinguished in anterior regions, though persists within tailbud chordamesoderm (G). A remarkably specific region of expression is seen within the dorsal region of the midbrain (tectum) at this stage (G, arrow), that persists at 24 hpf (H, arrow).
5.8.3 Antisense knockdown of *lama2, lama4* and *lama5* in WT embryos

I tested each of the MOs designed against *lama2, lama4* and *lama5*, by injecting into WT embryos, to determine if there is any loss-of-function phenotype for each of these genes. Initially 1.5ng, 3ng and 6ng were injected. Phenotypes described below and shown in Figure 5-9 represent the 3ng dose, unless stated otherwise (>100 embryos were injected for each MO).

*lama2* morphants appeared WT initially, and by 24 hpf were indistinguishable from control injected siblings. By 4 days post-fertilisation, however, the embryos had failed to hatch from the chorion and appeared dystrophic within trunk and tail regions. Those embryos that were manually de-chorionated showed reduced movements and were unable to swim, when gently prodded with forceps. These defects are very similar to the muscular dystrophy defects observed in zebrafish dystroglycan morphants (Parsons et al., 2002). Thus, it is likely that lack of *lama2* results in a muscular dystrophy phenotype, implicating this gene in normal development/function of zebrafish muscle. This phenotype is not unsurprising given the specific expression of *lama2* within the somites and given that lesions in human *LAMAl* give rise to congenital muscular dystrophy (see Introduction).

*lama4* morphants show no obvious morphological defects by 48 hpf and are indistinguishable from control morphant siblings. Some, however, do have a bend within the notochord following hatching, though there are no defects in notochord differentiation. *lama4* morphants show WT expression of *ehh* mRNA and laminin protein (Figure 5-9).

At 24 hpf *lama5* morphants show a striking lack of the yolk extension. There is a large reduction in fin folds, consistent with the strong expression of the mRNA in this tissue. Despite the strong chordamesoderm expression of *lama5* there are no morphological defects within the notochord and embryos fully elongate in the AP axis. *Lama5* morphants were analysed using immunocytochemistry to determine levels of laminin protein, and also by in situ hybridisation to monitor expression of the chordamesoderm marker *ehh*. Remarkably, expression of *ehh* is strongly maintained in *lama5* morphants at 28 hpf compared to control MO injected siblings. This was unexpected given that WT
levels and localisation of laminin protein is seen in these embryos and the notochord had a WT appearance with fully vacuolated cells (Figure 5-9).

5.8.4 Antisense knockdown of lama4 and lama5 in bal	extsuperscript{m190} embryos

The cloning of lama4 and lama5 cDNA fragments and identification of bal as lamal provides an excellent opportunity to investigate the roles of specific laminin isoforms through double knockdown of various chains. Thus, adult bal	extsuperscript{m190} heterozygous carriers were crossed and the progeny were injected with lama4, lama5 or control MO. This enables double loss-of-function phenotypes to be determined, and is preferential to double MO injections as the experiment is internally controlled. 25% of injected embryos will be homozygous WT for lamal and will have a phenotype identical to injection of MO into WT embryos. 25% of MO injected embryos will be homozygous mutant for lamal (i.e. bal mutants), and will have a double loss-of-function phenotype. 50% of will be heterozygous for bal.

Injection of lama4 MO into a bal cross resulted in 22.8% of embryos with a severe notochord defect, similar to gup and sly at 48 hpf, and 77.2% with a WT appearance (two experiments, n=136) (Figure 5-10). Several of these WT embryos contained a bent notochord, though this likely to be an artefact of the injection procedure as this was also seen within control-injected MO siblings. Embryos were analysed using immunocytochemistry for expression of laminin protein, and a reduction in levels surrounding the notochord was seen in bal mutants injected with lama4 MO, but not WT siblings or control injected MO siblings (either WT or bal). These results show that lama4 acts partially redundantly with lamal in control of notochord vacuolation, and is responsible for the WT notochord present in posterior regions of bal mutants.

Injection of lama5 MO into a bal cross (two separate experiments, n=241) resulted in three distinct classes of phenotype. 24.8% of embryos show a severe phenotype (class 1) by 48 hpf, that included blebbing of the epidermis, lack of fin folds, a large amount of cell death within the midbrain, and a lack of AP extension, possibly due to lack of mature notochord and somites. The majority of embryos (57.3%) showed an intermediate type of phenotype (class 2) in which the yolk extension failed to form, massive cell death was apparent within the midbrain and the notochord was severely
bent. 17.8% of the morphants had an almost WT appearance (class 3) apart from an absence of the yolk extension (identical to lama5 injections into a WT cross).

Genotyping of embryos using the RFLP was performed to confirm that the three distinct classes of phenotype seen reflected the homozygotes bal (class 1), heterozygotes bal (class 2), and WT (class 3). I tested four embryos from each class in order to confirm the embryos as either WT, heterozygous (bal<sup>m190</sup>/WT), or homozygous (bal<sup>m190</sup>/bal<sup>m190</sup>).

Embryos from each of these three classes were tested for their expression of laminin protein and ehh mRNA, using immunocytochemistry and in situ hybridisation respectively (Figure 5-10). All embryos showed WT levels of laminin immunoreactivity, and class 2 (bal<sup>m190</sup>/WT) and class 3 (WT) embryos showed normal localisation of the protein within the notochord BM and intersomitic boundaries. In those embryos with the severe class 1 phenotype (bal<sup>m190</sup>/bal<sup>m190</sup>) the laminin was severely disorganised, with very little localisation. Each of the embryos photographed were genotyped subsequent to the laminin immunocytochemistry analysis to confirm their identity.

Thus, both lama4 and lama5 function during notochord formation. Laminin α4 chain is required for vacuole inflation and acts partially redundantly to Laminin α1 (it is not used during anterior notochord differentiation). Laminin α5 chain functions specifically in control of gene expression within the chordamesoderm (probably as part of laminin 10, α5β1γ1). Therefore multiple laminin isoforms are present within the peri-notochordal BM that control different aspects of the notochord differentiation process. This explains why the bal/lama1 mutants are weaker than both gup/lamba1 and sly/lamc1, in regards to the notochord defects.
Figure 5-9 Injection of lama2, lama4 and lama5 MOs into WT embryos. Lama2 morphants at 48 hpf show a phenotype similar to dystroglycan MO, in that trunk and tail somites are dystrophic and embryos fail to swim correctly (A). Lama4 morphants do not show any obvious defects at 48 hpf (D). Both lama2 and lama4 morphants show WT expression of ehh mRNA at 28 hpf (F and I), compared to control injected morphants (C). Laminin immunoreactivity also appears normal at this stage in these morphants (E and H; control in B). Lama5 morphants fail to form the yolk extension (arrow, J). Although lama5 morphants contain a morphologically WT notochord (J), and laminin protein appears similar to WT embryos (K), these embryos fail to extinguish expression of ehh by 28 hpf (L).
**Figure 5-10** Injection of *lama4* and *lama5* MOs into a *bal* cross. The morphology of embryos at 48 hpf is shown (A-H). Molecular characterisation of morphants was performed using whole-mount immunocytochemistry for laminin protein (I, J, M, N Q and R), and in situ hybridisation for *ehh* (K, L, O, P, S and T). Injection of *lama4* MO into *bal* mutants results in an increased severity of the notochord defect as notochord within posterior regions is mutant (D), compared to control MO injected siblings (B). Using the laminin antibody it is apparent that *bal* mutants injected with *lama4* MO have reduced levels of laminin protein around the notochord (N). These embryos also maintain expression of *ehh* compared with control MO injected embryos (K and L) and *lama4* injected WT siblings (M and O). Injection of *lama5* MO into *bal* mutants results in a severe defect involving lack of AP extension, disruption of many tissues, including notochord, skin and fin-folds (G), as well as large amounts of cell death within the midbrain (G, arrow). In contrast, WT siblings injected with *lama5* MO only fail to form the yolk extension (F). The notochord appears to differentiate normally in these embryos as judged by vacuolation; however, these embryos maintain high levels of *ehh* expression (S), in contrast to control MO injected embryos (K and L).
5.9 EM of the peri-notochordal basement membrane

Laminin is one of the major components of BM and polymerises via the N-terminal short arms to form a sheet-like matrix that interacts with collagen via nidogen (Colognato and Yurchenco, 2000). We decided to investigate the peri-notochordal BM using transmission electron microscopy in both WT and mutant embryos. One would expect the loss of such a crucial component as laminin would affect the overall organisation of the BM. The results show that, indeed, the BM is disrupted in all three mutants (Figure 5-11 and Figure 5-12).

Electron micrographs of transverse sections, made at the posterior limit of the yolk extension, from 28 hpf and 32 hpf WT zebrafish embryos, reveal a number of features of the differentiated notochord. A thick BM completely surrounds the notochord and fibres running both parallel and perpendicular to the plane of section are seen within the well-organized BM. Notochord cells of gup or sly mutants are dramatically different. The BM surrounding the notochord in these mutants appears grossly disorganised and by 32 hpf is virtually undetectable (Figure 5-11). In the few places where BM can be seen, it is severely disorganized and in addition, there are fewer and smaller vacuoles than in WT notochord. Finally, we observed darkly stained, cells in and around the notochord, consistent with those undergoing apoptosis. For bal mutants, EM was performed on transverse sections at the posterior limit of the yolk extension on 28 hpf embryos. Notochord cells appear WT in character, as vacuolation seems to have occurred normally (Figure 5-12). However, the BM is more disorganised than in the WT sibling control with excess material spilling into adjacent intercellular spaces. Thus, although the posterior notochord in bal mutants appears WT under light microscope and contains WT levels and localisation of laminin protein, there are in fact disruptions to the organisation of the BM. These disruptions, however, are not sufficient to prevent notochord differentiation, probably because of the functions of lama4 and lama5.
Figure 5-11  Electron microscopy of gup and sly mutant notochord. Transverse sections through notochords of a WT (A and E) embryo at 28 hpf; (B and F) a gup mutant at 28 hpf; (C, G) a wild-type embryo at 32 hpf; (D, H) a sly mutant at 32 hpf. BM of notochordal sheath (between arrowheads) of WT (E) and gup (F), 28 hpf embryos are shown. In WT (E) the BM appears well-organised with distinct laminin organisation. Conversely in a gup mutant sibling (F) a thick disorganized layer is observed. Similarly, in the more mature notochordal sheath of a 32 hpf wild-type embryo (G) a well organised sheath is observed (between arrowheads). In a sly mutant sibling (H), however, the sheath is completely disorganised. Scale bar: (A-D) is 5 μm. (E-H) is 0.5 μm.
**Figure 5-12** Electron microscopy of *bal* mutant notochord. Transverse sections were made at the posterior end of the yolk extension in both *bal* (A, B) and WT notochord from 28 hpf embryos. In *bal* mutants cells in the posterior region of the notochord inflate the vacuole and appear WT in morphology (A). Under higher magnification, however, the BM seems disorganised with ECM spilling into intercellular spaces (arrow, B), compared to WT siblings (D). Scale bars: A and C (5μm), B and D (0.5μm).
5.10 In vitro culture of embryonic shield tissue

An in vitro system for monitoring notochord differentiation would be useful and would allow biochemical and cell biology studies of notochord formation. Thus, I developed a system to study notochord differentiation in culture. The shield transplantation technique developed in our lab, and described earlier in this thesis (genetic mosaic experiments), provides a system for the isolation of notochord progenitors. I cultured embryonic shields as tissue explants by directly transferring them to a tissue culture well.

An example of such an experiment is shown in Figure 5-13. I tested a variety of substrates for their ability to support notochord differentiation and found that pre-treatment of the tissue culture plastic with fibronectin was able to support cell attachment. I found that shield cells undergo cell movements reminiscent of convergent extension. After 24 hours in culture, large vacuoles are obvious within certain regions of the explant. A layer of ECM surrounds these regions, as seen using anti keratan-sulphate proteoglycan antibody MZ15, which marks differentiated notochord (Figure 5-13C).
**Figure 5-13 Explant culture of zebrafish shield tissue.** Zebrafish shield tissue is removed using a micropipette and transferred to tissue culture wells pre-treated with fibronectin. The whole shield explant is shown in (A). Cells at the periphery of the explant migrate away and may represent migratory hypoblast cells that give rise to prechordal plate (arrow). Higher power magnification of a region of the explant containing a block of notochord tissue (B). Notochord tissue forms within 24hrs of culture as a block of tissue and stains positive with the notochord marker MZ15 (C).
5.11 Discussion

Given the results from the positional cloning of *sly* it became apparent that the third member of this class of mutants, *gup*, might also encode a laminin chain. It was thus gratifying that a laminin gene, *lamb4*, mapped to a similar region as *gup*. The laminin β4 chain has not been reported thus far as being part of any isoform, but was identified from the sequencing of the human genome project. RH mapping, of a cDNA clone with homology to *lamb1*, also identified this laminin chain as a candidate for *gup*. So faced with two excellent candidates for the *gup* gene I tested each through MO injections. This showed that *lamb1* was the gene disrupted in *gup*. Subsequently a premature stop was detected within *lamb1* from *gup* mutants (Parsons et al., 2002).

Therefore, we have shown that the shared defects of the three mutant *bal*, *gup* and *sly* are due to lack of functional laminin 1 isoform (α1β1γ1). Electron microscopy of the notochord reveals disruption of the peri-notochordal BM in the mutants - as one might expect from loss of laminin. Almost certainly these fish will have subtle defects within organs other than the notochord, as BM is thought to be associated with all epithelial cells. There are no gastrulation defects in *bal*, *gup* or *sly*, and thus, there is probably no role for laminins during this process. Molecules such as collagen and fibronectin, and cell-cell interactions through cadherins and Eph-Ephrins etc. are probably sufficient to allow the major morphogenetic movements and only later during organogenesis does BM become more essential.

My preliminary results suggest that *lama1* may be regulated at the transcriptional level through some mechanism involving the β1 and γ1 chains. This would be intriguing as it would fit with several biochemical reports that suggest assembly of a β1γ1 heterodimer occurs prior to association of the α chain to form the heterotrimer (Goto et al., 2001; Matsui et al., 1995; Nomizu et al., 1996). The expression of the zebrafish *lama1*, *lamb1* and *lamc1* during early development suggests that cells adjacent to the notochord supply the Laminin α1 chain, while Laminin β1 and γ1 chains are supplied by both chordamesoderm and adaxial tissue (Parsons et al., 2002). When thinking in terms of both laminin assembly and BM assembly, the fact that *lama4* is expressed specifically within the chordamesoderm complicates matters. These chains could be acting to transport the β1γ1 dimer out of the chordamesoderm where formation of the α1β1γ1
trimer would occur extracellularly, although alternatively, different laminin isoforms may be supplied from neighbouring tissues. The non-autonomous nature of the mutation shows that the Laminin α1 chain must be supplied from non-notochordal sources. Such control might allow formation of BM at the interface of cell populations expressing different chains or isoforms. Interactions between different laminin isoforms within the same BM have not been studied.

The identification of bal, gup and sly, as components of the same molecule is a validation of the grouping of them together within the same phenotypic sub-class (Stemple et al., 1996). These three chains together will form the specific isoform laminin 1 (α1β1γ1) and so one can conclude that the shared defects between these mutants, (notochord differentiation and CNS development) are due to lack of this heterotrimer. An important observation is that the bal mutant phenotype is much weaker than both gup and sly. All of the bal alleles identified during the mutagenesis screens possess phenotypically WT posterior notochord (although the anterior limit where notochord differentiation is defective varies between alleles and within different genetic backgrounds). The polyclonal laminin antibody was used to analyse the localisation of laminins during development, both in WT embryos and within the laminin mutants. This highlighted a wide range of tissues where laminin is located during development, and clearly showed that there remains a large amount of immunoreactivity in bal mutants.

Analysis of the mRNA expression for zebrafish lama2 suggests that it is involved during muscle development. lama5 is expressed strongly within the chordamesoderm, suggesting that one or both might function redundantly to Laminin α1 and thus responsible for weak nature of the notochord defect within bal mutants. Lama4 and lama5 were studied by MO injection. Those embryos that lack lama1 genetically (bal mutants) and in conjunction lack lama4 due to injection of MO, show a notochord phenotype more similar to gup and sly, i.e. the whole notochord appears mutant morphologically, shows reduced laminin protein and maintains ehh expression, all phenotypes characteristic of gup and sly. The expression pattern of lama4 remains to be determined as the probe generated from the RACE product for this cDNA was ineffective.
Lama5 MO injected into WT embryos results in morphants that failure to extinguish ehh hedgehog expression, though the notochord cells do vacuolate and extension of the AP axis occurs normally. This suggests these processes are independently controlled. I have previously shown that bal/lama1 mutants do extinguish ehh expression, whereas gup and sly do not (see section 3.2). This shared phenotype when either laminin α5, β1 or γ1 chain are disrupted suggests that the laminin 10 isoform (α5β1γ1), specifically controls expression of ehh gene expression, and hence the duration of hedgehog signalling. Interestingly the double loss of lama1 and lama5 (i.e. lama5 MO into bal mutants) show an extremely severe phenotype that reveals a redundant function for the Laminin α1 and Laminin α5 in midbrain development and formation of the trunk and tail. These embryos need to be characterised further, by looking at earlier stages using markers of neural patterning (e.g. pax2.1, krox20, and emx1) and the axial mesoderm marker ntl to determine the primary defect responsible for the morphant phenotype.

5.12 Summary

- Genetic mapping of gup restricted the locus to 0.4cM from myoD.
- lamb1 and lamb4 map to this locus and are expressed during early development.
- MO injections identify lamb1 as gup.
- bal, gup and sly encode laminin α1, β1 and γ1, respectively.
- Laminin immunoreactivity is greatly reduced in bal, gup and sly.
- The shared defects of these mutants are due to lack of functional laminin 1.
- lama2, lama4 and lama5 are also expressed during early development.
- Laminin 2 (α2β1γ1) is necessary for muscle function.
- Laminin 8 (α4β1γ1) functions partially redundantly with laminin 1 during notochord formation.
- Laminin 10 (α5β1γ1) controls the extinction of ehh expression in the notochord.
- Lama1 and lama5 function redundantly during formation of the midbrain.
Chapter 6

ILK and Integrins
Chapter 6

Integrin-linked kinase (ILK) and Integrins

6.1 Introduction

Laminin proteins are necessary for notochord differentiation, but it is unclear how loss of laminin leads to this defect. One possibility is that without an organised perinotochordal BM the physiology of notochordal cells is disrupted and that apoptosis and control of gene expression are affected secondarily to this. Alternatively, there may be direct signalling via membrane-bound receptors such as integrins, that controls notochord differentiation. Laminins can signal through integrins to control the cytoskeleton, apoptosis, and gene expression. Each of these events occurs during notochord differentiation. In this final chapter I examine the role of integrins and a downstream component of the integrin signal transduction pathway called Integrin-linked kinase (ILK), during zebrafish development.

6.1.1 Laminin receptors

Many proteins have been shown to bind various laminin isoforms. Although dystroglycan and the integrins have been most intensively studied, several other molecules such as Lutheran, LAR, 67kD receptor and Galectin, can act as laminin receptors (Hughes, 2001; Moulson et al., 2001; O'Grady et al., 1998). Both dystroglycan and integrins are also thought to aid in the assembly of the BM (Schwarzbauer, 1999).
α-Dystroglycan is a well-characterised laminin receptor. It is expressed during early mammalian development, and later within muscle tissue (Durbeej et al., 1998). It was isolated as part of the dystrophin-glycoprotein complex (DGC) and is a peripheral membrane protein that associates with the membrane spanning β-dystroglycan which binds the cytoskeletal protein dystrophin. Defects in α-dystroglycan can lead to Duchenne muscular dystrophy (Durbeej et al., 1998). Dystroglycan was studied in our laboratory and seems to have no role in zebrafish notochord differentiation. Antisense knockdown of the mRNA using a MO results in morphants with a muscular dystrophy phenotype (Parsons et al., 2002).

Integrins are a family of transmembrane proteins that were first identified as receptors for fibronectin (Chen et al., 1985). Since then many integrins have been discovered, and they seem to be the major receptors by which cells attach to extracellular matrix, binding not only to fibronectin but also laminin, vitronectin and collagen, reviewed in (Hynes, 1992)). They are a family of membrane glycoproteins consisting of two subunits termed α and β. Each α subunit associates non-covalently with a β subunit. Although 14 α and 9 β subunits could potentially associate to give more than a hundred heterodimers, the actual diversity appears more restricted. Each integrin has a large extracellular domain, a single membrane spanning domain and a short cytoplasmic domain. Various members of the integrin family have been shown to bind laminin in in vitro assays, though the physiological relevance of some of these interactions is not completely understood, and very few studies have been performed to address the specificity of integrins for each of the laminin isoforms (Giancotti, 1997).

Many integrins containing the β1 subunit have been implicated as laminin receptors, though some can also bind collagen and fibronectin. Integrin α6 associates with either the β1 or β4 chain to form dimers that appear to be specific for members of the laminin family. Targeted disruption of the integrin α6 gene in mice is lethal and disrupts epithelial formation leading to skin blistering and CNS defects; α6β4 is thought to be restricted to epithelial hemidesmosomes. In Xenopus, interference of the integrin α6 chain using antisense techniques resulted in embryos defective in neurulation (Lallier et al., 1996). A separate study, also in Xenopus, showed expression of the integrin α3 chain restricted to involuting cells of the dorsal mesoderm during gastrulation and in the
presumptive notochord cells at the midline during neurula stages (Meng et al., 1997). Within the muscle, integrin α7β1 acts as a specific receptor for laminin, and can act through various laminin isoforms (Klaffky et al., 2001).

6.1.2 Integrin signalling and integrin-linked kinase

The cytoplasmic tails of integrins are not by themselves capable of transducing extracellular signals. Integrins operate is via adaptor proteins that engage with downstream components, such as the cytoskeleton, cytoplasmic kinases and other membrane receptors. Although alterations of gene expression occur through integrin signalling it is clear that the important changes affecting cell adhesion occur through alterations of the cytoskeleton, are mediated directly through locally acting cytosolic signalling systems (Hynes, 1992). An important mechanism used to achieve such control is the formation of large focal adhesion complexes through the clustering of receptors and signalling components. These are thought to form in part through a feedback mechanism involving actin stress fibres. A particularly well-studied protein tyrosine kinase central to this is focal adhesion kinase (FAK), reviewed in (Parsons et al., 2000).

A second kinase, integrin-linked kinase (ILK), was identified in 1996 by a yeast two-hybrid screen using the cytoplasmic tail of integrin β1 as bait. ILK contains three ankyrin repeats, a pleckstrin homology domain, and the serine/threonine kinase activity. The role of ILK during development has recently been addressed in Drosophila and C. elegans where a role in muscle development has been shown (Mackinnon et al., 2002; Zervas et al., 2001). These studies suggest that ILK may function at focal contacts as an adaptor rather than a kinase. The relationship between ILK and FAK is not entirely clear, though many important activities have been assigned to ILK. It may be an important therapeutic target, as it is has been shown to be elevated in cells mutant for the tumour suppressor PTEN, which is associated with >60% of all forms of solid tumour (Yoganathan et al., 2000).
6.2 Full-length cDNA of ILK

The gene structure of the human ILK gene was determined by analysis of the human genome and then used to characterise the six sequences from the zebrafish genome-sequencing project database. I designed twelve primers in both directions using each of the zebrafish exon sequences and used these to sequence a full-length ILK cDNA clone (AI883753) obtained from the RZPD EST database (Figure 6-1).

6.3 Expression of ILK mRNA

Integrin-linked kinase (ILK) is a molecule that may be a common point of convergence for a variety of integrin receptors. I generated an in situ probe to analyse ILK expression during zebrafish development (Figure 6-2). Intense staining during early cleavage stages indicates a maternal contribution. Widespread expression during shield stage shifts localisation during 70% epiboly to the chordamesoderm. This localised expression within the chordamesoderm at tailbud stages would be consistent with ILK being involved during these stages in transduction of a differentiation signal from surrounding laminins. At later stages expression is widespread through the brain and the gut. The axial staining seen at tailbud becomes adaxial to the notochord by the 12-somite stage, with chordamesoderm staining persisting only within the tailbud.

6.4 Antisense knockdown of ILK

Given the above results from analysis of the ILK mRNA expression a MO was designed against the ILK start of translation, and injected into WT embryos in order to establish any role in notochord differentiation. Injection of this MO gave a convergent extension phenotype, as shown in (Figure 6-3). At 5-somite stage the somites in ILK morphants are shorter and wider in the AP and medio-lateral axes respectively, which is a characteristic phenotype resulting from disruption of convergent-extension movements. The phenotype is strikingly similar to that of silberblick, knypek and trilobite mutants (Hammerschmidt et al., 1996; Solnica-Krezel et al., 1996). Later, at 24 hpf, morphants display a shortened body axes. The notochord in these embryos is differentiated, as judged by the vacuole inflation. Thus, it seems from this data that ILK is necessary for correct cell movements during gastrulation and is not required for notochord differentiation.
Figure 6-1  Zebrafish ILK sequence and alignment with homologues.
Figure 6-2  **Expression of ILK mRNA during zebrafish development.** Strong maternal expression is seen at the 4-cell stage (A). By 70% epiboly expression is seen restricted dorsally within the axial mesoderm (D-F) and this becomes more obvious by tailbud stage (lateral view G; dorsal view, H). At the 12-somite stage the expression is widespread within the brain (J). Within the chordamesoderm at this stage expression is down regulated, but can still be detected in adaxial cells (black arrow, K). Expression also remains within the tailbud mesoderm (white arrow, K). Widespread staining persists in the brain and tailbud mesoderm at 20-somite stage (L and M).
Figure 6-3  Injection of a MO against ILK. At 60% epiboly ILK morphants appear similar to control morphants (A and C). By tailbud stages, however, the ILK morphants show incorrect movement of the hypoblast anteriorly (B and D). Three examples of 5-somite stage ILK morphants are shown (F, H and I). A dorsal view of a 5-somite embryo shows that ILK MO-injected embryo (J) has wider (medio-lateral) and thinner (anterior-posterior) somites than control-MO injected siblings (G). At 28 hpf the ILK morphants show a shortened AP axis (M and N), though notochord vacuolation does occur (O).
6.5 Expression of integrin α6 and β4 mRNA

Given that ILK morphants show such a severe gastrulation defect we decided to study integrin subunits to test if these would have a more specific role in notochord differentiation. ESTs with homology to both integrin α6 and β4 were identified in the GenBank database (D0317Q8 and F0934Q8). cDNA clones were obtained and sequenced confirming their identity, however, neither clone contained a full-length cDNA. Fragments of an appropriate size for each cDNA were generated by PCR and subcloned into pCR2.1-TOPO, Invitrogen), and used to generate in situ hybridisation probes.

The results of the in situ for integrin α6 are shown in Figure 6-4. There seem to be low levels of maternal expression of integrin α6 and widespread zygotic expression by 70% epiboly with strongest levels within dorsal mesoderm. At both 10 and 15 somite stage there is widespread staining through the CNS and eyes and a strong staining of chordamesoderm. There is also weaker staining within the forming somites and tailbud at these stages. Expression within the chordamesoderm is extinguished by 20 somite stage though staining remains strong within the CNS. At 24 hpf the tailbud mesoderm and developing eyes both have strong expression.

Integrin β4 expression is shown in Figure 6-5. Specific expression within the chordamesoderm is not seen at any stage. There is, however, widespread expression within the CNS and somites at 10 and 20 somite stage. At 24 hpf there seems to be a general low level of expression throughout the trunk, possibly within the epidermis, CNS and eyes.

6.6 Antisense knockdown of integrin α6

Integrin α6 expression was enriched in chordamesoderm, making it a candidate for control of notochord differentiation. I performed a 5' RACE from the cDNA clone to obtain the 5' UTR, from which a MO was designed. I did not attempt MO knockdown of integrin β4 as its expression did not suggest an involvement in notochord differentiation. Injection of the integrin α6 MO alone did not give any phenotype. Notochord differentiated normally and embryos were morphologically identical to those
injected with the control MO (data not shown). It was recently shown in our lab, that there are two copies integrin β1 present in the zebrafish genome (integrin β1(a) and integrin β1(b)). Both of these are expressed within the chordamesoderm during development, though injection of MOs against each of these also does not result in any phenotype. However, co-injection of the α6 and β1(a) or β1(b) results in a severe convergent extension defect identical to that obtained with the ILK MO alone (M. Parsons, pers. comm.). As with ILK morphants, however, there was not a notochord differentiation defect as vacuole inflation seems to occur normally.
Figure 6-4  Expression of integrin α6 mRNA during zebrafish development. Low levels of maternal staining are seen during the early cleavage stages (A). Later, at 70% epiboly transcripts become localised to the dorsal side of the embryo within the axial mesoderm (E and F). At the 10-somite stage, there is broad expression within the brain (G and H) and strong staining within the chordamesoderm and developing somites (I and J). By 20-somite stage expression within the chordamesoderm and somites has been extinguished with the exception of the tail-bud. At 24 hpf there is expression throughout the brain with particularly strong levels within the lens (arrow, Q). Expression persists in the tailbud axial mesoderm (R).
Figure 6-5  Expression of integrin β4 mRNA during zebrafish development.
Staining within the CNS is fairly broad and seen through the 10-somite stage to 24 hpf (B, E, H and (K). No obvious restriction to the chordamesoderm is seen, and most expression at 20-somite stages within the trunk seems restricted to the somites (I and J). At 24 hpf staining appears to be broad and superficial, possibly within the skin (L).
6.7 Discussion

Given the requirement for laminin or a laminin-rich BM surrounding the chordamesoderm for notochord differentiation, it was important to determine whether the laminin protein itself is the differentiation signal, or some other component of the BM. Integrins are one of the major receptors for laminin and thus represent excellent candidates that could transduce a laminin signal. The results given here from the investigation of two integrin subunits and a downstream kinase (ILK) suggest that integrin signalling is not necessary for notochord differentiation.

Clearly both ILK and integrin α6 are expressed appropriately both temporally and spatially for a role in notochord differentiation. However, using MO injections to knockdown protein levels for each of these has revealed an obvious involvement of ILK-mediated integrin signalling during cell movements through gastrulation. This is perhaps not unexpected given the many previous reports implicating integrins signalling during gastrulation. There appears to be no requirement for either of these proteins during notochord differentiation (although early chordamesoderm marker gene expression was not studied) and so we tentatively conclude that laminin signalling through integrin receptors containing α6 and β1 subunits is not required for notochord differentiation.

6.8 Summary

- Integrin α6 and ILK are expressed within the chordamesoderm.

- Injection of a MO against ILK results in gastrulation defects, but notochord differentiation appears normal.
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7.1 Laminins and their receptors during zebrafish development

Over the past two decades many families of transcription factors have been identified that have provided the foundations of our understanding of how a cell becomes specified to a particular fate during embryonic development. For example, the homeobox proteins encoded by genes of the Hox cluster specify regional identity along the AP axis, while the basic helix-loop-helix factors MyoD and Neurogenin specify cells to a muscle or neural lineage respectively (Ma et al., 1996; Weintraub et al., 1991; Weintraub et al., 1989). Also, intercellular signalling pathways using secreted proteins such as, Wnt, FGF, Hedgehog and TGFβ families are used in many different contexts through all stages of embryogenesis. Although transcription factors and signalling pathways are major regulators of many questions remain as to how these molecules result in differentiation of cells and formation of tissues. What genetic and biochemical changes occur as a cell differentiates? What signalling systems are active, and how is the cytoskeleton organised? What effect does the cellular environment have on the cell? How can differentiated cells be reprogrammed or trans-differentiated into other cell types? And how are differing cell-types co-ordinated and organised to form complex tissues and organs? These issues are being addressed using a range of model systems, and they represent major un-resolved questions in cell and developmental biology.

During this investigation I have studied the process of cell differentiation during early embryonic development. The zebrafish notochord is a particularly convenient system for such a study. My approach has been to investigate, and to ascertain what gene
products are important during notochord formation by positional cloning of zebrafish mutants. I have characterised these genes and studied their role during development. This work has revealed an essential role for laminins in control of notochord differentiation.

7.1.1 Zebrafish genetics

Positional cloning of bal was made possible due to the rapid development of zebrafish genomics since the completion of the zebrafish screens in 1996. Expansion of the number of SSLP markers and mapped ESTs, mean that high-resolution genetic mapping and laborious chromosome walks are largely obviated. Zebrafish genomics promises another major leap forward with imminent completion of the zebrafish genome sequence (Fishman, 2001). Indeed the sequence data currently available is already benefiting many researchers, and enabled more rapid progress during this investigation.

Using a positional candidate approach we cloned gup. This approach will soon become the standard method for identification of mutant genes. A rough genetic interval was defined through low-resolution mapping and then two good candidate genes in the region were analysed. The progress made in antisense knockdown procedures through the use of morpholino chemistry enabled gup to be identified as lamb1, limiting the laborious search for a mutation within lamb4. Recapitulation of the mutant phenotype by MO injection has rapidly become a universal practice within the field when identifying and characterising mutant genes.

7.1.2 Laminin function in notochord differentiation

The results from the positional cloning and associated studies of bal, gup and sly provide compelling evidence that laminin 1 (α1β1γ1) is necessary for differentiation of the zebrafish notochord. Through the EM studies presented here we have shown that lack of this molecule disrupts the formation of the basement membrane (BM) surrounding the notochord. The absence of BM is directly due to lack of laminin, which polymerises to form an important BM layer adjacent to the cell membrane, and upon which the remaining components of the BM are organised. The fact that mutant notochords can be rescued by surrounding WT tissue and extinguish early marker gene expression suggests that notochord differentiation involves the transduction of some
signal. It is not clear from the data available whether it is the laminin molecule itself that is this signal, or whether the defect is secondary to this, due to lack of some other component associated with the BM. For example proteoglycans are present within the BM and are known to be important mediators of growth factor activity (Costell et al., 1999; Perrimon and Bernfield, 2000; Rubin et al., 2002). This remains perhaps the most important outstanding question concerning the role of laminin in notochord differentiation. We have addressed the role of various integrins as potential laminin receptors during notochord development. Two major problems exist. Firstly, the large number of integrins together with their ability to form numerous isoforms could result in such redundancy as to make comprehensive knockdown of all integrin activity difficult. Secondly, their roles during earlier stages in gastrulation movements complicate analysis of notochord differentiation. It was reasonable to expect that disruption of ILK might circumvent the redundancy issue, as it is thought to be a common signalling component of a variety of integrins. Disruption of ILK reveals a gastrulation phenotype where cells do not undergo convergent extension, but no obvious notochord differentiation defect is seen. Morphant embryos show a strikingly similar phenotype to previously identified convergent extension mutants such as trilobite, knypek and silberblick (Hammerschmidt et al., 1996; Heisenberg et al., 2000; Solnica-Krezel et al., 1996). One might expect there to be a cross talk between the machinery controlling cell attachments to substrates, with that of cell polarity. Identifying interactions between the integrins and the planar cell polarity pathway is an extremely important issue. Simultaneous disruption of α6 integrin and integrin β1 chains by MO injection produces an identical phenotype suggesting there is indeed redundancy among integrin chains during convergent extension movements. There are several integrins that remain to be examined before we can rule out there a role for integrin mediated signalling in notochord differentiation.

One issue that has been resolved through our investigations is why the bal mutants have a much weaker notochord phenotype than either gup or sly. This was noted at the time these three mutants were identified and didn’t seem to be an allele specific phenomenon, as every bal allele appears weaker in this regard. The identification of the mutations responsible for these phenotypes immediately suggested a simple hypothesis; that other α chains (hence other laminin isoforms) redundant to α1 are used during
notochord differentiation. Given this possibility I identified other laminin α chains and have shown that these are expressed during zebrafish development and are necessary for notochord differentiation, and correct formation of other tissues. By combining MO injection with bal mutants I found that both laminin 1 (α1β1γ1) and laminin 8 (α4β1γ1) are necessary notochord differentiation. The laminin α1 and α4 chains therefore have redundant roles during notochord differentiation. In addition I found that the laminin α5 chain is required during notochord differentiation, specifically for the extinction of echidna hedgehog expression. Thus, multiple laminin isoforms exist within the peri-notochordal BM that act independently to control different parts of the differentiation program. Why these separate chains/isoforms are used within the notochord sheath is unclear. One could imagine that these specific functions arise through different interactions or modifications that allow them to fulfil specific aspects of the differentiation program.

How do these results fit into our current model of notochord formation? It is clear that the homeobox genes bozozok and floating head are expressed in response to the action of β-catenin and nodal signalling. These two transcription factors are necessary for specification of the chordamesoderm, though their downstream effectors remain unknown. The T-box transcription factor, no tail, is required for the differentiation of the notochord and for the formation of posterior mesoderm. In Xenopus laevis, brachyury (the no tail homologue) has been shown to regulate transcription of Bix1-4, Wnt-11, and eFGF (Casey et al., 1998; Saka et al., 2000; Tada and Smith, 2000). Wnt-11 mediates convergent extension of the mesoderm (Heisenberg et al., 2000). The Bix homeodomain transcription factor family and FGF may act to regulate genes such as BM proteins specific to the mature notochord. The relationship between bozozok, floating head, and no tail is not well understood.

The changes occurring during the transition of chordamesoderm into notochord involve three major processes: (a) notochordal cells acquire their characteristic morphology, with large central vacuoles, surrounded by the peri-notochordal BM; (b) immature genes such as Shh are extinguished upon differentiation; and (c) apoptosis is prevented. These processes are all defective in bal, gup and sly mutants (Parsons et al., 2002; Stemple et al., 1996). A major goal of our lab is to understand each of these processes and their interrelationship. Laminin proteins may potentially directly control each of
these events through signalling via several known receptor systems. Integrins and
dystroglycan, the major types of laminin receptor are known to control cytoskeletal
organisation, alter gene expression, and function in regulation of programmed cell death
(Durbeej et al., 1998; Giancotti and Ruoslahti, 1999).

The large central vacuole is a structure peculiar to the notochord. It is not clear how this
vacuole forms, though one explanation might include deposition of carbohydrate-rich
molecules into the vacuole. This would attract sodium ions resulting in extensive water
intake through osmosis and inflation of the vacuole. A similar type of process gives rise
to hydrated gel-like matrix (largely glycosaminoglycans) of cartilage (Barry, 1990).
Concurrent with vacuole inflation large mechanical forces are generated, and it is likely
that major changes to the cytoskeleton occur in order to withstand this stresses.
Intermediate filaments are thought to act as a stress bearing structure within the cell and
it is likely they are present within the mature notochord for this reason. Intermediate
filament attachment to the underlying BM is mediated by hemidesmosomes that use
integrins as the transmembrane linker protein. In fact, the major hemidesmosome
integrin subunits are thought to be integrin α6 and β4 (Hynes, 1992). In Xenopus
cytogether have been noted within the notochord (Godsave et al., 1986). These are
also present surrounding the vacuole within the notochord of bony fish (Schmitz, 1998).

Focal adhesions contacts also use integrins as the membrane anchor, but differ in they
attach to the cytoskeleton via actin, which can give rise to adhesion belts and stress
fibres. It has recently been shown that the activated phosphorlyated form (Y397) of
FAK is present within the chordamesoderm and later localises to the peripheral regions
of the notochord cells (Henry et al., 2001). These authors did not however perform the
knockdown of this protein using a MO and so a necessity for this protein in notochord
differentiation has not been determined.

Apoptosis within mutant notochord cells suggests that they are not receiving some
survival signal, such as, a signalling molecule, attachment to the matrix, or a cell
autonomous program of events triggered through lack of mechanical support from the
BM. Apoptosis following loss of attachment to the extracellular matrix has been termed
anoikis (Frisch and Francis, 1994). Curiously, anoikis can be rescued by an activated
form of FAK (phosphorlyated at Y397) (Frisch et al., 1996), and so one possibility is
that within the zebrafish notochord activation of FAK serves to keep these cells alive. In \textit{bal}, \textit{gup} and \textit{sly} this cannot occur as there is no BM, and consequently cells would die. Integrins have been shown to directly regulate apoptosis through recruitment of caspase-8 to the cell membrane (Stupack et al., 2001). This process is death receptor-independent and is dependent on unligated integrins. It is distinct from anoikis as it is not a direct response to loss of adhesion (Stupack et al., 2001). Determining which type of apoptosis occurs in the notochord mutants is an interesting question.

The notochord is a major source of signals that pattern adjacent tissues. How are these signals shut down? The transformation of chordamesoderm into notochord is accompanied by extinction of transcription of genes such as \textit{sonic hedgehog}. Studying the various signalling processes in embryos with grossly disorganised extracellular matrix should shed light into how, and to what extent, signalling molecules are regulated by extracellular matrix (Briscoe and Ericson, 1999; Pons and Marti, 2000; Rubin et al., 2002). Each of the patterning roles for which the notochord has been implicated could be studied in this manner. For example, in terms of the DV patterning of the neural tube, vitronectin can enhance with Shh signalling during motor neurone specification (Pons and Marti, 2000).

Recently within our lab the gene disrupted in the notochord mutant \textit{sneezy (sny)} was identified as COPA (P. Coutinho, in preparation). COPA is part of the coatomer complex that is important for vesicle trafficking. The related mutants \textit{dopey} and \textit{happy} are defected in other components of the coatomer complex. In these mutants the defects within the notochord may be due to an inability to secrete either the components of the BM or processing of membrane associated receptors. EM studies of these mutants show that the BM is somewhat disorganised, though there does not seem to be a lack ECM export and there remains WT levels of laminin immunoreactivity surrounding the notochord in \textit{sny} (P Coutinho, pers. comm.). There are, however, striking ultrastructural defects in the Golgi and endoplasmic reticulum, and so processing of some component of the signalling machinery for notochord differentiation is more likely to explain the phenotype.
7.1.3 Further roles of Laminin during zebrafish development

The cloning and characterisation of bal, gup and sly immediately identified laminin-1 (α1β1γ1) as a component necessary for notochord differentiation. The bal mutants differ from gup and sly in several regards, leading us to investigate the possibility that another α chain associated with β1γ1 is responsible for the phenotypic differences. This proved correct as the cloning and analysis by RNA expression and MO knockdown of three other Laminin α chains (α2, α4, and α5) revealed specific functions for these laminin chains. I have shown that zebrafish lama2 is necessary for development/function of the muscle and knockdown of this protein results in embryos with similar defects to zebrafish dystroglycan morphants (Parsons et al., 2002). This result was expected since in humans mutation of LAMA2 can give rise to congenital muscular dystrophy (Helbling-Leclerc et al., 1995; Xu et al., 1994). Laminin 8 (α4β1γ1) and laminin 10 (α5β1γ1) are likely function redundantly with laminin 1 in notochord differentiation. An intriguing redundant role for Laminin α1 and α5 chains in brain development was also seen, wherein large cell death occurs within the midbrain of embryos lacking both these chains.

Laminin has been known for many years to promote neurite outgrowth and is commonly used as a substrate for in vitro studies of neurones. More recently, laminin 1 has been identified in a screen for genes important for axon guidance in the mouse (Leighton et al., 2001), and has also been shown to modulate the effects of netrin 1, altering its effect on growth cones from attractant to repellent (Hopker et al., 1999). It is not clear whether there is a specific role for laminin 1 in axon guidance, or whether the observed pathfinding defects are secondary to the morphological disruptions. This is difficult to determine without chain specific antibodies. Given the large size of the protein and with very many interaction domains known to exist there may be a level of post-translational control, possibly involving the family of matrix metalloproteases. One could imagine that particular regions/pathways are created through local changes in laminin structure. The expression of the lama5 is such a restricted manner within the tectum suggests that laminin isoforms containing Laminin α5 may function specifically in the formation of the tectum or retina-tectal pathfinding. Investigation of the axon tracts from the eye to tectum should therefore be performed in lama5 morphants to ascertain whether they are disrupted.
Recently it has been shown that, \textit{bal}^{wingnut} (an allele identified in the lab of Steve Wilson) has the axon guidance defect but does not contain any notochord phenotype (S. Wilson, pers comm.). Thus it should be possible to determine the region Laminin \( \alpha_1 \) specific to the neural as opposed to notochord function.

Both \textit{gup} and \textit{sly} lack intersegmental vessels, which form normally in \textit{bal} mutants, suggesting that another alpha chain associating with the \( \beta_1 \) and \( \gamma_1 \) is important for their development. The formation of these is thought to occur through migration of \textit{fli-1} expressing cells from the dorsal aorta (Fouquet et al., 1997). The recently published targeted deletion of mouse \textit{lama4} suggests that this gene is important for formation of microvessels (Thyboll et al., 2002). Thus, it would be interesting to look at the expression of \textit{fli-1} early on within the laminin mutants and morphants.

A conclusion from these results is that the \( \beta_1 \) and \( \gamma_1 \) chains are used broadly during early development and it is the \( \alpha \) chains, through restricted expression domains that give specific functions of the laminins. This is supported by expression and gene knockout studies in mouse and is somewhat expected since the C-termini of all of \( \alpha \) chains contain globular domains that interact with laminin receptors (Colognato and Yurchenco, 2000).

### 7.2 Future directions

Laminins are essential for both the development and normal function of organs. Their widespread use in these different contexts and subtleties of isoform functions means that there are many directions that future studies could take. Many important questions concerning this family of molecules remain. This investigation has focused on the differentiation of the notochord, for which there is a clear requirement for a functional BM. For other tissues, the effects may be subtler and it is likely that nearly all organs and tissues containing epithelia will be affected in some way in laminin mutants. The \( \alpha_1 \) and \( \beta_1 \) chains have yet to be studied by gene targeting in mouse, though the laminin \( \gamma_1 \) mouse knockout has an early lethal defect (see Introduction), likely due to lack of laminin 1 (\( \alpha_1 \beta_1 \gamma_1 \)). Hence, the zebrafish is an excellent vertebrate model for studying the specific function of the many laminin isoforms, as there is not an early requirement
for laminin 1, and replacement of mutant notochord with WT can be achieved easily through shield transplantations.

An immediate goal is to clone and investigate the mRNA expression and antisense loss-of-function phenotypes for all of the other known laminin genes. This would then enable identification of potential isoforms that are present during zebrafish development. Analysis of co-operative and regulatory mechanisms between the laminin genes should reveal interesting aspects of their biology. Though only preliminary data, I did find evidence of such regulation/feedback e.g. lack laminin β1 and γ1 chains results in reduced expression of α1 (section 5.6). Thus, these zebrafish mutants and the MO technology provide us with the opportunity for the first time to address this issue in a comprehensive manner. Most of the tools to carry this out are now available.

In some ways the roles of extracellular matrix molecules during construction of the vertebrate embryo has not received as much attention as, for example, proteins affecting cell fate specification. With the advent of genomics and microarray technology it should be possible to identify all of the genes that are direct targets of for any particular signalling system or transcription factor. Thus, the links between cell fate commitment and cell differentiation can be determined.

The phenotype of any fully differentiated cell represents a ‘set’ of specific proteins expressed at any particular time (the proteome). This will by definition include all of those structural components that give the cell its characteristic differentiated morphology. Extracellular matrix molecules represent an important class of these differentiation end-point proteins as they are vital for generation of tissue polarity and cohesion and therefore link the molecular biology of the cell to the biology of the organism.

In studies of notochord differentiation in zebrafish it will be interesting to discover what events occur between the point floating head is switched on and the formation of a mature notochord. Identifying all of the direct downstream genes of this transcription factor is therefore an important goal. There may also be, many components of the mature notochord BM that have yet to be identified. Identifying these through biochemical analysis would therefore be useful. Notochord BM can be easily extracted
by chemical treatments (D. Stemple, pers comm.), and 2D PAGE along with mass spectrometry would allow a comprehensive catalogue of these proteins.

The perinotochordal sheath is an ancient structure and is seen in the ascidian tadpole, *Ciona intestinalis*, which represents the most simplified chordate body plan (Miyamoto and Crowther, 1985). Recent studies in this organism have used subtractive libraries to identify genes downstream of *brachyury* that may be important in notochord differentiation, and large-scale EST and in situ screens have revealed many genes with notochord specific expression. Reassuringly these have included: Laminin β1 chain, COPA, FAK, talin, and collagen type IV α4 chain. There are well over 30 other genes identified in this manner that are of unknown function. A search of homologous genes within the zebrafish and their knockdown by MO is an obvious direction for future work. One particularly interesting gene with restricted notochord expression in *Ciona intestinalis* is LAR, a transmembrane protein tyrosine phosphatase, which has been shown to be a ligand for the laminin-nidogen complex. It is required during axis determination in Drosophila, possibly through its interaction with laminin (Frydman and Spradling, 2001; O'Grady et al., 1998).

Although the role of specific laminin isoforms has been addressed to some extent using mouse gene targeting approaches and studies of invertebrate mutants, there remains a massive gap in our understanding of why these various isoforms exist. There are distinct functional domains within each laminin isoform and it is likely that particular domains give rise to specific functional properties of each. Most work has concentrated on the biochemistry of the domains and mapping of the interacting sites for receptors and other ligands. My development of an in vitro system to analyse notochord differentiation may be of use for checking of the physiological significance of these.

There have also been interesting reports of post-translational modifications of the laminin chains, such as modification of the Laminin α4 chains by chondroitin sulphate (Bernhardt and Schachner, 2000). A chondroitin sulphate antibody has been used in zebrafish and interestingly shows protein surrounding the notochord and within the somite boundaries (Sasaki et al., 2001). A major goal of any future work in this area should be to tackle this problem and find out the important interacting molecules in vivo.
Identification of *doc* promises to reveal the signalling mechanism. The notochord phenotype of *doc* mutants (shown to be allelic to *gnome*) is similar to *bal*, *gup* and *sly*, but *doc* mutants lack the brain and eye phenotype. In genetic mosaic experiments *doc* was shown to act cell autonomously (Odenthal et al., 1996), and EM studies show that *doc* mutants possess a normal BM. Hence the *doc* gene might be expected to be a component downstream of the BM and possibly part of a signalling pathway.

Laminins are heterotrimers, and only a few studies have addressed the question of their assembly. This is an important issue as organisation and control of BM formation occurs widely though all stages/tissues during metazoan development. Genetic mosaics provide a method to examine trimer assembly. The expression patterns of the laminin 1 chain mRNAs (α1β1γ1) in zebrafish suggests that cells of different tissues can provide different chains, as α1 is not expressed in the chordamesoderm itself. Thus, assembly of the heterotrimer may be more complicated than the simplest scenario in which all three chains are derived from the same cell. There are further complications since lama5 is also expressed within the chordamesoderm. Moreover, the Laminin β4 chain is also present in the early embryo with broad expression. So how do these chains interact with α1, β1 and γ1, and what are the restrictions concerning chain assembly to give rise to the various isoforms? Clearly the situation is complicated and we are far from a complete understanding. Our results seem to contradict simple models for formation of the BM, which assume that all laminin chains are contributed from the cells that sit upon it. Formation of a BM at the boundary between two cell populations expressing different chains of the laminin trimer is an attractive hypothesis and can be fully investigated when chain specific antibodies for the zebrafish laminins become available.
7.3 Conclusion

In conclusion, I have studied the differentiation of the zebrafish notochord. I have characterised bal and gup mutants, which fail to form a mature notochord. Identification of the genes responsible for their phenotype has revealed a requirement for functional laminin 1 protein, and thus BM, in notochord differentiation. Characterisation of other laminin α chains has revealed roles for laminin α4 and laminin α5 chains in during notochord differentiation. Thus, multiple laminin isoforms are present within the perinotochordal sheath, and are supplied from cells of the chordamesoderm itself, and adjacent tissues. A major unresolved question arising from this finding is whether laminins within the BM act directly through receptor-mediated signalling to carry out the differentiation program, or whether some other component of the BM is necessary. The answer to this question will almost certainly be obtained through cloning of doc, a mutant with the same notochord differentiation phenotype as bal, gup and sly, but lacking any other defects.
Appendices

A1  SSLP primers used during the positional cloning of *bal*

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Appendices

A2  YAC end RH mapping primers

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R  CAGGCCAAATGGGTAA

165B10  T7 end
F  TTAAGCCGCAACCTAAT
R  GGTTTGGGGGAAGTA

5E7  T3 end
F  CCGCCATGCTCGTAA
R  GATGCATCTATGCCCAC

5E7  T7 end
F  GGACGTGTGCATGCTAGA
R  GCCCCATGCTAA

70F1  T7 end
F  GTGAAATATTTAAATCTGACT
R  GCCTTCTGCTGCTCCTAC

89A10  T7 end
F  TCCCAGCAGCAGAAAAATAGGT
R  GGTGGAGTCACATGAGGTT
A3 Primers used for cloning of full length *lama1* cDNA

- **5' RACE of *lama1* from exon in BAC 143J17 (F1 and F2)**

Two rounds of RACE were performed.

5LSP1  GAGCTCTCACACACATCTGACAC
5LSP2  CTGCTTACACAGGTCACAGTGAGAG
5LSP3  CAGACCTGCCTGTGGGGTTGCGTGA
5LSP4  ACGTGTTGGATCTCTCCATGCTCCAG

- **Probe for EST and phage library screening: (based on F2 sequence)**

F  CCAGTTCACAGGAGGAGGAG
R  GACACACCGAAGCAGAAACA

- **RH mapping of Sanger Centre Genomic sequences:**

**zfishK-a589a07.p1c (exon 26)**

F  CATCCGCTGCTAAAACATA
R  CCTTATTTGGGTGGGTGATG

**zfishK-a616e01.q1c (exon 60)**

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R  CCCTCGACGTGTAGTGTGTG
- **F3 long range PCR: (exon10 → exon 26)**
  
  F  
  GGTGTGTCAGATGTGTGTGAGAG  
  
  R  
  CACGGGTTCATCAGACACTG  

- **F4 long range PCR: (exon 26 → exon 60)**
  
  F  
  ACGGGTCCGTACTACAGCAG  
  
  R  
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- **Cloning F5  (3' RACE of lamal from exon 60 sequence)**

  3LSP1  
  GTTGTGGTCAGTGTGGACGGGATTG  
  
  3LSP2  
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- **A4  Primers for RFLP analysis**

  X18F  
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### Alignment of zebrafish *lama1* with human and mouse homologues

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**Appendices**
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A6  Sequencing of the full-length ILK cDNA

Genomic sequences identified through a TBLASTN of the human ILK against the zebrafish genome sequencing repository. Six exons were identified with high homology to the human ILK that were used as a source of primers for sequencing of the full-length zebrafish EST clone.

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<td>exon9 R</td>
</tr>
<tr>
<td>ILKSP9</td>
<td>CCAGGATAAGCATGGCAGAT</td>
<td>exon10 F</td>
</tr>
<tr>
<td>ILKSP10</td>
<td>CTGCCAGGACACTGAAATGA</td>
<td>exon10 R</td>
</tr>
<tr>
<td>ILKSP11</td>
<td>CACGATCATGTGCAATTGG</td>
<td>exon12 F</td>
</tr>
<tr>
<td>ILKSP12</td>
<td>CCCTCACATCTGCAAACCTCA</td>
<td>exon12 R</td>
</tr>
</tbody>
</table>
A7 Cloning and RH mapping of *lama2, lama4 and lama5*

• **RH mapping of lama2**

5’ RACE was performed from a *lama2* exon sequence within a genomic fragment (Z35725-a4760h04.p1c) to obtain 5’ UTR and start of translation in order to design a MO against *lama2*.

A2SP1  CGCTGTTCCAGATCGCAGGTCCTACA  
A2SP2  ATGTGGCGTTGGTCTTGATCTCTGC

The sequence of this cDNA was used to identify a *lama2* genomic fragment (Z35725-a4234f05.p1c). The following primers mapped this gene to LG20:

A2RH1  CTGCAGCAGGAATAAACCACA  
A2RH2  CAAGGTGACCCAGACTCACA

• **RACE and RH mapping of lama4**

5’ RACE was performed from exon sequence located within a genomic fragment (Z35723-a1410d04.q1c) identified within the zebrafish genome sequencing database, using two nested primers:

A4SP1  TGGAAGACAGGAGGCCAGGAGCTGA  
A4SP2  TGGCGAGTCCCATGTAGTCCCTCTT

This genomic fragment mapped *lama4* to LG20 using the following RH primers:

A4RH1  GCGTGACACACACGGTAAAGAA  
A4RH2  ACATGGGACTCGCCATAAAG

• **RACE and RH mapping for lama5**

200
5' RACE was performed from exon sequence located within a genomic fragment (Z35723-a1811e10.q1c) identified within the zebrafish genome sequencing database, using two nested primers:

A5SP1  CGTTGACCTGGTTGTGTTTGGC ACT
A5SP2  AGGGCTTTGCCACCAGCGTTC

A cDNA clone AA545720 was obtained from RZPD. A genomic fragment corresponding to the end sequence of this insert was identified (Z35723-a25d12.p1c) and the following primers mapped this gene to LG23:

A5RH1  TCTCTGCCACAGCCTCATT A
A5RH2  ATGGGTTGCTGCTGTAAGG
Appendices

References


Appendices


Appendices


Appendices


Appendices


