Role of p21 Activated Kinase in Zebrafish Lateral Line Development.

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Abstract

Migration of the posterior lateral line primordium is a process of directed response to a chemotactic signal. We have observed directional protrusions of the cells at the leading edge of migrating primordia. The p21 activated kinase (Pak) family of proteins are downstream effectors of the small GTPases Rac and Cdc42 and have been shown to play important roles in the regulation of cell adhesion and migration in many organisms. In order to understand the molecular mechanisms underlying directed cell migration in zebrafish we have identified and characterised two zebrafish *pak2* genes (*pak2a* and *pak2b*) and found that their functions are important for migration of the lateral line primordium. Pak2a and Pak2b have similar protein sequences suggesting similar functions in development.

Knock down of Pak2 using antisense morpholino oligonucleotides resulted in a variety of defects in zebrafish development including retarded migration of the posterior lateral line primordium and aberrant migration of primordial germ cells. Zebrafish *pak2* genes may function by dynamically regulating the F-actin reorganisation and focal adhesion formation required for directed migration. Differences in the phenotypes generated by knock down of Pak2a and Pak2b may be explained in part by the observation that Pak2a can bind to the adaptor protein Nck while Pak2b cannot.

Pak2b is required to establish and/or maintain F-actin organisation, cadherin-mediated adhesion and tissue integrity within the migrating lateral line primordium. We show that dynamic regulation of the actin cytoskeleton and cell adhesion is involved in the formation of rosette-like structures within the lateral line primordium which are deposited as a unit and represent the nascent neuromast precursors. We have also identified possible roles for Pak2a and Pak2b in regulating gene expression in early zebrafish development. Pak2 proteins may control the expression of several genes involved in mesoderm formation through regulation of transcriptional activators and repressors.

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For Rob

for always and forever

"Migratory is not something that is stuck together with toffee"

Douglas Adams, The Salmon of Doubt.

Abbreviations

AID	Autoinhibitory Domain
BLAST	Basic Local Alignment and Searching Tool
BMP	Bone Morphogenetic Protein
bp	Base Pair
BrdU	Bromodeoxyuridine
cAMP	Cyclic Adenosinemonophosphate
cDNA	Complementary DNA
CRIB	Cdc42/Rac Interaction Binding
DAB	3,3'- Diaminobenzidine
DAPI	4',6-Diamidino-2-phenyindole
dH_2O	Distilled Water
DMF	Dimethylformamide
DMSO	Dimethyl Sulfoxide
dNTP	Deoxyribonucleotide Triphosphate
dpf	Days Post Fertilisation
DTT	Dithiothreitol
ECM	Extracellular Matrix
EST	Expressed Sequence Tagged
EVL	Enveloping Layer
F-actin	Filamentous Actin
FAK	Focal Adhesion Kinase
FITC	Fluorescein isothiocyanate
G-actin	Glutenous Actin
$G\alpha_z$	G-Protein α_z subunit
$G\beta\gamma$	G-protein $\beta\gamma$ subunit
GDP	Guanosine Diphosphate

GFP	Green Fluorescent Protein
GIT1	G-protein-coupled receptor kinase-interacting protein
GST	Glutathione-s-transferase
GTP	Guanosine Triphosphate
GTPase	Guanosine Triphosphatase
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
hpf	Hours Post Fertilisation
IQGAP	IQ GTPase Activating Protein
LB	Luria-Bertani
MAP	Mitogen-activated Protein
MAPK	Mitogen-activated Protein Kinase
MEK	MAP or ERK kinase
MHC	Myosin Heavy Chain
MLC	Myosin Light Chain
MLCK	Myosin Light Chain Kinase
mo	Morpholino
MP	Muscle Pioneers
NCBI	National Centre for Biotechnology Information
NIMR	National Institute for Medical Research
NMP	Non-muscle Pioneers
PAGE	Polyacrylamide Gel Electrophoresis
Pak	p21 Activated Kinase
PBD	p21 Binding Domain
PBS	Phosphate Buffered Saline
PBT	Phosphate Buffered Saline and Triton-x-100
PBTw	Phosphate Buffered Saline and Tween20
PCR	Polymerase Chain Reaction
PDGF	Platelet Derived Growth Factor
PFA	Paraformaldehyde

PGC	Primordial Germ Cell
PIX	Pak Interacting Exchange Factor
PKA	Protein Kinase A
PKL	Paxillin Kinase linker
PSM	Presomitic Mesoderm
RZPD	Resource Centre Primary Database
SDF-1	Stromal-Derived Factor 1
SDS	Sodium Dodecyl Sulphate
SH3	Src3 Homology Domain
SSC	Saline Sodium Citrate
TCA	Trichloroacetic Acid
TY	Tryptone-Yeast
Wnt	Wingless type
YCL	Yolk Cytoplasmic Layer
YFP	Yellow Fluorescent Protein
YSL	Yolk Syncital Layer

Chapter One

Introduction

Embryonic development relies on highly regulated and coordinated migrations of cells. Understanding the mechanisms controlling cell migration will allow us to discover how different organs and tissues are formed by the coordinated movements of cells.

It has been well characterised that migration of adherent cells involves regulation of the actin cytoskeleton and the assembly and disassembly of cellular adhesions to the extracellular matrix (ECM) (Christopher and Guan, 2000). When cells move they alter this internal, actin skeleton to push the cell membrane out at the front and pull it in at the back (Cory and Ridley, 2002). Actin polymerisation at the cell front provides the protrusive force required for extension of membrane at the leading edge (Rottner *et al.*, 1999). Membrane extensions at the leading edge are known as lamellipodia and as the cell advances the lamellipodia form adhesions to the ECM, known as focal adhesions. These adhesions are disassembled at the trailing edge as the rear of the cell is pulled forward by contraction of actin-myosin fibres (Christopher and Guan, 2000). In addition, migration of a group of cells requires cell to cell adhesion to maintain integrity (Gumbiner, 1996).

Regulation of these factors depends on the cooperative effects of many intracellular signalling events resulting in the activation of proteins that can control actin dynamics and adhesion formation (Christopher and Guan, 2000). Many proteins are known to be involved in the regulation of the actin cytoskeleton and of these the roles of the small GTPases have been well described (see (Hall, 1998) for a review). The Rho family of small GTPases, Rac, Cdc42 and Rho, have different roles in

regulating the actin cytoskeleton: Cdc42 triggers the formation of the thin membrane protrusions, filopodia, Rho controls actin stress fibre formation and Rac regulates formation of lamellipodia and membrane ruffles, which contain small focal adhesions (Christopher and Guan, 2000). Although much is known of the effect of the small GTPases, less is known of their downstream effector molecules. In a screen carried out to look for proteins acting downstream of the small GTPases a novel kinase was identified that could bind with Rac and Cdc42 and mediate their effects on the cytoskeleton. As a result of this interaction it was named p21 activated kinase (Pak) (Manser *et al.*, 1994).

Work carried out in our lab aims to further elucidate control of cell migration during development using the zebrafish, *Danio rerio*, as our model organism. Embryonic zebrafish have a conspicuous sensory system present on their surface, the lateral line system. This system is formed by migration of lateral line primordia that deposit lateral line sensory organs at stereotypical positions. The easy identification and reproducible pattern of the lateral line system make it an excellent model for studying cell migration. The zebrafish has many features that make it an excellent system for studies in developmental biology, including the ability to obtain large numbers of embryos and a short life cycle. Of particular interest to the analysis of cell migration is the optical clarity of zebrafish embryos that permits direct observation of individual cells and cell movements within the developing embryo.

1.1 A model for cell migration – development of the zebrafish posterior lateral line system.

The lateral line is a mechanosensory system present on the skin of fishes and amphibians (Metcalfe *et al.*, 1985; Stone, 1933; Winklbauer and Hausen, 1983). It is composed of two organs, the mechanoreceptive neuromasts, that contain sensory hair cells like those found in the ear that act as water displacement detectors (Kornblum *et al.*, 1990), and the electroreceptive ampullary organs that respond to

weak electric fields (Baker and Bronner-Fraser, 2001). These organs are innervated by axons of the lateral line ganglion and function in various activities including obstacle avoidance and schooling behaviour. Lateral line organs are derived from two dorsolateral placodes (focal ectodermal thickenings) on the head of the embryo, these placodes give rise to the neuromasts of a particular line and the neurons that innervate it (Stone, 1922). Each placode is polarised such that one part gives rise to the cells of the migratory primordium while the other part gives rise to the lateral line ganglion (Stone, 1922). The migrating primordia deposit the precursors of the neuromasts over the head and along the body.

The embryonic zebrafish lateral line has two major components, anterior and posterior. The anterior lateral line comprises the neuromasts of the head, jaw and opercle and the sensory neurons of the anterior lateral line ganglion. The anterior system forms from a placode that is derived from the ectoderm rostral to the otic vesicle. The posterior lateral line system is formed from the posterior lateral line placode which is first detected caudal to the otic vesicle adjacent to the developing sensory ganglion (Metcalfe, 1985). The posterior lateral line consists of the neuromasts of the trunk and tail and the sensory neurons of the posterior lateral line ganglion (Metcalfe, 1985). Both the anterior and posterior lateral line systems comprise several branches. The major branch of the posterior lateral line is the lateral or midbody line, which is the first to arise (Metcalfe, 1985). It is the characterisation of this line that will be discussed in this study.

1.1.1 Migration of the posterior lateral line primordium.

Migration of the posterior lateral line primordium is first detectable at 20 hours post fertilisation (hpf), at this stage scanning electron microscopy of zebrafish embryos shows the primordium as an elongated elevation of the epidermis (Metcalfe, 1985).

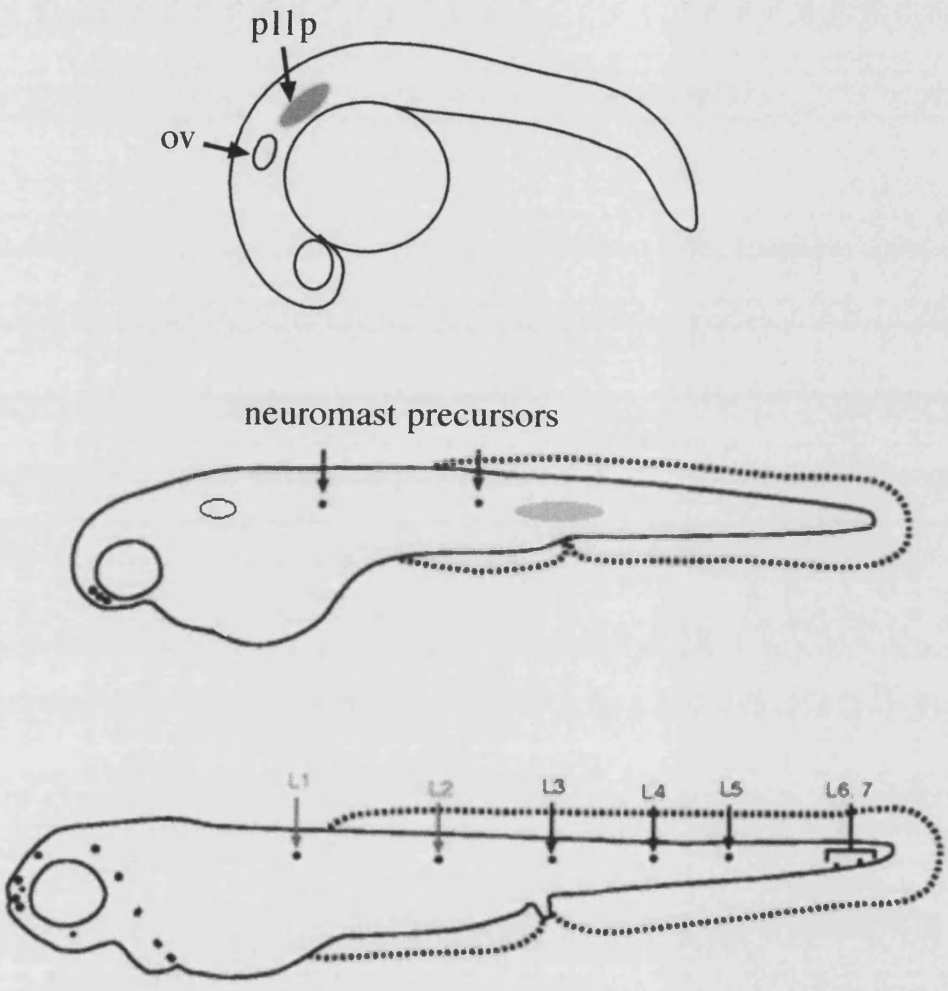


Figure 1.1

Figure 1.1: Development of the posterior lateral line system.

The posterior lateral line arises from a placode posterior to the otic vesicle which gives rise to the ganglion of the posterior lateral line and the migratory primordium. As the primordium migrates along the trunk and tail it deposits seven neuromasts at stereotyped positions. The neuromasts are innervated by nerve fibres of the lateral line ganglion. This schematic adapted from (Ledent, 2002) shows primordium migration and neuromast deposition from commencement of migration at approximately 22hpf, deposition of neuromast precursors at 36hpf, and differentiation of the full complement of posterior neuromasts at 72hpf. Abbreviations refer to: ov, otic vesicle; pllp, posterior lateral line primordium.

The primordium migrates caudally under the epidermis along the midbody line and migration is complete by 42hpf (Metcalfe, 1985). During this time seven neuromast precursors are deposited at stereotypically spaced intervals along the trunk and tail (Figure 1.1). The first five neuromast precursors are deposited along the horizontal myoseptum, an area in middle of the zebrafish trunk, parallel to the notochord and overlying the somites. The neuromasts along the horizontal myoseptum are found at somite borders (Gompel *et al.*, 2001). At the level of somite 30 the primordium moves ventrally and the final two neuromasts are deposited close to the tip of the tail. These neuromast precursors are deposited simultaneously when the primordium has reached the tip of the tail, stops migrating and splits into two, forming the sixth and seventh neuromast precursors (Gompel *et al.*, 2001). Deposited neuromast precursors mature into recognisable sensory neuromasts 6-9 hours after deposition (Metcalfe, 1985).

The neuromasts of the posterior lateral line system are innervated by the posterior lateral line sensory neurons which are found under the epidermis at the midline along the horizontal myoseptum (Metcalfe *et al.*, 1985). The sensory neurons and the migratory neuromast precursor cells arise from a common postauditory placode (Metcalfe, 1985). The growth cones of the sensory neurons comigrate with their innervation targets, the migratory lateral line primordium, and follow the migratory path of the primordium..

Gompel *et al* have described initial characterisation of the morphology and movement of the neuromast precursor cells of the zebrafish posterior lateral line primordium during migration and deposition. The posterior lateral line primordium is highly amenable to such studies as during its migration it is clearly distinguishable from the surrounding ectoderm as a stripe of small round cells that migrate as a tight group. The primordium is about 4-5 cells wide and 20-25 cells in length at the beginning of migration and extends over 2-3 consecutive somites. The number of cells within the lateral line primordium varies during migration, mitosis occurs within the migrating primordium and cells are lost during neuromast precursor deposition

(Gompel *et al.*, 2001). Deposition of the neuromast precursors is characterised by the slowing down of a group of cells at the trailing end of the primordium. The other cells in the primordium maintain their migration rate resulting in thinning of the primordium between the cells being deposited and those continuing their migration. The slowing down of the trailing cells is progressive as the neuromast precursor splits from the primordium about two somites posterior to the point at which deposition began. The neuromast comes to rest one or two somites caudal to the point of splitting. The final position of the neuromast varies between embryos and between left and right sides of a single embryo. Gompel *et al* (2001) investigated this variation using the somites, over which the neuromast are deposited, to plot the distance between consecutive neuromasts. In doing this they found that, although neuromast position varies, the spacing between adjacent neuromasts is absolute.

1.1.2 Guidance of the migration of the posterior lateral line primordium.

The molecular mechanisms governing the guidance of migration of the lateral line primordium are beginning to be elucidated. Ablation studies in axolotl have demonstrated that signals from the overlying ectoderm and underlying somites are required for proper migration of the lateral line primordium (Smith *et al.*, 1990). In zebrafish the homologue of the growth-cone repulsive molecule semaphorin III/D or collapsin 1, *semaZ1a*, has been shown to be expressed in the dorsal and ventral portions of the myotome, but not in the horizontal myoseptum, over which the primordium migrates. This observation suggested a *semaZ1a*-free zone acts to guide primordium migration by inhibiting movement of the lateral line primordium over the dorsal or ventral myotome and restricting the migration pathway to the horizontal myoseptum (Shoji *et al.*, 1998). Indeed in mutants where the horizontal myoseptum is absent, such as floating head, *semaZ1a* is uniformly expressed and the lateral line primordium migrates aberrantly along the ventral myotome (Shoji *et al.*, 1998).

Recent studies in zebrafish, looking at migration of the posterior lateral line primordium, have identified a trail of the chemokine Sdf1a (a zebrafish homologue of stromal cell-derived factor-1) along the horizontal myoseptum and expression of its receptor (*cxcr4b*) in the migrating cells of the lateral line primordium (David *et al.*, 2002). The inactivation of either the ligand or the receptor blocks primordium migration and in mutants where the horizontal myoseptum is absent the lateral line primordium migrates ectopically along an alternative, ventral trail of Sdf1a. These data provide strong evidence that correct migration of the posterior lateral line primordium along the horizontal myoseptum is directed by attractive, chemokine signalling and is restricted by inhibitory domains of SemaZ1a.

Whilst the guidance pathways of lateral line primordium migration are beginning to be understood, little is known of the molecular mechanisms that provide the physical, intracellular changes required to respond to such migration cues and control migration in the developing lateral line. The main aim of our work is to better understand how the cellular changes required to initiate and regulate the directed migration of the cells of the lateral line primordium are mediated. This was initially approached through detailed characterisation of the behaviour of the cells of the lateral line primordium during their migration and deposition. With a good understanding of normal cell behaviour in the migratory lateral line primordium we can approach investigations into the molecular mechanisms underlying this migration using loss-of-function analyses.

1.2 Regulation of cell migration.

The ability of a cell to migrate is dependant on the cooperative effects of many intracellular signalling events triggered through cell surface receptors, internal cellular mechanics and the extracellular matrix (Christopher and Guan, 2000). Migrating cells display a polarised morphology with distinct leading and trailing ends. At the leading edge polymerisation of the actin cytoskeleton leads to formation of membrane

protrusions, or lamellipodia, that extend in the direction of migration, while at the trailing end, contraction of the actin/myosin cytoskeleton causes the cell body to retract (Wittmann and Waterman-Storer, 2001; Zeng *et al.*, 2000). Cell motility requires the transmission of forces generated by actin movements inside the cell to the matrix outside through regulated formation and dissolution of cell-substrate adhesions (Beningo *et al.*, 2001). Cell to substrate adhesions are mediated by focal adhesions and during cell migration new focal adhesions are formed at the lamellipodia and broken down at the rear of the cell (Mitchison and Cramer, 1996). Thus regulation of cell migration can be discussed in two sections: organisation of the actin/myosin cytoskeleton in stimulating extension at the cell front and retraction at the cell rear, and regulation of focal adhesions.

1.2.1 The actin/myosin cytoskeleton – advancement and retraction.

At the leading edge of motile cells high levels of filamentous actin permits the formation of highly dynamic protrusive structures that contain dense arrays of actin filaments (Mitchison and Cramer, 1996). These membrane protrusions can be thin filopodia, such as those observed in neuronal growth cones, or sheet-like lamellipodia, characteristic of many motile cells. Amoeboid cells tend to protrude thicker processes termed pseudopods (Mitchison and Cramer, 1996). Formation and extension of protrusions requires polymerisation of actin filaments at the leading edge through elongation of existing filaments or nucleation of new filaments (Mitchison and Cramer, 1996). It has been demonstrated that lamellipodia extension is simply correlated with actin filament growth using fluorescently labelled actin filaments and that disruption of actin filaments using Cytochlasin D arrests the formation of protrusions (Theriot and Mitchison, 1991; Wang, 1985; Wessels, 1971).

Actin filaments are double helical polymers of globular subunits arranged head to tail resulting in a filament polarity and based on the pattern created by decoration with myosin, one end of the filament is described as barbed and the other

end pointed (Pollard and Borisy, 2003). The barbed end is favoured for growth and faced towards the edge of the cell.

The initial step in the formation of an actin filament is known as nucleation. Once the "nucleus" is established the filament will grow rapidly. The initial combination of actin monomers is unfavourable owing to the instability of actin dimers and trimers and is, therefore, the limiting step of actin polymerisation (Pollard and Borisy, 2003). Actin polymerisation is enhanced by factors that stimulate nucleation such as WASp (Wiskott-Aldrich Syndrome protein) and WAVE/Scar (Eden *et al.*, 2002). Active nucleation-promoting factors stimulate a complex known as Arp2/3 to initiate nucleation of a new actin filament, possibly by mimicking an actin trimer (Cory and Ridley, 2002).

Actin filaments then continue to grow through addition of monomer until stopped by the addition of capping proteins to the barbed end (Pollard and Borisy, 2003). Actin monomers in filaments are bound to ATP and filaments age by hydrolysis of their bound ATP to ADP and phosphate. Phosphate is slowly released and it's dissociation stimulates the ADF/cofilin proteins that promote actin filament depolymerisation and dissociation of ADP from the actin monomers (Pollard and Borisy, 2003). Profilin is the nucleotide exchange factor for actin, catalysing the exchange of ADP for ATP and returning the ATP-actin-profilin complex to the actin monomer pool, ready for another cycle of assembly (Pollard and Borisy, 2003). The profiling-actin complex fails to form nuclei and in the absence of preformed filaments will not assemble into filaments, however, profiling-actin complexes can readily add to free barbed ends of assembled filaments (Bubb *et al.*, 2003). A concentration of actin monomers is maintained at a level far from equilibrium by depolymerisation and capping and this large pool of monomers permits rapid growth at non-capped, barbed ends (Pollard and Borisy, 2003). Cofilin increases the rate of depolymerisation and therefore the size of the pool of actin monomers, while capping proteins bind the growing end of selected actin filaments to enhance the rate of polymerisation of

uncapped filaments, therefore making polymerisation more efficient (Pantaloni *et al.*, 2001).

The movement of actin filaments is powered by myosins, that function as motor proteins by hydrolysis of ATP. This process requires that the actin filament interacts with the myosin motor domain, the head domain, in such a way as to accelerate release of ADP and phosphate (de la Roche and Cote, 2001). The myosins are a large family of proteins which play a number of roles within cells depending on their type and localisation.

Cytoskeletal dynamics are primarily modulated by actin and myosin II and these interactions are modulated by the phosphorylation state of the regulatory light chain of myosin II (rMLC). (Sanders *et al.*, 1999). Studies in *Dictyostelium* have demonstrated that phosphorylation, and therefore activation, of myosin II filaments is critical for contraction at the rear of a migrating cell (Zeng *et al.*, 2000). Phosphorylation of rMLC, by myosin light chain kinase (MLCK), stimulates the actin activated ATPase activity of myosin II and contraction (Wirth *et al.*, 2003). The reverse reaction is catalysed by myosin light chain phosphatase (MLCP), which, dephosphorylates the rMLC and results in relaxation in smooth muscle cells (Wirth *et al.*, 2003). Myosin phosphorylation is also regulated by Caldesmon, a thin filament associated protein that inhibits the actin-activated ATPase activity of myosin II and may be involved in the regulation of tension development (Wirth *et al.*, 2003). The actinomyosin cytoskeleton is subject to continuous remodelling in response to extracellular signals and actin and myosin can be rapidly assembled or disassembled to allow the cell shape changes associated with directed movement (van Leeuwen *et al.*, 1999).

1.2.2 Focal adhesions.

The formation of protrusions initiates migration, but for migration to occur the protrusions must be stabilised by attaching to the underlying substratum (Geiger and

Bershadsky, 2001). Attachments to the underlying substratum, the ECM, are mediated by the integrin family of heterodimeric receptors. Engagement of integrin receptors with their extracellular ligands leads to the formation of well-defined structures that link the ECM to the cytoplasmic actin cytoskeleton. Sites of ECM-integrin adhesion are dynamic, heterogeneous structures that vary in size and organisation (Parsons *et al.*, 2000).

Initial adhesions are located at the cell perhiphery and the leading edge of motile cells. They are highly tyrosine phosphorylated and contain $\alpha_v\beta_3$ integrin. The cytoplasmic proteins, Paxillin and Talin are initially recruited into these adhesion, followed by Vinculin and Focal Adhesion Kinase (FAK) (Zaidel-Bar *et al.*, 2003). The recruitment of Vinculin marks the transition from an initial adhesion to a 'focal complex', so named because the distance between the ECM and the adhesion is decreased to become 'tight' or 'focal' (Galbraith *et al.*, 2002). Recruitment of these binding proteins is induced by ligand binding or aggregation of integrin receptors (Parsons *et al.*, 2000).

The formation of focal complexes, i.e. the recruitment of Vinculin, is rapid, 10 to 40 seconds, and stabilises the adhesion so that cytoskeletal force can be applied to the ECM. This is critical for cell migration and requires Vinculin (Galbraith *et al.*, 2002). Proteins associated with actin filament assembly may also be found within focal complexes, such as, vasodilator-stimulated phosphoprotein (VASP) and the Arp2/3 complex, as well as the actin binding protein α-actinin (Brindle *et al.*, 1996; Zaidel-Bar *et al.*, 2003).

Focal complexes are short lived and highly dynamic. Maturation of a focal complex into the more stable focal adhesion is Rho-dependant and requires localised membrane retraction. This retraction produces tension that induces the recruitment of other adhesion proteins including, the focal adhesion specific protein, Zyxin and the actin filament binding protein, Tensin (Chen *et al.*, 2000; Zaidel-Bar *et al.*, 2003). Zyxin may regulate actin filament organisation and acts as a docking protein in binding α-actinin, indeed an increase in the level of α-actinin is associated with formation of

focal adhesions (Reinhard *et al.*, 1999). The clustering of these proteins contributes to adhesion and to integrin-mediated signalling. Associated proteins such as FAK as well as MAPK act upstream in promoting cell proliferation and migration (Boudreau and Jones, 1999).

The size and maturity of intergrin-ECM adhesions depends on their position within the cell. At the leading edge of a migrating cell new contacts are formed, established contacts are maintained between the body of the cell and the ECM, and at the trailing end old contacts are weakened and broken (Galbraith *et al.*, 2002). At the cell front Paxillin-containing focal complexes remain small and these nascent attachments serve as traction points for the propulsive forces that drive the cell forward. As the cell moves forward the nascent adhesions are turned over and reformed at the new leading edge (Webb *et al.*, 2002). In contrast, under the body of the cell and at the cell rear, adhesion to the ECM is via mature, stable focal adhesions. Unlike focal complexes at the leading edge, focal adhesions are not released by simple dissociation of the component proteins. As the cell migrates the linkage between cytoplasmic adhesive proteins and the integrin receptors at the rear of the cell are initially maintained. As the rear of the cell retracts through contraction of the actin-myosin cytoskelton, the intracellular adhesion proteins, which are under tension by virtue of their interaction with the cytoskeleton, are eventually pulled towards the cell body and break contact with the integrin receptors (Webb *et al.*, 2002). In this way cell contacts are released at the trailing edge and the cell moves forward.

1.3 The small GTPases.

Dynamic regulation of the actinomyosin cytoskeleton and focal adhesions during cell migration requires the interaction of many proteins. The Rho family of small GTPases have emerged as key regulators of such activities (see /(Hall, 1998) for a review). GTPases alternate between an active "on" state when GTP bound, and an

inactive "off" state when GDP bound. In the "on" state GTPases are able to bind and activate specific effector proteins (Lodish *et al.*, 1995). Activation of GTPases is stimulated by extracellular ligands binding to cell surface receptors such as PDGF in fibroblasts (Hall, 1998) or G-protein linked receptors in *Dictyostelium* (Chung *et al.*, 2000). This activation occurs in two steps, release of GDP, accelerated by the action of guanine nucleotide exchange factors, and binding of GTP, which occurs spontaneously. Inactivation occurs by hydrolysis of GTP to GDP through the action of GTPase activating proteins (Lodish *et al.*, 1995).

The Rho family of small GTPases comprises Rac, Cdc42 and Rho and all three members have important roles in regulating the actin cytoskeleton and focal adhesions. The small GTPases can also regulate with each other as Cdc42 can activate Rac, and Rac can activate Rho (Hall, 1998). Studies in neutrophils have demonstrated specific roles for Rac and Rho in coordinating leading edge extension and trailing edge retraction respectively and these two activities inhibit each other (Xu *et al.*, 2003). By opposing each others function Rac and Rho act maintain polarisation of the migrating cell (Xu *et al.*, 2003). Additionally, studies in *Dictyostelium* have characterised an action of Rac on polymerisation of the actin since expression of dominant negative Rac results in loss of the well defined F-actin rich leading edge, failure to extend pseudopodia and poor motility (Chung *et al.*, 2000). In addition expression of constitutively active Rac leads to a loss of directed motility and extension of aberrant lateral pseudopods (Chung *et al.*, 2000). A similar role for Rac has been described in other systems. Treatment of fibroblast cells with agents that increase levels of GTP bound Cdc42 and Rac leads to *de novo* formation of F-actin networks (Hall, 1998). This indicates that Rac and Cdc42 integrate signalling pathways leading to actin polymerisation and this results in the formation of lamellipodia and filopodia respectively (Parsons *et al.*, 2000). The roles of Rac and Cdc42 in actin filament formation are two-fold. Firstly Rac and Cdc42 can promote actin nucleation through the activation of WAVE, by Rac, and WASp, by Cdc42, which in turn activate the Arp2/3 complex (Eden *et al.*, 2002). Secondly, Rac and Cdc42 can

activate Pak, which in turn phosphorylates and thereby activates LIM kinase resulting in inhibition of Cofilin-induced disassembly of actin filaments (Edwards *et al.*, 1999).

In contrast to the extension activities of Rac and Cdc42, activation of Rho leads to the formation of actin stress fibres which have focal adhesions at their terminus (Parsons *et al.*, 2000). Studies in monocytes have shown that RhoA is required for tail end retraction by regulating contraction of the actinomyosin cytoskeleton (Worthylake *et al.*, 2001).

In addition to their effects on the actinomyosin cytoskeleton the Rho family of GTPases regulate cell migration through control of substrate contact dynamics (Rottner *et al.*, 1999). Active Rac can induce the formation of focal adhesions, possibly through stimulation of actin polymerisation and clustering of integrins (Geiger and Bershadsky, 2001). Rac induced focal adhesions are short lived and rapidly turned over during cell migration (Rottner *et al.*, 1999). The influence of Rho-mediated myosin contraction is required to form mature and stable focal adhesions (Rottner *et al.*, 1999). This action of Rho is mediated by the downstream kinase, Rock, which acts to promote actinomyosin-driven cell contraction (Rottner *et al.*, 1999). In agreement with the studies in *Dictyostelium*, Rac and Rho pathways can also antagonise each other in mammalian cells, in this case during regulation of focal adhesion formation (Rottner *et al.*, 1999).

1.4 p21 activated kinase.

Although much is known of the effect of the small GTPases, less is known of their downstream effector molecules. The p21-activated kinase (Pak) was identified in a screen for binding partners of the small GTPases, Rac and Cdc42 and it was shown that the serine/theronine protein kinase activity of Pak could be stimulated by binding of activated, GTP-bound Cdc42 and Rac (Manser *et al.*, 1994). Identification of a downstream target for Rac and Cdc42 showed that they act like other GTP-binding proteins in stimulating a target/effector protein and suggested an analogy to the well

characterised Ras signalling pathway where the small GTPase Ras directly binds the serine/theronine protein kinase Raf (Bagrodia and Cerione, 1999). The possibility of a Rac/Cdc42/Pak signalling pathway lead to identification of downstream components and a better understanding of how the effects of Rac and Cdc42 on the cytoskeleton are achieved.

1.4.1 The p21 activated kinase family.

The Pak family of proteins are serine/theronine kinases and six members have been found in humans to date (Abo *et al.*, 1998; Dan *et al.*, 2002; Knaus and Bokoch, 1998; Yang *et al.*, 2001). Pak homologues have also been identified in *Dictyostelium*, yeast, *Drosophila, Xenopus laevis* and mouse amongst others (Bagrodia *et al.*, 1995; Chung and Firtel, 1999; Gulli *et al.*, 2000; Hing *et al.*, 1999; Islam *et al.*, 2000). The Pak family can be grouped into two classes based on the conservation of structure, Group I (Pak1, Pak2, Pak3) and Group II (Pak4, Pak5, Pak6). The Group I Paks share a number of defining structural characteristics: Firstly a Rac/Cdc42 interaction site or p21 binding domain (PBD) in the N-terminal region, and secondly a C-terminal kinase domain (Knaus and Bokoch, 1998). Overlapping the PBD of Group I Paks is a sequence implicated in autoinhibition, the autoinhibitory domain or inhibitory switch (AID/IS), which inhibits catalytic activity in the absence of activating stimuli (Lei *et al.*, 2000). In the inactive state Pak proteins are found as dimers. Binding of GTP-Rac or Cdc42 to the PBD relieves autoinhibition of the catalytic domain through disruption of the dimer and conformational change, permitting kinase activity and autophosphorylation (Lei *et al.*, 2000). Pak1 proteins can also be activated by interaction with sphingosine or related long chain sphingoid bases in a similar manner to GTPase-mediated activation (Bokoch *et al.*, 1998).

The N-terminal PBDs of the Group II Paks are less than 40% identical to the Group I PBDs, however they remain able to bind to activated Rho family proteins (Jaffer and Chernoff, 2002). The Group II Paks do not posses an identifiable

autoinhibitory domain and despite the ability to bind small GTPases the kinase activity of Group II Paks does not appear to be stimulated by interaction with activated Rho GTPases. However, truncated versions of both Pak4 (Abo *et al.*, 1998) and Pak6 (Yang *et al.*, 2001) containing only the catalytic domains, have greater kinase activity than the full-length proteins. The kinase domains of Group II Paks are even more diverged and these differences imply that Groups I and II Paks may be regulated differently and may have different downstream effectors (Jaffer and Chernoff, 2002). These results suggest that the Group II Paks probably are regulated intramolecularly but by a different mechanism (Jaffer and Chernoff, 2002).

Activation of Group I Pak is dependent on cell adhesion to the ECM and this dependence is related to the inability of Rac to activate Pak in non-adherent cells. Growth factor stimulation of adherent cells leads to Rac localisation at the plasma membrane where it can activate Pak, however in non-adherent cells Rac remains in the cytosol where even when GTP bound it is unable to activate Pak (del Pozo *et al.*, 2000).

1.4.2 Protein interactions and activities of Pak.

Investigations into the proteins interacting with the Pak family is a highly active field of research, as a result many proteins have been described that bind, activate or are activated by Pak (see (Kumar and Vadlamudi, 2002) for a review). In this thesis we will focus on the role of Pak in the regulation of cell adhesion and cell migration through protein interactions. However, some other Pak interactions will be discussed. A summary of some of these interactions can be found in Figure 1.2.

Figure 1.2

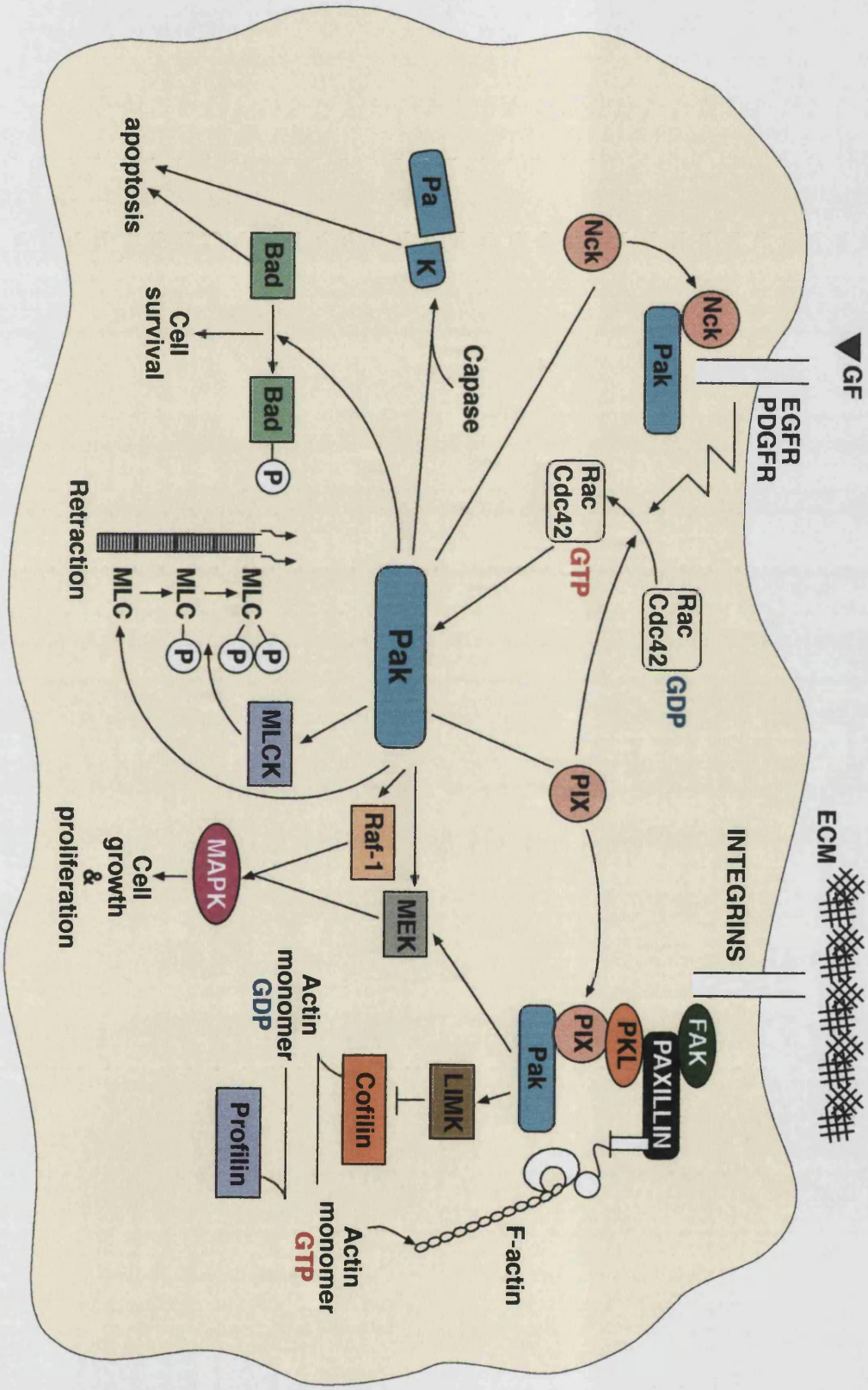

GF
EGFR
PDGFR
Nck
Pak
Nck
Nck
Pak
Rac
Cdc42
GTP
Rac
Cdc42
GDP
PIX
PIX
PKL
FAK
PAXILLIN
INTEGRINS
ECM
F-actin
Actin
monomer
GTP
Actin
monomer
GDP
Profilin
Cofilin
LIMK
Pak
Pak
Pa
K
Capase
Bad
Bad
P
Cell
survival
apoptosis
Retraction
MLC
MLC
P
MLC
P
P
MLCK
Raf-1
MEK
MAPK
Cell
growth
&
proliferation

Figure 1.2: Summary of known Pak interactions.

The Group I family of Pak proteins interact with a large number of proteins and have influences on many different signalling pathways. Pak can be recruited to growth factor receptors by interaction with Nck (Bokoch *et al.*, 1996; Galisteo *et al.*, 1996) and focal adhesions via binding to PIX (Brown *et al.*, 2002). Recruitment to the cell membrane enhances activation of Pak by GTP bound Rac or Cdc42 (Lu *et al.*, 1997; Lu and Mayer, 1999). In adherent cells active Pak can promote cell growth through the MAPK pathway (Howe and Juliano, 2000b). Pak can also act upstream in cell survival pathways, through phosphorylation of Bad and apoptosis pathways by caspase induced cleavage into an N-terminal region (Pa) and a constitutively active kinase domain (K). Pak also regulates the actinomyosin cytoskeleton by inhibiting the action of Cofilin through LIMK (Edwards *et al.*, 1999) and stimulating contraction of the actin-myosin cytoskeleton (Felsenfeld *et al.*, 1991; Kiosses *et al.*, 1999; Sells *et al.*, 1999).

Abbreviations refer to: Pak, p21 activated kinase; GF, growth factor; EGFR, epidermal growth factor receptor; PDGFR, Platelet-derived growth factor receptor; GDP, guanine diphosphate; GTP, guanine triphosphate; PIX, Pak interacting exchange factor; PKL, paxillin kinase linker; FAK, focal adhesion kinase; LIMK, LIM-domain kinase; F-actin, filamentous actin; MLC, myosin light chain; MLCK, myosin light chain kinase; MAPK, mitogen-activated protein kinase; MEK, MAP or ERK kinase; ECM, extracellular matrix; P, phosphorylation.

In addition to the PBD the N-terminal region of Group I Paks, described as the regulatory domain, comprises an acidic region and a number of proline-rich sites of interaction with Src3 homology (SH3) domain containing proteins (Knaus and Bokoch, 1998). In addition to the three proline-rich SH3 domain binding sites in the N-terminal regulatory domain, protein interaction occurs through the kinase domain and a binding site for the G-protein βγ-subunit at the extreme C-terminus. The most N-terminal SH3-binding site binds the adapter protein Nck and targets Pak to the plasma membrane, this relocation is associated with increased activity of Pak by virtue of the high levels of active Rac at the membrane (Lu *et al.*, 1997). SH3-domain binding sites are usually characterised by a PxxP motif, where P is a proline residue and x any residue (Bokoch *et al.*, 1996). The binding site specific for the SH3-domain of Nck has been further characterised as a consensus binding sequence of PxxPxRxxS[20] (Chong *et al.*, 2001). Autophosphorylation of serine residues within this motif (Ser[19] or Ser[20]) has been shown to cause a loss of affinity between Pak and Nck that results in dissociation of Pak for the membrane (Chong *et al.*, 2001).

Specific protein binding at the second proline-rich motif has not been described. The third proline-rich motif is atypical (PPxxxPRP) and binds the Pak-interacting exchange factor, PIX (Manser *et al.*, 1998). Interaction of PIX with Pak is also negatively regulated by serine phosphorylation at residues close to the C-terminus of the PIX binding domain (Zhao *et al.*, 2000a). Binding of PIX recruits Pak to sites of focal adhesion through an interaction with Paxillin kinase linker (PKL) (Brown *et al.*, 2002) and this interaction will be discussed in more detail later in this Chapter.

The C-terminal kinase domain is highly conserved and is autophosphorylated in response to activated Rac/Cdc42 binding or lipid interaction (Bokoch *et al.*, 1998; Lei *et al.*, 2000). In this state the kinase domain can activate a series of downstream effectors including: Raf1, which allows its maximal activation by Ras (King *et al.*, 1998), and MEK1, allowing stable interaction of MEK1 and Raf1 (Frost *et al.*, 1997). Both Raf and MEK are upstream components of the MAPK cell proliferation signalling

pathway, therefore Pak activity can trigger this pathway. Phosphorylation of other proteins by the Pak kinase domain also has inputs on a number of other pathways.

The mitogen-activated protein kinase (MAPK) cascade is activated by a wide variety of extracellular signals and is an essential component of pathways that regulate cell division, motility and differentiation. It has been shown that activation of the MAPK cascade by soluble factors is inhibited in non-adherent cells (Howe and Juliano, 2000b). Expression of a constitutively active Pak mutant stimulates anchorage-independent cell growth through the MAPK pathway (Vadlamudi *et al.*, 2000). Inhibition of the cAMP-dependent protein kinase A (PKA) also permits anchorage-independent stimulation of the MAPK pathway via Pak. In non-adherent cells PKA inhibits Pak and activation of the MAPK pathway resulting in inhibition of cell cycle progression (Howe and Juliano, 2000a).

Members of the Pak family also have been shown to act in cell survival pathways to either promote or inhibit apoptosis. Pak1 is able to phosphorylate the death agonist Bad, a member of the Bcl-2 family of proteins that augment cell death (Schurmann *et al.*, 2000). This phosphorylation alleviates Bad inhibition of the cell survival signals Bcl2 and Bcl_{XL} and prevents activation of downstream members of the apoptotic pathway (Schurmann *et al.*, 2000). Promotion of cell survival has also been demonstrated by the Group II Pak, Pak4 (Gnesutta *et al.*, 2001).

An opposing role has been described for Pak2, which, can generate the morphological changes associated with apoptosis including the formation of apoptotic bodies. These changes are induced by a constitutively active kinase fragment generated from caspase-mediated cleavage of Pak2 (Rudel and Bokoch, 1997). However expression of a constitutively active form of Pak2 increases phosphorylation of Bad and protects cells from cell death induced by ectopic expression of Bad (Jakobi *et al.*, 2001). Therefore Pak2 can act as both a pro- and anti-apoptotic factor depending on the mode of activation (Kumar and Vadlamudi, 2002).

The binding site for the $G\beta\gamma$ subunit, located at the extreme C-terminus of Pak was initially characterised in the yeast homologue of Pak, Ste20 (Leeuw *et al.*, 1998).

Studies in neutrophils have shown that signalling through G-protein coupled receptors and activation of Pak is important for regulating the actinomyosin cytoskeleton (Xu *et al.*, 2003) and recent studies are beginning to establish functional roles for the action of vertebrate Paks in the G-protein signalling pathway (Li *et al.*, 2003).

1.4.3 Pak regulates the cytoskeletal changes required for cell migration.

One of the foremost roles for the Pak family of proteins is the regulation of cell migration. As we have already discussed, cell migration requires polarised rearrangement of the actin/myosin cytoskeleton. The actin cytoskeleton is highly dynamic and the rates of polymerization and depolymerization are important determinants of cell motility and the formation of specialized structures. Actin polymerization drives formation and extension of lamellipodia at the leading edge of motile cells, while the actin-based molecular motor myosin provides the traction forces necessary for cell movement and directs contraction at the rear of migratory cells (Edwards *et al.*, 1999).

Initial evidence that Pak regulates the cellular cytoskeleton came from studies in Swiss 3T3 fibroblasts. Microinjection of certain activated mutants of Pak into these cells resulted in formation of polarised lamellipodia and membrane ruffles like those induced by Rac and Cdc42 (Sells *et al.*, 1997). Pak has also been demonstrated to be colocalised with the filamentous actin at sites of membrane ruffles (Dharmawardhane *et al.*, 1997). The chemotactic ability of *Dictyostelium* cells was used to examine the roles of the Rac effector PAKa in regulation of the assembly of F-actin in cell movement. Wild-type chemotaxing *Dictyostelium* cells are polarised with a leading edge enriched in F-actin. Activation of F-actin assembly and pseudopod extension is regulated by Rac1B which has been shown to bind PAKa, implicating Pak in regulation of the actin cytoskeleton (Chung *et al.*, 2000).

In fission yeast the Pak homologue, Shk1 (Ste-20 homologous kinase 1), is essential for cell viability acting as the effector of Cdc42. Shk1 is targeted to sites of

actin deposition and in the absence of Shk1 there are defects in localisation of F-actin, disruption of the actin cytoskeleton and loss of morphological polarity (Kim *et al.*, 2001). In budding yeast the Pak-like kinase Cla4 is required for effecting Cdc42-induced polarised bud growth, acting in two ways to promote actin polymerisation. Cla4 binds the formin Bni1, which acts as a general regulator of actin polymerisation, as well as acting in a positive feedback loop by activating Cdc42 (Gulli *et al.*, 2000). In mammalian cells actin polymerisation can be regulated through the kinase activity of Pak. Active Pak phosphorylates and thereby activates LIM kinase resulting in inhibition of Cofilin-induced disassembly of actin filaments (Edwards *et al.*, 1999).

Pak-mediated polarisation of the actin cytoskeleton also plays a crucial role in growth cone guidance and neurite outgrowth. The *Drosophila* homologue of Pak is important for regulating axon guidance in the eye and the loss-of-function of DPak results in photoreceptor cell axon projection defects. DPak binds to Dock, the *Drosophila* homologue of Nck, and recruits DPak to the plasma membranes of growth cones. The Dock/Pak complex is then thought to transduce signals from the guidance receptors to control directional movement of growth cones by modulation of the actin cytoskeleton (Hing *et al.*, 1999). Pak proteins have also been shown to play a role in nerve growth in cell culture as membrane targeted Pak1 induces neurite outgrowth from PC12 cells (Daniels *et al.*, 1998).

In addition to the regulation of actin, Pak can also regulate myosin and contraction of the actinomyosin cytoskeleton. Pak proteins have been shown to phosphorylate and activate myosin molecules (Chew *et al.*, 1998). In endothelial cells Pak2 has been shown to phosphorylates myosin II regulatory light chain at serine^{19} in a Ca independent manner and this induces cell retraction of endothelial cells (Zeng *et al.*, 2000). In mammalian fibroblasts overexpression of Pak1 was accompanied by increased MLC phosphorylation (Sells *et al.*, 1999). Further more, the *Drosophila* Pak homologue, DPak, phosphorylates the regulatory light chain of *Drosophila* non-muscle myosin II and activates myosin II function and activated DPak induces increased levels of phosphorylated light chain (Crawford *et al.*, 2001).

Intriguingly, the converse has also been demonstrated, in that Pak can phosphorylate and inactive MLCK preventing MLCK-mediated phosphorylation of myosin regulatory light chains (Goeckeler *et al.*, 2000; Sanders *et al.*, 1999). In *Escherichia coli* Pak was found to inhibit activation of MLCK and phosphorylation of MLC (Rudrabhatla *et al.*, 2003) and in mammalian kidney cells where Pak phosphorylated MLCK leading to decreased MLCK activity and decreased MLC phosphorylation (Sanders *et al.*, 1999).

In *Dictyostelium* PAKa colocalizes with myosin II to the posterior of polarized, chemotaxing cells via its N-terminal domain and is required for maintaining directional cell movement, suppressing lateral pseudopod extension, and proper retraction (Chung and Firtel, 1999). This requirement for *Dictyostelium* PAKa results from the ability of PAKa to maintain myosin filaments. PAKa functions to prevent the phosphorylation of the tail domain of myosin molecules, thereby preventing disassembly of myosin filaments (de la Roche and Cote, 2001). These studies show that Pak can act on the actin/myosin cytoskeleton in a number of ways to regulate many aspects of cell migration in a number of tissue types.

1.4.4 Pak regulates cell adhesion.

In addition to regulation of the actin/myosin cytoskeleton, cell migration requires dynamic assembly and disassembly of adhesions between cells and the ECM. The physical link between the ECM and the actin cytoskeleton is formed by the action of a cluster of adaptor and signalling proteins, known as focal adhesions (Wehrle-Haller and Imhof, 2002). During cell migration nascent focal adhesions within protrusions of the cell membrane make the initial exploratory contacts with the cellular environment. Mature focal adhesions form at this leading edge and provide the necessary traction force to pull the cell forward while focal adhesions at the rear of the cell are released allowing the cell to move forward (Wehrle-Haller and Imhof, 2002).

Evidence for the involvement of Pak in the regulation of focal adhesion cycling during cell migration stems from the observation that Pak is localised to sites of focal adhesion. As described earlier, the third proline-rich motif of the Pak regulatory domain binds the Rac/Cdc42 guanine nucleotide exchange factor PIX. As well as initiating a positive feedback loop to promote GTP binding to Rac and Cdc42, PIX acts to target Pak to focal adhesions. This is achieved through a multiple step process where PIX binds the Paxillin kinase linker protein (PKL or $p95^{Pkl}$) which then binds Paxillin, a component of focal adhesion complex (Brown *et al.*, 2002). The adaptor protein Nck may also play a role in recruiting PAK to focal adhesions as well as to the plasma membrane (Bokoch *et al.*, 1996; Galisteo *et al.*, 1996). Recruitment of Pak/PIX to sites of focal adhesion also has an important influence on regulation of the actin cytoskeleton. PIX can induce membrane ruffling when associated with activation of Rac1 by the PIX/PAK complex this suggests a role for PIX in Cdc42 to Rac1 signalling and regulation of the cytoskeleton (Manser *et al.*, 1998).

It is clear that Pak is part of the multi-protein focal adhesion complex, however, the precise role of Pak at focal adhesions is less clear. In *Drosophila* DPak dependent focal complexes are found at the leading edge of cells undergoing dorsal closure where Pak is thought to drive the morphological changes required for dorsal closure (Harden *et al.*, 1996). Cell culture assays have shown that dominant negative mutations of Pak result in an increase in focal adhesions. Intriguingly a gain or loss of focal adhesions is observed in cells expressing constitutively active Pak1 (^{CA}Pak) depending on cell-type (Kiosses *et al.*, 1999; Manser *et al.*, 1997). In endothelial cells expression of ^{CA}Pak1 results in an increase of focal adhesions (Kiosses *et al.*, 1999) where as in epithelial cells expression of ^{CA}Pak leads to a dramatic decrease in focal adhesions (Manser *et al.*, 1997). These observations suggest that Pak acts in a highly regulated manner to regulate turnover of focal adhesions.

In migratory cells it has been proposed that Pak functions to disassemble focal adhesions at the trailing edge while forming peripheral Rac/Cdc42 adhesions at the leading edge, thereby promoting cell migration (Kiosses *et al.*, 1999). Evidence for

Pak influence of dissociation of focal adhesions results from PIX-induced coupling of Pak activity to the G-protein-coupled receptor kinase-interacting protein known as GIT1 (Zhao *et al.*, 2000b). Overexpression of GIT1 in fibroblasts or epithelial cells causes a loss of Paxillin from focal adhesions and stimulates cell motility. Additionally, GIT directly couples to FAK and together GIT1 and FAK cooperate to promote motility both by directly regulating focal adhesion dynamics and by the activation of Rac (Zhao *et al.*, 2000b).

While the major adhesion mechanism for controlling migration of an individual cell is clearly the adhesive interaction between the cell and the matrix over which it moves, the migration of a group of cells, such as the lateral line primordium, is more complex. We have previously described that a morphological prepattern can be observed within the lateral line primordium and that this is maintained during migration. In order for cells to migrate as a group while preserving an organised pattern, regulated cell to cell adhesion is required. One of the most important and ubiquitous types of adhesive interactions required for the maintenance of tissue architecture is the cadherin-mediated cell to cell adhesions (Gumbiner, 1996). The most well studied of these adhesions are those found in epithelial cells that are mediated by E-cadherin (Gumbiner, 1996). Clustering of the transmembrane E-cadherin molecules triggers an association with the actin cytoskeleton that stabilises the adhesion (Jamora and Fuchs, 2002). E-cadherin is linked to the cytoskeleton by the binding of β-catenin which in turn binds α-catenin, which, either binds to actin directly or indirectly through association with actin binding proteins such as Vinculin (Pokutta and Weis, 2002). This cadherin/catenin/actin complex is known as the adherens junction (Braga, 2002).

Evidence is emerging for the involvement of Pak in the cadherin-mediated adherens junctions. In *Drosophila* it has been shown that the mushroom-bodies-tiny (Mbt) protein, a homologue of the Group II family of Pak proteins, can be recruited to adherens junctions (Schneeberger and Raabe, 2003). In addition, one of the upstream activators of Pak, Rac, is recruited to cadherin-mediated adhesion sites and Rac has

been shown to regulate these adhesions. Expression of a constitutively active Rac mutant results in a greater accumulation of E-cadherin and β-catenin at sites of cell contact and an inactive Rac mutant inhibits this accumulation (Nakagawa *et al.*, 2001). The function of Rac in regulating cadherin-mediated cell adhesions relates to their dynamic rearrangement during cell movements. E-cadherin can be tethered to either the actin cytoskeleton, via α-catenin, or to the small GTPase effector IQGAP (Fukata and Kaibuchi, 2001). Activated Rac (and Cdc42) positively regulate cadherin-mediated cell adhesion by inhibiting the interaction of IQGAP with β-catenin (Fukata and Kaibuchi, 2001). As of yet it is unclear whether this regulation involves Pak, although, Pak has been demonstrated to function as an upstream activator of Rac in addition to its downstream effector role (Obermeier *et al.*, 1998).

The fate of E-cadherin once it is delivered to the basolateral cell surface, and the mechanisms which govern its participation in adherens junctions, are not well understood. Surface biotinylation and recycling assays have shown that cell surface E-cadherin is actively internalized and is then recycled back to the plasma membrane. This indicates that a pool of surface E-cadherin is constantly trafficked through an endocytic recycling pathway and that this may provide a mechanism for regulating the availability of E-cadherin for junction formation in development (Le *et al.*, 1999). Rac-mediated regulation of the actinomyosin cytoskeleton is required for formation of endocytic vesicle formation. Rac is also required for the formation of pinocytic vesicles and Pak localises to sites of pinocytic vesicles, suggesting that Pak mediates the effects of Rac on the cytoskeleton required for vesicle formation (Dharmawardhane *et al.*, 1997). Pak can also regulate endocytosis through activation of the unconventional myosins (Yamashita and May, 1998) and inhibition of Pak activity blocks endocytic pathways (Buss *et al.*, 1998; Dharmawardhane *et al.*, 2000).

1.4.5 Pak is a component of the heterotrimeric G-protein signalling pathway.

In addition to the many activities of Pak mediated through the N-terminal regulatory and C-terminal kinase domains are those mediated by the Gβγ binding site in the non-catalytic carboxy-terminus. In the budding yeast homologue of Pak, Ste20, this binding site is involved in transmitting the mating-pheromone signal from the βγ-subunits of a heterotrimeric G-protein to a downstream kinase cascade (Leeuw *et al.*, 1998). In mammalian tissues G-protein-coupled receptors have a potent ability to stimulate Pak activity (Daniels and Bokoch, 1999). It has also been shown that Pak1 can phosphorylate the unique Gα subunit $G\alpha_z$ decreasing its affinity for Gβγ subunits and sustaining activity of $G\alpha_z$ (Fan *et al.*, 2000). The role of this Pak1-induced prolonged activation is not known, but may be related to cellular differentiation and transformation events as active $G\alpha_z$ can inhibit adenylyl cyclase and the production of cAMP (Ho and Wong, 2001). The activity of the cAMP-dependent protein kinase A (PKA) contributes to anchorage-dependant signalling. PKA can inhibit Pak in non-adherent cells and prevent Pak from activating the MAPK pathway, resulting in inhibition of cell cycle progression (Howe and Juliano, 2000a). In addition, in vertebrates the active $G\alpha_z$ can inhibit Eya activity, a component of the *Pax-Six-Eya-Dach* regulatory network which is an important inducer of myogenic genes and myogenesis (Kawakami *et al.*, 2000). Eya is an intrinsically cytosolic protein and is translocated into the nucleus by interaction with Six, whereupon transcription of downstream genes is activated. Association of Eya with Six and translocation to the nucleus is inhibited by activated $G\alpha_z$ (Fan *et al.*, 2000). Therefore Pak-induced signalling through G-protein subunits may also regulate gene transcription.

Of particular interest, in terms of a possible role for Pak in regulating migration of the posterior lateral line primordium, is the recent work revealing that directional sensing requires Gβγ-mediated activation of Pak1 (Li *et al.*, 2003). Pak is known to be important for chemotaxis-induced cell migration from studies in *Dictyostelium* where PAKa, is required for maintaining directed cell migration, suppressing lateral pseudopod extension, and retraction of the posterior of chemotaxing cells (Chung and

Firtel, 1999). Studies using smooth muscle tracheal cells have shown that Pak activity is also required for PDGF-induced chemotactic cell migration (Dechert *et al.*, 2001) and that stimulation of neutrophils with chemoattractants results in rapid activation of Paks (Huang *et al.*, 1998). Dissection of this pathway by Li *et al* has revealed that chemoattractant bound receptors activate heterotrimeric G-proteins and release Gβγ proteins that recruit Pak1 via a direct interaction. PIXα is also recruited by its association with Pak in order to activate Cdc42 at the membrane. The active Cdc42 then activates Pak and mediates the downstream cytoskeletal changes required for the directional sensing, cell polarisation and persistent directional migration (Li *et al.*, 2003). Studies in chemotaxis-activated neutrophils have demonstrated that Pak is colocalised with the F-actin found at sites of membrane ruffling and lamellipodia formation, indicating that the role of Pak in mediating chemotactic response is to reorganise the actin cytoskeleton (Dharmawardhane *et al.*, 1999). This is similar to studies in neutrophils revealing that activation of G-protein associated receptors leads to release of G-subunits that play specific and opposing roles in inducing actin filament extension at the leading edge and retraction of actinomyosin filaments at the training end (Xu *et al.*, 2003). It appears likely that this pathway will also involve Pak.

1.4.6 Negative regulators of Pak activation.

The apparent multiplicity of Pak structure and Pak-mediated signalling pathways suggest that Pak activity must be tightly regulated. As Pak activation is adhesion-dependant loss of cell adhesion can lead to inactivation of Pak (del Pozo *et al.*, 2000). Additionally, specific proteins with an inhibitory action on Pak are being identified. In the fission yeast, *Schizosaccharomyces pombe*, Skb15 (Shk1 kinase binding protein 15), which shares homology with the Gβ-subunit related WD repeat (tryptophan-aspartate repeat) proteins, functions as an inhibitor of Shk1, the fission yeast homologue of Pak, by negatively regulating kinase activity (Kim *et al.*, 2001). Loss of Skb15 is lethal and results in severe deregulation of the actin and microtubule

cytoskeletons (Kim *et al.*, 2001). A homologous protein in mammalian cells, hPIP (human Pak/PLC-interacting protein 1) interacts with the N-terminal domain of Pak1 and may act to stabilise Pak protein in the inactive dimeric conformation (Xia *et al.*, 2001).

As Pak activation requires phosphorylation of serine and theronine residues throughout the protein it is intuitive that dephosphorylation would lead to inactivation. Two serine/theronine phosphatases have been identified that efficiently inactivate Pak, POPX1 and POPX2 (Partner Of PIX) (Koh *et al.*, 2002). POPX phosphatases form a trimeric complex of POPX-PIX-Pak and act directly on autophosphorylated Pak to block the phenotypic effects of Pak (Koh *et al.*, 2002).

1.5 Approaches.

The aim of the work described in this thesis was to understand the molecular mechanisms that regulate cell migration during embryonic development using the formation of the zebrafish lateral line as the model. We set out to analyse in detail the behaviour of the cells within the lateral line primordium during development of the posterior lateral line system. The possible role of Pak in lateral line development will be studied in loss of function analyses. Observation of cell behaviour, both in normal development and following loss of Pak function, will help to elucidate possible mechanisms through which zebrafish Pak proteins may regulate cell migration. Biochemical approaches will also be used to study Pak protein interactions and analyse the effects of loss of function.

Chapter Two

Materials and Methods

2.1 Zebrafish embryo collection and staging.

2.1.1 Zebrafish embryo collection.

Zebrafish (*Danio rerio*) embryos were produced by natural mating and raised in fish water (0.03g/L red sea salt, 2mg/L methylene blue) or 0.3x Danieau solution (1x Danieau solution: 58mM NaCl, 0.7mM KCl, 0.4mM $MgSO_4$, 0.6mM $Ca(NO_3)_2$, 5mM HEPES, pH7.6). Embryos were generally raised at 28°C, however, temperatures ranging between 18°C and 30°C were used.

2.1.2 Embryo staging.

Embryos were staged in accordance with the criteria provided in (Kimmel *et al.*, 1995). Stages are stated according to morphological feature or in hours-post-fertilisation (hpf) or days-post-fertilisation (dpf).

2.2 DNA Techniques.

2.2.1 Preparation of fresh competent cells.

DH5α cells were used to inoculate 1ml of 2xTY broth (16g Difco Tryptone, 10g Yeast Extract, 5g NaCl in 1L distilled water, dH_20) and incubated overnight at 37°C at 250 revolutions per minute (rpm) in an orbital shaker. From this culture 0.5ml was used to inoculate 100ml 2xTY and incubated until an optical density (OD_{595}) of 0.34

was reached. Cells were pelleted by centrifugation at 3,000rpm for 5 minutes. The pellet was resuspended in 17ml of Hanrahans Buffer (HBT: 10mM $CaCl_2.6H_20$, 45mM $MnCl_2$, 100mM RbCl, 3mM Hexamino-III-colbalt chloride, 10mM MES pH6.3) and incubated on ice for 30 minutes. Cells were pelleted again by centrifugation at 3,000rpm for 10 minutes. Supernatant was removed and replaced with 4ml HBT and 140µl Dimethylformamide (DMF) and kept on ice for 5 minutes, then 140µl of 40mM Dithiothreitol (DTT) was added and left for 10 minutes. A further 140µl DMF was added and after 5 minutes the cells were ready to use.

2.2.2 Transformation of competent cells.

Fresh competent cells were used for "blunt end" ligations. For routine transformations and "sticky end" ligations stocks of competent cells stored at -80°C were used.

DH5α competent cells were dispensed (50µl) into pre-chilled 1.5ml Eppendorf tubes to which 1-5µl of DNA solution was added and mixed gently. Cells were incubated on ice for 20 minutes and heat shocked for two minutes at 42°C. For transformation 500µl (200µl for ligations) of Luria-Bertani (LB) broth was added and the cells were allowed to recover at 37°C for 20 minutes. Fifty to 100µl of cells were plated onto LB-agar plates containing an appropriate antibiotic (ampicillin; amp or kanamycin; kan). Bacterial plates were incubated upside down at 37°C overnight.

2.2.3 Small scale preparation of DNA.

Overnight cultures of 3ml LB broth, grown from single colonies and containing the appropriate antibiotic, were pelleted in 1.5ml Eppendorf tubes by centrifugation at 12,000rpm for 1 minute. Supernatant was removed and preparation of DNA was carried out using the QIAprep Spin Mini-prep Kit (Qiagen) following manufacturer's protocol. DNA was generally eluted with 30µl EB buffer (Qiagen) and stored at -20°C.

2.2.4 Large scale preparation of DNA.

Overnight cultures of 100ml LB or 2xTY broth containing the appropriate antibiotic were pelleted in 50ml Falcon tubes by centrifugation at 3,000rpm for 15 minutes. Supernatant was removed and preparation of DNA was carried out using the Plasmid Maxi Kit (Qiagen) following manufacturer's protocol. DNA pellets were generally resuspended in 200-400µl sterile low TE (0.1mM Ethlenediaminetetraacetate (EDTA), 10mM Tris) and stored at -20°C.

DNA and RNA were quantified by spectrophotometry at 260nm; an OD reading of 1 equates to 50µg/ml double stranded DNA, 40µg/ml of RNA or 35µg/ml single stranded DNA. The ratio of readings at 260nm and 280nm provided an estimate of purity of the preparation; pure preparations achieve a ratio of 1.8 for DNA and 2.0 for RNA.

2.2.5 Preparation of DNA fragments for sub-cloning.

Plasmid DNA was cut using restriction enzymes according to the manufacturer's instructions (Roche, New England Biolabs) using the appropriate supplied buffer. Enzyme volumes never exceeded 10% of the total reaction volume. Approximately 100ng was run on a 1% agarose gel to confirm completion of enzymatic digest.

To remove proteins from nucleic acid solutions an equal volume of Tris-saturated phenol was added and the sample was vortexed thoroughly and centrifugated at 13,000rpm for 2 minutes. The aqueous phase was removed to a clean tube and extracted with an equal volume of chloroform by vortexing and centrifugation at 13,000rpm for 2 minutes. The aqueous phase was again removed to a clean tube for ethanol precipitation.

DNA was precipitated by adding 3M NaOAc (pH5.5) to a final concentration of 0.3M and 2.5 volumes of 100% ethanol, the reaction was then left at -20°C to -80°C for 1 hour or longer. DNA was pelleted by centrifugation at 13,000rpm for 10 minutes.

The pellet was washed in 70% ethanol, air dried and resuspended in low TE or DNase- and RNase-free water (Sigma).

2.3 Generation of Constructs.

Three types of construct were used to analyse zebrafish Pak function: yellow fluorescent protein tagged full length Pak2a and Pak2b constructs (Pak2a^{FL}YFP and Pak2b^{FL}YFP), yellow fluorescent protein tagged truncated Pak2a and Pak2b constructs (Pak2a$^{\Delta c}$YFP and Pak2b$^{\Delta c}$YFP) and C-terminal truncated Pak2a and Pak2b GST fusion proteins (Pak2a$^{\Delta c}$GST and Pak2b$^{\Delta c}$GST). Fluorescently tagged constructs were designed for *in-vivo* injection of RNA and the GST fusion proteins were used to screen for zebrafish Pak2a and Pak2b specific antibodies (carried out by Lynne Fairclough, Division of Developmental Biology, NIMR) and protein pull down assays. Constructs were created by amplification of cDNA using primers containing restriction sites and ligation into the relevant vectors. All constructs were sequenced to ensure no mutations were generated by PCR.

2.3.1 Incorporation of a restriction enzyme site using PCR.

PCR primers (Oswell) were designed by hand to amplify the desired DNA sequence and incorporate a restriction enzyme site. Care was taken to avoid high G/C content, hairpin, palindromic or repetitive sequences. Sequences were generally 33 base pairs (bp), including a 21bp gene specific sequence, 6bp corresponding to the 6-cutter restriction enzyme site and 6bp flanking the restriction site to aid enzymatic digestion.

Primer stocks were diluted to 100μg/ml before use. Annealing temperatures were calculated by the average T_m for a primer pair minus 5°C (T_m data provided by Oswell). The annealing temperature was reduced by a further 2-8°C to account for the restriction enzyme site and flanking sequence which will not anneal. PCR reaction

solution contained: 1µl 10mM dNTP mix, 5µl Pfu buffer (Stratagene), 2µl Pfu polymerase (Stratagene), 50ng DNA, 2µl primer 1, 2µl primer 2 and RNase-/DNase-free water to 50µl. The PCR cycle was 3 minutes at 95°C followed by 30 cycles of 95°C for 1 minute, 48-60°C for 30 seconds and 72°C for 4 minutes and final elongation was at 72°C for 10 minutes.

2.3.2 Gel purification of DNA.

If required, following restriction digest or PCR, DNA fragments were gel purified. Samples of up to 50µl were run on a 1% agarose gel with 1xTBE (0.89M Tris Borate pH8.3 and 20mM Na_2EDTA concentrate; National Diagnostics) diluted 1:10 with distilled water (dH_20) containing Ethidium Bromide (EtBr). DNA bands were cut out from the gel and purified using the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's protocol. Samples were eluted using 30µl EB buffer (Qiagen).

2.3.3 Ligation of DNA fragments.

DNA fragments were produced and host vectors prepared by restriction enzyme digestion. If required 5' overhangs were end-filled with Klenow polymerase (Promega) prior to ligation. To a 50µl restriction digest, 1µl polymerase buffer (Promega), 1µl Klenow polymerase, 2µl 2mM dNTP and 6µl RNase-/DNase-free water were added and incubated at 37°C for 30 minutes. DNA fragments were purified using S300HR columns (Amersham) or by gel extraction (section 2.3.2). DNA fragments were ligated into linear vectors using the Rapid DNA Ligation Kit (Roche) according to manufacturers instructions. A 1:3 ratio of vector to insert was used.

2.3.4 Generation of full length YFP tagged Pak constructs.

Full length Pak2a and Pak2b YFP constructs were generated by amplification of the full length coding region including the 5' untranslated region against which the

morpholino sequences were designed. Primers against *pak2a* were designed to incorporate Bgl II and Sal I restriction sites at the 5' and 3' termini respectively (underlined):

5' end: 5' GGA CTC AGA TCT ACC CTT CTG AGT GTG TTT CCC 3'

3' end: 5' GGT ACC GTC GAC TGG TAA CGG TTA CTC TTC ATT GC 3'.

Primers against *pak2b* were designed to incorporate EcoRI and BamHI restriction sites at the 5' and 3' termini respectively (underlined):

5' end: 5' AGC TTC GAA TTC TGT CTC CTC ATC CTG ATC ATG 3'

3' end: 5' ACC GGT GGA TCC GAG TAG CGG TTG TTC TTC ATG GC 3'.

PCR products were gel purified and ligated into pEYFP-N1 (Clontech) at the corresponding restriction sites, before the YFP sequence. The Pak-YFP fusion sequences were then excised from pEYFP-N1 by digestion with Bgl I (Pak2a) or BamHI (Pak2b) and NotI and were sub-cloned into the BamHI and XhoI sites of pCS2+ following blunting of the NotI and XhoI sites using Klenow DNA polymerase (Promega).

2.3.5 Generation of dominant negative Pak constructs.

Dominant negative Pak proteins were generated by removal of the majority of the C-terminal kinase domain and comprised amino acids 1-241 and 1-263 of Pak2a and Pak2b respectively. Primers were designed to truncate Pak2a and Pak2b 43 amino acids into the N-terminus of the kinase domain. Primers at the 5' terminus incorporated an EcoRI restriction site (underlined) adjacent to the start codon:

Pak2a 5' AGC TTC GAA TTC ACC ATG TCT GAC AAC GGC GCG CTG 3'

Pak2b 5' AGC TTC GAA TTC ACC ATG TGT GAT AAT GGC GAT GTG 3'.

The 3' primer was designed against a homologous region of the kinase domain of Pak2a and Pak2b (one amino acid difference, therefore, 50% of primers were designed to have T and 50% C at position Y) and incorporated a BamHI restriction site (underlined).

5' GAC CGG TGG ATC CCG GAT GAT CAG CTC TTT YTT GGG CTG 3'.

PCR products were gel purified and ligated into pEYFP-N1 (Clontech) at the corresponding restriction sites, before the YFP sequence. The Pak$^{\Delta C}$YFP fusion sequences were then excised from pEYFP-N1 by digestion with EcoRI and NotI. Pak$^{\Delta C}$YFP fusion sequences were sub-cloned into the EcoRI and XhoI sites of pCS2+ following blunting of the NotI and XhoI sites using Klenow DNA polymerase (Promega).

2.3.6 Generation of GST-Pak fusion protein constructs.

Glutathione-S-transferase (GST) tagged Pak proteins were designed to find proteins or antibodies that only interact with the N-terminal regulatory domain. PCR primers were designed to truncate the Pak proteins N-terminal to the kinase domain at aa236 of Pak2a and aa259 of Pak2b.

Primers at the 5' terminus incorporated a BamHI restriction site (underlined) adjacent to the start codon:

Pak2a 5' CCG CGT GGA TCC ATG TCT GAC AAC GGA GAG CTG 3'

Pak2b 5' CCG CGT GGA TCC GTC TGT GAT AAT GGC GAT GTG 3'

Primers at the N-terminal to the kinase domain incorporated an EcoRI restriction site (underlined):

Pak2a 5' CAC GAT GAA TTC TCC AAT ACT GAC AAT GGT TCT 3'

Pak2b 5' CAC GAT GAA TCC GTC TCC AAT GCT GAC TCT GGT 3'

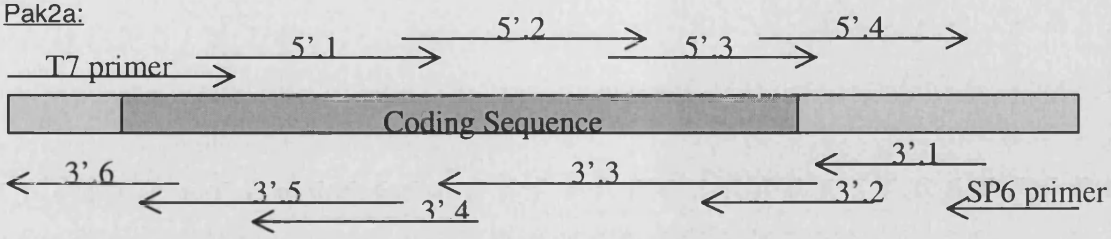
PCR products were gel purified and ligated into pGEX6.1 (Amersham) at the corresponding restriction sites, following the GST sequence.

2.4 DNA sequencing.

Zebrafish *pak2a* and *pak2b* cDNAs in pSPORT1 were obtained from the expressed sequence tagged (EST) sequences. Initial sequencing was carried out

using standard pSPORT T7 and SP6 sequencing primers and the sequences obtained were used to design further, sequence specific, primers as listed below.

Pak2a:



T7: 5' GTAATACGACTCACTATAGGGC **SP6:** 5' GAATTTAGGTGACACTATAGA

5'.1: 5' GCTCTAAACCCCTGCCCTCCG **3'.1:** 5' CCGGTGTTCTGTCAGATTGTT

5'.2: 5' GCCATTGCAGACACAGATGGC **3'.2:** 5' GGAGAGACATCAGTAGAAACG

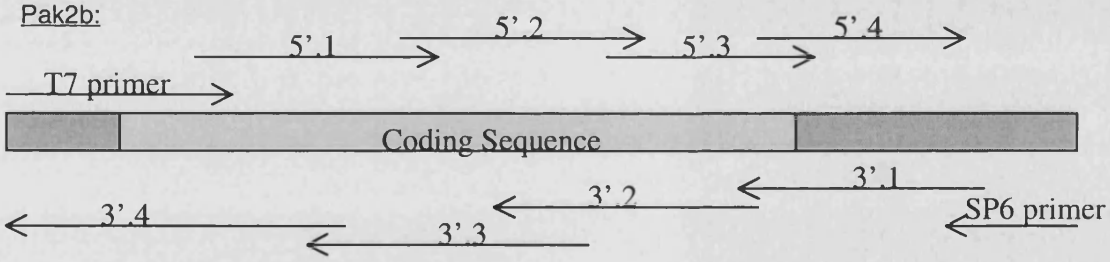
5'.3: 5' GCTCAAATTGCTGCTGGCTGC **3'.3:** 5' GGCAAGACACGGTGATTGTGC

5'.4: 5' GCTGCCTGGAGATGGATGTGG **3'.4:** 5' GCTCATCTCCAACCAAGAAGC

3'.5: 5' GCTTAATAGCAACCTCTTGGC

3'.6': 5' CCCACATGTATAGTGTGCTCG

Pak2b:



T7: 5' GTAATACGACTCACTATAGGGC **SP6:** 5' GAATTTAGGTGACACTATAGA

5'.1: 5' GCGCAAGCGCAACAAGATCTA **3'.1:** 5' GAATAACAACAAAGGCACGAG

5'.2: 5' GCCGTCGTCTCCAGTCAAAGC **3'.2:** 5' GGAAGTCTCGGAAGATTGGCG

5'.3: 5' CCATCAAACAGATCAACCTGC **3'.3:** 5' CGATCTGAGCTTCATCCATGC

5'.4: 5' GCAACCAATGGCACTCCAGAG **3'.4:** 5' CGCTCTTAGTGTGTTCAGGCC

2.4.1 Cycle sequencing PCR.

Cycle sequencing PCR was performed using the ABI PRISM Big Dye Terminator Kit (Perkin Elmer) according to manufacturer's instructions. Briefly, 0.5μg DNA was combined with 2μl Big Dye, 3μl 5x PCR buffer (1M KCl, 1M Tris-HCl pH8.4, 1M $MgCl_2$, 100mM dATP, 100mM dTTP, 100mM dGTP, 100mM dCTP, 0.8μg/ml BSA), 5μl Primer 1, 5μl Primer 2 and RNase-/DNase-free water to 20μl, in a 0.5ml thin-walled bubble-cap tube. Annealing temperatures were calculated as described in section 2.3.1. PCR was cycled 25 times at 96°C for 30 seconds, 45°C for 15 seconds and 60°C for 4 minutes.

2.4.2 Automatic sequencing.

PCR samples were prepared for sequencing by adding 2μl 3M NaOAc pH5.5, 1μl glycogen and 58μl ethanol. Samples were placed on ice for 15 minutes and then centrifugated at 13,000rpm for 30 minutes. The pellet was washed in 70% ethanol with brief vortexing. Samples were pelleted again with centrifugation for 15 minutes. All ethanol was removed and pellets were left to air dry. Once dry, pellets were resuspended in 4μl ABI dye (Perkin Elmer) and denatured at 95°C for 3 minutes. On an acrylamide gel (18g Urea, 5ml 10x TBE, 5ml Long Ranger acrylamide, 250μl 10% ammonium persulphate, 25μl TEMED in 50ml dH_2O) 2μl of sample was loaded. Samples were run on the ABI Prism 377 DNA sequencer (Perkin Elmer).

2.4.3 Sequence analysis.

Sequences were manipulated using Sequencher (Gene Codes) and MacVector (Oxford Molecular Groups). Analysis of sequences was performed using BLAST (NCBI). Praline was used for protein sequence alignment (Heringa, 1999), along with CLUSTAL through MacVector.

The Sanger Centre zebrafish genome site was also used for sequence searches and comparisons (http://www.sanger.ac.uk/Projects/D_rerio/).

2.5 RNA techinques.

2.5.1 Preparation of total RNA from zebrafish embryos.

One hundred embryos at 24hpf were homogenised in 1ml TRIzol reagent (Life Technologies). Following a 5 minute incubation at room temperature 200µl of chloroform was added and the sample was shaken for 5 minutes before centrifugation at 12,000rpm for 20 minutes at 4 °C. The aqueous phase was transferred to a fresh tube and RNA was precipitated with 500µl isopropyl alcohol by incubation at room temperature for 10 minutes. After centrifugation at 12,000rpm for 15 minutes at 4 °C the supernatant was discarded and the pellet washed in 70% ethanol, air dried and resuspended in 20µl RNase-/DNase-free water. RNA was stored at -80°C.

2.5.2 Northern Blot analysis.

High stringency detection of RNA transcripts was achieved using specific DNA probes. DNA probes were radiolabelled using the New Megaprime Kit (Amersham) according to the manufacturer's protocol with some amendments. In brief, 0.5µg DNA was denatured in the presence of 5µl primer in a total volume of 10µl at 95-100°C for 5 minutes. At room temperature 10µl labelling buffer including dNTPs (Amersham), 2µl Klenow polymerase (Promega), and RNase-/DNase-free water to 45µl was added and the mixture briefly spun down to the bottom of the tube. Finally 5µl $dCTP^{32}$ was added and the reaction was incubated at 37°C for 10 minutes to 1 hour. The reaction was stopped by addition of 2µl 0.5M (EDTA). Unincorporated nucleotides were removed by filtration through S300HR columns (Amersham). Probes were denatured at 95-100°C for 5 minutes before use.

Total RNA was separated on a formaldehyde gel and transferred to a membrane as previously described (Sambrook *et al.*, 1989). Briefly, morpholinopropanesulphonic acid (MOPS) running buffer (10x MOPS; 0.2M MOPS pH7.0, 50mM NaAc, 1mM EDTA pH8.0) and a formaldehyde gel were prepared (1.5% Agarose, 1x MOPS, 2.2M formaldehyde in dH_2O). RNA samples were prepared for loading by mixing a maximum of 4.5µl (20µg) RNA with 2µl 10x MOPS, 3.5µl formaldehyde, 10µl formamide and 1µl EtBr (800µg/ml) and incubating at 55°C for 15 minutes. Samples were loaded with the addition of 2µl sterile loading buffer (50% glycerol, 1nM EDTA, 0.4% bromophenol blue and 0.4% xylene cyanol) and separated by electrophoresis. RNA was transferred from the gel to a nylon blotting membrane (Electran; BDH) overnight under alkaline conditions.

After RNA transfer the membrane was briefly washed in 5x Saline Sodium Citrate (SSC) and dried between blotting paper. The RNA was then cross-linked in a UV transluminator and the membrane baked at 80°C for 30 minutes before a 3 hour incubation at 50°C in pre-hybridisation buffer (5xSSC, 1/10 Denharts, 50% formamide, 0.1% Sodium Dodecyl Sulphate (SDS), 0.1% sodium phosphate and 100µg/ml sonicated and denatured salmon sperm DNA). P^{32} labelled DNA probes were denatured and added directly to the pre-hybridisation buffer and membranes were further incubated overnight at 68°C. Membranes were then washed for 20 minutes at room temperature in 1x SSC, 0.1% SDS, followed by three washes of 20 minutes each at 68°C in 0.2x SSC, 0.1% SDS. Binding of probes was visualised by exposing the membrane to OMAT X-ray film (Kodak).

2.5.3 Whole mount *in situ* hybridisation.

Linearised plasmids containing zebrafish cDNA were used as templates in the synthesis of dioxygenin (DIG) (Roche) labelled anti-sense probes. A table of probes and restriction digest sites for synthesis of anti-sense RNA can be found in Table 2.1. Following synthesis, template was removed by treatment with RNase-free DNase I

(Promega) and RNA was purified by filtration through S300HR columns (Amersham). Probe was then diluted in 100µl hybridisation buffer (50% formamide, 5x SSC pH6.0 adjusted with 1M citric acid, 50µg/ml yeast RNA, 50µg/ml heparin, 0.1% Triton-x-100) and stored at -20°C. Probe integrity and approximate concentration was determined by agarose gel electrophoresis.

In situ hybridisations were performed essentially as described (Xu and Wilkinson, 1998). However proteinase K (PK) treatment was not carried out on embryos as this damages superficial structures of the embryo including the lateral line. Probes were used at 0.5-10% of total hybridisation solution depending on expression levels. Following detection of probe embryos were rinsed in PBT (Phosphate buffered saline and 0.1% Triton-x-100) and refixed in 4% paraformaldehyde (PFA) in PBS for 30 minutes or longer at room temperature. Embryos were then stored in 70% glycerol/PBS at 4°C.

2.5.4 Preparation of capped RNA for microinjection.

In vitro transcription of capped RNA was carried out in a 50µl reaction volume; 5µl 10x transcription buffer (Roche), 5µl rNTPs (1mM GTP and 10mM ATP, CTP, UTP), 5µl 5mM m7G(ppp)G RNA cap structure analogue (New England Biolabs), 2µl RNasin (Promega), 2µl RNA polymerase (SP6; Roche, T7, T3; Promega), 2µg purified linear DNA and RNase-/DNase-free water to 50µl. The reaction was incubated at 37°C for 3 hours or longer. DNA template was removed with 1µl RNase-free DNase I (Promega) for 30 minutes at 37°C. RNA was purified by water-saturated phenol/chloroform extraction and filtration through S300HR columns (Amersham). Aliquots of RNA were stored at -80°C. For micro-injections 3nl RNA was used undiluted or diluted up to 1:5 and was injected at one-cell stage.

2.6 Protein analysis

2.6.1 *In vitro* translation.

Proteins were translated *in vitro* using the TNT Rabbit Reticulocyte Translation System (Promega). Translation was performed using the manufacturer's protocol. Briefly, 25µl TNT rabbit reticulocyte lysate was combined with 2µl reaction buffer, 1µl TNT RNA polymerase, 1µl amino acid mixture minus methionine, 0.04mCi ^{35}S methionine, 40 units RNase inhibitor, 1µg DNA template and RNase-/DNase-free water to 50µl. The reaction was incubated at 30°C for 2 hours. Translation products were separated by SDS-PAGE (see section 2.6.4). Protein gels were fixed for 10 minutes (45% methanol, 45% dH_2O and 10% acetic acid) and vacuum dried. Radiolabelled protein bands were detected using a phosphoimager or by exposure to Biomax film (Kodak).

2.6.2 Preparation of protein extracts from embryos.

Whole cell extracts were prepared from zebrafish embryos at stages 50% epiboly to 48hpf, in lysis buffer (1x cell lysis buffer; (Cell Signalling Technology), 1x protease inhibitor; (Roche) in dH_2O) using 10µl per embryo. For some experiments whole cell extracts were further fractionated by centrifugation at 12,000rpm for 10 minutes at 4°C, into a pellet containing proteins linked to the cytoskeleton and the supernatant. Extracts were stored at -80°C.

2.6.3 Preparation of GST-fusion protein.

Betty Bennett (Division of Developmental Biology, NIMR) carried out preparation of GST-fusion protein. In brief, 90ml of a 100ml overnight culture (grown in 2xTY containing chloramphenicol, (cpl) and amp) was added to a 1L medium (amp and cpl) and grown for 1 hour at 37°C. After 1 hour 1ml 1M Isopropyl β-D-thiogalactopyranoside (IPTG) was added and growth continued for 4 hours. Cells

were then pelleted by centrifugation at 3,000rpm for 15 minutes and washed with phosphate buffered saline (PBS). Pellets were resuspended in 80ml ice-cold MTPBS (150mM NaCl, 16mM Na_2HPO_4, 4mM NaH_2PO_4, pH7.3) containing proteinase inhibitors and 100µl lysozyme (50mg/ml). To the cell suspension, 48µl Phenylmethylsulfonyl Fluoride (PMSF; 10mg/ml in isopropanol) was added and incubated on ice for 15 minutes. Samples were sonicated (Roth Scientific, "Vibra-Cell") in 48µl 1M DTT and 2ml 10% Sarkosyl using large tips at 70% power for five 10 second bursts. Samples were then centrifugated at 16,000rpm for 20 minutes. Supernatants were saved and Triton-x-100 (Sigma) was added to a final concentration of 1%. To purify the GST-fusion protein 2ml 50% glutathione beads (Sigma) were added and the mixture was incubated at 4°C for 30 minutes. The beads were then pelleted at 1,000rpm for 5 minutes and washed with PBS twice. The GST-fusion protein was then eluted with 2ml glutathione elution buffer (5mM glutathione, 50mM Tris pH8) and dialysed at 4°C against 3L PBS, changing buffer once. The purified protein was mixed with an equal volume of glycerol and stored at -80°C. For pull down assays the GST-fusion protein was retained on the beads and stored at 4°C in the presence of 0.1% sodium azide.

2.6.4 Protein separation by sodium dodecyl sulphate polyacrylamide (SDS-PAGE) gel electrophoresis.

SDS-PAGE gels were prepared essential as described (Sambrook *et al.*, 1989). A 10-12% acrylamide resolving gel (13.3ml 30% (v/v) bis-acrylamide (37:5:1 acrylamide:bis; Bio-Rad), 10ml 1.5M Tris pH8.8, 200µl 20%SDS, 400µl 10% (w/v) ammonium persulphate (APS), 16µl NNN'N'-tetramethylethylenediamine (TEMED) and 16.1ml dH_2O) was poured and overlaid with isopropyl alcohol. Following polymerisation the alcohol was removed and replaced by a 5% acrylamide stacking gel (1.3ml 30% (v/v) bis-acrylamide (37:5:1 acrylamide:bis; Bio-Rad), 1ml 1M Tris

pH6.8, 40µl 20% SDS, 80µl 10% APS, 8µl TEMED and 5.5ml dH_2O) into which a comb was inserted to form sample wells.

Protein samples were prepared by addition of an equal volume of 2x sample loading buffer (100mM Tris.HCl, 200mM DTT, 4% SDS, 20% Glycerol, 0.2% Bromomethylblue, 20% β-mercapto-ethanol) and denatured by boiling for 5 minutes. Samples were then loaded into wells and elecrophoresed in Tris-glycine electrophoresis buffer (25mM Tris base, 250mM glycine, 0.1% SDS in dH_2O).

2.6.5 Western Blot analysis.

Following SDS-PAGE electrophoresis, separated protein samples were transferred onto polyvinylidene difluoride (PVDF) membrane (Bio-Rad). The membrane was permeablised by immersion in methanol and washed in transfer buffer (10mM Cyclohexylamino-propanesulfonic acid (CAPS; Sigma), 10% methanol in dH_2O) before being laid over the protein gel. Proteins were transferred to the membrane by electrophoresis.

The membrane was blocked in milk (10% non-fat milk powder in PBS) for one to two hours at 37°C before incubation with primary antibody in 1% milk powder for one hour at room temperature (details of antibodies used, source and dilution factor can be found in Table 2.2). After two rinses in PBTw (0.1% Tween20 (Sigma) in PBS), the membrane was washed once for 15 minutes and twice for 5 minutes in PBTw. The membrane was then incubated for one hour at room temperature in 1% milk with a 1:3000 dilution horseradish peroxidase-conjugated secondary antibody (Amersham). Horseradish-peroxidase (HRP) signal was detected using the ECL detection kit (Amersham) and exposed to Hyperfilm (Amersham) according to manufacturer's instructions.

Membranes were rinsed and stored in PBTw at 4°C. To reuse, the membrane was incubated in 1x stripping buffer (10x stripping buffer (Chemicon) in PBS) for 10 minutes at room temperature and washed three times in PBTw for 5 minutes.

2.6.6 GST fusion protein pull down assay.

The Pak2-GST fusion proteins were prepared as described in section 2.6.3. Protein extracts from 100 embryos were combined with 100µl of a 50% slurry of GST fusion protein and glutathione-agarose beads and incubated overnight at 4°C with gentle rotation. The beads were then pelleted by centrifugation (12,000rpm for 10 minutes at 4°C) and washed three times with lysis buffer. Samples were resuspended in an equal volume of 2x sample loading buffer, boiled for 5 minutes and separated on a 10% SDS-PAGE gel.

2.6.7 Immunoprecipitation (IP) analysis.

Detection of proteins interacting with the Pak2$^{\Delta c}$YFP proteins was achieved using immunoprecipitation. Whole cell extracts from 50 embryos injected with Pak2a$^{\Delta c}$-YFP or Pak2b$^{\Delta c}$YFP were precleared by incubation with 5µl of protein-A sepharose beads for 1 hour at 4°C, with gentle rotation. In 150µl lysis buffer, 5µl anti-GFP antibody (Clontech) was bound to 40µl protein-A sepharose beads by incubation for 1 hour at 4°C, with gentle rotation. Beads were removed from the embryonic protein extracts by centrifugation at 12,000rpm for 10 minutes at 4°C, the supernatant was then combined with the antibody-bead slurry and incubated overnight at 4°C with gentle rotation. The beads were then pelleted by centrifugation (12,000rpm for 10 minutes at 4°C) and washed three times with lysis buffer. Samples were resuspended in an equal volume of 2x sample loading buffer, boiled for 5 minutes and separated on a 10% SDS-PAGE gel.

2.7 Cell staining techniques.

2.7.1 Whole mount immunocyctochemistry.

A range of fixation conditions were employed for whole mount immunocyctochemistry to ensure the best possible antibody penetration and signal strength. The antibodies used and fixatives can be found in Table 2.2. Generally embryos were either fixed for 45 minutes at room temperature in 4% paraformaldehyde (PFA) or 2% Trichloroacetic acid (2% TCA in PBS) or for 10 minutes in 100% methanol. For staining with the F59 antibody (Devoto *et al.*, 1996) embryos were fixed in 4% PFA and stored at least overnight in methanol and subsequent washes were performed using PBDT (PBS, 0.2% Triton-x-100 and 1% DMSO).

Fixed embryos were washed in PBT (0.1% Triton-x-100 in PBS) and blocked in 5% goat serum (GS) in PBT for two hours or longer at room temperature. Embryos were then incubated in 2% GS/PBT containing primary antibodies overnight at 4°C with gentle shaking. Following incubation embryos were washed extensively in PBT at room temperature before overnight incubation in 2% GS/PBT containing secondary antibodies conjugated with Fluorescein, Cy3 or Cy5 (Stratech; 1:400 dilution). 4',6-Diamidino-2-phenyindole (DAPI) counterstain was added (0.2µg/ml) and incubation continued for one hour at room temperature. Embryos were washed thoroughly in PBT, stored and mounted in 70% citifluor/PBS (Citifluor).

For antibody staining following *in situ* hybridisation embryos were refixed for 20 minutes in 4% PFA/PBS and washed in PBT. Immunostaining was carried out as above but a horseradish peroxidase (HRP) conjugated secondary antibody (Amersham) was used. HRP activity was visualised in 3,3'-Diaminobenzidine (DAB) solution (one DAB fast tablet (Sigma) dissolved in 5mls 0.1M Tris pH7.6) 6µl 30% H_2O_2 was added to catalyse the reaction. The reaction was stopped by washing three times in PBT and refixing in 4% PFA/PBT for 20 minutes. Stained embryos were stored at 4°C in 70% glycerol/PBT.

2.7.2 Staining for endogenous alkaline phosphatase activity.

Embryos were fixed at three days post fertilisation with 4% PFA/PBS for 15 minutes at room temperature. Fixed embryos were then washed twice with PBT and three times with BCL buffer (0.1M Tris.HCl pH9.5, 50mM $MgCl_2$, 0.1M NaCl and 0.1% Triton-X-100). Endogenous alkaline phosphatase was detected with NBT/BCIP solution (4.5µl/ml NBT, 3.5µl/ml BCIP, (Roche) in BCL buffer). After 8-12 minutes the reaction was stopped by washing with PBT and embryos were refixed for 20 minutes in 4% PFA/PBS. Embryos were then washed twice in PBT and stored in 70% glycerol at 4°C.

2.7.3 Phalloidin labelling of actin filaments.

Embryos were fixed in 4% PFA/PBS for 1.5 hours at room temperature, washed 3 times for 5 minutes in PBT and blocked in 5% GS/PBT for one hour at room temperature. Embryos were then incubated in 2% GS/PBT containing a 1:500 dilution Cy3-conjugated phalloidin (5µg/µl; Sigma) for a further hour at room temperature and in the dark. Excess phalloidin was removed with PBT washes and embryos were stored and mounted in 70% citifluor/PBS.

2.7.4 Assay for cell proliferation and apoptosis.

Detection of proliferative and apoptotic cells was performed simultaneously. Proliferating cells were identified using BrdU incorporation essentially as described (Gray *et al.*, 2001). DNA fragmentation in apoptotic cells was detected using the terminal deoxynucleotide transferase-mediated dUTP nick-end labelling (TUNEL) method. The ApopTag kit (Serologicals Corporation) was used with a protocol adapted from the manufacturer's instructions. Embryos were treated in 10mM BrdU/10% DMSO in 0.3x Danieau solution for 30 minutes at 28°C. After washing twice in 0.3x Danieau solution, for 15 minutes, the embryos were allowed to continue development at 28°C for four hours. Embryos were then fixed in 4% PFA/PBS for 1

hour at room temperature, transferred to 100% methanol and stored at -20°C overnight. Following rehydration, embryos were re-fixed in chilled ethanol:glacial acetic acid (2:1) for 10 minutes at -20°C and washed 3 times for 5 minutes in PBT. Following incubation in 75µl of equilibration buffer at room temperature for one hour, the terminal transferase reaction was performed in 17µl working strength TdT enzyme (70% reaction buffer:30% TdT enzyme) with incubation overnight at 37°C. The reaction was stopped by washing embryos six times for 30 minutes at 37°C in stop/wash buffer diluted 1:35 in distilled water. Embryos were then blocked for one hour at room temperature in blocking solution (2mg/ml Bovine serum albumin, 5% GS/PBT) and incubated overnight in 47% anti-DIG-rhodamine:53% blocking solution at 4°C. Antibody was removed with PBT washes and embryos were not refixed.

To detect proliferating cells using BrdU, embryos were washed once and incubated in 2N HCl for 1 hour at 37°C. Following three 15 minute washes with PBT, embryos were blocked in 5% GS/PBT and incubated at 4°C overnight in 2%GS/PBT containing 1:400 anti-BrdU (Sigma) and other primary antibody if required. After extensive washing in PBT embryos were incubated overnight at 4°C with 1:400 fluorescently conjugated secondary antibodies (FITC and/or Cy5). DAPI counterstain (1µg/ml) was added and incubation continued for 1 hour at room temperature. Embryos were washed 10 times in PBT and mounted in citifluor.

2.7.5 Whole mount embryo staining with vital dye.

Cell behaviour and migration of the lateral line primordium was followed using live zebrafish embryos stained with Bodipy-Sphingomyelin FL (Molecular Probes). Embryos were dechorionated by hand and stained at 24hpf in 2% Bodipy (2.5µg/µl Bodipy-Sphingomyelin/DMSO in 0.3x Danieau solution). Embryos were kept in the dark and placed on an orbital shaker at 150rpm for 2 hours. Embryos were rinsed 3 times for 5 minutes in 0.3x Danieau solution and allowed to develop at 28°C for three hours.

For time-lapse microscopy embryos were anaesthetised with Tricane (Kimmel *et al.*, 1995) and then immobilised on a microscope slide (BDH) with a drop of 3% methyl cellulose. Embryos were covered with 0.3x Danieau solution, sealed using silicone grease (Dow Coring) and a cover slip.

2.8 Design and preparation of morpholino oligonucleotides.

2.8.1 Morpholino oligonucleotide design.

Morpholino oligonucleotides were obtained from Gene Tools, LLC. Sequences were selected based on the manufacturer's recommendation of 25mer antisense oligonucleotides with 50%G/C and A/T content and no predicted hairpins. Four consecutive guanine nucleotides were avoided. Each morpholino sequence was tested by BLAST at NCIB for representation elsewhere in the zebrafish genome.

The *pak2a* morpholino was designed against the 5'UTR immediately adjacent to the predicted translation initiation methionine (ATG). The *pak2b* morpholino was designed against the 5'UTR including the initiation site. Selected sequences were as follows:

pak2a: 5'–GACAGGGAAACACACTCAGAAGGGT–3'

pak2b: 5'–CACACATGATCAGGATGAGGAGACA–3'

2.8.2 Preparation of morpholino oligonucleotides for microinjection.

Morpholino oligonucleotides at 300mM were solubilised in 63µl RNase-/DNase-free water. The resulting stock solutions were diluted to working concentrations of 1:8 to 1:32 in morpholino dilution buffer (200mM KCl, 5mM HEPES, pH 7 and 5µg/ml phenol red) at 1:16 dilution this corresponded to a concentration of approximately 1.5ng/nl. Embryos were injected with 3nl of diluted morpholino oligonucleotide.

2.9 Microscopy techniques.

2.9.1 Photomicrography.

Bright field low power images of whole embryos were taken on a stereomicroscope (Leica MZ FLIII) with a Hamamatsu Digital camera using Openlab software (Improvision). Flat mounted embryos were imaged using the Zeiss Axiophot microscope with a Jenoptik ProgRes C14 camera attachment and Openlab software.

2.8.2 Confocal microscopy.

Fluorescent images of flat mounted embryos and timelapse of live embryos were collected using the Leica SP confocal microscope and accompanying software. Timelapse movies and still images were processed with the Imaris (Bitplane AG), softWoRx (AppliedPrecision), Photoshop Image Ready (Adobe) and Quicktime (Apple) software packages.

2.8.3 Electron microscopy

Electron microscopy was performed by Elizabeth Hirst (Electron microscopy lab, NIMR). Whole zebrafish embryos were dechorionated manually and fixed overnight with 2% glutaraldehyde, 2% PFA in 0.1M sodium cacodylate buffer, pH7.2 (SCB). Embryos were then washed for 10 minute in SCB and post fixed for 1 hour in 1% osmium tetroxide, SCB. Following a second wash with SCB embryos were stained *en bloc* with 1% aqueous uranyl acetate for 1 hour. Samples were dehydrated through a graded ethanol series followed by two changes of propylene oxide over 20 minutes and embedded in Epon resin (Agar Scientific). Ultra thin, 50nm 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.

Gene	Reference	Enzyme	RNA pol
pak2a	RZPD	NotI	T7
pak2b	RZPD	NotI	T7
nkx5-1	(Adamska *et al.*, 2000)	EcoRI	T7
papc	(Yamamoto *et al.*, 1998)	ApaI	T7
myoD	(Weinberg *et al.*, 1996)	BamHI	T7
shh	(Krauss *et al.*, 1993)	HindIII	T7
fli-1	(Brown *et al.*, 2000)	XbaI	T3
eya-1	(Hammond *et al.*, 2002)	EcoRI	T7
col IIa	(Yan *et al.*, 1995)	HindIII	T3
ptc	(Concordet *et al.*, 1996)	BamHI	T3
lst-1	(Inoue *et al.*, 1994)	XbaI	T3
nanos	(Koprunner *et al.*, 2001)	NotI	T7

Table 2.1: ***In situ* hybridisation probes.**

Origin of cDNA used for *in situ* hybridisation, the restriction enzyme used to linearise the plasmid and the RNA polymerase required to generate antisense RNA probes.

Antibody	Original source	Clonal origin	fixation	Dilution immuno	Dilution Wb.
F59	(Crow and Stockdale, 1986; Miller *et al.*, 1989)	m	PFA	1:50	-
Acetylated tubulin	Sigma	m	PFA	1:1000	-
S100	DAKO	p	PFA	1:500	-
β-catenin	Sigma	m/p	PFA	1:500	1:1000
E-cadherin	BD Biosciences	m	PFA	1:500	1:2000
Paxillin	BD Biosciences	m	TCA	1:200	1:10,000
Vinculin	Sigma	m	TCA	1:100	1:200
1025	Simon Hughes, KCL	m	PFA	1:50	-
4D9 (en)	(Ekker *et al.*, 1992; Hatta *et al.*, 1991; Patel *et al.*, 1989)	m	PFA	1:200	-
BrdU	Sigma	m	PFA	1:400	-
GFP	Clontech	p	-	-	1:500
PIX	(Koh *et al.*, 2001)	p	-	-	1:500
α-catenin	Sigma	p	-	-	1:1000
Nck	Oncogene	m	-	-	1:200
Rac	Upstate	m	-	-	1:1000
Cdc42	Santa Cruz	p	-	-	1:500

Table 2.2: Antibodies.

Antibodies used in this work, original source, clonal origin (m; monoclonal, p; polyclonal), fixation method and dilution factor for immunocytochemistry (immuno) and Western blot (Wb).

Chapter Three

Development of the embryonic posterior lateral line system

3.1 Introduction.

We are interested in understanding the molecular mechanisms underlying cell migration in zebrafish, using development of the lateral line primordium as a model system. The lateral line primordium is readily identifiable and shows directional migration along a specific pathway. A number of factors have been shown to be important for regulating cell migration. Firstly, directed migration of cells requires polarised organisation of the actin cytoskeleton in order to drive extension of lamellipodia at the leading edge and contraction at the trailing edge (Wittmann and Waterman-Storer, 2001). Failure to generate a polarised actin cytoskeleton, extend a single lamellipodia and retract the trailing edge results a loss of persistent directed migration (Sells *et al.*, 1999). Secondly, traction forces generated by focal adhesions are necessary to link cells to the extracellular matrix and provide the grip required for cells to migrate. Migration proceeds with the regulated formation and dissolution of these focal adhesions (Wittmann and Waterman-Storer, 2001). Finally, the maintenance of cell to cell adhesion is important for the correct migration of a group of cells. Cell to cell adhesion in epithelial cells, like the lateral line primordium cells, is initiated by E-cadherin-mediated adherens junctions (Nagafuchi, 2001). Adherens junctions have been shown to be critical in maintaining cell adhesion, tissue integrity and polarisation of cells in *Drosophila* (Cox *et al.*, 1996).

To gain a better understanding how migration of the lateral line primordium is controlled, we observed cell behaviour during migration of the lateral line primordium using confocal microscopy. Time-lapse analyses were focused specifically on the behaviour of cells at the trailing end during neuromast deposition and on the protrusive activity of cells at the leading edge. Within the cells of the lateral line primordium, the actin cytoskeleton, cell to cell adhesions and cell to ECM adhesions were visualised by detecting the localisation of F-actin, adherens junction proteins, β-catenin and E-cadherin, and the focal adhesion protein, Paxillin. The results of these studies will allow us to better interpret the role of the actin cytoskeleton cell adhesion and the proteins that regulate them might have in controlling migration of the lateral line primordium.

3.2 Characterisation of cellular organisation within the lateral line primordium.

The zebrafish primary posterior lateral line consists of seven mechanosensory neuromasts deposited by the posterior primordium during migration along the trunk and tail. Work from our lab and others has shown that a molecular and morphological prepattern exists within the lateral line primordium that presages and correlates with the deposition of cells to form neuromasts. Itoh *et al* have shown that the immature sensory hair cells of nascent neuromasts can be readily identified within the migrating primordium by the restriction of the proneural gene, *zath1*, and neurogenic genes, *deltaA*, *deltaB* and *notch3* (Itoh and Chitnis, 2001). Expression of a third member of the delta family of genes, *deltaD*, has also been recognised to demarcate nascent neuromasts (Q, Xu; pers. comm.).

We aimed to further characterise this prepatterning by investigating the organisation of cells within the migratory primordium, the role of cell adhesion in maintaining the prepatterning and real time observations of nascent neuromast patterning during primordium migration and neuromast deposition. By fluorescently

labelling the cell membranes of live embryos, with bodipy sphingomyelin FL, we were able to directly observe the organisation of cells within the primordium using confocal microscopy.

Shortly after migration was initiated, cells of the trailing end and of the mid region of the primordium were clustered into distinct repetitive groups. The groups of cells were organised into circular structures whose cells were broad at the outer face of the circle and narrowed to a point at the centre, thus giving them a rosette-like appearance (Figure 3.1a). The rosettes spanned the width of the primordium and at the centre were characterised by a concentration of membrane label (Figure 3.1b). Rosette-like structures were not observed in the leading edge of the primordium, where cells were tightly packed, but randomly organised.

At different stages of development 2-4 rosette-like structures could be identified within the primordium. This mirrored the expression patterns of neurogenic, and other genes, that have been shown to become restricted to 2-4 single cells or small clusters within the primordium. Additionally, gene expression was increasingly restricted towards the trailing end while molecular prepatterning was not observed in the cells of the leading edge (Gompel *et al.*, 2001; Itoh and Chitnis, 2001). These restricted patterns of gene expression have been shown to correspond to the neuromast precursors, therefore it seemed likely that the rosette-like structures observed within the primordium represented nascent neuromast precursors.

The absence of organisation of the leading edge cells into rosettes suggested that neuromast precursors were determined in an anterior to posterior manner, with anterior neuromasts determined first. This was in agreement with the observation of more tightly organised neuromast precursors at the anterior, or trailing end, intuitively the end from which neuromast precursors are deposited. Thus we have demonstrated that cellular patterning of the neuromast precursors is established within the primordium, prior to their deposition.

Figure 3.1

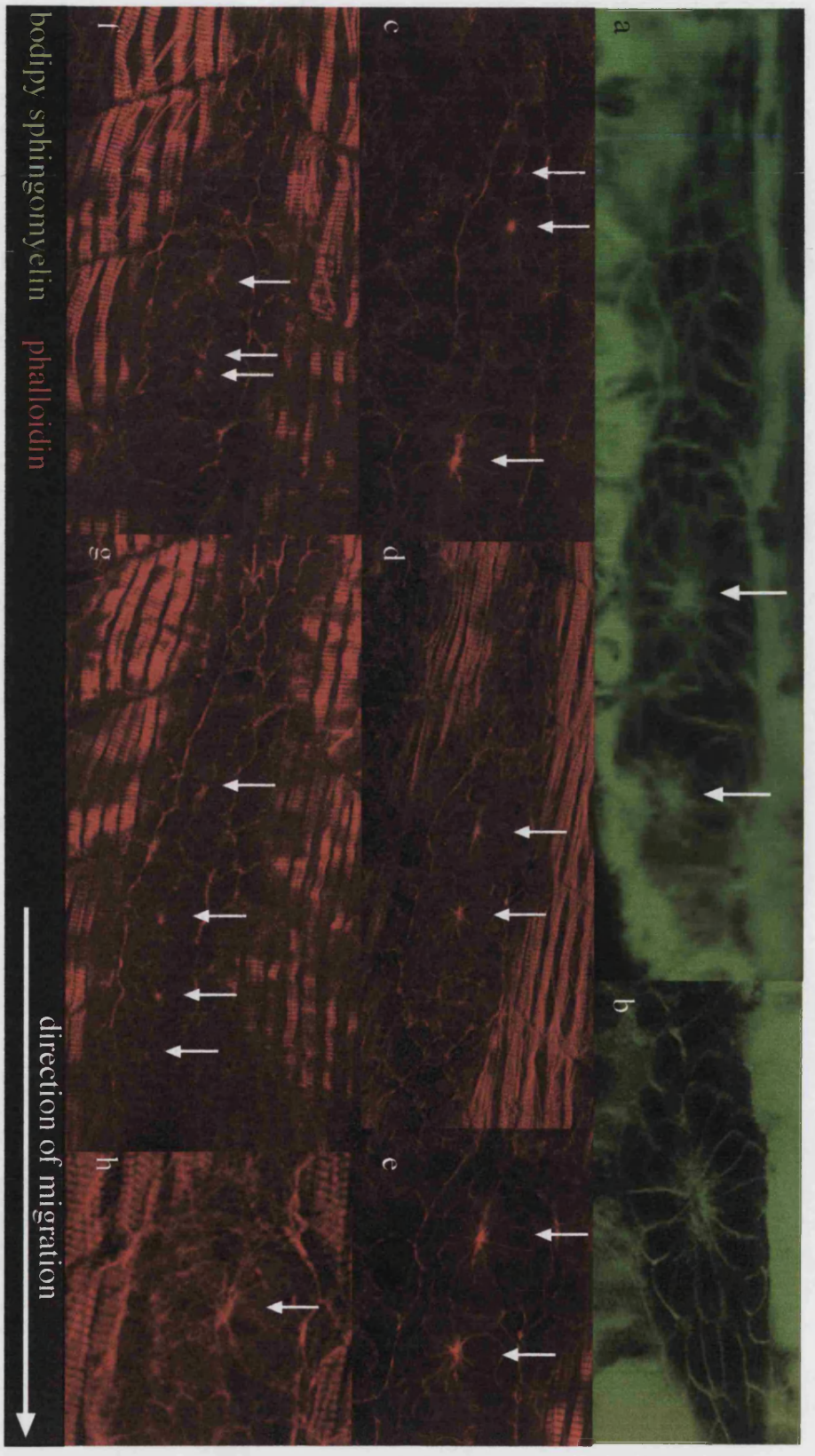

bodipy sphingomyelin
phalloidin
direction of migration
a
b
c
d
e
f
g
h

Figure 3.1: Visualisation of a morphological prepattern within the posterior lateral line primordium.

Confocal sections of the migrating primordium in 28hpf embryos stained with bodipy sphingomyelin FL (a,b). Two to three membrane clusters, corresponding to nascent neuromasts, are identified by arrows (a). The rosette-like structure is maintained in deposited neuromast precursors (b). Filamentous F-actin was visualised by taking confocal sections of embryos stained with TRITC-phalloidin, F-actin foci are indicated by arrows (c-h).

Figure 3.2

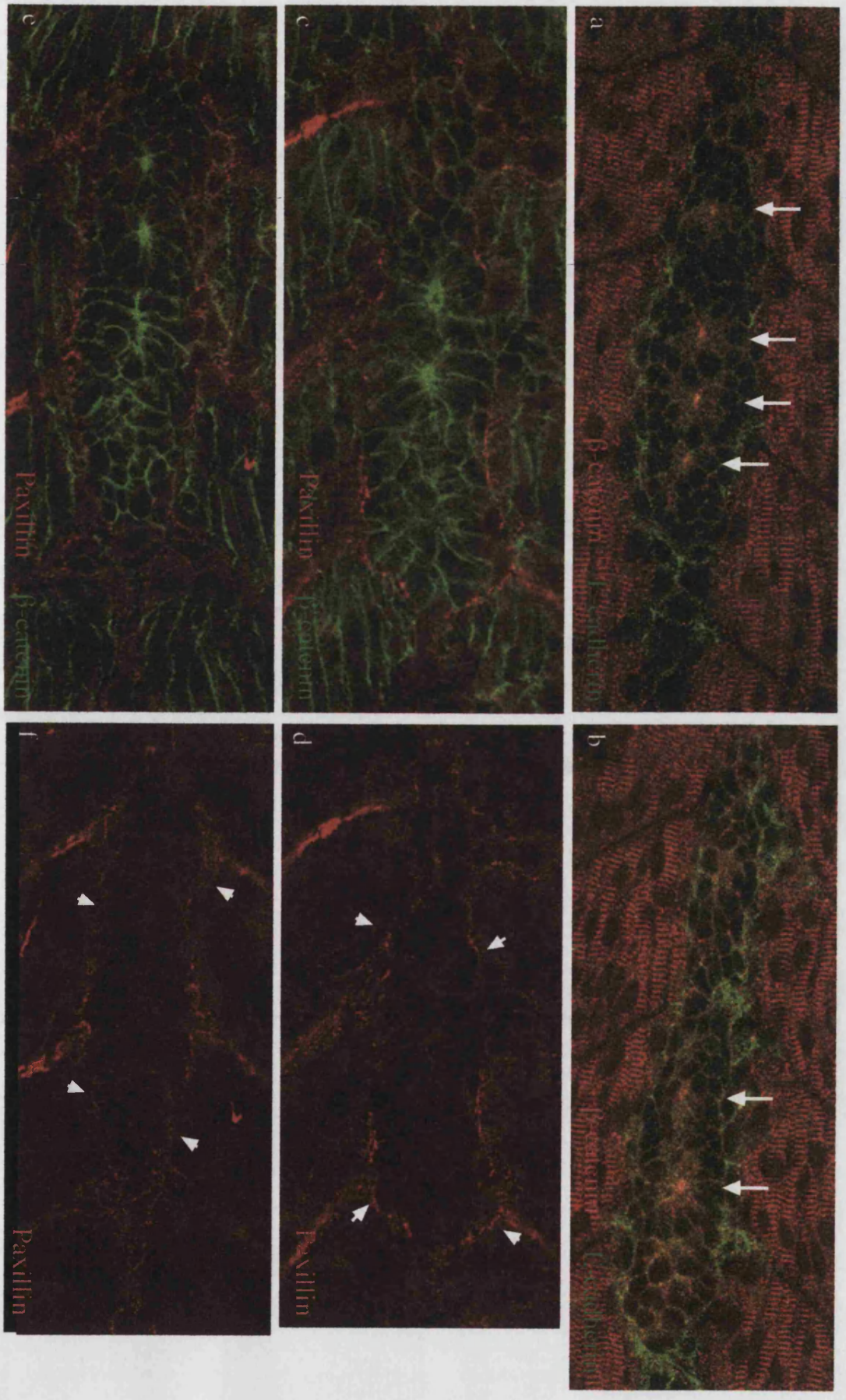

direction of migration
a
β-catenin
b
Paxillin
c
Paxillin
β-catenin
d
Paxillin
e
Paxillin
β-catenin
f
Paxillin

Figure 3.2: Distribution of adhesion molecules in the migratory primordium.

β-catenin and E-cadherin proteins were visualised by fluorescent immunocytochemistry in 24hpf to 28hpf embryos and were found clustered at the apex of the rosette-like structures (a-b). β-catenin protein (red) was localised to cell membranes and concentrated at the centre of the rosette (a-b; arrows). E-cadherin protein (green) was associated with membranes surrounding the primordium (a-b) and within cells of nascent neuromasts was polarised towards the apex (a-b, arrows). It is also noteworthy that, within cells of the surrounding epithelium, E-cadherin protein was polarised towards the face in contact with the lateral line primordium.

Panels c-f show localisation of paxillin protein (red) within the primordium using anti β-catenin antibody (green) to aid visualisation of the primordium (c,e). Paxillin protein was localised to the outer edge of the primordium (d,f; arrowheads).

The actin cytoskeleton plays an important role in maintaining cell shape and contacts with other cells and the ECM. As cells of the nascent neuromast precursors were observed to form a tightly organised pattern within the lateral line primordium even during deposition, we were interested in visualising actin cytoskeleton localisation in these cells. Actin cytoskeleton was visualised by staining embryos with fluorescently labelled phalloidin, which, binds to the filamentous actin (F-actin). F-actin was found to be distributed around the cell membranes within the primordium and a high concentration of F-actin was localised at the focal point of each rosettes (Figure 3.1c-h). Actin clustering at a site where cells are in close contact is indicative of high levels of adhesion complexes. This suggested that the cells of the neuromast precursors are tightly held together at a central point where the cells meet.

Adhesion of cells to one another or to the ECM is essential to the formation and maintenance of tissue structure and are certain to be required for the formation of nascent neuromast rosettes and maintenance of these structures during migration of the primordium and deposition. Linking the cytoskeleton of adjacent cells confers tensile strength (Braga, 2002), which would be required to maintain the shape of neuromast precursors throughout migration and particularly during deposition, during which the cells are under a great deal of mechanical stress (see section 3.3). Many adhesion complexes form links to the actin cytoskeleton (Braga, 2002). It is of interest to know the adhesion molecules involved in the clustering of F-actin at the central point of nascent neuromasts.

In epithelial tissues, adherens junctions form the first cell to cell contacts (Pokutta and Weis, 2002). Adherens junctions are multi protein complexes mediated by cadherin molecules (Braga, 2002). In the adherens junctions of epithelial cells β-catenin is required to link E-cadherin to α-catenin, which, in turn binds the actin cytoskeleton (Gumbiner, 1996). We used fluorescently conjugated antibodies to visualise the localisation of E-cadherin and β-catenin proteins in the lateral line primordium. β-catenin was found to exhibit a very similar pattern of localisation to that of F-actin where protein was present at the membranes of all cells within the lateral

line primordium and was concentrated at the apical tips of rosettes (Figure 3.2a-i). In the more randomly organised cells of the leading edge no focal clustering of β-catenin protein was observed. However, distribution of the β-catenin protein appeared to be polarised in some cells at the leading edge, possibly representing an early step in the clustering of cells into rosettes.

High levels of E-cadherin protein were detected throughout the epidermis including the migrating primordium (Figure 3.2a-b). Expression of E-cadherin indicated that cells of the lateral line primordium are epithelial in nature. E-cadherin protein was distributed in a similar pattern to β-catenin with higher levels of the E-cadherin protein present at the foci of neuromast precursors (Figure 3.2a-b). Taken together these data suggest that nascent adherens junctions may be involved in establishing and maintaining the structure of neuromast precursors.

3.3 Characterisation of migration of the primordium.

It is known that the driving force for cell migration is reorganisation of the actin cytoskeleton, which includes the protrusions of lamellipodia at the cell front and retraction of the cell rear (Wittmann and Waterman-Storer, 2001). The retraction of the cell rear is regulated by the release of adhesive contacts to the extracellular matrix (Ballestrem *et al.*, 2000). Focal adhesions provide a structural link between the extracellular matrix and the intracellular actin cytoskeleton (Turner, 2000). Focal adhesions are mediated by intergrins and form a complex of proteins including Paxillin. Using Paxillin antibody for immunostaining we have shown that Paxillin protein was localised to sites where the primordium was in contact with the membrane of cells surrounding the membrane and the overlying epidermis (Figure 3.2c-f). Higher levels of paxillin were observed around the leading edge of the primordium compared to around the trailing end (Figure3.2d).

Time-lapse analysis was carried out to study cell behaviour of the leading edge of the migrating primordium. By focusing on the leading cells of the lateral line

primordium we have found that cells behave differently depending on their relevant position within the primordium. There were two main types of cell behaviour in terms of motility and shape changes. At the leading edge of the migrating primordium cells were more loosely associated with each other and were actively changing their shape. These cells were seen to be actively extending and contracting both laterally and in the direction of migration (Figure 3.3). Away from the leading edge cells underwent little shape changes if any, and appeared to migrate as a tightly packed group, any elongation that occurred was in the direction of migration. Over a 30 minute time course looking at the leading edge, the primordium could be seen to narrow. It is possible that the primordium narrowed as neuromast precursors were deposited and cells at the trailing end elongated (Figure 3.4). The primordium may have broadened again as the neuromast precursor was released and cells at the trailing end retracted, much like the action of an elastic band being stretched and released.

This hypothesis was confirmed by time lapse analysis focusing on the trailing end during the deposition of neuromast precursors. Deposition of the neuromast precursors was marked by slowed movement of the rosette at the trailing end in relation to the rest of the primordium. Time lapse studies revealed that the deposition process was gradual and deposition was completed when the neuromast precursor became stationary while the remainder of the primordium continued to migrate away. During deposition contacts were maintained between the cells of the depositing neuromast precursor and the remainder of the primordium and this resulted in elongation of cells at the boundary (Figure 3.4 180'). Formation and dissolution of contacts between the neuromast precursor and the primordium appeared to be dynamic. Eventually the cell contacts were lost when the newly deposited neuromast precursor pinched off from the primordium and elongated cells returned to their normal shape (Figure 3.4 220').

Figure 3.3

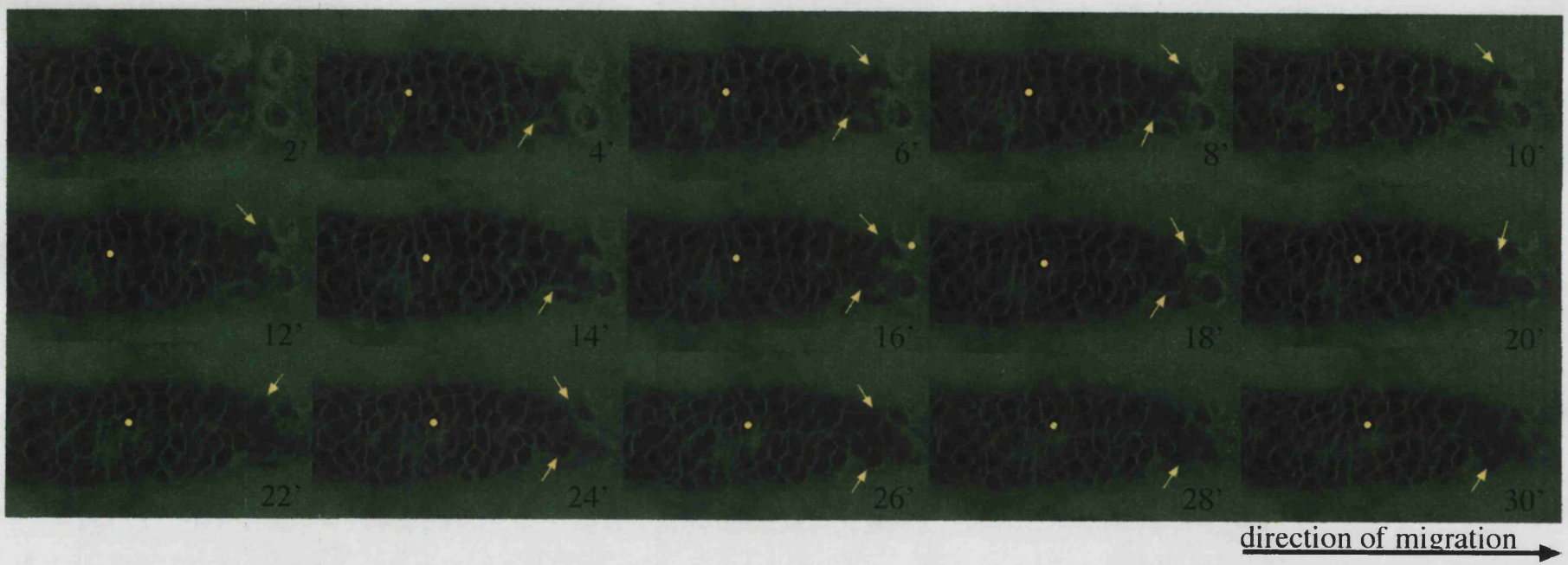


Figure 3.3: Time lapse series focusing on the leading edge of the migrating posterior lateral line primordium.

Time lapse analysis showed that cells at the leading edge of the primordium were highly active and explorative and elongated in the direction of migration (arrows highlight two cells which are particularly active during this series). Cells within the primordium and away from the leading edge were rigid in shape and that there was no explorative activity of the cells at the sides of the primordium into the surrounding tissue (yellow dot highlights an unchanging cell). Embryos were live stained with bodipy sphingomyelin and times are shown in minutes.

Figure 3.4

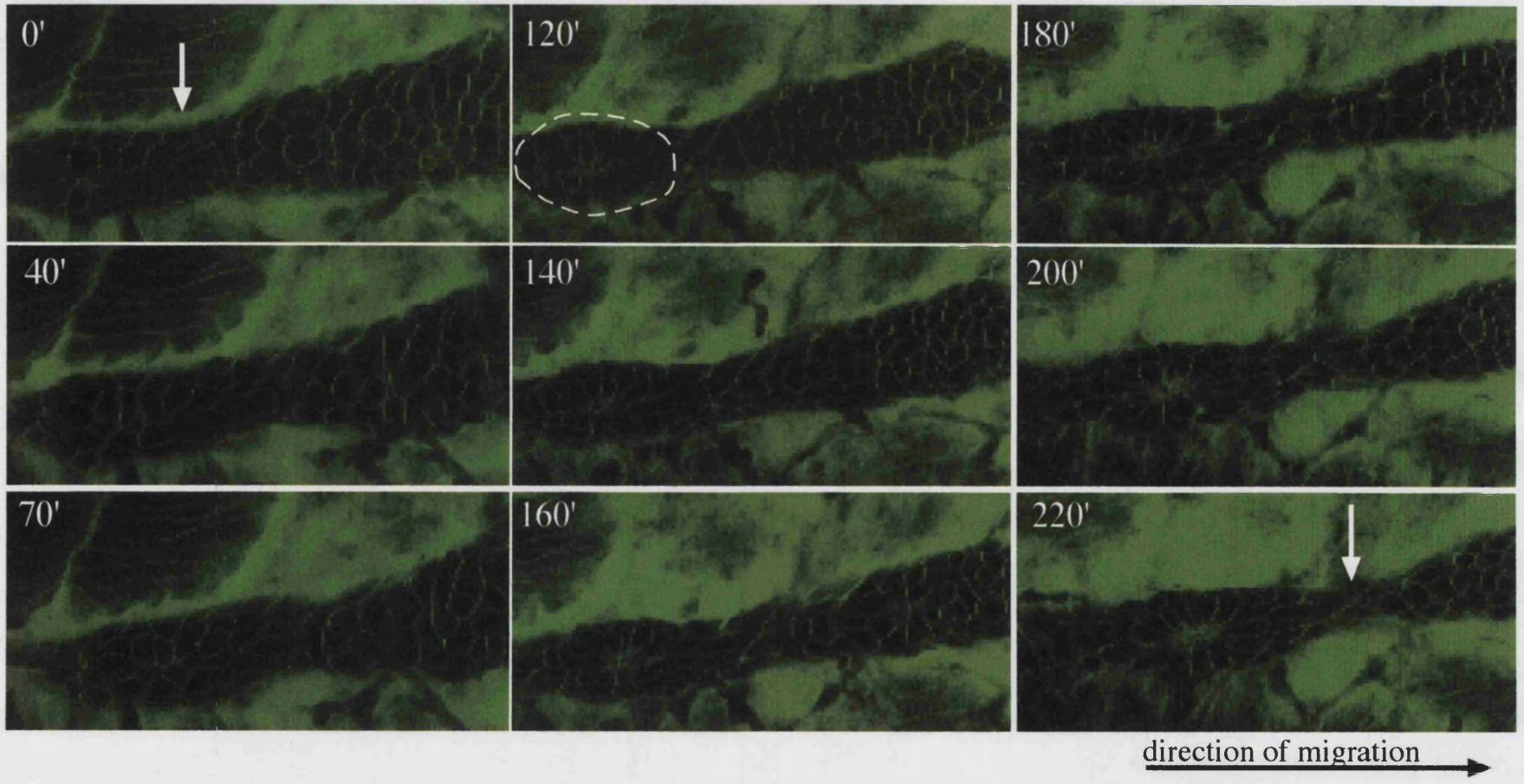


Figure 3.4: Time lapse series showing posterior lateral line primordium migration and neuromast precursor deposition.

A rosette-like structure was visible at the at the trailing end (white dashed circle). Arrows identify changes in cell shape between the migrating primordium and the depositing trailing neuromast precursor. Embryos were live stained with bodipy sphingomyelin and times are shown in minutes.

Following deposition of a rosette-like neuromast precursor a new rosette could be observed to take shape within the primordium. We observed that the rosette-like structure of neuromast precursors was maintained throughout deposition despite what appeared to be the exertion of large stress forces between the cells of the migrating primordium and the depositing neuromast precursor. Maintenance of this structure is likely to require the interaction and support generated by cell adhesion and the dynamic regulation of adhesion both within and surrounding the primordium. This would permit migration of the primordium and deposition of neuromast precursors, while maintaining the rosette structure of nascent neuromast and the organisation of the primordium.

3.4 Sensory organs of the lateral line system.

Neuromast precursors are deposited by the lateral line primordium at somite borders along the trunk or more ventrally at the tail as shown in the schematic (Figure 3.5A). The neuromast precursors differentiate into mature mechanosensory organs approximately 8 hours after their deposition (Metcalfe, 1985). Structurally, the differentiated neuromasts consist of a centrally elongated strip of sensory hair cells surrounded by a peripheral zone of mantle and supporting cells (Abbate *et al.*, 2002).

We have previously observed that the mantle cells can be visualised by the use of an alkaline phosphatase substrate as a result of the high level of endogenous alkaline phosphatase activity (unpublished observations). Analysis of this activity showed that high levels were only detected in differentiated neuromasts, after 3dpf, and the activity was restricted to the outer, mantle support cells (Figure 3.5B).

We also characterised specific markers of the sensory hair cells. The sensory hair cells are so named because of their apical hair-like projections. The apical bundle of sensory "hairs" consists of a single kinocilium and tens to hundreds of hexagonally packed stereocilia (Hackney and Furness, 1995). The kinocilium is composed of microtubles arranged in a classical 9+2 array and is located at the periphery of the

apical bundle (Cernuda-Cernuda and Garcia-Fernandez, 1996). The stereocilia contain a core of cross-linked actin filaments and are generally arranged in rows, the heights of successive rows increasing in the direction of the kinocilium (Hackney and Furness, 1995). This arrangement of stereocilia and kinocilum provides the hair cell with directional polarity (Cernuda-Cernuda and Garcia-Fernandez, 1996).

The microtubules of the kinocilium in the vertebrate ear have been shown to contain posttranslationally acetylated α-tubulin (Ogata and Slepecky, 1995). Analysis of the localisation of acetylated α-tubulin protein in 3dpf zebrafish embryo showed labelling of the hair-like projections of the sensory cells and of nerve fibres of the lateral line ganglion innervating the individual neuromasts (Figure 3.5: C).

To label the hair cell itself we used another molecular marker. Calcium ions play a key role in the physiology of the vertebrate sensory hair cell, and in mechanosensory transduction. The large family of EF-hand, Ca^{2+} binding, S100 proteins are present in a wide range of cells including the sensory and secretory cells of the vertebrate inner ear (Fermin and Martin, 1995). Hair cells of the fish ear are known to exhibit strong immunoreactivity to the S100 antibody (Saidel *et al.*, 1990) and recently the S100 protein has been shown to be a marker for sensory hair cells of the lateral line system in teleosts (Abbate *et al.*, 2002). Staining of zebrafish embryos at 3dpf, when all neuromasts of the embryonic posterior lateral line have differentiated, the S100 antibody specifically labelled the central cluster of sensory hair cells (Figure 3.5: C). In combination, staining of S100 and acetylated tubulin permit excellent visualisation of the sensory cells, including the projections, of the lateral line organs. These markers are valuable tools to study the formation of the lateral line system.

Figure 3.5

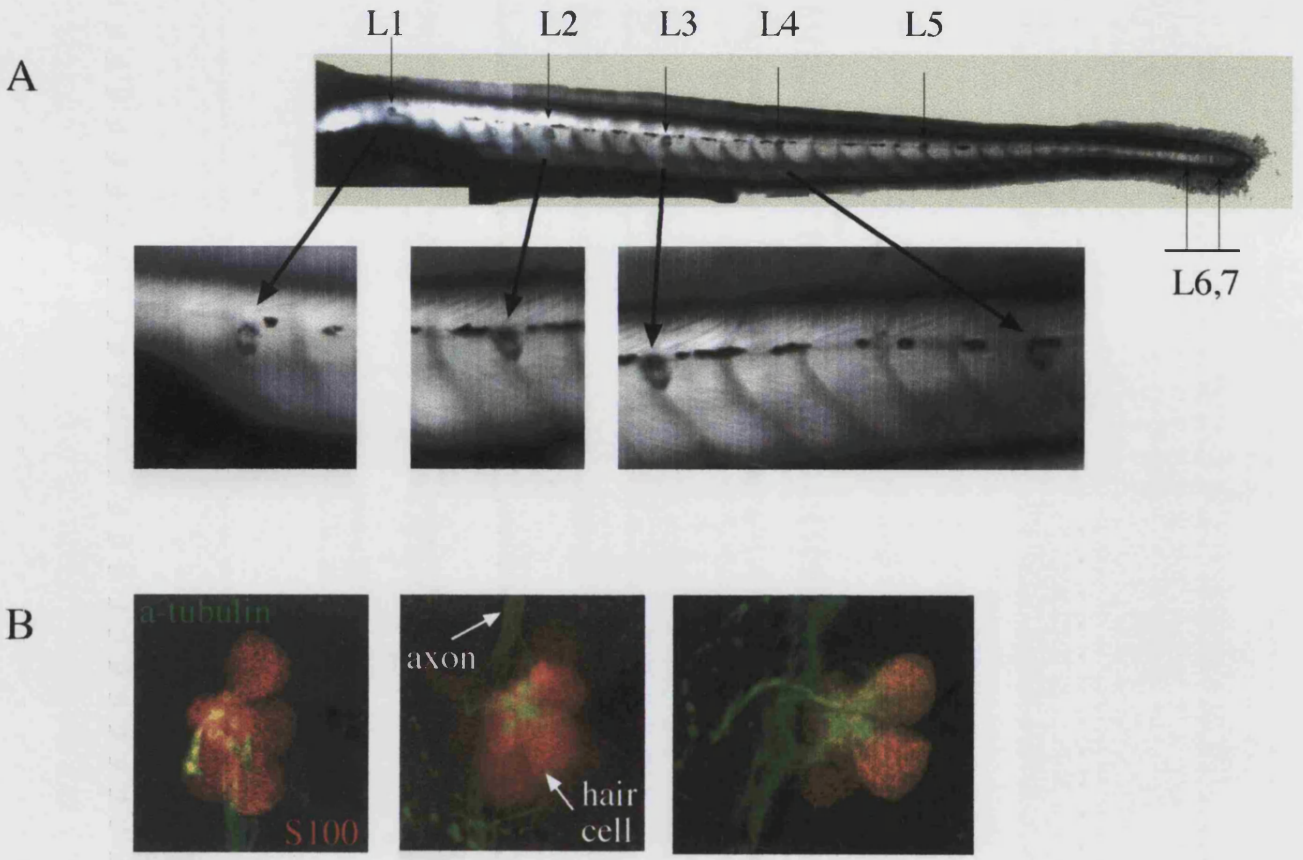


Figure 3.5: Development and visualisation of the sensory neuromasts.

A: Mantle cells of the differentiated neuromasts can be visualised by detection of the high levels of endogenous alkaline phosphatase at 3dpf. Neuromasts are labelled according to (Ledent, 2002).

B: Confocal images of the mechanosensory hair cells stained with S100 (red), the stereocilia and posterior lateral line axons (axon) are labelled with acetylated tubulin (green) at 3dpf.

3.5 Discussion

Using bodipy sphingomyelin to label cell membranes in live embryos we have been able to visualise the cellular organisation and cell behaviour of the posterior lateral line primordium during migration and deposition of neuromast precursors. Time lapse imaging has demonstrated that nascent neuromasts are readily identifiable within the lateral line primordium by their distinctive rosette-like structure. It is evident that this cellular organisation is established prior to neuromast deposition. This is in agreement with work by Gompel *et al* (2001) who show that the primordium is dynamically prepatterned, and that clusters of cells corresponding to presumptive neuromasts are already defined within the primordium several hours before their deposition. Consistent with this prepattern is the expression pattern of the proneural gene, zath1, and of neurogenic genes, notch3, deltaA, deltaB and deltaD. Within the primordium the expression patterns of these genes appear to correlate with the cells of the neuromast precursors (Itoh and Chitnis, 2001; personal communication, Dr Q.Xu). Two further genes have been identified that also exhibit expression patterns that reflect the prepatterning of the primordium into neuromast precursors, these genes have been initially characterised as an epithelial glycoprotein and a chemokine receptor (Gompel *et al.*, 2001).

It is, as yet, unknown how the lateral line primordium becomes patterned to form the neuromast precursors, although there are two main possibilities. Firstly, the formation of the neuromast precursor rosettes may be induced by factors external to the primordium, such as the underlying somites or growth cones of the lateral line nerve. Secondly, it is possible that prepatterning of the primordium cells into neuromast precursors is intrinsic to the primordium itself. The fact that neuromast precursors are patterned in a stepwise manner, with only one to two neuromast rosettes visible within the primordium at any one time, suggests an intrinsic mechanism, or at least intrinsic differences in the competency of primordium cells to respond to external cues.

It has been proposed that the primordium undergoes a process known as metamerisation, similar to that described for presomitic mesoderm during somitogenesis (Gompel *et al.*, 2001; Pourquie, 2000). By this method a continuous strip of cells, the primordium, is segregated into discrete clusters, the neuromast precursor rosettes. However, this possibility is still conjecture and as yet it is unclear how the lateral line primordium is prepatterned and what the molecular controls might be that govern it. It this work we chose to focus on the mechanisms regulating migration of the primordium as a whole and the mechanisms that maintain the prepattern of the primordium once it has been established.

Time-lapse analysis also showed that the rosette structure of nascent neuromasts is maintained during deposition despite great elongation of the cells at the posterior of the neuromast and the anterior of the primordium. The gradual deposition and release of the neuromast precursors observed in this work is in agreement with previous findings (Gompel *et al.*, 2001).

Maintenance of the rosette structure within the primordium and during deposition can be ascribed to strong focal clustering of adhesion molecules. Adherens junctions play pivotal roles in cell and tissue organisation by mediating cell adhesion (Tepass, 2002). By interdigitation of stable adhesive elements between adjacent cells, adherens junctions maintain overall tissue architecture (Gumbiner, 1996). We have provided evidence that adherens junctions may play a role in maintaining integrity of the lateral line primordium and nascent neuromasts. Components of adherens complexes; β-catenin, E-cadherin and F-actin are found at the focal point of the nascent neuromast rosette. Additionally the asymmetric distribution of E-cadherin and β-catenin protein within cells at the leading edge of the primordium suggests that the formation and clustering of adherens junctions functions to draw cells together to form the nascent neuromast rosettes. Cadherin-mediated adhesion may also provide tensile strength to the primordium in order to permit the cells to migrate as a group.

Cadherin-mediated cell adhesions are dynamically rearranged during cell movements by tethering of E-cadherin to either the actin cytoskeleton via α-catenin or

to small GTPase effectors (Fukata and Kaibuchi, 2001). Activated Rac and Cdc42 positively regulate cadherin-mediated cell adhesion by promoting formation of the E-cadherin-β-catenin-α-catenin complex (Fukata and Kaibuchi, 2001). This provides an interesting mechanism through which Pak may regulate formation of cadherin-mediated adhesion.

The presence of higher levels of Paxillin around the primordium indicates that the focal adhesions may be involved in regulating migration. As focal adhesions have been shown to transmit strong forces and serve as traction points to aid cell migration (Webb *et al.*, 2002) it is likely that the focal adhesion sites serve as traction points for the propulsive forces that would move the lateral line primordium along the trunk. It is interesting that Paxillin was distributed differentially around the primordium with higher levels at the leading edge and low levels at the trailing end. In this sense the primordium can be thought of as one giant polarised cell. Higher levels of Paxillin at the front of this "cell" is consistent with the idea of new focal adhesions being actively formed at the leading edge as the primordium migrates. At the rear of the primordium, the focal adhesions are disassembled and this is reflected in the lower levels of Paxillin observed around the trailing end.

The p21 activated kinase, Pak, is a key regulator of focal adhesions as well as effecting the GTPase-mediated regulation of the cytoskeleton (Frost *et al.*, 1998). Pak is able to regulate the cytoskeleton and focal adhesion formation through interaction with a number of downstream targets including LIM-Kinase by inhibiting dissociation of actin filaments (Edwards *et al.*, 1999) and Myosin Light Chain Kinase by promoting phosphorylation of MLC and cell retraction (Kiosses *et al.*, 1999; Rudrabhatla *et al.*, 2003; Sells *et al.*, 1999). Pak has also been demonstrated to localise to focal adhesions. The binding of Pak to the guanine nucleotide exchange factor, PIX, recruits Pak to focal adhesion sites through interaction with Paxillin (Brown *et al.*, 2002). Once recruited Pak functions to promote disassembly of the focal adhesion (Frost *et al.*, 1998; Manser *et al.*, 1997; Zhao *et al.*, 2000b). This activity of Pak may

be crucial in regulating turnover of focal adhesions to facilitate migration of the lateral line primordium.

Another role for Pak in migration of the lateral line primordium lies with the ability of Pak to regulate the actin cytoskeleton. Paks are effectors of Cdc42 in pathways that regulate the organisation of the cortical actin cytoskeleton and hPak1 co-localizes with F-actin to cortical actin structures (Dharmawardhane *et al.*, 1999; Eby *et al.*, 1998). Therefore zebrafish Pak may play a role in inducing clustering of F-actin at the centre of nascent neuromast precursors. Furthermore Pak can induce the rapid formation of polarised filopodia and membrane ruffles observed in motile cells (Sells *et al.*, 1997). Migration of the lateral line primordium is likely to require formation of polarised extensions and as a consequence the activity of Pak.

Pak is involved in many pathways that could be important during lateral line primordium migration in zebrafish, the challenge is to assess which, if any, of these are critically important. The work described in this thesis aims to establish whether Pak is important for the migration of the posterior lateral line primordium and provide direction for future studies to dissect the role that Pak may play.

Chapter Four

Identification and characterisation of zebrafish p21 Activated Kinase.

4.1 Introduction

Cell migration involves regulation of the actin cytoskeleton and the assembly and disassembly of cellular adhesions to the ECM (Christopher and Guan, 2000). Pak proteins are key regulators of the actin cytoskeleton and focal adhesions. Studies in cell culture as well as in yeast and *Drosophila* have indicated that Pak has a crucial role in controlling cell migration. We were interested in identifying the zebrafish homologues of *pak* and characterising their role in development and particularly in the formation of the lateral line system. This chapter describes the identification, sequence analysis and characterisation of zebrafish *pak*.

4.2 Identification of zebrafish p21 activated kinase genes.

No *pak* genes have previously been described in zebrafish. A BLAST search of the EST database (NCBI) was carried out using the full-length *Xenopus pak1* cDNA sequence. This search identified four highly related zebrafish sequences with homology to X*pak1* (Genbank accession numbers: AI545254, AI878231, AI722617 and AI877550).

High quality sequence data provided by the NCBI for the ESTs was limited to less than 500bp. Conceptual translation produced short sequences which showed homology to either the 5' terminus of xPak1, including the 5' untranslated region

(UTR) and the start codon (AI545254, AI878231), or homology to the 3' terminus, including the stop codon (AI722617 and AI877550). These four EST clones were requested from the RZPD Resource Centre/Primary Database (Heubnerweg 6, D-14059 Berlin: RZPD clone identification: MPMGp609P0214, MPMGp609M0634, MPMGp609C0529 and MPMGp609P1133).

4.3 Sequence analysis of the zebrafish *pak* cDNAs.

Zebrafish *pak* EST clones were sequenced as described in Chapter Two. Sequence analysis identified clones MPMGp609P1133 (AI877550) and MPMGp609C0529 (AI722617) as partial cDNA sequences comprising part of the Pak C-terminal kinase domain and were not pursued further. However, the clones MPMGp609P0214 (AI545254) and MPMGp609M0634 (AI878231) contained the complete open reading frame and the predicted translation products suggested proteins of 517aa and 539aa respectively. Alignment of these two protein sequences showed a high number of shared identities (88%) however, divergence between the sequences was observed in the N-terminus where MPMGp609M0634 contained small insertions (Figure 4.1).

The Pak family of proteins comprises six known members, Pak 1-6, which are dived into two groups according to their structure and activation mechanism (see reviews: (Jaffer and Chernoff, 2002; Knaus and Bokoch, 1998)). Each of the putative zebrafish Pak protein sequences were aligned with known human sequences for each of the family members (data not shown). Both of the zebrafish sequences shared the highest number of conserved identities with human Pak2 (hPak2) with 87% and 85% for MPMGp609P0214 and MPMGp609M0634 respectively. Identification of both zebrafish sequences as homologues of Pak2 was further confirmed by high homology with sequences from rabbit, rat and *Xenopus laevis* (Figure 4.2, sequences obtained from the NCBI database). No zebrafish Pak homologues of the other members of the Pak family were identified from the RZPD search, therefore work in this thesis focuses

only on zebrafish homologues Pak2. Comparison of the DNA sequences of the zebrafish *pak* cDNAs revealed differences that suggest they are likely to be encoded by two different *pak2* genes, MPMGp609P0214 shall be referred to as *pak2a* and MPMGp609M0634 as *pak2b*.

Recent sequencing of the zebrafish genome by the Sanger Centre has identified a zebrafish homologue of *pak2* (assession number CAD59176). The predicted translation product of this gene is 517aa in length. Alignment of the translated sequences of the 517aa Pak2a and CAD59176 showed them to be identical at 516 of 517 amino acid positions (data not shown). This further confirmed the identification of *pak2a* as a zebrafish homologue of *pak2* and showed sequencing carried out in this study to be accurate and correct. At the time of writing no zebrafish sequence identical to the longer *pak2b* had been identified by the Sanger Centre.

4.4 Characterisation of *pak2a* and *pak2b* genes.

Northern blotting analysis was employed to detect and determine the size of *pak2a* and *pak2b* transcripts in zebrafish embryos. Total RNA was prepared from 24hpf zebrafish embryos and was separated on a formaldehyde gel then transferred to nylon blotting membrane. DNA probes specific to *pak2a* or *pak2b* coding regions were radiolabelled and used to probe the membrane. The sizes of RNA transcripts detected were determined by comparison with the bands representing the ribosomal subunits. Specific transcripts of 3kb in length were detected for both *pak2a* and *pak2b* (Figure 4.3A).

Figure 4.1

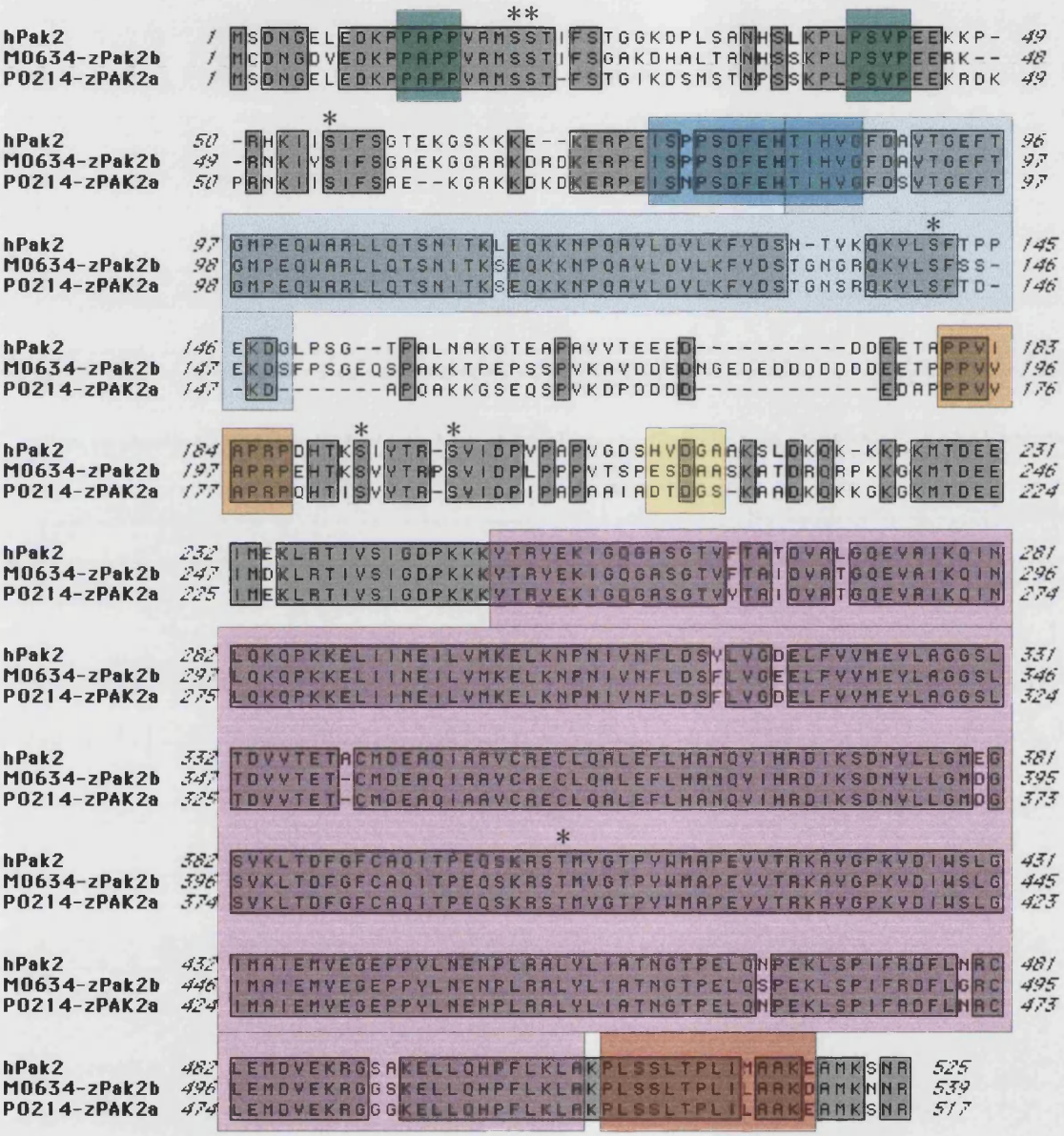


Figure 4.1: Alignment of zebrafish Pak2a, Pak2b and hPak2 proteins.

Alignment of the two novel zebrafish Pak sequences (Pak2a and Pak2b) with human Pak2 (hPak2). The different domains are individually highlighted. Protein interaction sites in the N-terminal regulatory domain were conserved, including the conventional and unconventional SH3 domain binding motifs (green and orange respectively) and the Rac/Cdc42 binding site (dark blue). The auto-inhibition sequence (light blue) was also conserved. The C-terminal kinase domain (pink) showed strong homology to the human sequence. The putative binding site for the G-protein subunit, Gβ is also highlighted (red). Sites of autophosphorylation are marked with asterisks.

Figure 4.2

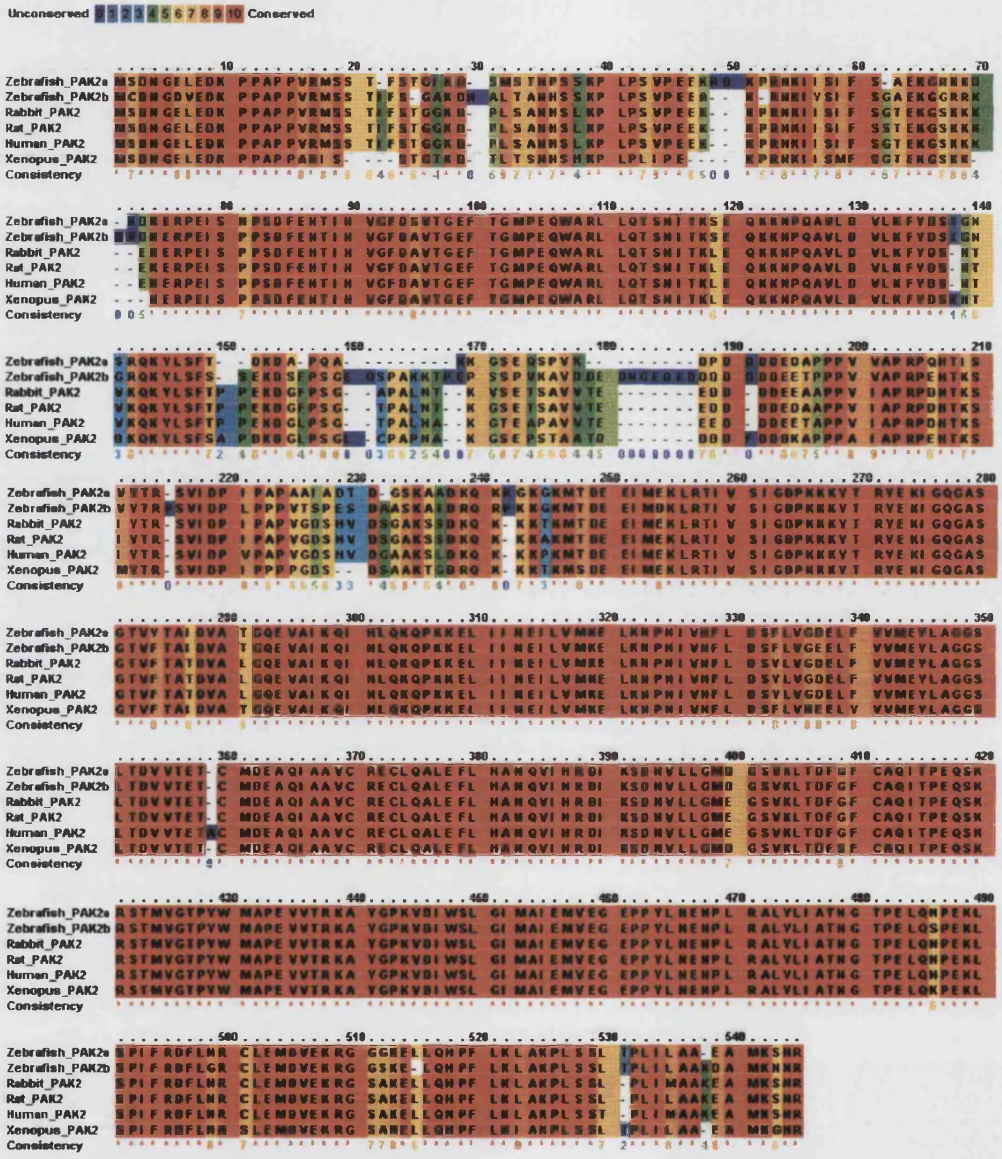

Unconserved 1 2 3 4 5 6 7 8 9 10 Conserved
Zebrafish_PAK2a
Zebrafish_PAK2b
Rabbit_PAK2
Rat_PAK2
Human_PAK2
Xenopus_PAK2
Consistency

Figure 4.2: Protein sequence alignment of zebrafish Pak2a and Pak2b with human, rat, rabbit and *Xenopus laevis* Pak2.

Protein sequences obtained from the NCBI database and alignment carried out using PRALINE (Heringa, 1999). Colour shows amino acid conservation with red corresponding to highly conserved and dark blue corresponding to unconserved. Consistency gives a measure (out of 10) of conservation at a particular amino acid between protein sequences. A "*" indicates all amino acids are in agreement and "0" indicates no amino acid agreement.

Full length cDNA corresponding to zebrafish *pak2a* and *pak2b* were transcribed and translated *in vitro* using the TNT rabbit Reticulocyte Translation System (Promega). Synthesis of Pak2a protein resulted in two products with molecular weights of 62kD and 59kD and synthesis of Pak2b protein also resulted in two products with a similar separation in molecular weight, 67kD and 65kD (Figure 4.3B). The molecular weights of the protein products are in agreement with the size of protein predicted from the DNA coding region. For both genes the lower molecular weight products may be the result of a translation from a second initiation site found 48bp downstream of the predicted primary initiation site. It is not known if the second initiation site is utilised *in vivo*.

4.5 Comparative analysis of zebrafish Pak2a and Pak2b domains

The group I family of Pak proteins are characterised by the presence of an N-terminal regulatory domain and a C-terminal serine/theronine kinase domain. The N-terminal regulatory domain of Pak2 contains three binding sites recognised by proteins with Src-Homology-3 (SH3) domain and the p21 binding domain (PBD). SH3-domain binding sites are generally characterised by a PxxP motif, where P is a proline residue and x any residue (Bokoch *et al.*, 1996). The adapter protein Nck binds to the first of the proline-rich domains and associates Pak with the membrane, this association is sufficient to increase activation of Pak (Galisteo *et al.*, 1996; Lu and Mayer, 1999). The consensus binding sequence for Nck has been identified as PxxPxRxxS (Chong *et al.*, 2001). This sequence is conserved in Pak2a and Pak2b (Figure 4.1: green). Pak proteins have also been shown to contain a third, unconventional, SH3 binding site (PPxxxPRP) which binds the Pak-interacting exchange factor, PIX (Manser *et al.*, 1998). Binding of PIX recruits Pak to sites of focal adhesion through an interaction with Paxillin kinase linker (PKL) (Brown *et al.*, 2002).

Figure 4.3

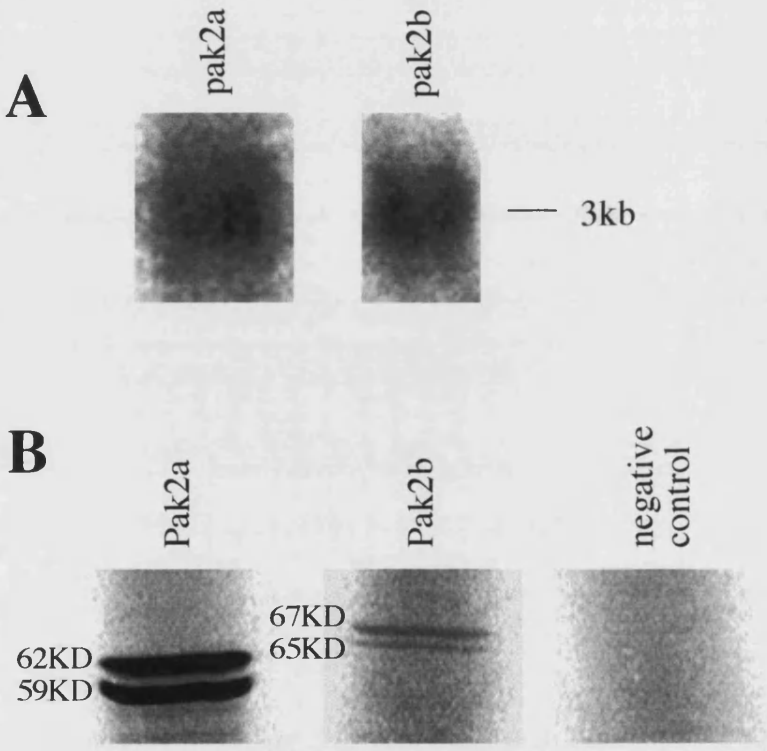

A
pak2a
pak2b
3kb
B
Pak2a
Pak2b
negative control
62KD
59KD
67KD
65KD

Figure 4.3: Size of *pak2a* and *pak2b* RNA transcripts and molecular weight of proteins.

A: Detection of *pak2a* and *pak2b* RNA transcripts by Northern blot analysis. Transcript size was determined by comparison to ribosomal RNA subunits, 16S: 1.6kb and 28S: 4kb.

B: Protein products of *pak2a* and *pak2b* cDNAs synthesised by *in-vitro* transcription and translation. The negative control was generated by carrying out the reaction in the absence of DNA template. The molecular weight of the protein products were determined by comparison with molecular weight markers.

The consensus site for PIX binding was also conserved in the zebrafish Pak2a and Pak2b sequences (Figure 4.1: orange).

The PBD is the site of Pak activation through the binding of the small GTPases, Rac and Cdc42. Analysis of the protein structure of Pak1 identified amino acids 70-113 as the PBD (Lei *et al.*, 2000) and this corresponds to an identical region in Pak2a and Pak2b . Lei *et al* further characterised this domain by alignment of group I Pak family members (Pak1-3) with the related Wiskott-Aldrich Syndrome proteins, WASP and N-WASP based on NMR structure. Using this approach they identified the minimal motif required for Cdc42/Rac-interaction binding (CRIB) as ISxPxxFxHxxHxGxD. Among Group I Pak proteins, including zebrafish Pak2a and Pak2b, this motif can be more explicitly described as ISxPSDFEHTIHVGFD (Figure 4.1, dark blue).

The Pak family of proteins exhibit autoregulation through binding of an autoinhibitory (AI) sequence (Figure 4.1, light blue) with the kinase domain (Figure 4.1, pink). This interaction maintains Pak in an inactivate conformation in the absence of binding of Rac or Cdc42. Within the autoregulatory region key residues have been identified that contribute to the interface between the inhibitory sequence and the kinase domain (Lei *et al.*, 2000). The key residues, Leu^{107}, Glu^{116} and Asp^{126} are conserved in both zebrafish Pak2 proteins. Release of autoinhibition permits autophosphorylation of Pak proteins at a number of stereotyped positions including six serine residues in the N-terminal domain and one tyrosine residue within the kinase domain (Figure 4.1, asterisks). The phosphorylation of Ser^{19} is unique to Pak2 and its phosphorylation has been shown to negatively regulate binding of the Nck SH3 domain (Zhao *et al.*, 2000a). The number and position of these residues within the zebrafish Pak proteins agrees with the published work for hPak2 (Chong *et al.*, 2001).

It is interesting to note that Pak2b contains an insertion of 10, mostly acidic, amino acid residues in the region between the AI sequence and the PIX binding SH3 domain (Figure 4.1, aa177-186). In addition, there is divergence in amino acid residues immediately following the Nck consensus binding domain in Pak2b (Figure

4.2, aa positions 25 and 30). It is not clear what effect, if any, these differences have on the activity of Pak2b.

In the C-terminal domain Pak proteins contain a serine/theronine kinase domain and a binding site for the heterotrimeric G-protein β-subunit (Gβ) (Leeuw *et al.*, 1998). The kinase domains of zebrafish Pak2a and Pak2b were highly conserved when compared to each other and to the Pak2 protein sequences of other species (Figure 4.2). The putative Gβ interaction site was conserved in both zebrafish Pak2 proteins (Figure 4.1, red). The Gβ interaction site, initially identified in the budding yeast PAK homologue Ste20, is implicated in signalling from heterotrimeric G proteins to mitogen-activated protein (MAP) kinase cascades. In mammalian systems G-protein-coupled receptors have a potent ability to stimulate Pak activity (Daniels and Bokoch, 1999) and Gβγ-mediated Pak1 activation of Cdc42 is required for efficient chemotactic directional sensing (Li *et al.*, 2003).

The high level of conservation of zebrafish Pak2 suggests they may function in a similar manner to the other Pak2 proteins.

4.6 Identification of proteins interacting with Pak2a and Pak2b.

Sequence comparisons show that zebrafish Pak proteins are highly conserved in many regions including the kinase domain, PBD and Nck and PIX binding sites. However interesting differences adjacent to the Nck and PIX binding sites are present in Pak2b. Pull down experiments using the GST fusion proteins were carried out to verify the binding of known interacting proteins. The GST fusion proteins were generated by fusing the N-terminal regulatory domain of Pak2a or Pak2b with the GST protein (Pak2a$^{\Delta c}$GST and Pak2b$^{\Delta c}$GST). Whole cell extracts were prepared from 15-somite stage embryos and incubated with the Pak-GST fusions, or GST alone. Proteins not bound to the Pak-GST fusion protein were washed from the glutathione beads with four rinses in lysis buffer containing 2x phosphatase inhibitor.

Beads with proteins specifically bound to Pak2a or Pak2bGST were resuspended in lysis buffer and run on an agarose gel. Western blot analysis was then carried out and interacting proteins were detected by specific antibodies.

The small GTPases, Rac and Cdc42, were both found to bind the zebrafish Pak-GST fusion proteins (Figure 4.4). The interaction with Rac/Cdc42 suggests that Pak2a and Pak2b are able to act as their effectors during zebrafish development.

Interaction of proteins known to bind Pak through the conserved SH3-domain binding sites was investigated. The adapter protein, Nck, was predicted to interact with both Pak2a and Pak2b by virtue of a conserved binding motif at the N-terminal SH3 binding site. Available sequence data from the zebrafish genome has indicated two Nck genes and the predicted molecular weights of zebrafish Nck are around 45kD. Western blot analysis of zebrafish embryo extract using the Nck antibody detected bands of; 54, 51, 46, 42 and 35kD (data not shown). Western blot analysis of Nck proteins pulled down by Pak2a$^{\Delta c}$GST and Pak2a$^{\Delta c}$YFP showed that Pak2a may interact with two isoforms of Nck with molecular weights of 48kD and 42kD.

Intriguingly we found that no Nck proteins were pulled down by the Pak2b$^{\Delta c}$GST fusion protein (Figure 4.4). This was confirmed by immunoprecipitation using bead immobilised anti-GFP antibody bound to the Pak2b$^{\Delta c}$YFP fusion protein. Western blot analysis of proteins bound to Pak2b$^{\Delta c}$YFP also revealed no interaction with Nck (data not shown). These two fusion proteins are constructed differently, the GST is fused to the N-terminus to generate the Pak2b$^{\Delta c}$GST fusion, whereas the YFP protein is fused to the C-terminus of Pak2b$^{\Delta c}$YFP. Therefore the failure of these Pak2b fusion proteins to bind Nck is likely to represent a real result. Thus we have shown that Pak2a, but not Pak2b, can bind Nck.

Interaction of Pak2a and Pak2b with PIX was explored using an antibody against the serine-rich C-terminus of hβ2PIX (Koh *et al.*, 2001). This revealed binding of a number of proteins of different molecular weights. The βPIX antibody is specific for a human protein of 68kD. In zebrafish, genome sequencing has identified three isoforms of βPIX with predicted molecular weights of 75kD, 68kD and 62kD. Western

blot analysis of zebrafish embryo extract using the βPIX antibody detected bands at: 79, 74, 68, 62, 57 and 48kD (data not shown), this may indicate the presence of further βPIX isoforms or that the antibody may show some unspecific binding.

Western blot of proteins pulled down by Pak2a$^{\Delta c}$GST, Pak2b$^{\Delta c}$GST or GST alone detected bands at: 74, 68, 62, 60, 57, 48 and 45kD (Figure 4.4). Although the two larger isoforms (74 and 68kD) appeared to be pulled down by control GST protein a higher affinity of binding was observed between these isoforms and Pak2a$^{\Delta c}$GST or Pak2b$^{\Delta c}$GST. The binding of the larger βPIX isoforms to GST may represent unspecific interaction between the βPIX isoforms and GST. There was low affinity binding of the 62kD protein to both Pak2a$^{\Delta c}$GST and Pak2b$^{\Delta c}$GST and of the 60kD isoform to Pak2b$^{\Delta c}$GST, βPIX proteins of these molecular weights were detected in Western blot analysis of total embryo extract. Pak2a$^{\Delta c}$GST was also found to bind, with high affinity to a βPIX protein of 57kD, while Pak2b-GST failed to bind this isoform. Intriguingly detection of βPIX proteins after pull down using Pak2a$^{\Delta c}$GST or Pak2b$^{\Delta c}$GST revealed strong bands at 48kD, a band of this size was also identified in total embryo extract. A strong band was also detected at 45kD in the Pak2a$^{\Delta c}$GST pull down sample which could not be seen in total embryo extract. These proteins are significantly smaller than the hβ2PIX of 68kD and it is not clear precisely what these small bands represent.

It will be interesting to find out what functional differences are conferred by the differences in protein interactions observed between Pak2a and Pak2b, particularly in relation to the inability of Pak2b to bind Nck.

Figure 4.4

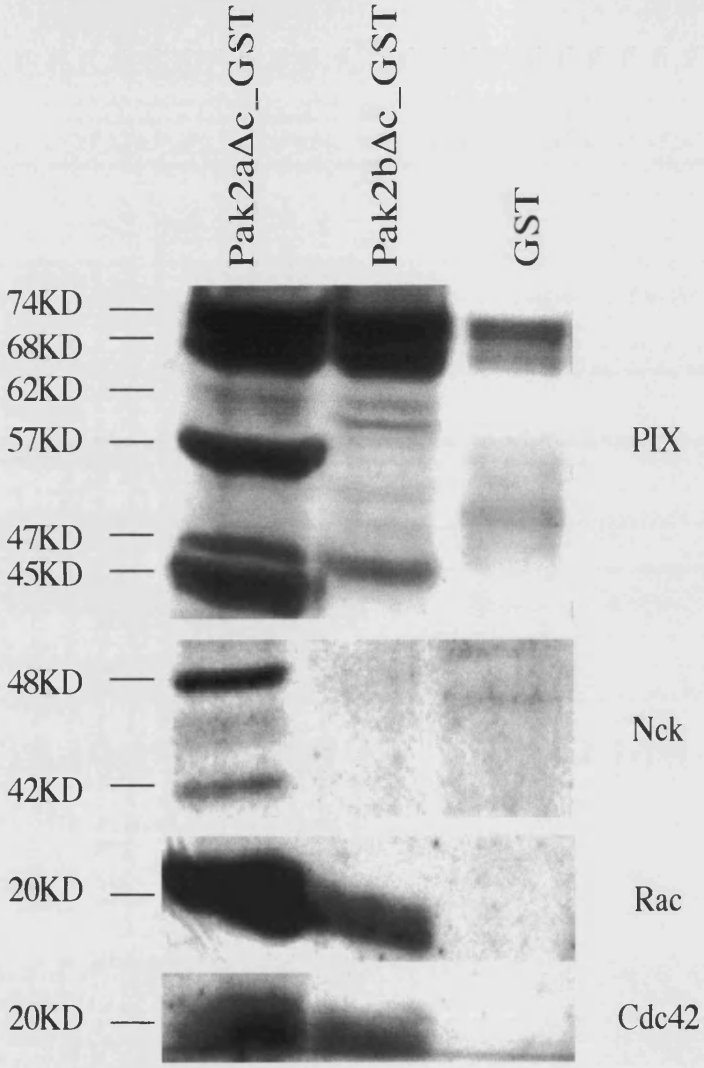

Pak2aΔc_GST
Pak2bΔc_GST
GST
74KD
68KD
62KD
57KD
47KD
45KD
48KD
42KD
20KD
20KD
PIX
Nck
Rac
Cdc42

Figure 4.4: Pull down assays showing proteins that interact with Pak2a and Pak2b.

Fusion proteins, Pak2a$^{\Delta c}$GST, Pak2b$^{\Delta c}$GST or GST alone (control), were immobilised on beads and incubated with whole cell embryonic extract (15 somite stage). Interacting proteins were detected by Western blot analysis using antibodies against proteins known to interact with Pak: PIX, Nck, Rac and Cdc42. These results were consistent over two repeats.

4.7 Expression of the zebrafish *pak2* genes during embryogenesis.

In order to gain insight into the function of the zebrafish *pak2* genes it is necessary to analyse their spatial and temporal expression pattern. Expression of the zebrafish *pak2* genes was investigated by whole mount *in situ* hybridisation during different stages of development.

4.7.1 Expression pattern of *pak2a.*

Whole mount *in situ* hybridisation showed the presence of maternal *pak2a* transcripts throughout the embryo at blastula stage (Figure 4.5a,b) and zygotic expression was ubiquitous during early stages of gastrulation (data not shown). At the tailbud stage a higher level of the transcripts was detected in the notochord and there was weak labelling of the epidermis, this staining became more marked at the 10-somite stage (Figure 4.5c,d). From the 15-somite stage, the expression in the notochord began to be restricted posteriorly and all epidermal cells expressed high levels of *pak2a* (Figure 4.5k). Dynamic expression of *pak2a* in the developing notochord, neural tube and epidermis was examined in detail by looking at transverse sections over a range of embryonic stages (Figure 4.5p-r).

At the 10-somite stage higher levels *pak2a* transcripts were detected in the eye, apical and basal surfaces of the neural tube and in epidermal tissue abutting the yolk (Figure 4.5c-e). Flat mount preparations showed that *pak2a* transcripts were enriched in the tailbud (Figure 4.5h). Expression of *pak2a* was observed throughout the developing brain from 10-somite stage and the expression was clearly visible at the 26-somite stage (Figure 4.5i). Expression in the brain and other anterior structures decreased in accordance with the general decrease in transcript level from 28hpf.

At the 26-somite stage a strong band of *pak2a* expression was observed in stripes through the central region of the somites, particularly in the more mature

somites (Figure 4.5j). The posterior lateral line primordium could be distinguished at the 26-somite stage, however, levels of transcripts within this tissue were comparable to the surrounding epidermis. From 28hpf transcript levels decreased throughout the embryo with the exception of epithelial cells (Figure 4.5m). At 28hpf and 32hpf staining in epithelial cells was enriched over somitic boundaries. Interestingly *pak2a* transcripts were highly expressed in epithelial cells adjacent to the posterior lateral line primordium (Figure 4.5n,o). This expression was maintained as the primordium migrated along the trunk. Expression of *pak2a* appeared to be down regulated within the posterior lateral line primordium.

4.7.2 Expression pattern of *pak2b.*

A low level of maternal *pak2b* transcripts was detected ubiquitously at blastula stage and the zygotic expression remained weak and ubiquitous up to the tailbud stage. Like the expression pattern of *pak2a*, *pak2b* transcripts were detected on the apical and basal surface of the neural tube from the 5-somite stage and the expression was increase in embryos at the 10-somite stage (Figure 4.6a,d).

Expression of *pak2b* was also detected in the anterior to posterior extent of the notochord at the 5-somite stage, however, the expression was restricted to the posterior at the 10-somite stage. The down regulation of expression in the notochord occurs earlier with *pak2b* than *pak2a*. At the 15-somite stage there was enrichment of *pak2b* transcripts in the adaxial cells and expression in the notochord was restricted to the tailbud (Figure 4.6f,g). At the 26-somite stage *pak2b* transcripts in the mature somites were detected at the same location as *pak2a*, forming stripes in the centre of the somites (Figure 4.6h). This pattern was maintained at 24hpf after which it could not be detected.

Figure 4.5

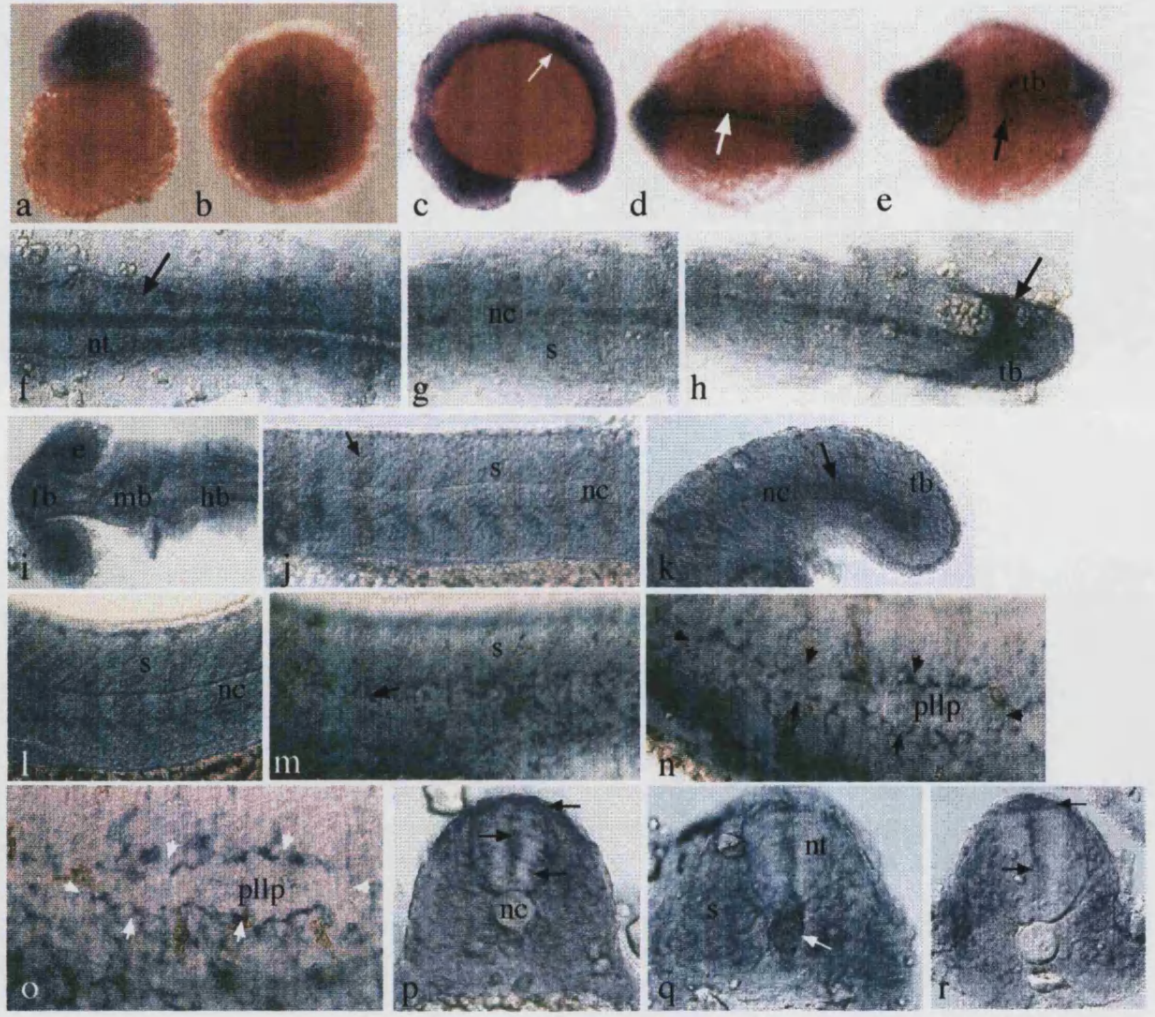

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Figure 4.5: *In situ* hybridisation analysis of *pak2a* expression during zebrafish development.

Expression of *pak2a* visualised by *in situ* hybridisation is shown in whole embryos at the following stages: blastula (a,b), 10-somite (c-h, p,q), 20-somite (r), 26-somite (i-k), 28hpf (n) and 32hpf (o) and in transverse, hand cut sections of approximately one-somite in thickness, at 10-somite (p,q) and 20-somite (r) stages. Arrows indicate tissues with high levels of *pak2a* transcripts. Embryos are orientated anterior to the left except *a*, animal to the top and *b*, a view of the animal pole and transverse sections (p-r). Abbreviations refer to: nt, neural tube; nc, notochord; s, somites; e, eye; fb, forebrain; mb, midbrain; hb, hindbrain; tb, tailbud; pllp, posterior lateral line primordium.

Figure 4.6

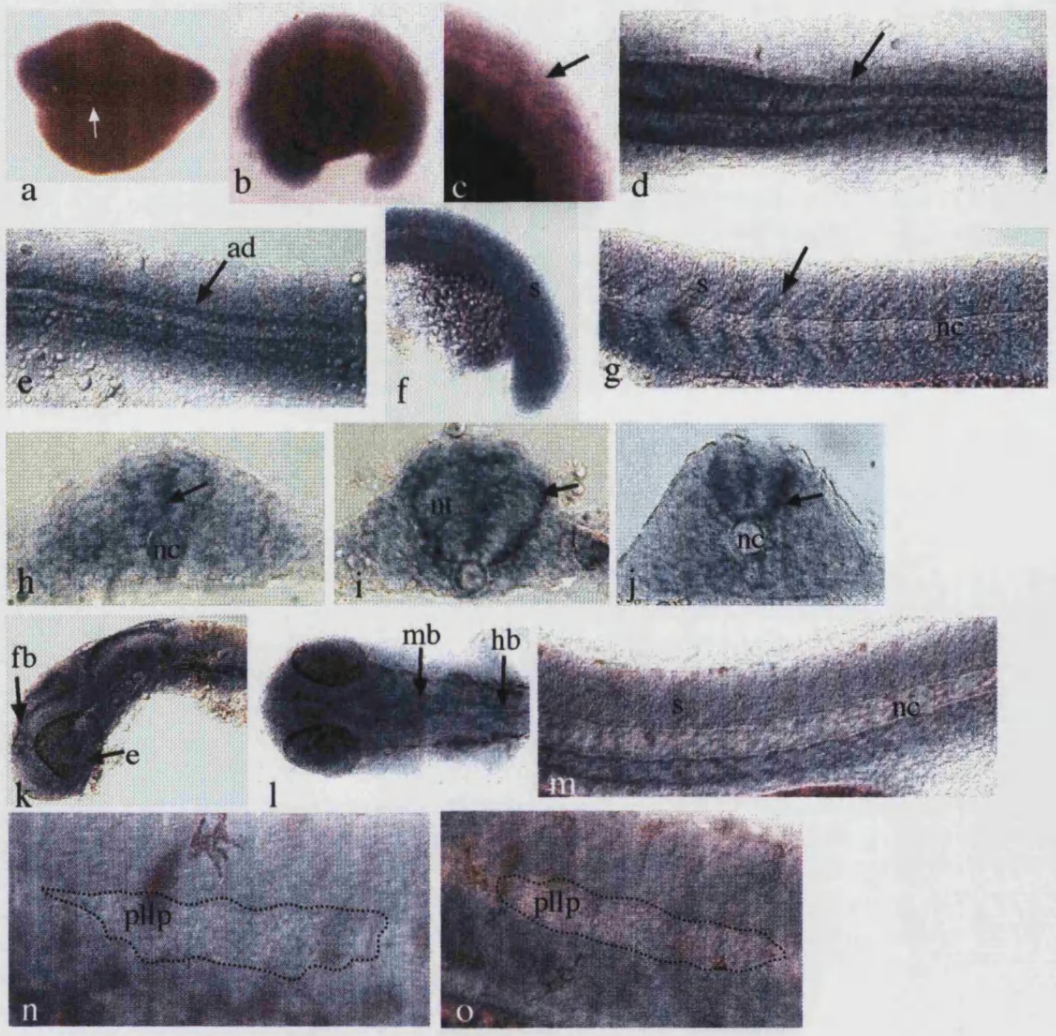


Figure 4.6: *In situ* hybridisation analysis of *pak2b* expression during zebrafish development.

Expression of *pak2b* visualised by *in situ* hybridisation is shown in whole embryos at the following stages: 5-somite (h), 10-somite (a-d,i,j), 15-somite (e,f), 26-somite (g), 28hpf (k,n) and 32hpf (l,m,o) and in transverse hand cut sections of approximately one-somite in thickness, at 5-somite (h) and 10-somite (i,j) stages. Arrow indicate tissues with high levels of *pak2b* transcripts. Embryos are orientated anterior to the left, expect transverse sections (h-j). Abbreviations refer to: ad, adaxial cells; nt, neural tube; nc, notochord; s, somites; e, eye; fb, forebrain; mb, midbrain; hb, hindbrain; tb, tailbud; pllp, posterior lateral line primordium.

Transverse sections of embryos at the 5- and 10-somite stages showed enrichment of *pak2b* transcripts in the lateral regions of the neural tube and on the apical surface (Figure 4.6: i). This was maintained at later stages and down regulated by 32hpf. Sections also showed that, unlike *pak2a*, there was no increased localisation of *pak2b* transcripts in the epidermal cells.

From 28hpf there was a general decrease in the level of *pak2b* transcripts although the expression remained in the brain, eyes, epidermis and somitic tissue (Figure 4.6k-m). The striking pattern of *pak2a* expression in epithelial cells surrounding the posterior lateral line primordium was not observed with *pak2b*. Expression of *pak2b* in the primordium was indistinguishable from that in the surrounding cells (Figure 4.6n,o).

4.8 Discussion.

This chapter described identification and characterisation of the two novel zebrafish homologues of *pak2*. The genes, *pak2a* and *pak2b*, were shown to be actively transcribed during embryogenesis (Figure 4.3A). *In-vitro* transcription and translation analysis indicated that the *pak2a* and *pak2b* cDNAs encoded proteins of approximately 62kD and 67kD respectively (Figure 4.3B). This is in agreement with published data for the molecular weights of the rat Group I Pak proteins (Pak1: 68kD, Pak2: 62kD, Pak3: 65kD) (Knaus and Bokoch, 1998).

Pak2a and Pak2b protein sequences showed very high homology to Pak2 proteins identified in other species (rat, rabbit, *Xenopus*, human) with specific domains including the proline rich SH3 binding domains, PBD and CRIB motif, kinase domain and Gβ subunit binding site highly conserved (Figures 4.1 and 4.2).

Zebrafish Pak2a$^{\Delta c}$GST and Pak2a$^{\Delta c}$GST proteins were found to bind to the small GTPases, Rac and Cdc42. Interaction of Pak with Rac and Cdc42 has been shown to be important for regulating organisation of the actin cytoskeleton (Eby *et al.*, 1998), therefore the pull down result indicates that both Pak2a and Pak2b proteins

could act as downstream mediators of Rac and Cdc42 in regulating the actin cytoskeleton. Interaction of Pak2a and Pak2b with SH3-domain proteins showed interesting differences. While Pak2a$^{\Delta c}$GST was able to bind to two isoforms of Nck, Pak2b$^{\Delta c}$GST appeared unable to bind to Nck. Differences were also apparent in the binding of βPIX isoforms to Pak2a$^{\Delta c}$GST and Pak2b$^{\Delta c}$GST proteins in the intensity, size and number of βPIX proteins pulled down. Variation between Pak2a and Pak2b in the ability to interact with Nck and βPIX was surprising as binding motifs for these proteins were absolutely conserved in both proteins. One possible explanation for these differences results from the amino acid divergence at residues flanking the Nck and PIX binding sites of Pak2b. Single amino acid differences around the Nck binding site (aa6,7, 25 and 29) and the insert of 10, mostly acidic, amino acids in the region immediately preceding the PIX binding site in Pak2b could affect the conformation of the Pak2b protein. Changes to the conformation of Pak2b may then affect the binding of Nck or PIX by altering the accessibility of the binding sites.

The expression patterns of *pak2a* and *pak2b* were in agreement with observations of *pak2* localisation in other species. In rat and *Xenopus pak2* expression was found to be ubiquitous (Jaffer and Chernoff, 2002; Souopgui *et al.*, 2002). Higher levels of X*pak2* transcript have been noted in the neural plate and later in the brain, eye and tailbud. The enrichment of X*pak2* in the brain, eye and tailbud was mirrored in zebrafish *pak2a* and *pak2b* expression in early stages of development. An interesting pattern of *pak2a* expression was observed surrounding the migrating lateral line primordium. Although the cells of the posterior lateral line primordium expressed low levels of both *pak2a* and *pak2b*, there were high levels of *pak2a* transcripts in the epithelial cells surrounding the lateral line primordium. It is interesting to note that during dorsal closure in *Drosophila* the *pak* homologue, DPak, is enriched in epidermal cells flanking the amnioserosa and associated with the focal adhesions at the leading edge of these migrating cells (Harden *et al.*, 1996). We have shown that high levels of the focal adhesion protein Paxillin are found surrounding the posterior lateral line primordium. These data raise the interesting possibility that

Pak2a may be associated with focal adhesions and could be involved in regulating migration of the posterior lateral line primordium. Pak can be recruited to focal adhesions by means of binding to the PIX/PKL complex (Brown *et al.*, 2002). PIX binds to Pak proteins through the atypical SH3 motif that is conserved in Pak2a and Pak2b, suggesting that both zebrafish Pak proteins would be able to interact with PIX. Pull down analysis with the Pak2a$^{\Delta c}$GST and Pak2b$^{\Delta c}$YFP showed that Pak2a and Pak2b were able to bind to PIX, however Pak2a and Pak2b bound a number of isoforms with differing affinity.

In addition to PIX, Nck is also thought to play a role in recruiting Pak to sites of focal adhesion complexes (Zhao *et al.*, 2000a) and Nck can increase activation of Pak by co-localisation of Pak with its activator Rac (Lu *et al.*, 1997). Binding of Nck to Pak2a implies that zebrafish Pak2a could be activated by Nck-directed recruitment to the membrane. The finding that Pak2a binds to Nck but Pak2b does not raises some interesting questions regarding the regulation of focal adhesion complexes by zebrafish Pak2 and the activation of Pak2a and Pak2b.

In summary Pak2a and Pak2b are likely to be involved in regulation of the actin cytoskeleton and formation of focal adhesions during zebrafish development by virtue of interactions with proteins such as Rac/Cdc42, Nck and PIX. We have found significant differences in the binding of Pak2a and Pak2b to Nck and PIX. The ubiquitous nature of *pak2a* and *pak2b* gene expression suggests that these proteins may act in a number of tissues during development including the formation of the posterior lateral line system. We will employ a loss-of-function approach to investigate the role of Pak2a and Pak2b during zebrafish development, in particular during the development of the posterior lateral line system.

Chapter Five

Role of Pak in development of the posterior lateral line system.

5.1 Introduction

In order to investigate the role of Pak2a and Pak2b in zebrafish development we have primarily utilised a morpholino knock down approach and analysed the loss-of-function phenotypes. This chapter describes the general phenotype of *pak2a* and *pak2b* morpholino-mediated knockdown and detailed analysis of the posterior lateral line development. We utilised the techniques described in Chapter Three to follow migration of the lateral line primordium, patterning of cells within the lateral line primordium, deposition of neuromast precursors and their differentiation. Furthermore we explored the effect of Pak2 knock down on cadherin-mediated adherens junctions and focal adhesions by immunocytochemistry and Western blot analysis.

5.2 Morpholino knock down of Pak2a and Pak2b.

Morpholinos are chemically modified oligonucleotides with base-stacking abilities similar to those of natural genetic material. Morpholinos have been shown to bind to and block translation of mRNA by hindering translational initiation (Nasevicius and Ekker, 2000). This approach makes *in vivo* targeting highly predictable and reduces non-specific effects. Injection of morpholino into zebrafish embryos at the one-cell stage can effectively knock down expression of the target gene, providing a useful method for loss-of-function experiments.

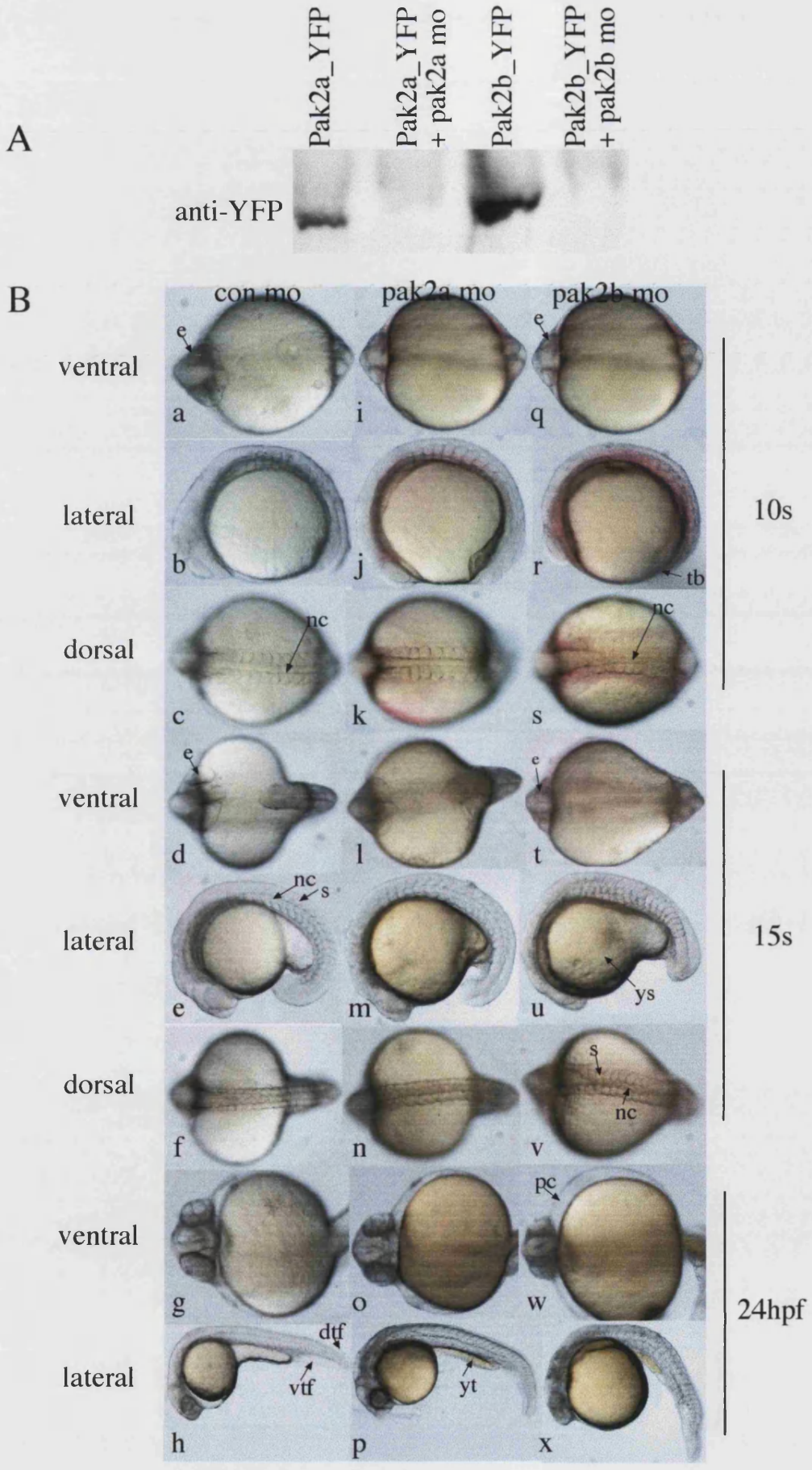
Morpholino sequences were designed to target the 5' untranslated region (UTR) immediately adjacent to the translation initiation methionine (ATG) of Pak2a and against the 5'UTR including the initiation site of Pak2b (Chapter Two). Both morpholino sequences were tested by BLAST at NCBI for representation elsewhere in the EST database and shown to be unique. As no antibody was available to specifically identify zebrafish Pak2a or Pak2b we generated RNA expression constructs. The constructs comprised the full length *pak2a* or *pak2b* gene, including the 5' UTR to which the morpholino was designed, fused to the yellow fluorescent protein (YFP). This construct could be utilised to evaluate the knock down effect of the morpholinos on protein translation by co-injection of Pak2a^{FL}YFP or Pak2b^{FL}YFP RNA with the corresponding morpholino oligonucleotide. The strength of fluorescence in embryos co-injected with morpholino was considerably reduced when compared to embryos injected with either Pak2a^{FL}YFP or Pak2b^{FL}YFP RNA alone (data not shown). This result was quantified by Western blot analysis of whole cell extract prepared from embryos injected with *pak2a^{FL}YFP* or *pak2b^{FL}YFP* RNA alone and together with the corresponding morpholino. Pak2a^{FL}YFP and Pak2b^{FL}YFP protein was detected using an anti-GFP antibody which could detect YFP. The levels of Pak2a^{FL}YFP and Pak2b^{FL}YFP were significantly reduced in embryos co-injected with morpholino (Figure 5.1A). As a result it is evident that translation of *pak2a* and *pak2b* was effectively blocked by their specific morpholino sequences.

Initial experiments were carried out to define the dose of morpholino required to produce a specific phenotype. Stock morpholino oligonucleotides (300mM), dissolved in 63µl RNase- and DNase-free water, were further diluted from 1:8 down to 1:32 and 3nl was injected into embryos at one-cell stage. With both *pak2a* and *pak2b* morpholinos a morphological phenotype was observed following injection of 1:16 dilution, equivalent to 2ng/nl. The phenotypes of embryos injected with *pak2b* morpholino were more severe than those observed in embryos injected with *pak2a* morpholino.

5.2.1 General phenotype of morpholino-mediated knock down of *pak2a* and *pak2b.*

At early stages the development of the *pak2a* and *pak2b* morpholino injected embryos proceeded normally and as for control morpholino injected embryos (data not shown). The morphological shield was formed and gastrulation appeared normal in *pak2a* and *pak2b* morpholino injected embryos, although the process was occasionally slightly delayed in embryos injected with *pak2b* morpholino. At advanced epiboly stages the forerunner cells were present and by the tailbud stage embryos injected with *pak2a* or *pak2b* morpholino had completed epiboly and the notochord anlagen was present. As morpholinos do not affect levels of maternal protein the absence of phenotype at early stages may result from activity of maternal Pak2, which would be degraded by later stages.

Defects began to be visible from the 10-somite stage, whereupon the head appeared smaller and in embryos injected with *pak2b* morpholino a convergent extension defect was visible (Figure 5.1i,j and q,r). This was characterised by a reduction in advancement of the tailbud over the yolk sac (Figure 5.1r). A mild convergent extension defect was apparent in embryos injected with *pak2a* morpholino from the 15-somite stage. This was characterised by a shortened body axis and at later stages by a ventral curl of the tail, posterior to the yolk tube (Figure 5.1p). Ventral curling of the tail was more pronounced in embryos injected with *pak2b* morpholino (Figure 5.1x). In both cases the somites at the bent area of the tail were compacted and more "u" shaped than the classical chevron shaped and many "u" and irregular shaped somites were present in embryos injected with *pak2b* morpholino (Figure 5.1m,p and u,x). The morphology of the notochord appeared normal in embryos injected with *pak2a* morpholino, however, the notochord exhibited kinks in *pak2b* morpholino injected embryos (Figure 5.1u).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B
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pak2b mo
ventral
lateral
dorsal
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lateral
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ventral
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Figure 5.1

Figure 5.1: Phenotypes of embryos injected with pak2a and pak2b morpholino.

A: Morpholino-mediated knock down of the Pak2a and Pak2b YFP-fusion proteins shown using Western blot analysis. Embryos were injected with *pak2a*-YFP/*pak2b*-YFP alone or co-injected with the corresponding morpholino.

B: Images of live embryos injected with 3nl control (a-h), *pak2a* (i-p) or *pak2b* (q-x) morpholino at a 1:16 dilution, equivalent to 4.5ng. Embryos are shown at the 10-somite stage (a-c, i-k, q-s), 15-somite stage (d-f, l-n, t-v) and at 24hpf (g,h,o,p and w,x). Embryos are shown from ventral, lateral and dorsal views and are orientated anterior to the left. Tissues are labelled by the following abbreviations; e, eye; tb, tailbud; nc, notochord; s, somite; ys, yolk sac; pc, pericardium; dtf, dorsal tail fin; vtf, ventral tail fin; yt, yolk tube.

Posterior constriction of the yolk sac to form the yolk tube was defective, resulting in a thin yolk tube (Figure 5.1m,p and u,x). The heart was small and resembled an early heart tube (data not shown). The heart rate was slow and circulation was poor or not evident and as a result there was swelling of the pericardium (Figure 5.1o,w). A swelling was also present over the mid and hindbrain ventricles in embryos injected with *pak2a* morpholino (Figure 5.1p).

At later stages of development the epidermis of embryos injected with *pak2a* or *pak2b* morpholino had a necrotic appearance and the dorsal and ventral tail fins were greatly reduced or absent (Figure 5.1p,x). Embryos injected with *pak2a* morpholino were able to hatch from their chorions and swim and a large percentage survived until 5dpf. Some embryos injected with *pak2b* morpholino were able to hatch and swimming was hampered by the highly curved tail. Approximately 50% of embryos injected with *pak2b* morpholino survived to 5dpf. Survival beyond this stage was not investigated.

5.3 Effect of Pak2 knock down on development of the posterior lateral line.

As discussed previously Pak has been shown to play important roles in the control of cell migration. We have demonstrated that development of the posterior lateral line represents a valuable model system for studying cell migration in zebrafish development. The superficial location and characteristic patterning of the lateral line primordium presents a useful system to investigate the effects of *pak2* morpholino-mediated knock down on cell migration *in vivo*.

5.3.1 Neuromast number and position in morpholino injected embryos.

Detection of the endogenous alkaline phosphatase activity was used to identify the mantle cells of mature neuromasts in order to analyse pattern formation in the lateral line system. In normal development the primary posterior lateral line consists of five neuromasts along the midline and two neuromasts in a more ventral position at the tail (Figure 5.2a,f).

In a large percentage of embryos injected with *pak2a* morpholino mature neuromasts were present along the full length of the trunk and tail. However, the number and position of the neuromasts was disrupted and in 63% of embryos analysed (n=27) there was a reduction in the number of neuromasts. Most common (35%) was the loss of one neuromast and increased spacing between either the first and second neuromasts (Figure 5.2c) or between the second and third neuromasts (Figure 5.2b). Staining with the differentiated hair cell marker, S100, and nuclear counter-staining did not identify any further neuromasts.

A more severe phenotype was observed in embryos injected with *pak2b* morpholino. In the majority of cases (85%, n=70) no mature neuromasts were detected by staining for endogenous alkaline phosphatase activity (Figure 5.2d,e). However when using the antibodies against S100 and acetylated tubulin, differentiated hair cells could be observed in the anterior trunk of some *pak2b* morphant embryos (Figure 5.2n-p). Closer observation revealed these S100 labelled hair cells were surrounded by tightly packed support-like cells that in some cases showed weak alkaline phosphatase activity (Figure 5.2m). In general, only one neuromast was identifiable in this manner, however as many as three could be observed in a small number of embryos (12%). Therefore injection of *pak2a* or *pak2b* morpholino results in a loss of differentiated neuromasts.

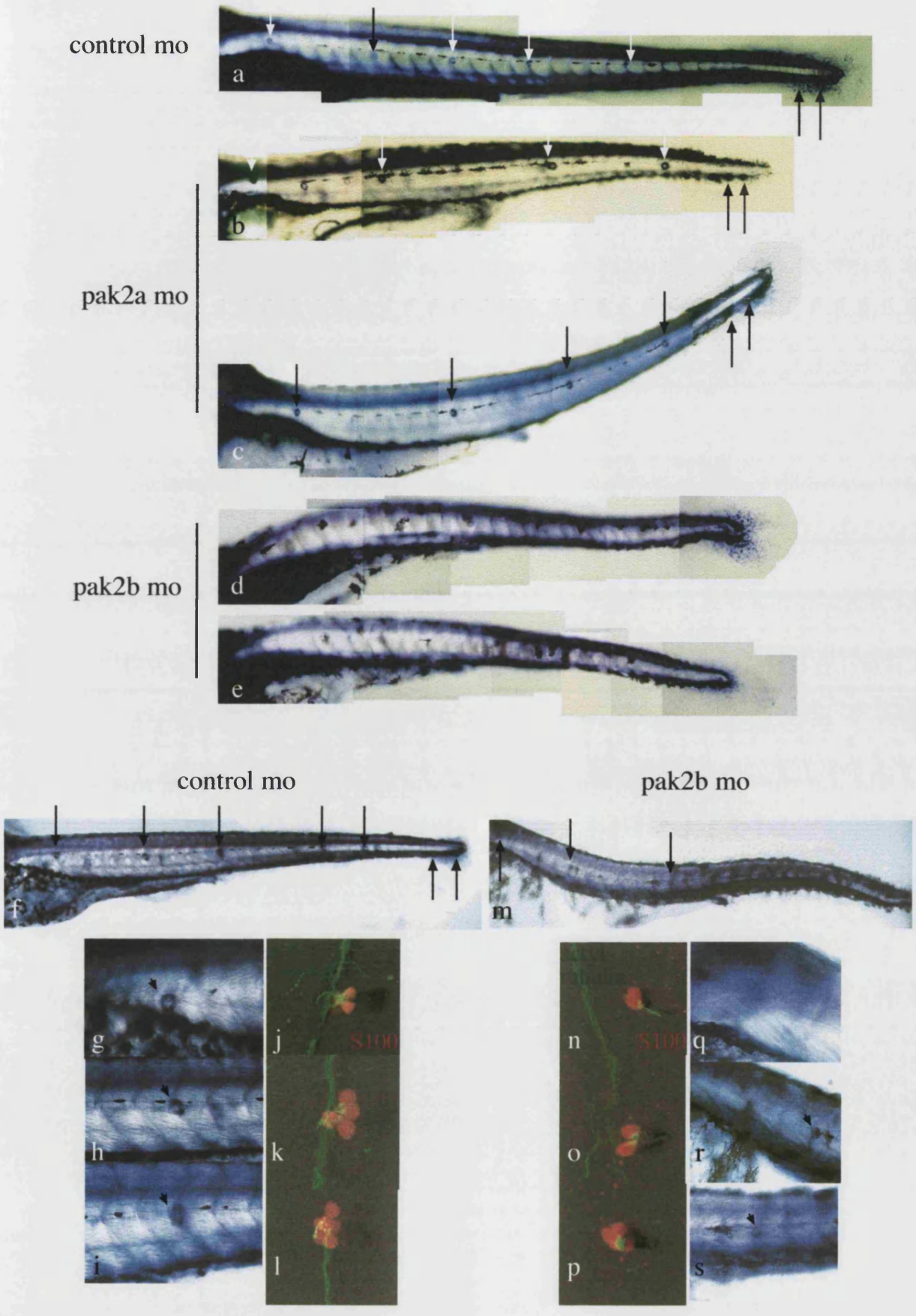
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Figure 5.2

Figure 5.2: Effect of Pak2 knock down on the number and position of mature neuromasts in the posterior lateral line system.

Differentiated neuromasts were visualised by staining for endogenous alkaline phosphatase activity at 3dpf. The number and position of neuromasts was compared between control embryos and embryos injected with 4.5ng morpholino. Positions of the posterior neuromasts are indicated by arrows in control embryos (a,f-i), embryos injected with *pak2a* morpholino (b,c) and embryos injected with *pak2b* morpholino (d,e,m-p).

Differentiated hair cells were detected by S100 (red) and acetylated tubulin (green) antibodies in control (j-l) and upon the anterior trunk of *pak2b* (n-p) morpholino injected embryos at 3dpf.

5.3.2 Migration of the posterior lateral line primordium in morpholino injected embryos.

While alkaline phosphatase activity and S100 staining revealed the defects in the formation of the posterior lateral line in terms of the position and number of mature neuromasts, we needed to look at earlier stages to understand how these defects arose. One possible explanation for the reduction in the number and the anterior position of neuromasts in embryos injected with *pak2b* morpholino is defective migration of the posterior lateral line primordium. The position of the primordium in embryos injected with *pak2a* or *pak2b* morpholino over a series of stages was visualised by whole mount *in situ* hybridisation using molecular markers of the posterior lateral line primordium. The markers, *nkx5-1* and *eya-1*, were selected for their complete temporal coverage of posterior lateral line development and broad expression in many cell types of the lateral line system. *Nkx5-1*, a homologue of the *Drosophila NK* homeobox genes, is expressed in the posterior lateral line from the 4-somite stage (11.3hpf) and is maintained in the migratory primordium throughout it's passage along the trunk and tail (Adamska *et al.*, 2000). Zebrafish *eya1* is a member of the *eyes absent*-like family of transcriptional co-activators, and is expressed in the lateral line placodes, lateral line ganglion, migratory primordium and is maintained in neuromast precursors following deposition (Sahly *et al.*, 1999).

Knock down of Pak2a or Pak2b did not have any effect on the specification of the lateral line primordium as a focal thickening of *nkx5-1* and *eya1* positive cells could be detected in embryos injected with *pak2a* or *pak2b* morpholino (data not shown). Detection of the primordium at stages throughout migration revealed that while the primordium migrated normally in embryos injected with *pak2a* morpholino, knock down of Pak2b resulted in retarded migration. Distance of the lateral line primordium migration was measured in relation to the number of somites it had moved over. In embryos injected with *pak2b* morpholino the rate of migration of the primordium was severely affected and much reduced.

Figure 5.3

Figure 5.3: Migration of the lateral line primordium is retarded in embryos injected with *pak2b* morpholino.

Whole mount *in situ* hybridisation of *nkx5-1* was carried out with control embryos (a-e) or embryos injected with 4.5ng *pak2b* morpholino (f-j) over a range of stages; 24hpf (a,f), 26hpf (b,g), 28hpf (c,h), 30hpf (d,i) and 32hpf (e,j). The migration of the primordium was also investigated using bright field microscopy, (k) control, (l-o) *pak2b* morpholino injected. The position of the primordium is denoted by the number of the underlying somites.

The primordium typically stopped in the mid-trunk and did not reach the tail end of the embryo (Figure 5.3f-j). This defect in primordium migration is manifest at later developmental stages, at 3dpf a cluster of cells, indicative of the lateral line primordium, was detected in the mid-trunk region by labelling of cell nuclei with DAPI. Co-labelling of the lateral line ganglion with acetylated tubulin revealed that this cell cluster was associated the termination of the lateral line ganglion (data not shown).

The primordium was often smaller and of irregular shape in embryos injected with *pak2b* morpholino compared with control morpholino (Figure 5.3a-e and i,j). These defects in migration, shape and primordium size have also been observed morphologically in live embryos by virtue of the distinctive thickening of the primordium (Figure 5.3k-o).

5.3.3 Real-time analysis of migration of the posterior lateral line primordium in pak2b morpholino injected embryos.

In order to gain a better understanding of the defects in the developing lateral line when *pak2* translation was attenuated, we looked at migration of the primordium in real time. Time-lapse analysis was focused mainly on embryos injected with *pak2b* morpholino, as these embryos showed retardation in the migration of the primordium. The lateral line primordium was visualised in live embryos injected with morpholino using bodipy sphingomyelin or co-injection of gapGFP to label cell membranes. The gapGFP fusion protein is specifically targeted to the cell membranes (Moriyoshi *et al.*, 1996; Okada *et al.*, 1999) and was used as an alternative to bodipy to aid visualisation of activity at the leading edge. Injection of gapGFP after the one cell stage produced mosaic expression and labelling of only a fraction of cell membranes, which enhanced visualisation of cell activity. This technique was also used in control embryos and did not affect normal migration of the primordium (data not shown).

Figure 5.4

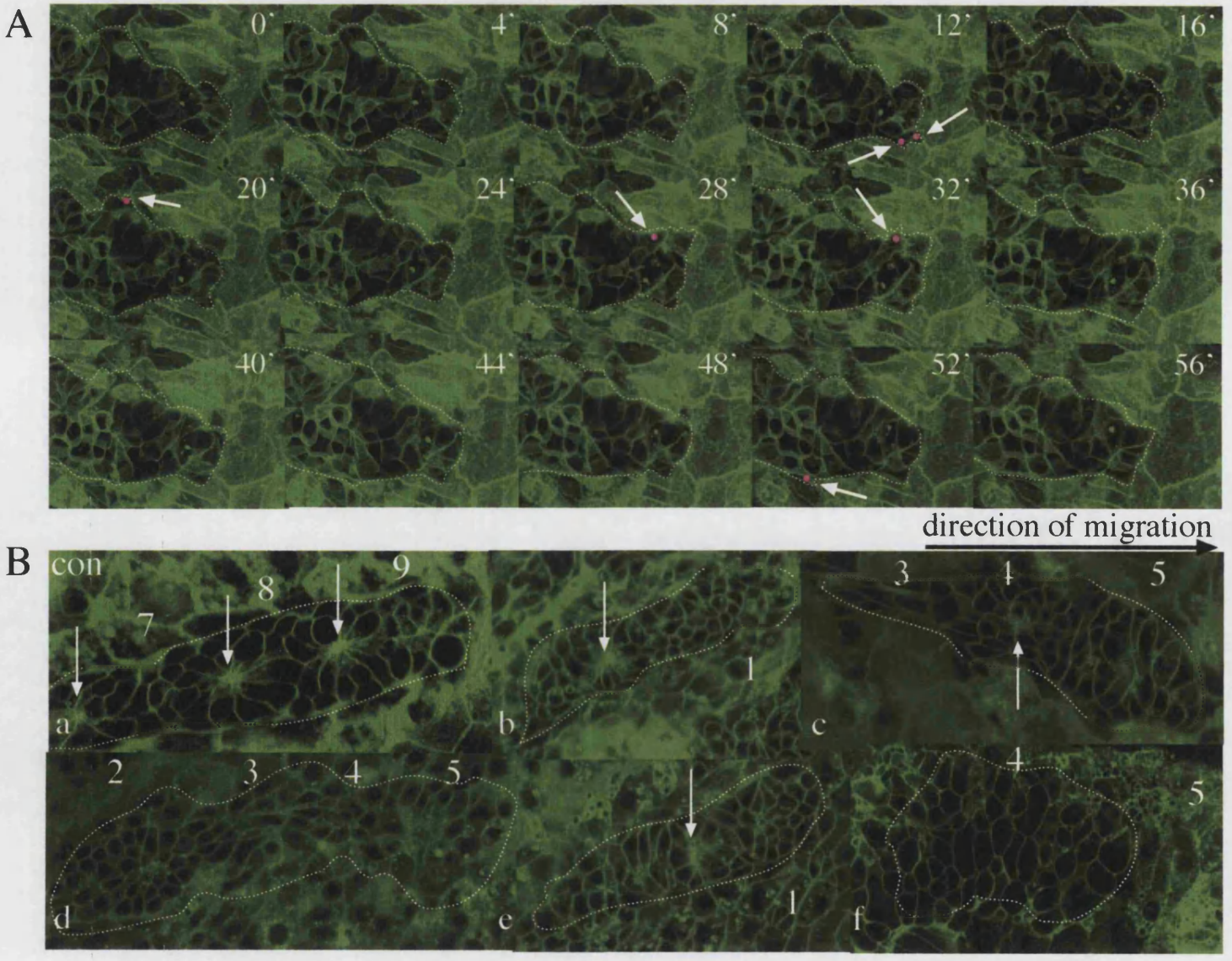
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Figure 5. 4: Effect of Pak2b knock down on lateral line migration.

A: Time lapse series showing typical cell behaviour at the leading edge of the migrating posterior lateral line primordium following injection of 4.5ng *pak2b* morpholino. Images shown are consistent with observations of pllp migration in more than 15 pak2b morpholino injected embryos. Cell membranes were visualised in live embryos by injection at the one cell stage with gapGFP. Times shown are in minutes. White dashed lines outline the posterior lateral line primordium. Arrows highlight lateral extension of cell membrane from labelled cells (pink).

B: Confocal sections showing disruption to the cellular organisation of the posterior lateral line primordium of embryos injected with 4.5ng *pak2b* morpholino (b-f) compared to a control (a). Primordia of embryos injected with *pak2b* morpholino exhibited a loss of cellular patterning and the rosette-like structures of nascent neuromasts were not detected or were poorly organised (arrows). Numbers indicated the position of the primordium in relation to the underlying somites.

The Pak family of proteins have emerged as important regulators of the changes to the cytoskeleton required for cell migration (Kiosses *et al.*, 1999). Time-lapse analysis was used to look at the activity of cells at the leading edge of the primordium comparing embryos injected with *pak2b* morpholino to uninjected controls. We have already described that, in a normal environment, cells at the leading edge of the migrating primordium are loosely associated with each other and actively change their shape, extending and contracting both laterally and in the direction of migration. Away from the leading edge cells undergo fewer shape changes and migrate as a tightly packed group, with elongation only in the direction of migration (Figure 3.3). When embryos were injected with *pak2b* morpholino the cells within the body of the primordium were rigid in shape but, unlike control embryos, explorative cell membrane protrusions at the sides of the primordium and into the surrounding tissue was observed (Figure 5.4A). At the leading edge of the primordium cell behaviour in embryos injected with *pak2b* morpholino was comparable to that in control embryos. However, despite the fact that cells at the leading edge were highly active and explorative, extending membrane processes both laterally and posteriorly, very little migration of the primordium was observed (Figure 5.4A). This loss of forward motility can be clearly observed in the time lapse series, by looking at the position of the primordium within the field of view. In control embryos the primordium visibly travels across the field of view in a 30 minute time period (Figure 3.3). However in embryos injected with *pak2b* morpholino the position of the primordium within the field of view is more or less unchanged over a 60 minute time period (Figure 5.4A). Furthermore, when position of the primordium, relative to the underlying somites, is compared in control and *pak2b* morpholino injected embryos it is clear that primordia in control embryos have migrated 4 to 8 somites further than the primordia of embryos injected with *pak2b* morpholino at the same developmental stage (Figure 5.5b-f). Thus it appears that knock down of Pak2b does not effect the motility of the cells of the lateral line primordium *per se*, but results in a loss of directed migration.

Interestingly the organisation of cells within the lateral line primordium was disrupted in embryos injected with *pak2b* morpholino, and to a lesser extent in embryos injected with *pak2a* morpholino. At early stages of migration the rosette-like structure of nascent neuromasts could be identified (Figure 5.4b,c), however as migration proceeded, cells of the primordium became progressively less organised and "rosettes" could no long be identified (Figure 5.4d,f). Furthermore the cells within the primordia of embryos injected with *pak2b* morpholino lost the characteristic teardrop shape and cell shape became randomised and irregular (n=20). These observations suggested that embryos injected with *pak2b*, and to a lesser extent *pak2a* morpholino, were unable to maintain the cellular prepattern of the primordium as it migrates which may indicate an inability to dynamically rearrange cellular adhesions during migration of the lateral line primordium.

5.3.4 Effect of knock down on primordium cellular organisation and adhesion molecules.

The observation of disrupted cellular organisation in the primordium of embryos injected with *pak2a* or *pak2b* morpholino raised the possibility of a role for Pak2a and Pak2b in establishing or maintaining the cellular prepattern of the lateral line primordium. We have shown that components of adherens junctions, E-cadherin and β-catenin, and focal adhesions, Paxillin, as well as F-actin have a characteristic pattern of localisation in the migrating lateral line primordium and we proposed that regulation of the actin cytoskeleton, adherens junctions and focal adhesions would have critical roles in controlling migration of the posterior lateral line primordium (Chapter Three). These roles would include directed migration of the primordium through polarisation of the actin cytoskeleton, focal adhesion-mediated traction forces to "pull" the cells of the lateral line primordium along their migratory pathway and dynamic cell to cell adhesions to maintain the close association of primordium cells throughout migration and during neuromast deposition. Analysis of the lateral line

primordia of embryos injected with morpholino, particularly *pak2b* morpholino, revealed disruption to cell shape, a lack of rosette-like structures and loss of directed migration of the primordium indicating an effect on regulation of the actin cytoskeleton and adhesion molecules.

It is known that Pak can play a role in the regulation of focal adhesion sites, through its association with Paxillin (Brown *et al.*, 2002) and affect Cdc42-mediated organisation of the actin cytoskeleton (Eby *et al.*, 1998). Dynamic regulation of adherens junctions is known to require Rac and Cdc42 activity (Fukata and Kaibuchi, 2001).

Immunostaining experiments were carried out to investigate the localisation of β-catenin, E-cadherin and Paxillin proteins and F-actin. Using fluorescently labelled phalloidin to label F-actin we found that F-actin was clustered in the primordium of embryos injected with *pak2a* morpholino in a similar manner to that observed in controls (Figure 5.5c,d). Although the clusters were often less dense than in the control embryos, three to four foci could be identified within the primordium of embryos injected with *pak2a* morpholino. However, in embryos injected with *pak2b* morpholino only one focal cluster of F-actin could be identified although occasionally a second very much reduced cluster was seen (Figure 5.5e,f). The major cluster in the primordium of embryos injected with *pak2b* morpholino was always located in the trailing end, corresponding to the first nascent neuromast precursor. This is consistent with the finding that one to two mature neuromast precurors can be detected in embryos injected with *pak2b* morpholino (Figure 5.2d,e,m). This suggests that *pak2b* may be required to maintain cellular organisation of subsequent neuromast precursors.

We analysed the localisation of the adhesion molecules that are associated with the actin cytoskeleton, β-catenin and E-cadherin, in embryos injected with *pak2a* or *pak2b* morpholino (Figure 5.5i-l). We have previously shown that, like F-actin, β-catenin and E-cadherin were clustered at the apex of the rosette-like structures of nascent neuromast precursors (Chapter Three). In the embryos injected with *pak2a*

morpholino clusters of β-catenin and E-cadherin could be observed at the foci of rosette-like structures (Figure 5.5i,j). However, in more than 50% of cases the clusters of β-catenin and E-cadherin were less concentrated and elongated, rather than focused to a point in the leading edge of the primordium (n=15). As the leading edge is where the most cellular activity is occurring elongation of the β-catenin and E-cadherin cluster suggests dynamic maintenance of the cluster is defective. Expression of E-cadherin in the epithelial cells surrounding the primordium did not appear to be affected by the knock down of Pak2a.

Disruption to the patterning of β-catenin and E-cadherin within the primordium of embryos injected with *pak2b* morpholino was similar to that observed for F-actin localisation. Only one focal cluster of β-catenin and E-cadherin proteins could be detected following injection of *pak2b* morpholino (n=15). Like F-actin, this cluster was found in the trailing end of the primordium (Figure 5.5k,l). In the absence of β-catenin and E-cadherin clusters, the cells within the primordium were randomly organised and less tightly packed resulting in irregular primordium shapes, such as broadening of the primordium at the leading edge. The E-cadherin expression in the cells surrounding the lateral line primordium did not appear to be significantly affected by the knock down of Pak2b.

As Pak is known to be an important regulator of focal adhesion formation we were interested to discover whether the expression of the focal adhesion protein, Paxillin, was disrupted following knock down of Pak2a or Pak2b. In normal embryos Paxillin is clustered around the edge of the lateral line primordium (Chapter Three) and this pattern was not affected by injection of control morpholino. However, in embryos injected with either *pak2a* or *pak2b* morpholino the level of Paxillin around the primordium was reduced and in embryos injected with *pak2b* morpholino it was frequently difficult to detect the presence of Paxillin protein (Figure 5.6). This suggested that Pak2b is required to form focal adhesions around the migrating lateral line primordium.

Figure 5.5

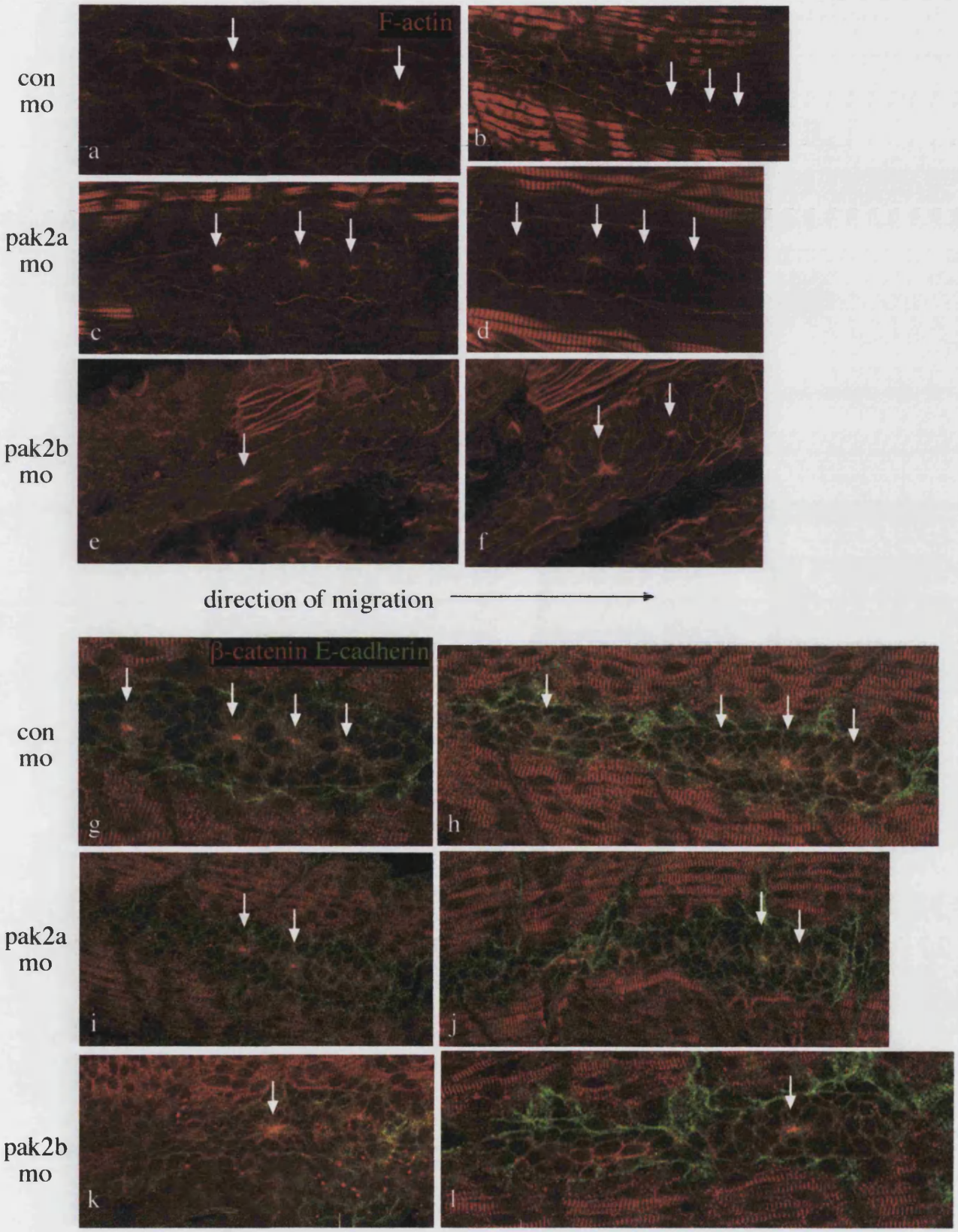

F-actin
con mo
pak2a mo
pak2b mo
a
b
c
d
e
f
direction of migration
β-catenin E-cadherin
con mo
pak2a mo
pak2b mo
g
h
i
j
k
l

Figure 5. 5: The cellular organisation of the lateral line primordium is disrupted in *pak2a* or *pak2b* morpholino injected embryos.

Embryos were labelled with Cy3-conjugated phalloidin to visualise F-actin (a-f). Images shown are generated from a stack of sections through the lateral line primordium at 24hpf. Embryos were injected with 4.5ng of control (a,b), pak2a (c,d) or pak2b (e,f) morpholino. Arrows indicate the centre of the rosette-like structure of nascent neuromast precursors.

Localisation of β-catenin (red) and E-cadherin (green) proteins was also visualised using fluorescent immunostaining at 24hpf (g-l). images shown are confocal sections of one plane through the lateral line primordium. Embryos were injected with 4.5ng control morpholino (g,h), *pak2a* morpholino (i,j) or *pak2b* morpholino (k-l). Arrows indicate foci of rosette-like structure of nascent neuromast precursors.

5.4 Effect of morpholino-mediated knock down of Pak2 on levels of adhesion proteins.

Immunocytochemical analysis of adherens junction and focal adhesion proteins demonstrated a disruption to their localisation in embryos injected with *pak2a* and *pak2b* morpholino oligonucleotides. In particular we noted a decrease in the intensity of Paxillin staining surrounding the lateral line primordium of embryos injected with the *pak2b* morpholino (Figure 5.6). We were interested to discover whether the changes to adhesion protein level observed by immunostaining were indicative of a significant effect of Pak2 knock down on the levels of adhesion proteins associated with the cytoskeleton. Whole cell extracts from embryos injected with control, *pak2a* or *pak2b* morpholino were separated into 1% Triton-soluble "cytosolic" and insoluble "cytoskeleton" fractions using a well established method of fractionation (Reiter *et al.*, 1985). Western blot analysis was then carried out to assay for levels of various adhesion proteins (Figure 5.7A). We investigated the levels of adherens junctions proteins, E-cadherin, β-catenin and α-catenin, the focal adhesion protein, Paxillin and Vinculin, which is associated with both types of adhesion (Pokutta and Weis, 2002; Turner, 2000).

Western blot analysis revealed clear differences in the fraction enriched in cytoskeleton between the levels of proteins associated with the cytoskeleton in morpholino injected embryos and in controls (Figure 5.7A). Quantitative analysis of the bands was carried out by measuring and comparing the density and the density of bands detected in control and morpholino injected embryos was compared. A general increase in the levels of all adhesion proteins assayed was detected in embryos injected with *pak2a* morpholino (Figure 5.7B). The greatest increases were observed for β-catenin and α-catenin with two fold increases and Vinculin, which, was detected at a level three times greater than the control level. The levels of Paxillin and E-cadherin were slightly increased at a 1.1 fold greater density than the control (Figure 5.7B).

Figure 5.6

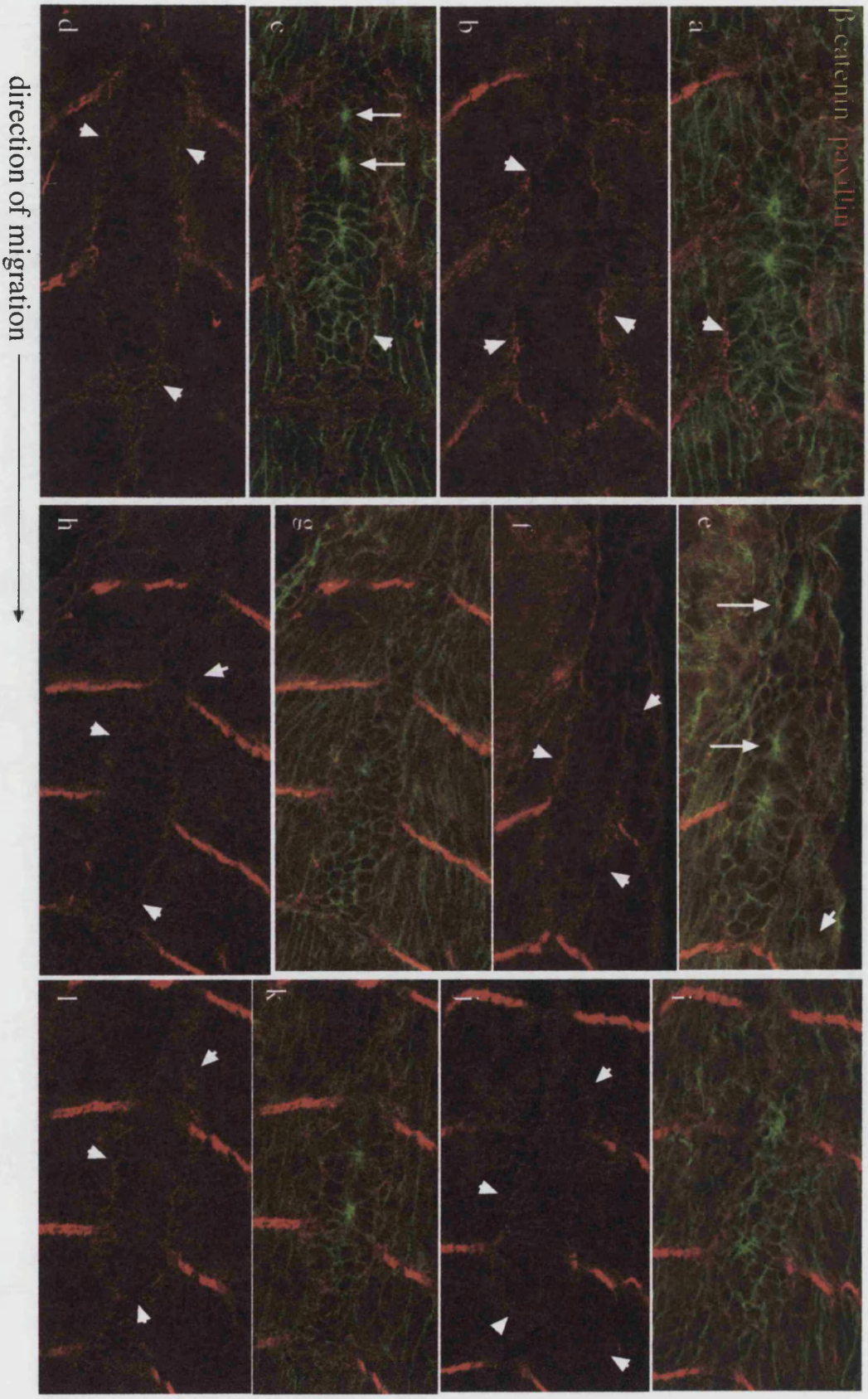

β-catenin/paxillin
control mo
pak2a mo
pak2b mo
direction of migration
a
b
c
d
e
f
g
h
i
j
k
l

Figure 5.6: Levels of focal adhesion protein surrounding the primordium are decreased in morpholino injected embryos.

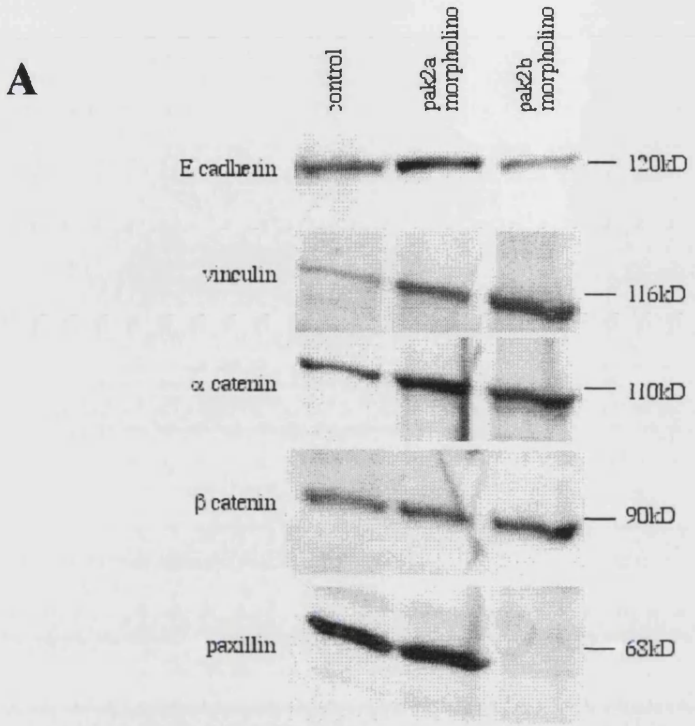
The level of Paxillin protein surrounding the lateral line primordium in 24hpf embryos injected with 4.5ng of either *pak2a* (e-h) or *pak2b* morpholino (i-l) is reduced when compared to the control (a-d). Localisation of Paxillin protein (red) was detected by immunostaining and visualised as confocal sections. The anti β-catenin antibody (green) to aid visualisation of the primordium (a,c,e,g,i,k). The edge of the lateral line primordium is indicated by arrowheads.

Levels of cadherin-mediated adhesion proteins were also increased in embryos injected with *pak2b* morpholino, particularly α-catenin and Vinculin. In comparison E-cadherin and Paxillin protein levels were decreased, only a very low level of Paxillin protein was associated with the cytoskeleton in *pak2b* morpholino injected embryos (Figure 5.7A). These results show that Pak2a and Pak2b have significant roles in regulating the levels of adhesion molecules associated with the cytoskeleton, however, these two Pak proteins are clearly acting in different ways.

5.5 Effect of knock down on cell death and proliferation within the posterior lateral line primordium.

Previous work in our lab has shown that deposition of the correct number of neuromast precursors during normal lateral line development requires cell proliferation (Q. Xu, pers. comm.). Members of the Pak family of proteins have been shown to have roles in both cell proliferation and survival and in promoting apoptosis (Rudel and Bokoch, 1997; Schurmann *et al.*, 2000). Following injection of *pak2a* morpholino we have observed a reduction in size of the lateral line primordium (Figure 5.3) and a reduction in the number of neuromasts deposited (Figure 5.2). This prompted us to look at patterns of apoptosis and proliferation within the lateral line primordium of *pak2a* morpholino injected embryos to discover whether changes to such patterns could contribute to the defects observed.

Figure 5.7


A
control
pak2a morpholino
pak2b morpholino
E cadherin
vinculin
α catenin
β catenin
paxillin
120kD
116kD
110kD
90kD
68kD

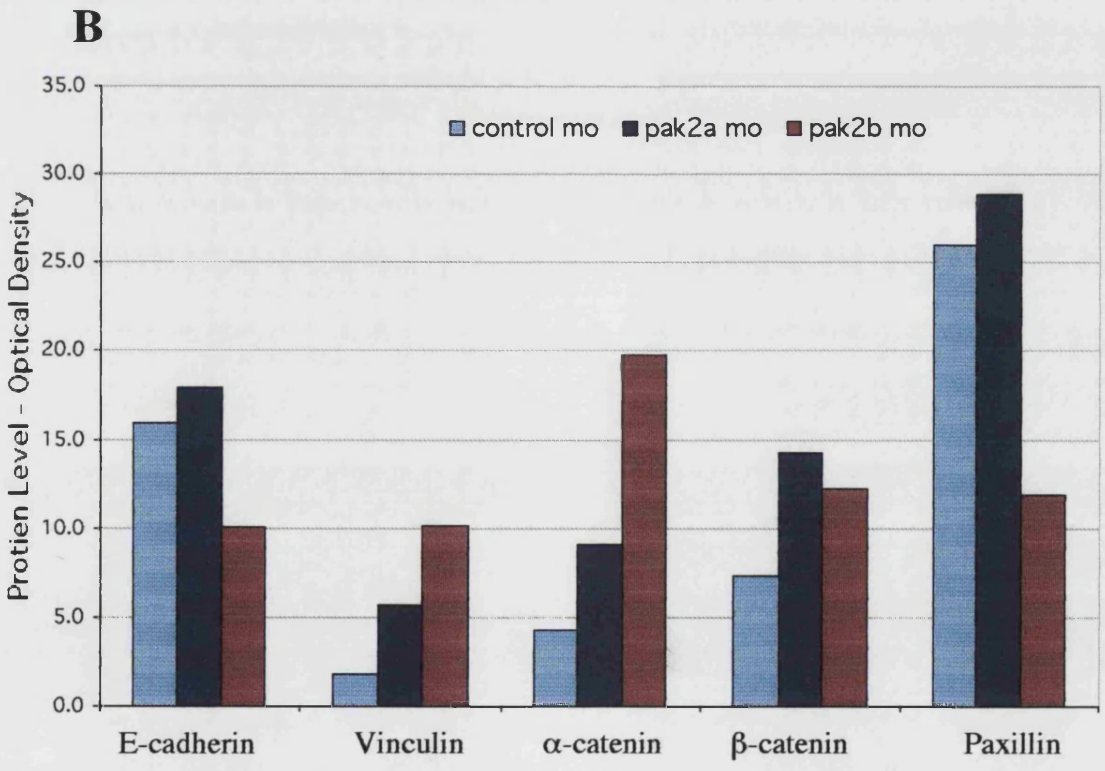
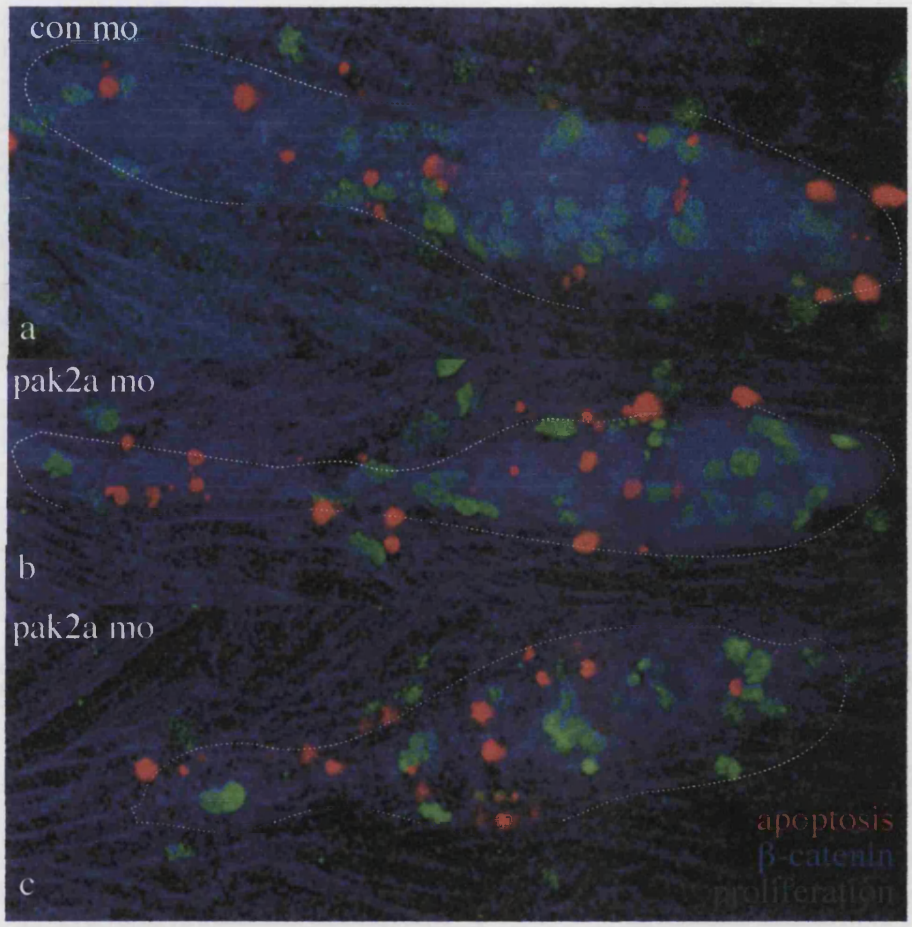

B
control mo
pak2a mo
pak2b mo
Protien Level - Optical Density
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
E-cadherin
Vinculin
α-catenin
β-catenin
Paxillin

Figure 5.7: Levels of cytoskeleton associated adhesion proteins are altered in *pak2a* and *pak2b* morpholino injected embryos.

A: Western blot analysis showing changes in the levels of cytoskeleton associated α-catenin, β-catenin, E-cadherin, Paxillin, and Vinculin in embryos injected with 4.5ng control, *pak2a* or *pak2b* morpholino. Cytoskeleton-enriched extracts were prepared from 50 embryos and protein bands were detected with specific antibodies as indicated.

B: Protein levels were quantified by measuring the optical density of the bands detected by Western blot analysis. This graph illustrates the average optical density, recorded over two experiments, for embryos injected with 4.5ng of control, *pak2a*, or *pak2b* morpholino.

Figure 5.8


con mo
a
pak2a mo
b
pak2a mo
c
apoptosis
β-catenin
proliferation

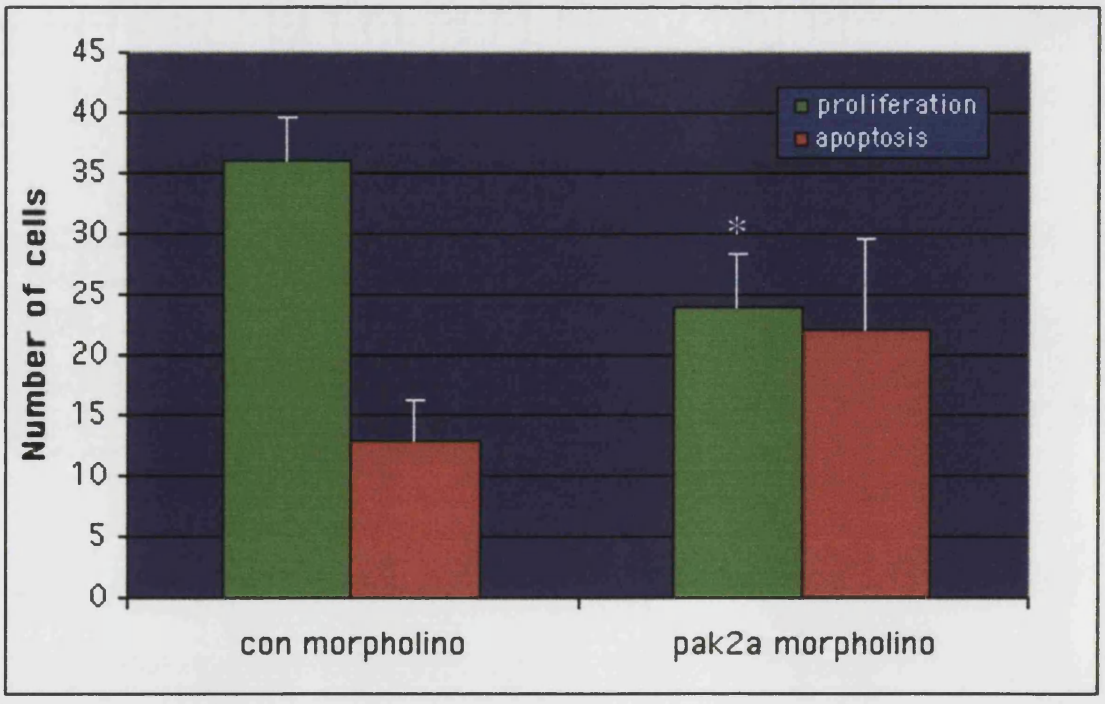

45
40
35
30
25
20
15
10
5
0
Number of cells
proliferation
apoptosis
*
con morpholino
pak2a morpholino

Figure 5.8: Cell proliferation is reduced in embryos injected with *pak2a* morpholino.

Numbers of proliferating and apoptotic cells within the lateral line primordium of 24-28hpf embryos injected with 4.5ng of control (a) or *pak2a* morpholino (b,c) were investigated in tandem. Proliferating cells were identified by incorporation of BrdU and labelled using a fluorescently conjugated anti-BrdU antibody (green). Apoptotic cells were labelled using a fluorescent TUNEL method (red). Visualisation of the lateral line primordium was aided by co-staining with anti-βcatenin antibody (blue). Average numbers of proliferating and apoptotic cells in the posterior lateral line primordium were calculated from four embryos and shown as a bar graph. Error bars indicate standard deviation from the mean. A student's t-test was used to calculate whether the two sample means were equal, p values less than 0.05 were taken to be significant and an asterisk indicates a significant change.

The number of cells undergoing proliferation or programmed death in the lateral line primordium was investigated by tandem detection of DNA fragmentation in apoptotic cells using the TUNEL technique and detection of BromodeoxyUridine (BrdU) incorporation into the S-phase DNA of proliferating cells. Our results showed that there was a increase in the number of cells undergoing apoptosis within the primordium of embryos injected with *pak2a* morpholino compared to embryos injected with control morpholino ($p=0.08$). Concomitantly we detected a significant decrease ($p=0.03$) in the number of proliferating cells in the primordium of embryos injected with *pak2a* morpholino (Figure 5.8).

This significant reduction in cell proliferation in the primordium of embryos injected with *pak2a* may be sufficient to reduce the number of cells within the primordium and lead to a decrease in the number of neuromasts deposited from the primordium.

5.6 Effect of Pak2 knock down on migration of the primordial germ cells.

Directed cell migration in response to chemotatic signals is important for many biological processes, in particular the migration of lymphocytes in immune response (Muller *et al.*, 2001). Recent studies in zebrafish development have shown that the chemokine *sdf-1* and its receptor *cxcr4* are involved in the guidance of cell migration in development of the lateral line (David *et al.*, 2002). Migration of the primordial germ cells (PGCs) also requires the activity of the chemokine Sdf-1 and it's receptor cxcr4 and the migratory pathway of the germ cells is well characterised (Knaut *et al.*, 2003). The defect in migration of the lateral line primordium observed in embryos injected with *pak2b* morpholino could result from a defect in the chemotatic response. We investigated this possibility by analysing the migration of PGCs in embryos injected with *pak2a* or *pak2b* morpholino.

We used the *nanos-like1* gene (*nanos*), which is expressed in the germ plasm and primordial germ cells (PGCs) of the zebrafish, as a molecular marker to follow movement of the PGCs during development (Koprunner *et al.*, 2001). At the dome stage of development PGCs originate at random positions along the edges of the embryo, then converge to form two lateral stripes and migrate towards the dorsal midline (Figure 5.9a,b). During somitogenesis, strong *sdf-1* expression domains at the level of the third and eighth somites, act to attract PGCs. The PGCs finally converge at a point just posterior to the yolk sac at 24hpf where the future gonad will form (Figure 5.9e,f).

When we looked at the location of the *nanos* positive PGCs in embryos injected with either *pak2a* or *pak2b* morpholino we found a number of defects. Firstly, the initial convergence of PGCs at early stages was disrupted or delayed and cells were scattered over the embryos rather than aligned to form the lateral stripes in 75% of *pak2a* morpholino injected embryos (n=8) and 80% of *pak2b* morpholino injected embryos (n=15) (Figure 5.9g,h and m,n). At the 10-somite stage PGCs could still be observed in the posterior most region of embryos injected with *pak2a* or *pak2b* morpholino (Figure 5.9j and p).

By 24hpf ectopic PGCs were found in 66% of *pak2a* morpholino injected embryos (n=15) and in 83% of *pak2b* morpholino injected embryos (n=12). Ectopic PGCs could be found in the head and dorsal midline of embryos injected with either *pak2a* or *pak2b* morpholino (Figure 5.9k,l and q,r). This suggested that PGCs were unable to respond correctly to the chemotactic signals emanating from somites. It was interesting to note that defects in PGC migration were seen in both *pak2a* and *pak2b* morpholino injected embryos as migration of the lateral line primordium is not significantly affected in *pak2a* morpholino injected embryos. Further work is required to characterise the role of zebrafish Pak2 in regulating directed cell migration in response to chemotactic signals during development.

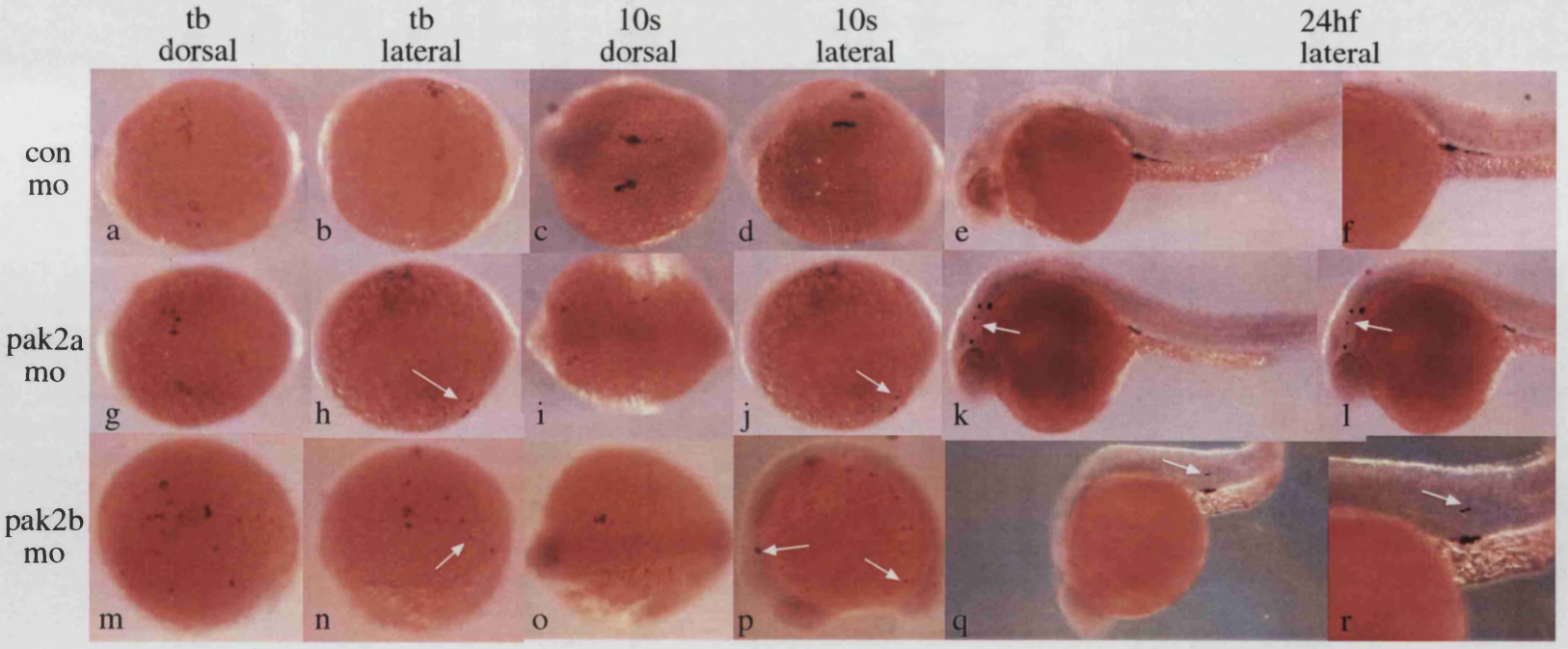
5.7 Discussion.

We have shown that morpholino-mediated knock down of Pak2a and in particular Pak2b, has striking effects on the migration of the lateral line primordium and on cell adhesion proteins within the primordium and throughout the whole embryo. Our results compare favourably with the defects that have been observed in cell culture systems following a decrease in Pak activity. Such studies have shown that Pak activity has significant effects on cell adhesion, contractility (Kiosses *et al.*, 1999) and the polarisation of the actin cytoskeleton required for directed migration (Eby *et al.*, 1998; Sells *et al.*, 1999).

Observations of embryo morphology following injection of *pak2a* or *pak2b* morpholino has revealed a possible role for Pak2 in extension of the zebrafish body axis. Morpholino injected embryos have a shortened body axis and compacted somites, consistent with a defect in convergent extension movements. In *Xenopus laevis* convergent extension of axial mesoderm during gastrulation was found to be dependent on the regulated activity of Rho and Rac (Tahinci and Symes, 2003). During convergent extension in *Xenopus*, cells of the future trunk mesoderm elongate and extend cytoplasmic protrusions laterally. These protrusions contact neighbouring cells and create traction and force enabling the cells to intercalate between one another (Tahinci and Symes, 2003). It is possible that Pak, as a downstream effector of Rac, is acting to mediate the converging and extending activities of cells in both *Xenopus* and zebrafish.

We have also begun to elucidate a role for Pak2b in controlling migration of the posterior lateral line primordium as time-lapse analysis of embryos injected with *pak2b* morpholino revealed retarded migration of lateral line primordium. We have shown that while no directed migration was observed, the ability of lateral line primordium cells to extend processes was not affected. This is in agreement with previous work demonstrating that Pak is not required for the formation of lamellipodia or membrane protrusions (Sells *et al.*, 1999).

Figure 5.9


tb dorsal
tb lateral
10s dorsal
10s lateral
24hf lateral
con mo
pak2a mo
pak2b mo
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n
o
p
q
r

Figure 5.9: Migration of the primordial germ cells (PGCs) is disrupted in embryos injected with *pak2a* or *pak2b* morpholino.

Migration of PGCs was visualised by *in situ* hybridisation using the molecular marker, *nanos*. Embryos were injected with 1.5ng control (a-f), *pak2a* (g-l) or *pak2b* (m-r) morpholino and the location of PGCs investigated at tailbud (a,b,g,h,m,n), 10-somite stage (c,d,l,j,o,p) and 24hpf (e,f,k,l,q,r). Arrows highlight ectopic PGCs.

However, Pak is known to regulate other aspects of cell migration control, for example, Pak can regulate cell migration through myosin-dependant contraction of the cell rear. Pak has been shown to phosphorylate myosin light chain (MLC), and phosphorylation of MLC by Pak2 has been demonstrated to induce cell retraction (Kiosses *et al.*, 1999; Sells *et al.*, 1999; Zeng *et al.*, 2000). In the lateral line primordium, knock down of Pak2b, may be acting to inhibit retraction of the trailing edges accompanied by a failure to form new adhesions at the leading edge. This could result in a primordium that remains static. The ability of the lateral line primordium to migrate normally in embryos injected with *pak2a* morpholino may suggest some redundancy in Pak2a function or significant functional differences between Pak2a and Pak2b. Functional differences of the two zebrafish Pak2 proteins may be a reflection on differences in amino acid sequence and interacting proteins, as we have identified significant differences in the binding of Nck and PIX isoforms between Pak2a and Pak2b (Chapter Four).

An additional pathway through which Pak activity may exert influence on migration of the lateral line primordium is the chemotaxis signaling pathway. Chemoattractants are known to stimulate signaling pathways that involve Rho family GTPases. The p21-activated kinases are specific targets of activated GTP-bound Rac and Cdc42, and have been proposed as regulators of chemoattractant-driven actin cytoskeletal changes in fibroblasts (Dharmawardhane *et al.*, 1999). In *Dictyostelium discoideum* the Pak homologue, PAKa, is also required for maintaining the direction of cell movement, suppressing lateral pseudopod extension, and proper retraction of the posterior of chemotaxing cells (Chung and Firtel, 1999). Recent work has shown that efficient chemotaxis requires directional sensing and cell polarization and this process utilises a signalling pathway involving αPIX-associated Pak, the Gβγ subunit and Cdc42. This Gβγ-PAK1/αPIX/Cdc42 pathway is essential for the localization of F-actin formation to the leading edge, directional sensing, and the persistent directional migration of chemotactic leukocytes (Li *et al.*, 2003). Migration of both the lateral line primordium and the primordial germ cells is dependant on chemotactic signalling

(David *et al.*, 2002; Knaut *et al.*, 2003) and we have shown that injection of *pak2a* morpholino disrupts PGC migration and injected of *pak2b* morpholino disrupts migration of both tissues. Analysis of PGC migration in morpholino injected embryos revealed some PGCs in ectopic locations, suggesting an inability to respond correctly to the chemotatic signals. Taken together the defects in lateral line primordium migration and PGC migration indicate a role of zebrafish Pak2 in mediating the chemotactic response.

We have also shown that development of the lateral line primordium requires dynamic regulation of adhesion molecules. Our analysis of protein localisation has indicated that components of adherens junctions are required to maintain the rosette structure of nascent neuromast precursors during migration of the primordium. We also demonstrated that the actin cytoskeleton is polarised within the nascent neuromasts such that high levels of cortical actin are found at the focal points of these rosettes. Furthermore the focal adhesion protein, Paxillin, is found at high levels surrounding the lateral line primordium leading us to suggest that these adhesions are dynamically formed and disassembled as the primordium migrates to provide the traction forces for primordium migration. There is strong evidence for the involvement of Pak proteins in the regulation of focal adhesions. In addition Pak proteins may regulate cadherin-mediated adhesions through interactions with F-actin or by activation of Rac.

Pak is known to regulate localisation of F-actin, which, in turn, stabilises the membrane localisation of part of the adherens complex, the E-cadherin-β-cateinin complex which binds to actin via α-actinin or Vinculin (Dharmawardhane *et al.*, 1997; Pokutta and Weis, 2002). Additionally active Rac can regulate E-cadherin-mediated adherens junctions by promoting their dissociation, through endocytosis of E-cadherin from the membrane and metalloproteinase induced cleavage of E-cadherin. Alternatively Rac can stabilise these adhesion by promoting association of the E-cadherin-β-catenin complex with α-catenin as a result of inhibiting the association of the E-cadherin-β-catenin complex with IQGAP (Fukata and Kaibuchi, 2001). As Pak

acts downstream of Rac and Cdc42 and can positively regulate their activity, it is possible that Pak may also regulate adherens junctions. In support of this it has been shown that *Drosophila* Mbt, a group II Pak protein, can be recruited to adherens junctions and associates with the *Drosophila* homologue of β-catenin, Armadillo (Schneeberger and Raabe, 2003). In agreement with this we observed that the rosette-like structures were largely lost from embryos injected with *pak2b* morpholino, as revealed by disrupted localisation of F-actin, β-catenin and E-cadherin.

Disruption to the cellular patterning of the primordium was also observed during time-lapse analysis of *pak2b* morpholino injected embryos, during which the cells of the rosette-like structures became dissociated and lost the characteristic teardrop shape. Together with the disruptions to adherens junction proteins and F-actin, these observations suggest that Pak2b is required to maintain the cellular prepattern of the primordium as it migrates by dynamic rearrangement of cellular adhesions during migration of the lateral line primordium.

Analysis of the levels of cadherin-mediated adhesion protein associated with the cytoskeleton in morpholino injected embryos, further aided characterisation of the action of Pak on regulation of this type of adhesion. In embryos injected with *pak2a* morpholino an increase in the level of all adhesion molecules tested was detected. Increased adherens junction components associated with the cytoskeleton in embryos injected with *pak2a* morpholino suggests that Pak2a normally functions to dissociate adherens junctions. A possible mechanism for this is through Pak-mediated control of actin and/or Myosin during macropinocytosis.

It has been shown that Pak co-localises to areas of membrane ruffling and pinocytic vesicles, where, it co-localises with polymerised actin (Dharmawardhane *et al.*, 1997). In addition, inhibition of endogenous Pak blocks growth factor induced macropinocytosis while injection of active Pak enhances Platelet-derived growth factor (PDGF) stimulated macropinocytosis in fibroblast cells (Dharmawardhane *et al.*, 2000). The force for vesicle budding, movement and the protrusion and retraction of membranes is thought to require ATP-dependant interactions between myosin family

proteins and actin (Buss *et al.*, 1998). Myosin VI has been shown to be recruited into surface ruffles and may play a role in macropinocytosis and transport of internalised vesicles (Buss *et al.*, 1998). Pak3 is able to phosphorylate Myosin VI and active it's motility (Yoshimura *et al.*, 2001). Additionally, studies in *Dictyostelium* have demonstrated that Pak can directly phosphorylate and activate Myosin I, another Myosin protein thought to be play a role in macropinocytosis (Buss *et al.*, 1998; de la Roche and Cote, 2001). Therefore Pak2a may function in an endocytosis pathway to cycle E-cadherin away from the membrane and disassociate adherens proteins.

Unlike *pak2a* morpholino injected embryos, levels of E-cadherin were decreased in embryos injected with *pak2b* morpholino, this may suggest an opposing role for Pak2b in maintaining E-cadherin at the membrane, possibly through activation of Rac and therefore inhibition of the E-cadherin-β-catenin-IQGAP complex (Fukata and Kaibuchi, 2001). However this data is preliminary and additional experiments are required to confirm this possibility. The increased levels of other cadherin-mediated proteins, β-catenin, α-catenin and Vinculin, in the cytoskeletal fraction of *pak2b* morpholino injected embryos does suggest that both Pak2a and Pak2b normally act to dissociate cadherin-mediated adhesions.

In relation to regulation of focal adhesions, Pak is known to play an important role and is recruited to focal adhesions by PIX (Brown *et al.*, 2002; Manser *et al.*, 1997; Turner, 2000), indicating that zebrafish Pak2 and may be involved in the formation of the focal adhesions surrounding the lateral line primordium. Morpholino-mediated knock down indeed revealed a role in the formation of focal adhesions. Levels of the focal adhesion protein, Paxillin, surrounding the lateral line primordium were slightly reduced in embryos injected with *pak2a* morpholino and largely absent in embryos injected with *pak2b* morpholino. The dramatic decrease in levels of Paxillin associated with the cytoskeleton in embryos injected with *pak2b* morpholino was confirmed by Western blot analysis. Taken together the immunocyctochemical and molecular data indicate that Pak2b is required for dynamic regulation of focal adhesions during lateral line primordium migration by inducing their formation or

stabilisation. In respect to Pak2a, the data from Western blot showing an increased level of Paxillin associated with the cytoskeleton is in disagreement with the reduced staining of Paxillin observed surrounding the lateral line. This may reflect a tight regulation of focal adhesion turnover by Pak2a that results in increases and decreases of focal adhesions when Pak2a activity is removed. Increases in Vinculin in both *pak2a* and *pak2b* morpholino injected embryos may also reflect an increase in focal adhesions, where this protein is also found. Alternatively differences in levels of Paxillin and Vinculin may reflect the different regulation of focal adhesions at the leading edge and trailing end. Pak is known to play a role in the dissociation of focal adhesions (Frost *et al.*, 1998) and the increased levels of Paxillin and Vinculin in embryos injected with *pak2a* morpholino suggests that Pak2a also functions to disassemble focal adhesions.

In summary, an important role for zebrafish Pak2 proteins, in many aspects cell migration is emerging. From dynamic regulation of the actin cytoskeleton and cell adhesion to responding to chemotactic signals and regulation of cell proliferation and apoptosis. Further studies will help to elucidate the precise role that Pak2 is playing in these aspects of zebrafish development.

Chapter Six

Requirement of pak2 in early zebrafish development

6.1 Introduction

The embryos injected with *pak2a* or *pak2b* morpholino were truncated along the anterior to posterior axis, presented small anterior structures curled tails and defects in somite formation. Defects in somite formation could be observed in embryos injected with *pak2b* morpholino as early as the 10-somite stage when somites were of irregular shape and organisation and extended medially. At later stages the somites of the tail region of both *pak2a* and *pak2b* morpholino injected embryos were narrow and compacted forcing them into a "u" shape. In order to further characterise the morphological defects in early embryogenesis we looked at the expression patterns of various molecular markers in embryos injected with *pak2a* or *pak2b* morpholino using *in situ* hybridisation and immunocytochemistry. We also studied adhesion complexes in the early embryos since Pak plays an important role in regulating cell to cell adhesion.

To complement our investigations into Pak function using morpholino-mediated knock down we have recently generated constructs to express a truncated form of Pak2a and Pak2b proteins. The Pak2a$^{\Delta c}$YFP and Pak2b$^{\Delta c}$YFP constructs were comprised of the N-terminal regulatory domain fused with yellow fluorescent protein at the C-terminus to permit visualisation of the subcellular localisation of these proteins. It has previously been postulated that expression of truncated Pak proteins retaining the CRIB motif act to titrate out endogenous GTP-bound active Rac and Cdc42

(Osada *et al.*, 1997). This titration would inhibit the functions of the molecules that rely on an interaction with Rac and/or Cdc42 for their activation, including the group I family of Pak proteins. In addition Pak2$^{\Delta c}$YFP proteins could act to sequester proteins that interact with the N-terminal regulatory domain including Nck and PIX. It is most likely that the Pak2$^{\Delta c}$YFP will act in a dominant negative manner however their precise mode of action remains to be tested. Although the work using Pak2$^{\Delta c}$YFP proteins is preliminary it nevertheless provides a means to increase our understanding of the phenotypes of *pak2a* and *pak2b* morpholino injected embryos.

6.2 Effect on *pak*2 knock down on somite development.

To investigate the effect of morpholino mediated knockdown of Pak2 on somite development we looked at the expression of *myoD*. The myogenic basic helix-loop-helix transcription factor *myoD* is essential for establishment of muscle cell precursors and their differentiation. During somitogenesis *myoD* is expressed in the adaxial cells and in a dynamic temporal pattern in the somites (Weinberg *et al.*, 1996). Expression of *myoD* in the somites and adaxial cells appeared to be normal in embryos injected with either *pak2a* or *pak2b* morpholino and the somitic boundaries could be observed. However, in 55% (n=139) of *pak2a* morpholino injected embryos and 15% (n=40) of *pak2b* morpholino injected embryos, ectopic *myoD* expressing cells were present in the midline (Figure 6.1b,c). The ectopic *myoD* expressing cells were evident from the 10-somite stage in embryos injected with either *pak2a* or *pak2b* morpholino. In *pak2b* morpholino injected embryos the ectopic *myoD* expressing cells were no longer detected at the 14-somites stage as opposed to the 20-somite stage in embryos injected with *pak2a* morpholino. In normal development *myoD* expression has begun to be down-regulated in the older somites at the 20-somite stage (Weinberg *et al.*, 1996). The ectopic *myoD* expressing cells were most frequently found in the anterior midline, adjacent to the more mature somites. Transverse sections have revealed that the ectopic *myoD* expressing cells were located ventral to

the notochord and in the most severe phenotype the two lateral somitic expression domains were fused under the notochord (Figure 6.1d,e).

To test whether the ectopic *myoD* expressing cells were able to differentiate into muscle we used the monoclonal antibody, A4-1025, which recognises an epitope that is conserved among the myosin heavy chain (MHC) isoforms that are present in all striated muscle (Blagden *et al.*, 1997). Normally striated muscle fibres are found in the lateral somitic tissue and are orientated in an anterior to posterior direction. However in embryos injected with *pak2a* morpholino, ectopic muscle fibres were present at the ventral midline. Some of the fibres were orientated in a lateromedial direction and crossed medially from their normally lateral somitic locations (Figure 6.1h). By looking at different focal planes it was clear that these defective muscle fibres occupied a ventral location, under the notochord and contained *myoD* expression (Figure 6.1h).

Pak plays an important role in regulating cell migration in many cell types (Dechert *et al.*, 2001; Kiosses *et al.*, 1999) therefore we investigated the possibility that ectopic *myoD* expressing cells may result from a defect in cell migration. The skeletal muscle of the trunk is derived from the dorsal myotome of the zebrafish somite (Stickney *et al.*, 2000). There are two populations of cells in the myotome, lateral presomitic cells, which comprise the non-adaxial segmental plate cells and differentiate to form fast muscle, and the adaxial cells. Adaxial cells form adjacent to the notochord, express high levels of *myoD* and differentiate into slow muscle fibres (for recent reviews of somite development see, (Christ *et al.*, 1998; Keller, 2000; Pourquie, 2001; Stickney *et al.*, 2000). The adaxial cells are known to contain two sub-populations, muscle pioneers (MP) and non-muscle pioneers (NMP). MPs remain in contact with the notochord where they demarcate the prospective horizontal myoseptum, whereas NMPs move away from this medial position to form slow muscle fibres on the lateral surface of the somite. Initially the NMP cells extend dorsoventrally adjacent to the notochord before travelling radially through the lateral somite (Devoto *et al.*, 1996).

Figure 6.1

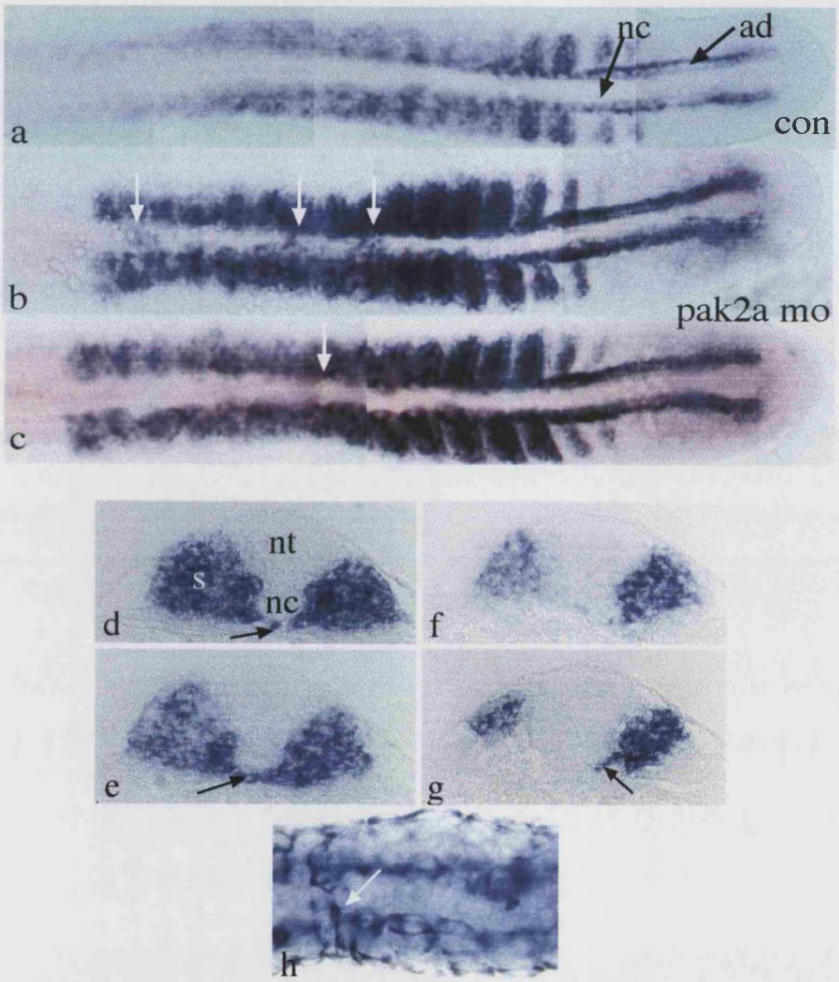


Figure 6.1: Ectopic myoD expressing cells at the ventral midline of pak2a morpholino injected embryos.

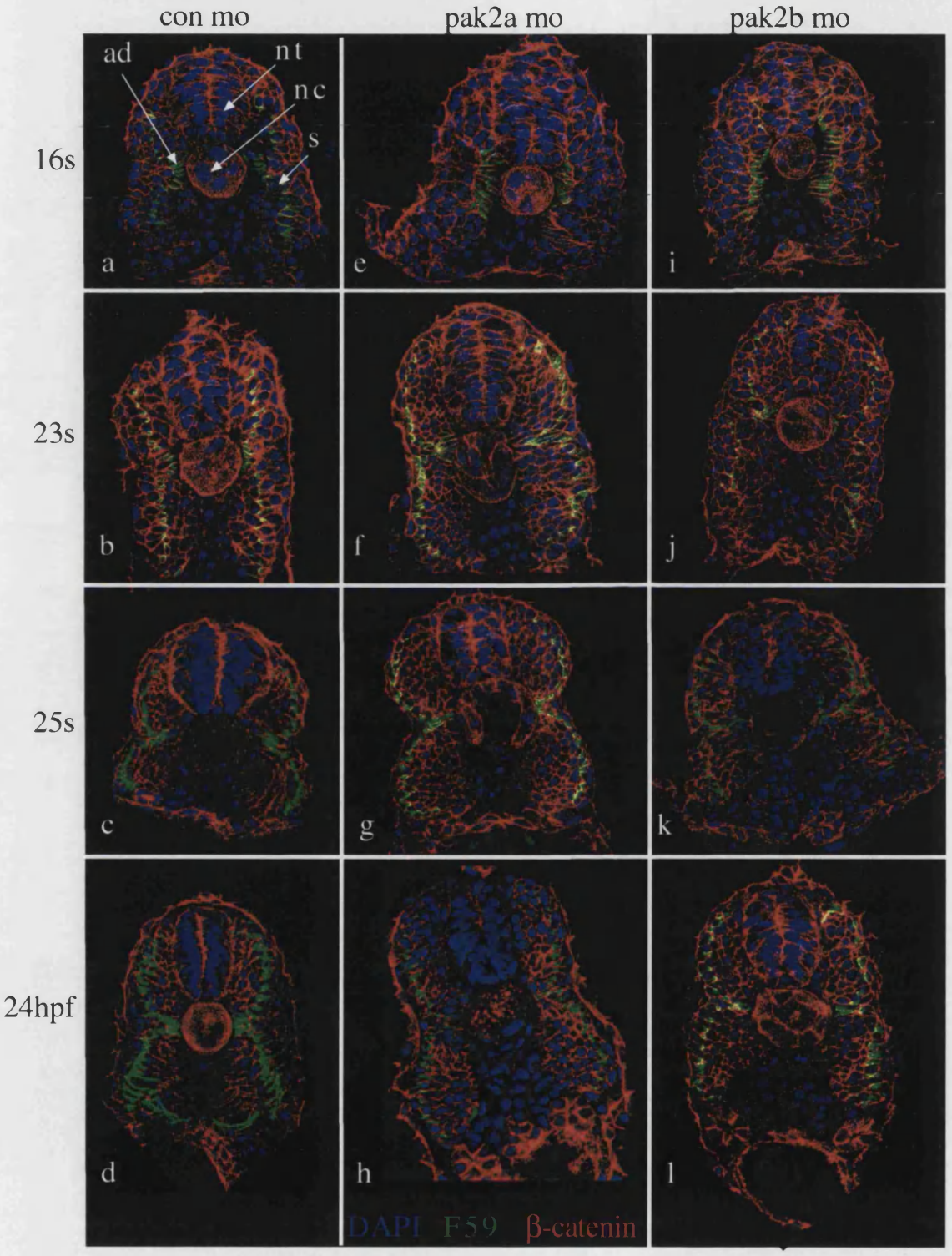
Expression of *myoD* was visualised at the 16-somite stage by *in situ* hybridisation of control (a) and *pak2a* morpholino injected (4.5ng) embryos (b,c). Transverse sections (d-g) show ectopic *myoD* expressing cells ventral to the notochord. Differentiated muscle fibres were visualised with the A4-1025 antibody and revealed ectopic ventral fibres in embryos injected with *pak2a* morpholino (h). Arrows highlight ventral ectopic *myoD* and A4-1025 labelled cells. Embryos are orientated anterior to the left (a-c,h). Abbreviations refer to, s, somite; ad, adaxial cells; nc, notochord and nt, neural tube.

A defect in migration of NMP cells, such that they migrate ventrally rather than laterally, presents one possible explanation for the ectopic *myoD* expressing cells observed in embryos injected with *pak2a* or *pak2b* morpholino. To address this possibility we looked at the position of slow muscle cells using the antibody F59, a specific marker of adaxial cells which strongly labels slow muscle precursor cells (Devoto *et al.*, 1996). Adaxial cells were observed to differentiate normally, adjacent to the notochord in embryos injected with either *pak2a* or *pak2b* morpholino (Figure 6.2e,i). In morpholino injected embryos the adaxial cells then extended dorsoventrally and could be observed to move laterally through the somites in the distinctive hour glass shape described by Devoto *et al* (Figure 6.2b-d,f-h,j-l). By 24hpf F59-positive cells were detected on the lateral surface of the somites in all embryos no F59-positive cells could be detected in the ventral midline of morpholino injected embryos (Figure 6.2h,l). This result also suggested that the ectopic muscle fibres found in the ventral midline were fast muscle fibres.

We also analysed the MP subpopulation of the adaxial cells using an antibody against a specific marker of MP cells, Engrailed (Devoto *et al.*, 1996). We found that the MP population was unaffected in morpholino injected embryos and the number and position of the Engrailed positive cells was normal (data not shown). Taken together these results indicated that the ventral ectopic *myoD* expressing cells could not result from aberrant migration of the slow muscle precursor cells.

6.3 Effect on Pak2 knock down on somitic cell adhesion.

In was clear from the analysis of F59 staining that the general organisation of the somites was disrupted in embryos injected with *pak2a* and particularly *pak2b* morpholino. Somitic cells were loosely packed and the general shape of the somitic blocks was irregular (Figure 6.2). Roles for Pak in cell adhesion have been well characterised and we have shown that knock down of Pak2 in zebrafish affects cell adhesion (Chapter Five).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l
DAPI F59 β-catenin

Figure 6.2

Figure 6.2: Migration of slow muscle precursors is normal in morpholino injected embryos.

Slow muscle cells differentiate adjacent to the notochord before moving laterally through the somite. In embryos injected with 4.5ng of control (a-d), *pak2a* (e-h) or *pak2b* (i-l) morpholino. Slow muscle cells were labelled with the marker F59 (green) at 16-somites (a,e,i), 23-somites (b,f,j), 25-somites (c,g,k) and 24hpf (d,h,l). Somites and other midline tissues were visualised using the nuclear counterstain, DAPI (blue), and cell membrane label, β-catenin (red). Following antibody staining transverse sections were cut by hand and visualised by confocal microscopy. Abbreviations refer to: nt, neural tube; nc, notochord; s, somite and ad, adaxial cells.

One of the major steps in somitogenesis is the development anterior-posterior polarity of cells in the presomitic mesoderm (PSM). This is achieved through the activity of a number of transcription and signalling factors and by development of cell adhesion properties within the somite (Rhee *et al.*, 2003). In zebrafish, somites form by alignment and polarisation of their border cells and new homotypic adhesions between boundaries cells is required to establish somite segmentation (Crawford *et al.*, 2003). Initiation of strong homotypic cell to cell adhesion has been shown to be mediated by cadherins (Gumbiner, 1996). During zebrafish somitogenesis a member of the cadherin family, *protocadherin C (papc)*, is expressed in the anterior PSM (Yamamoto *et al.*, 1998). In addition *papc* is expressed in the unsegmented paraxial mesoderm including adaxial cells, newly formed and forming somites. Expression of *papc* is down regulated in the anterior somites including the adaxial cells (Yamamoto *et al.*, 1998). Perturbing the activity of Papc in mouse has been shown to disrupt somitogenesis (Rhee *et al.*, 2003).

Injection of *pak2a* or *pak2b* morpholino disrupted the expression pattern of *papc*. Ectopic *papc* expressing cells were observed anterior to the normal expression domain and were found in high numbers in the anterior adaxial cells (Figure 6.3b-e, h-m). A small number of ectopic cells were also found in the ventral midline and within the segmented somites. Ectopic cells were detected in 53% of *pak2a* morpholino injected embryos (n=62) and 40% of embryos injected with *pak2b* morpholino (n=20).

Interestingly an ectopic band of cells, which appeared to represent the second most recently segmented somite, could also be detected in embryos injected with either *pak2a* or *pak2b* morpholino (Figure 6.3c,d). These ectopic cells may indicate an inability to downregulate *papc* expression at the correct time. In addition to ectopic cells, disruption to the segmentation of the paraxial mesoderm was observed. In 20% of embryos injected with either *pak2a* morpholino (n=62) and 5% of embryos injected with *pak2b* morpholino (n=20) bands of *papc* expression in the developing somites were fused (Figure 6.3b,h,l).

Figure 6.3

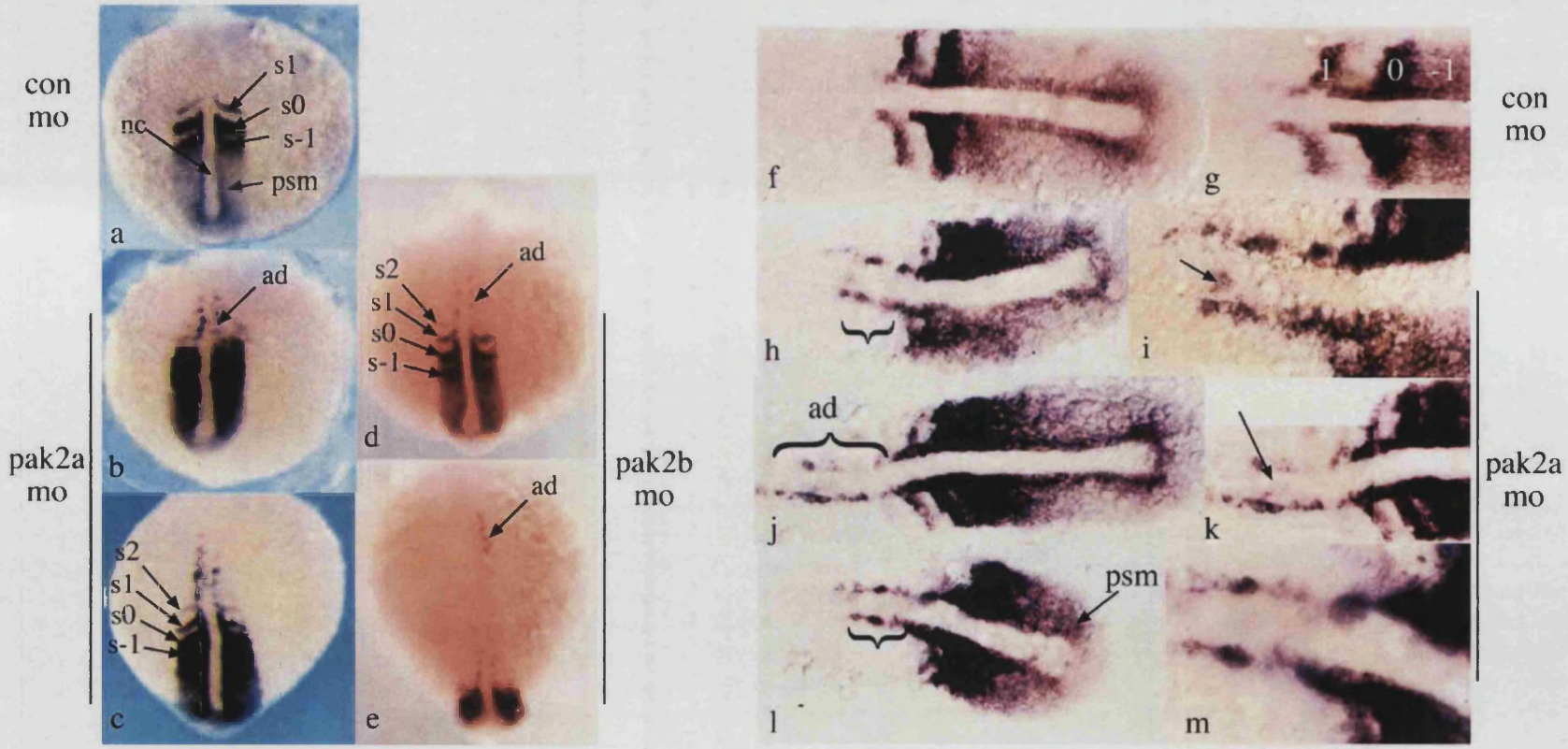
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Figure 6.3: Aberrant expression of *papc* in embryos injected with *pak2a* morpholino or *pak2b* morpholino.

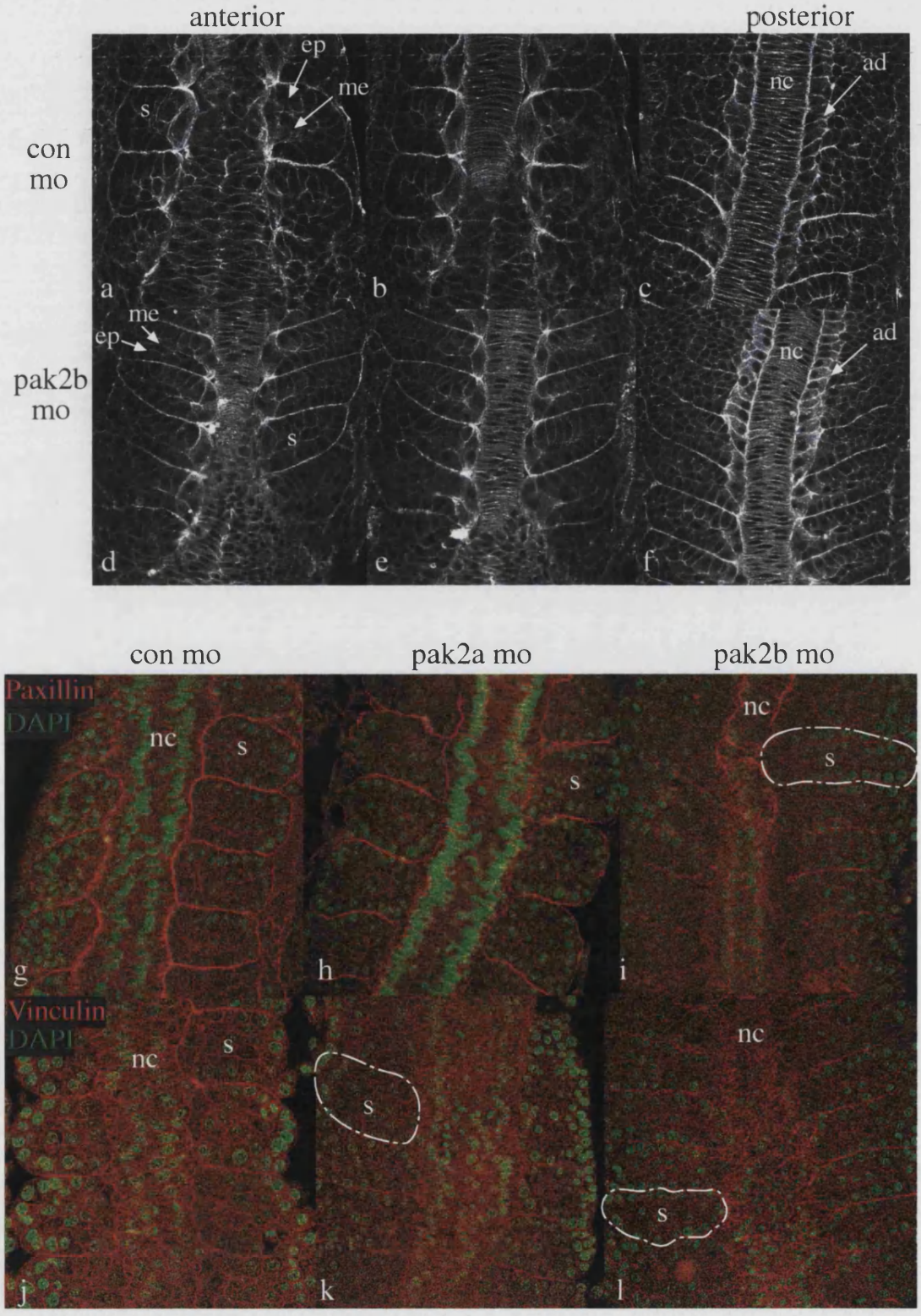
Expression of *papc* was visualised by *in situ* hybridisation at the 16-somite stage in embryos injected with 4.5ng control (a,f,g), *pak2a* (b,c h-m) or *pak2b* (d,e) morpholino. Somites are labelled according to convention, second segmented somite (s2), newly segmented somite (s1), forming somite (s0), presumptive somite (s-1). Ectopic expression is highlighted with brackets or arrows. Embryos are shown as whole mount (a-e, anterior to the top) or flat mount (f-m, anterior to the left). Abbreviations correspond to: nc, notochord; psm, presomitic mesoderm; ad, adaxial cells.

In the majority of *pak2a* morpholino injected embryos fusing of bands was also accompanied by ectopic expression of *papc* (Figure 6.3b,h,l). The fused pattern of *papc* expression showed that development of distinct somites is affected by Pak2 knock down. It has been suggested that stabilisation of somite boundaries requires deposition of matrix proteins and adhesion of somite border cells to these proteins (Crawford *et al.*, 2003) and focal adhesion proteins mediate the attachment of cells to the ECM (Parsons *et al.*, 2000). Therefore we further investigated the somitic defect in embryos injected with either *pak2a* or *pak2b* morpholino by analysis of the levels and distribution of F-actin and two focal adhesion proteins, Paxillin and Vinculin, at the somite boundaries.

Phalloidin labelled F-actin was found to be distributed around the cell membranes of all tissues at the midline and very high levels were found along the lateral surfaces of the notochord and surrounding the somites (Figure 6.4a-c). The levels of F-actin surrounding the somites was not uniform, levels of actin cable were higher at somite-somite boundaries than on the lateral surface of the somite, and the strongest site of F-actin localisation was at the interface between the adjacent somites and between somites and the notochord (Figure 6.4a). This general pattern of F-actin localisation was not disrupted by morpholino-mediated knock down of Pak2a or Pak2b (Figure 6.4d-f, Pak2a knock down not shown). The somites shape of embryos injected with *pak2b* morpholino appeared compacted with narrowing in the anterior-posterior direction and mediolateral extension. Cells within the somites also appeared small and compacted although both the cuboidal outer ring of epithelial cells and the inner loosely packed mesenchymal cells could be observed.

During zebrafish somitogenesis high levels of Paxillin have been observed at the somite boundaries (Crawford *et al.*, 2003). Western blot analysis has demonstrated a significant decrease in the levels of cytoskeletal associated Paxillin in embryos injected with *pak2b* morpholino (Chapter Five).

Figure 6.4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pak2b mo
Paxillin
DAPI
Vinculin
DAPI
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Figure 6.4: Reduced levels of focal adhesion proteins in embryos injected with either *pak2a* or *pak2b* morpholino.

Distribution of F-actin at the midline was visualised by staining embryos with phalloidin at the 10-somite stage in control morpholino (a-c) or *pak2b* morpholino injected embryos (4.5ng). Confocal sections of the midline are shown in an anterior (a/d) to posterior series (c/f). Levels of focal adhesion proteins, Paxillin (g-i) and Vinculin (j-k) were visualised by fluorescent immunocytochemistry at the 10-somite stage. The amount of proteins detectable was reduced in embryos injected with either *pak2a* (h,k) or *pak2b* morpholino (i,l) compared to embryos injected with control morpholino (g,j). The nuclear stain, DAPI (green) was used as a counterstain with antibodies against the focal adhesion proteins (red). Embryos are orientated posterior to the top (a-f) or anterior to the top (g-l). Abbreviations indicate: s, somite; nc, notochord; ad, adaxial cells; ep, somitic epithelium; me, somitic mesenchyme.

In agreement with this observation we found that levels of Paxillin protein at the somite boundaries of embryos injected with *pak2a* morpholino were decreased and very low levels of Paxillin were detected at the somite boundaries of embryos injected with *pak2b* morpholino (Figure 6.4h,i).

Decreased levels of Paxillin at the somite boundaries of embryos injected with either *pak2a* or *pak2b* morpholino suggested that focal adhesion formation at the somite boundaries was decreased following knock down of Pak2. Further evidence for this was provided by analysis of Vinculin distribution. Vinculin is a structural protein that links the actin cytoskeleton to adhesion complexes and is directly bound to Paxillin within focal adhesions (Turner, 2000). Cytoskeleton associated Vinculin was similarly distributed to F-actin and Paxillin, with high levels at the somite boundaries (Figure 6.4j). Cytoplasmic Vinculin could also be observed throughout the midline. Following morpholino-mediated knock down of *pak2a* of *pak2b* levels of Vinculin at the somite-somite and somite-notochord boundaries were reduced, however cytoplasmic protein could be detected at levels comparable to the control (Figure 6.4j-l).

Electron microscopy was used to further investigate the midline of embryos injected with *pak2b* morpholino and revealed interesting defects in the organisation of midline tissues. Sections were analysed from two embryos injected with *pak2b* morpholino. *Pak2b* morpholino was used since the more severe morphological phenotype of somite development was observed in these embryos. Embryos were fixed and stained at the 15-somite stage and processed for electron microscopy. In control embryos cells were tightly packed together within the somites (Figure 6.5b,c), however, in embryos injected with *pak2b* morpholino somitic cells were loosely packed and cells appeared rounded up (Figure 6.5e,f). Additionally, in some sections of *pak2b* morpholino injected embryos the round somitic cells had apparently detached from the somite block (Figure 6.5: g). This rounding up of cells is characteristic of a loss of cell adhesion (Leffers *et al.*, 1993; Luber *et al.*, 2000). The data obtained by electron microscopy provided further evidence that cell adhesion within somites was disrupted following knock down of Pak2.

Figure 6.5

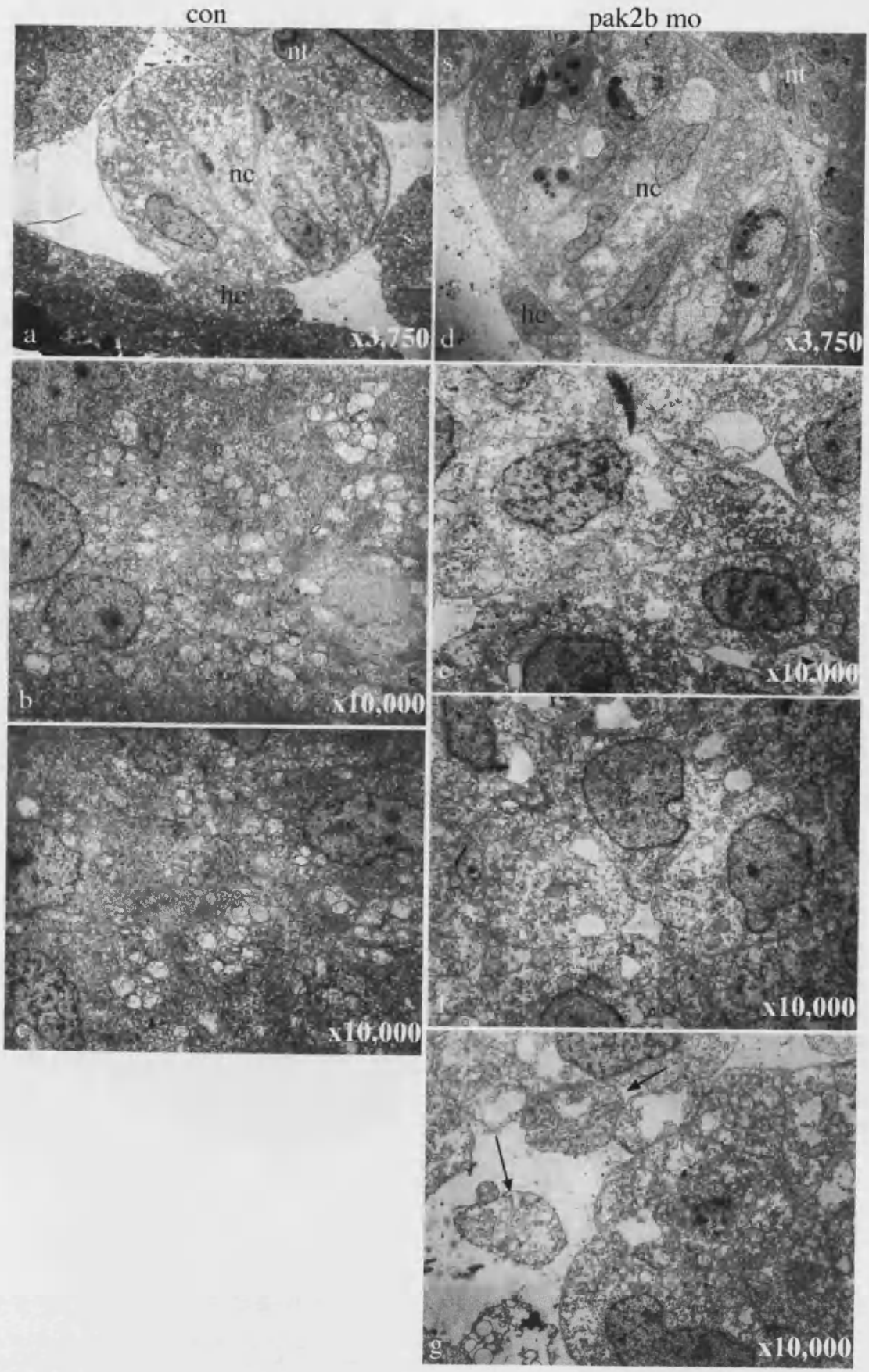
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Figure 6.5: Loss of cell adhesion in *pak2b* morpholino injected embryos.

Transverse sections of control embryos (a-c) and embryos injected with 4.5ng *pak2b* morpholino (d-g) were viewed under an electron microscope. Midline tissues are shown at x3,750 magnification and somites are shown at x10,000 magnification. Apparently loose and rounded up somitic cells are indicated with arrows (g). Abbreviations refer to: nt, neural tube; nc, notochord; s, somite; hc; hypochord.

6.4 Effect on Pak2 knock down on ventral midline tissues.

The ectopic *myoD* expressing cells observed in the ventral midline may indicate defects in the formation of ventral midline structures. Using molecular markers we investigated whether ventral tissues, such as the hypochord and dorsal aorta were formed normally in embryos injected with either *pak2a* or *pak2b* morpholino. The hypochord is a transient rod-like structure identifiable as a single row of cuboid shaped cells directly ventral to the notochord (Latimer *et al.*, 2002). There is some debate as to whether the hypochord is derived from the mesoderm (Appel *et al.*, 2003; Latimer *et al.*, 2002) or the endoderm (Cleaver *et al.*, 2000; Eriksson and Lofberg, 2000). However, recent work suggests that hypochord precursors arise from the lateral edges of the organizer in zebrafish, closely associated with midline precursors and the paraxial mesoderm and that signalling from paraxial mesoderm induces a subset of neighbouring midline precursors to develop as hypochord, rather than as notochord (Latimer *et al.*, 2002).

We carried out *in situ* hybridisation of embryos injected with either *pak2a* or *pak2b* morpholino using *collagen type IIa (col2a)* a molecular marker of the hypochord (Yan *et al.*, 1995). In addition to the hypochord, *col2a* is expressed in the developing notochord and floorplate. In embryos injected with either *pak2a* or *pak2b* morpholino expression of *col2a* in the floor plate and notochord was unaffected (Figure 6.6c-f). However, *col2a* expression in the hypochord revealed fragmentation and many areas of non-expressing cells (Figure 6.6c-f). At the 16-somite stage an incomplete hypochord was observed in over half of embryos injected with *pak2a* (n=40) or *pak2b* morpholino (n=40) and loss of *col2a* expression was generally restricted to the anterior and mid sections of the hypochord. This phenotype remained similar at 24hpf.

The hypochord is intimately associated with the notochord so when the notochord moves dorsally at 15-16 somite stages the hypochord moves with it, detaching itself from the underlying endoderm and leaving a space between itself and the endoderm.

Figure 6.6

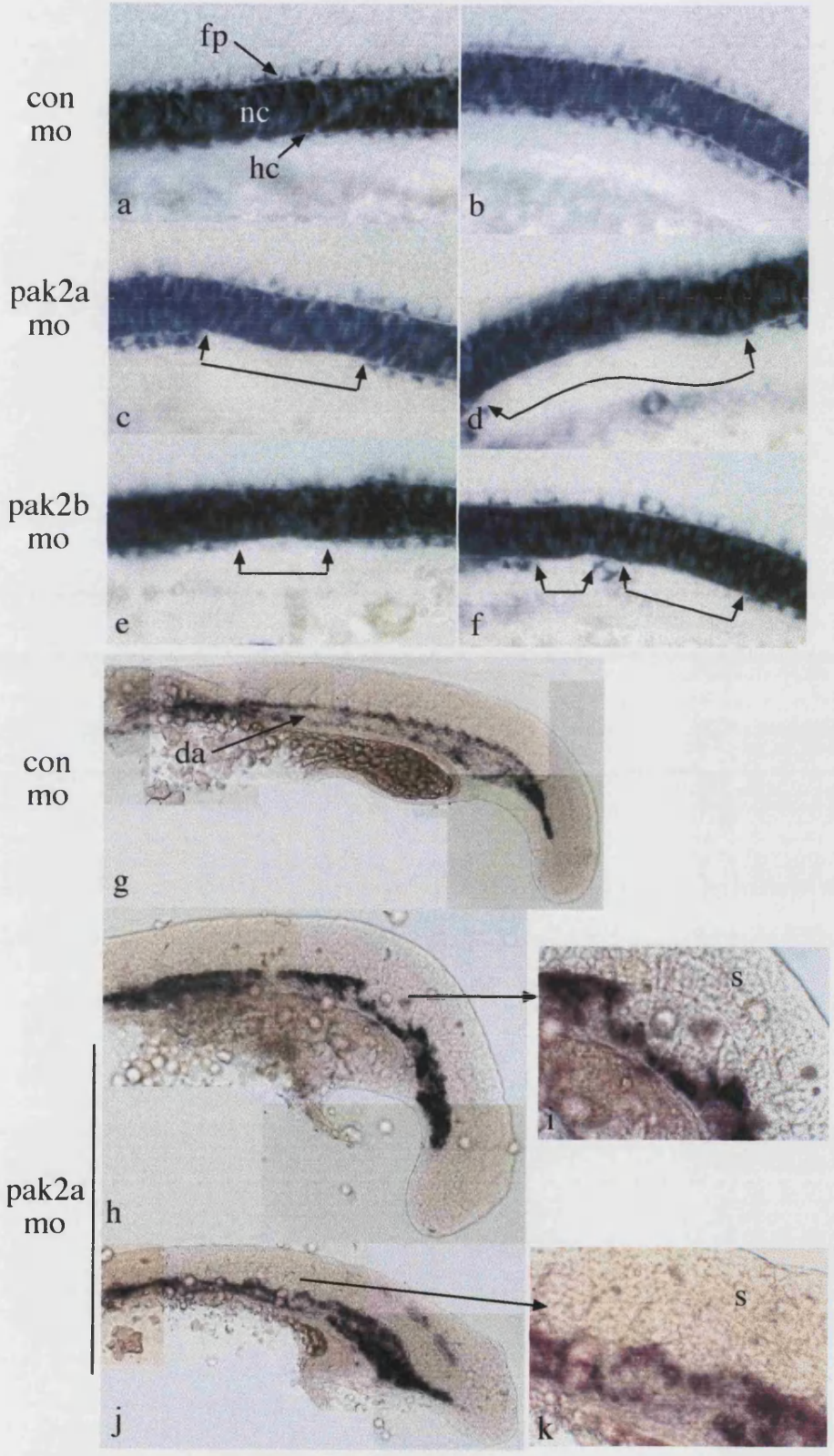
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Figure 6.6: Disruption to the formation of the hypochord and dorsal aorta in embryos injected with *pak2a* or *pak2b* morpholino.

Expression of *collagen type IIa* was investigated 16-somite stage embryos that had been injected with 4.5ng of control (a.b), *pak2a* (c,d) or *pak2b* morpholino (e,f). Loss of *col2a* expression in the hypochord is indicated by linked arrows (c-f). Vasculature, including the dorsal aorta was labelled using the molecular marker *fli-1* at 22hpf in control (g) and *pak2a* (h-k) morpholino injected embryos. Disrupted expression of *fli-1* is indicated by (]) and shown at high magnification (i,k). Abbreviations represent: fp, floor plate; nc, notochord; hc, hypochord; da, dorsal aorta; and s, somite.

This space is thought to be important for recruitment of the angioblasts by hypochord signals, the formation of blood islands and eventually formation of the dorsal aorta (Eriksson and Lofberg, 2000). *Fli-1* is an ETS-domain transcription factor expressed in the developing vasculature, including the dorsal aorta, and in sites of blood formation (Brown *et al.*, 2000). *In situ* hybridisation of embryos injected with either *pak2a* or *pak2b* morpholino to look at *fli-1* expression revealed a similar phenotype to that seen with *col2a.* The dorsal aorta was present following knock down of *pak2a* or *pak2b*, however, gaps were observed at the dorsal boundary of *fli-1* expression (Figure 6.6h-j). An incompletely formed dorsal aorta was observed in 44% of *pak2a* morpholino injected embryos (n=30) and 66% of embryos injected with *pak2b* morpholino (n=20). The width of the gaps ranged in size and could be observed from the anterior to the posterior of the dorsal aorta. By morphological analysis it appeared that in some sections of the aorta, adjacent to the notochord, *fli-1* expressing cells were replaced by somitic cells (Figure 6.6i,k).

6.5 Effect of Pak2a$^{\Delta c}$YFP and Pak2b$^{\Delta c}$YFP on early development of zebrafish embryos.

6.5.1 Localisation of Pak2a$^{\Delta c}$YFP and Pak2b$^{\Delta c}$YFP.

Pak2a$^{\Delta c}$YFP and *pak2b$^{\Delta c}$YFP* RNAs were injected at the one-cell stage and the localisation of yellow fluorescent protein and the morphology of injected embryos was analysed. Localisation of Pak2a$^{\Delta c}$YFP and Pak2b$^{\Delta c}$YFP protein was initially investigated at the shield stage. The morphological shield is formed by the involution of cells at the marginal region, forming the germ ring, and the convergence of cells towards the dorsal midline results in the formation of the morphological shield (Kimmel *et al.*, 1995). At this stage a number of cell types can be identified. The enveloping layer (EVL) is a superficial layer, one-cell thick, that covers the deep cells of the blastoderm and is attached by the vegetal rim to the yolk cell.

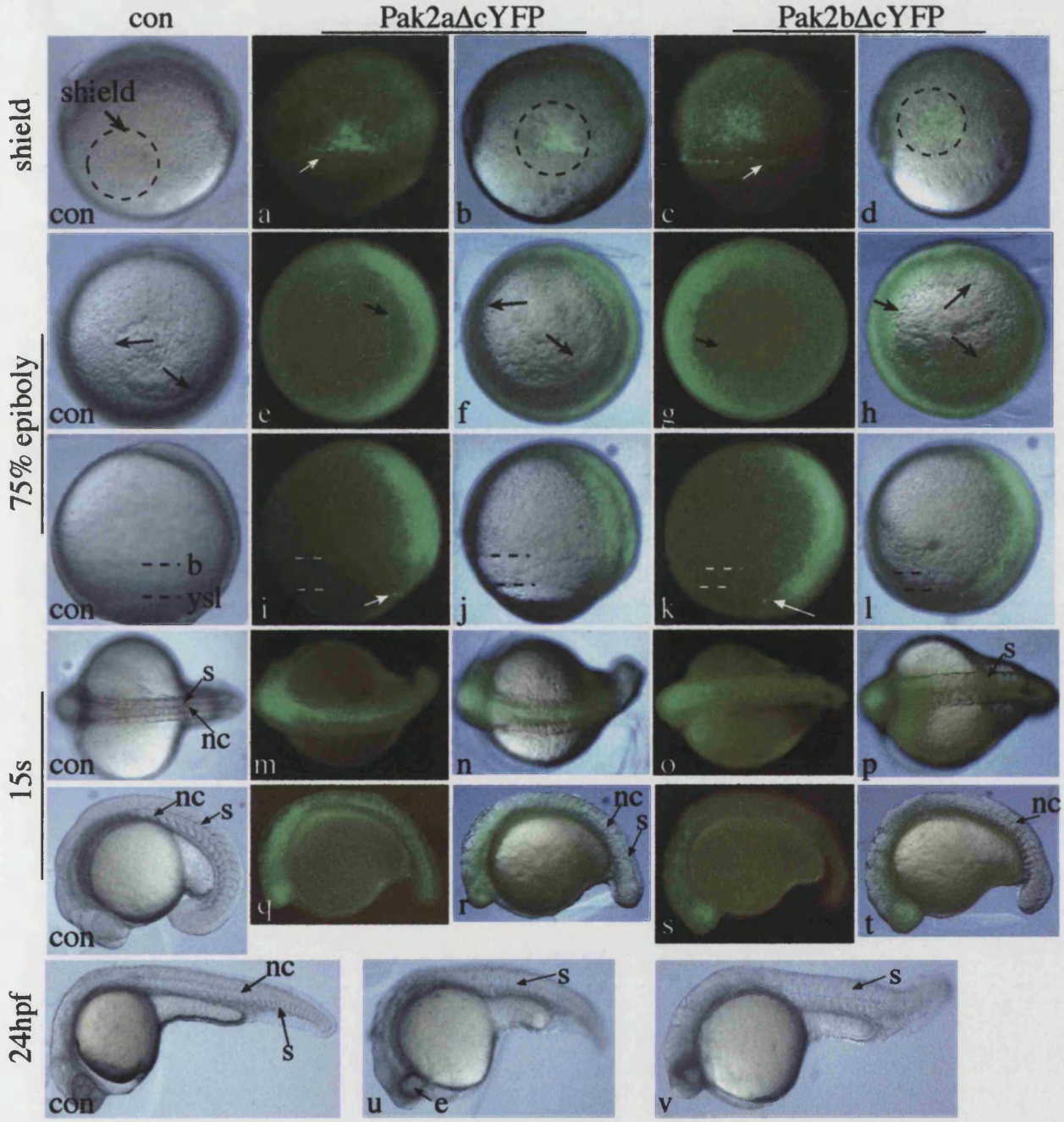
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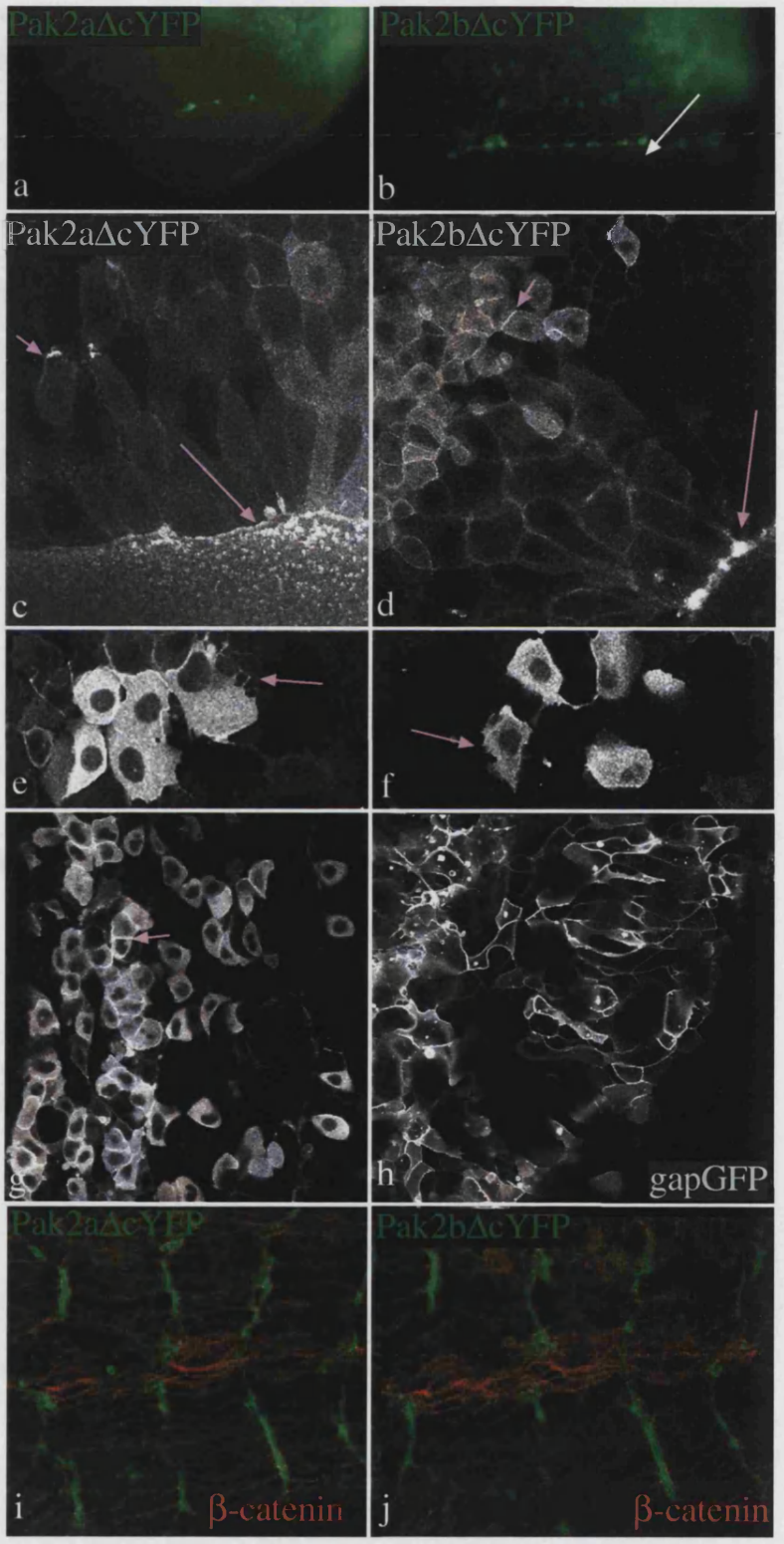
Figure 6.7

Figure 6.7: Localisation of Pak2a$^{\Delta c}$YFP and Pak2b$^{\Delta c}$YFP protein and morphology of injected embryos.

Pak2a$^{\Delta c}$YFP and Pak2b$^{\Delta c}$YFP proteins were highly concentrated at the boundary between the yolk syncitial layer (YSL) and the yolk cytoplasmic layer (YCL) as indicated by white arrows at shield stage (a-d) and 75% epiboly (i,k). The morphological shield is indicated by a circle (b,d: lateral view). The marginal rim of the YSL is indicated by arrows (e-h: vegetal view) or the lower dashed line (i-l: lateral view). The upper dashed line represents the boundary of the blastoderm. Pak2aDcYFP and Pak2bDcYFP protein localisation and morphology of injected embryos is shown at the 15-somite stage (m-p: dorsal view) and (q-t: lateral view) and 24hpf (u,v). Abbreviations correspond to: b, blastoderm; ysl, yolk syncitial layer; nc, notochord; s, somite; e, eye.

Also present are, the anuclear yolk cytoplasmic layer (YCL), that surrounds the bulk of the yolk mass, and the yolk syncytial layer (YSL), that forms between the YCL and the blastoderm (Solnica-Krezel and Driever, 1994; Solnica-Krezel *et al.*, 1995). Within the blastodermal cells of the morphological shield we detected high levels of Pak2a$^{\Delta c}$YFP and Pak2b$^{\Delta c}$YFP protein, however this may simply reflect the greater volume of cells at this position. Interestingly at the boundary between the YSL and YCL, a ring of Pak2a$^{\Delta c}$YFP and Pak2b$^{\Delta c}$YFP protein was observed at a high level. This ring was maintained as epiboly proceeded and until the YSL fully covered the yolk cell (Figure 6.7 and 6.8). The position of the ring corresponded to a highly active site of endocytosis (Betchaku, 1986; Cooper and D'Amico, 1996; Solnica-Krezel and Driever, 1994; Solnica-Krezel *et al.*, 1995). Epiboly of the surface of the YSL has been explained, in part, by endocytosis of the YCL resulting in expansion of the YSL and loss of the YCL (Betchaku, 1986).

Using confocal microscopy to visualise cellular localisation, Pak2a$^{\Delta c}$YFP and Pak2b$^{\Delta c}$YFP proteins were found in the cytoplasm as well as at the membrane. At the membrane, high levels of protein were visualised along cell boundaries and concentrated in bright spots, which may represent focal adhesion sites or forming membrane protrusions (Figure 6.8g,j). In agreement with our observations at low magnification, Pak2a$^{\Delta c}$YFP and Pak2b$^{\Delta c}$YFP were highly concentrated at the YSL/YCL boundary (Figure 6.8g,j). In contrast high levels of gapGFP were not detected at the YSL/YCL boundary (data not shown). At later stages higher levels of Pak2a$^{\Delta c}$YFP and Pak2b$^{\Delta c}$YFP proteins could be observed at the somite boundaries (Figure 6.8c,f). Confocal microscopy at the 15-somite stage revealed Pak2a$^{\Delta c}$YFP and Pak2b$^{\Delta c}$YFP protein within the cytoplasm but not within the nucleus and concentrated at the membrane where two cells were in contact (Figure 6.8h,i,j). Time-lapse analysis revealed that cells expressing Pak2a$^{\Delta c}$YFP or Pak2b$^{\Delta c}$YFP were able to produce membrane ruffles and extend lamellipodia (Figure 6.8h,k).


Pak2aΔcYFP
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Pak2bΔcYFP
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Pak2aΔcYFP
c
Pak2bΔcYFP
d
e
f
g
h
gapGFP
Pak2aΔcYFP
i
β-catenin
Pak2bΔcYFP
j
β-catenin

Figure 6.8

Figure 6.8: Confocal analysis of Pak2a$^{\Delta c}$YFP and Pak2b$^{\Delta c}$YFP localisation.

High levels of Pak2a$^{\Delta c}$YFP and Pak2b$^{\Delta c}$YFP were localised to the boundary between of the yolk syncitial layer and yolk cytoplasmic layer. Localisation at this boundary at 75% epiboly is shown in live embryos at low magnification (a,b) and in confocal sections (c,d). High levels of Pak2a$^{\Delta c}$YFP and Pak2b$^{\Delta c}$YFP protein were detected at the membranes where cells contact (d,g) and in polarised clusters at the membrane (c,d). Extension of membrane protrusions, lamellipodia, were visible in cells expressing Pak2a$^{\Delta c}$YFP (e) and Pak2b$^{\Delta c}$YFP (f). Localisation of Pak2a$^{\Delta c}$YFP and Pak2b$^{\Delta c}$YFP were compared to gapGFP, a membrane-associated GFP construct, as a control (h). At 28hpf higher levels of Pak2a$^{\Delta c}$YFP and Pak2b$^{\Delta c}$YFP were localised to the intersomitic boundaries (i,j).

6.5.2 Morphology of embryos injected with *pak2a$^{\Delta c}$YFP* or *pak2b$^{\Delta c}$YFP* RNA .

The gastrulation movements of involution and formation of the morphological shield all occurred normally in embryos injected with either *pak2a$^{\Delta c}$YFP* or *pak2b$^{\Delta c}$YFP* (Figure 6.7a-l). However, the process of epiboly was disrupted, from approximately the 70% epiboly stage a vegetal protrusion of the yolk occurred and there were problems in completing the closure of the yolk plug, particularly in embryos injected with *pak2b$^{\Delta c}$YFP*. From the tailbud stage, defects in the formation and patterning of the body axis could be observed. At the 15-somite stage the notochord was absent or difficult to distinguish and the body axis had failed to extend normally (Figure 6.7r,t). The mesoderm was frequently unsegmented, particularly in the anterior region (Figure 6.7r,t,u,v). Segmented somites were visible in the posterior region of the trunk although these were small and "u" shaped. By 24hpf the tail region was short and highly curled and necrosis began to be evident (Figure 6.7u,v).

Development of the eyes was also affected, particularly in embryos injected with *pak2a$^{\Delta c}$YFP* (Figure 6.7u). The eyes were small and poorly developed and embryos injected with *pak2a$^{\Delta c}$YFP*, showed mild cyclopia, suggesting defects in the ventral forebrain development. *Pak2a$^{\Delta c}$YFP* and *pak2b$^{\Delta c}$YFP* injected embryos were not viable and embryos with morphological phenotype did not survive to 5dpf.

The defects in gross morphology of embryos injected with *pak2a$^{\Delta c}$YFP* and *pak2b$^{\Delta c}$YFP* were similar, although more severe, to those observed following injection of *pak2a* or *pak2b* morpholino. Defects were further analysed using the same panel of *in situ* markers used to investigate midline defects in *pak2a* and *pak2b* morpholino injected embryos.

6.5.3 Molecular analysis of embryos injected with *pak2a$^{\Delta c}$YFP* or *pak2b$^{\Delta c}$YFP* RNA.

We investigated the midline defects of *pak2a$^{\Delta c}$YFP* and *pak2b$^{\Delta c}$YFP* injected embryos using the molecular markers of somites, *myoD* and *papc*, and the notochord, hypochord and floor plate, *col2a*.

The disrupted pattern of *myoD* expression reflected the morphological segmentation defects with fusing of the somites. We also observed ectopic *myoD* expressing cells at the ventral midline (Figure 6.9c-h). This ectopic expression of *myoD* in ventral cells, also observed following injection of *pak2a* or *pak2b* morpholino, was observed more frequently in embryos injected with *pak2b$^{\Delta c}$YFP* (Figure 6.9g,h).

A defect in somite segmentation was also revealed by detection of *papc* which is expressed within the presomitic and somitic mesoderm. Expression is detected in bands that demarcate the next somite to be segmented (s-1) the somite undergoing segmentation (s0) and the most recently formed somite (s1). Most commonly all three bands were fused (Figure 6.9o), however, there was also specific fusion of the s0 and s-1 bands (Figure 6.9m,p). Interestingly, in approximately 10% of embryos injected with *pak2b$^{\Delta c}$YFP* (n=18) a weak fourth band of papc expression could be detected, suggesting that *papc* expression was maintained in the second most recently formed somite. This maintenance of *papc* in the second somite was also observed in *pak2a* and *pak2b* morpholino injected embryos. Maintenance of *papc* expression in adaxial cells of the somitic mesoderm, as detected in embryos injected with either *pak2a* or *pak2b* morpholino, was also noted in embryos injected with *pak2a$^{\Delta c}$YFP* or *pak2b$^{\Delta c}$YFP* (Figure 6.9k,l). Ectopic papc was also observed in the somitic tissue of embryos injected with *pak2b$^{\Delta c}$YFP* (Figure 6.9n). Consistent with the observation that formation of the notochord was defective in embryos injected with *pak2a$^{\Delta c}$YFP* or *pak2b$^{\Delta c}$YFP* we noted that *papc* expressing mesodermal cells were able to encroach into the midline.

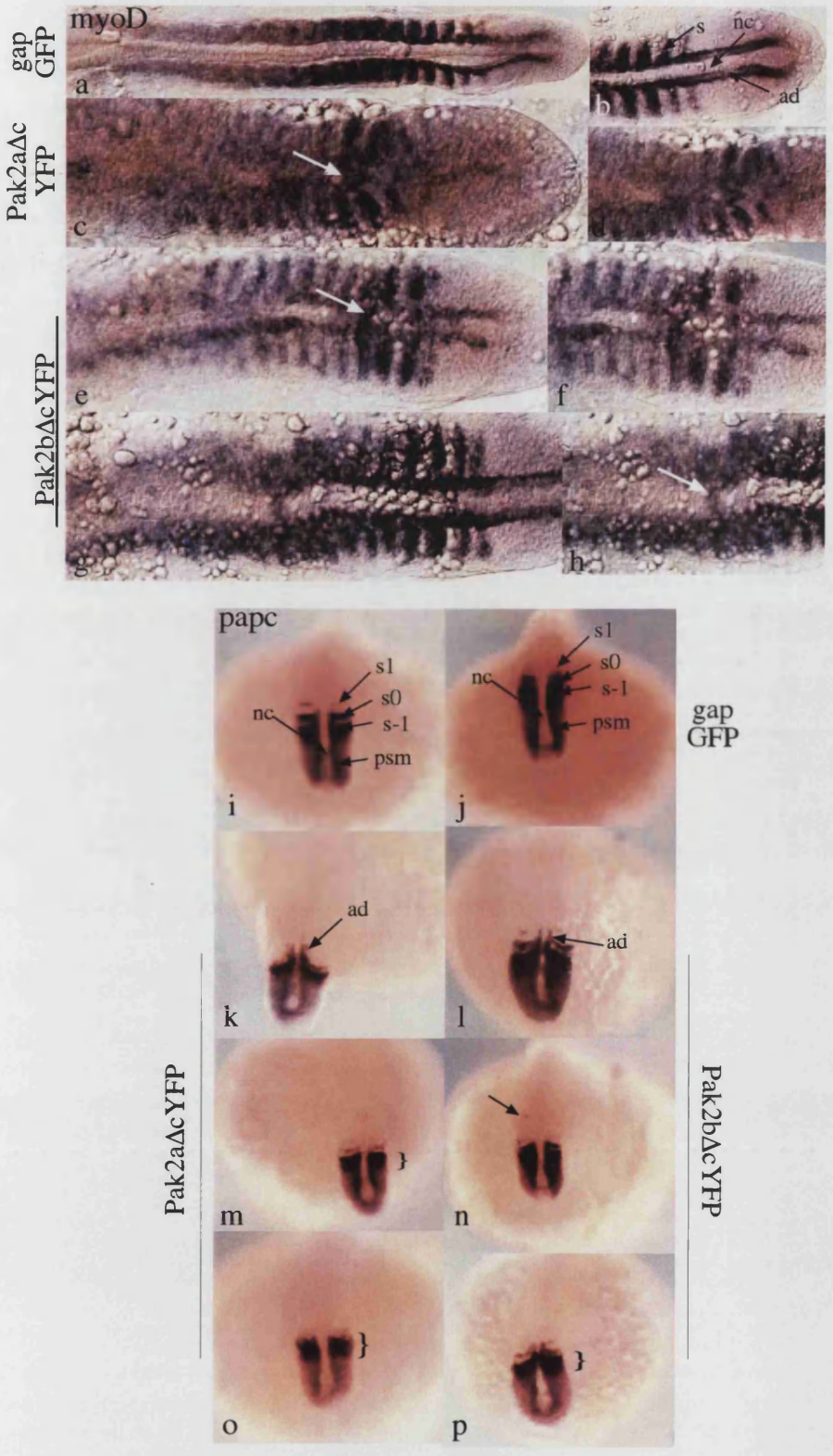
myoD
gap GFP
Pak2aΔc YFP
Pak2bΔcYFP
a
b
c
d
e
f
g
h
s
nc
ad
papc
s1
s0
s-1
psm
nc
ad
i
j
k
l
m
n
o
p
gap GFP
Pak2aΔcYFP
Pak2bΔcYFP

Figure 6.9

Figure 6.9: Aberrant expression of somitic genes in embryos injected with *pak2a$^{\Delta c}$YFP* or *pak2b$^{\Delta c}$YFP*.

Disruptions to the expression pattern of *myoD* were observed in 15-somite stage embryos injected with *pak2a$^{\Delta c}$YFP* (c,d) and *pak2b$^{\Delta c}$YFP* (e-h) when compared with gapGFP as a control (a,b). Arrows identify sites of ectopic *myoD* expression. Aberrant expression of *papc* was also observed in 15-somite stage embryos injected with *pak2a$^{\Delta c}$YFP* (k,m,o) and *pak2b$^{\Delta c}$YFP* (l,n,o). Somites are labelled according to convention, newly formed somite (s1), forming somite (s0), presumptive somite (s-1). Fused bands of expression are indicated by brackets and ectopic cells are identified by arrows. Embryos are shown as whole mount with anterior to the top. Abbreviations correspond to: nc, notochord; s, somite; psm, presomitic mesoderm; ad, adaxial cells.

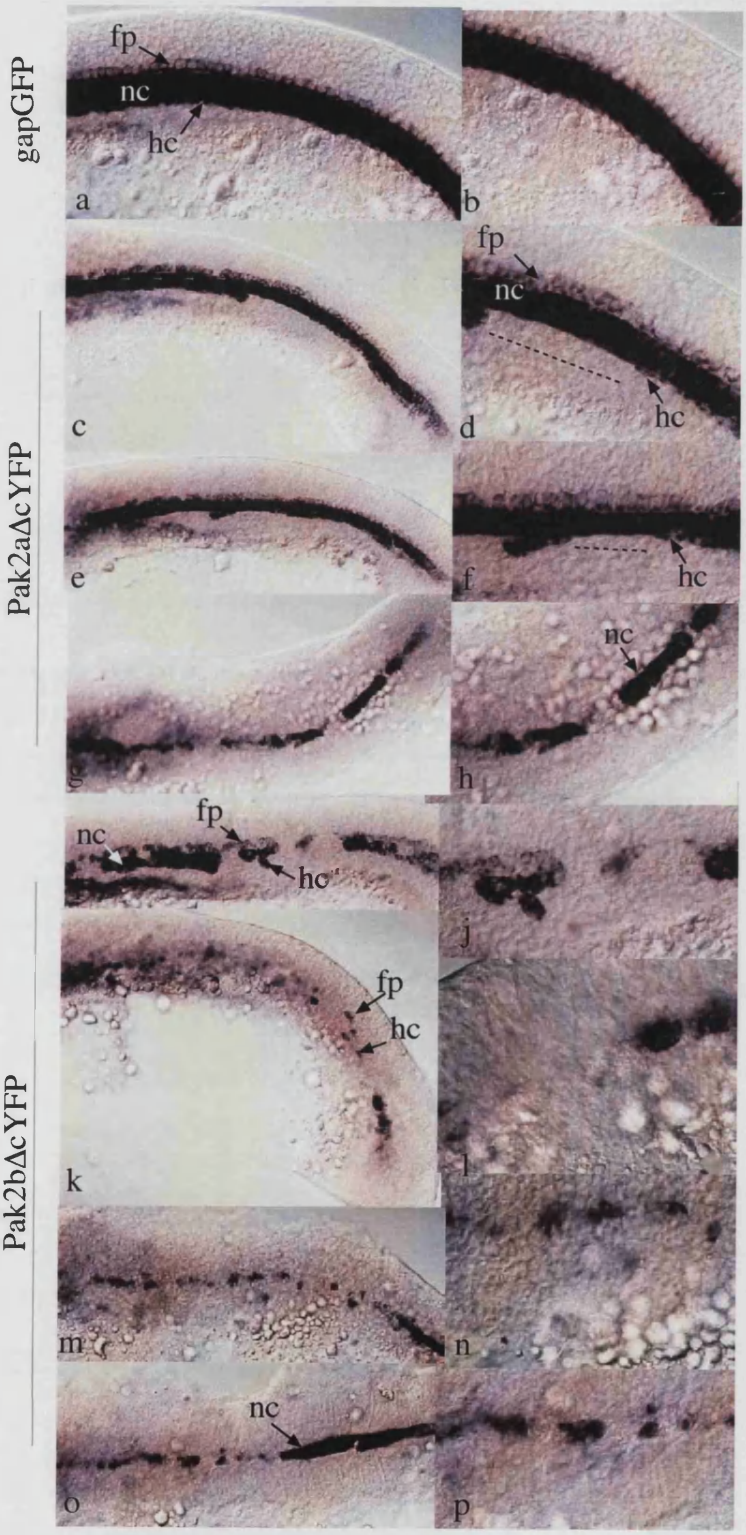

Figure 6.10

Figure 6.10: Fragmentation of the midline tissues in embryos injected with *pak2a*$^{\Delta c}$*YFP* or *pak2b*$^{\Delta c}$*YFP*.

Figure 6.10: Fragmentation of the midline tissues in embryos injected with *pak2a$^{\Delta c}$YFP* or *pak2b$^{\Delta c}$YFP*.

Disruptions to the formation of the midline tissues were observed in embryos injected with *pak2a$^{\Delta c}$YFP* (c-j) and *pak2b$^{\Delta c}$YFP* (k-p) when compared to gapGFP injected controls (a,b). Midline tissues were visualised using expression of *col2a* as a marker at the 15-somite stage. Gaps in the hypochord are indicated with a dotted line. Abbreviations refer to: fp, floorplate; nc, notochord; hc, hypochord.

To investigate the development of the notochord, as well as to compare expression to that observed in embryos injected with either *pak2a* or *pak2b* morpholino, we looked at the localisation of the notochord, floorplate and hypochord marker, *col2a*. Expression of *col2a* revealed striking defects in the formation of midline tissues particularly in embryos injected with *pak2b$^{\Delta c}$YFP*. In embryos injected with *pak2a$^{\Delta c}$YFP* defects were similar to those seen in embryos injected with either *pak2a* or *pak2b* morpholino, such that gaps were present along the hypochord (Figure 6.10c-f). However these gaps were also accompanied by disorganisation of the floorplate cells, a phenotype that was not observed in embryos injected with either *pak2a* or *pak2b* morpholino. Additionally a more severe phenotype could be observed in half of the affected embryos, in which gaps in floorplate and notochordal expression were observed, particularly in embryos injected with *pak2b$^{\Delta c}$YFP* (Figure 6.10g-p). The gaps in expression in the three tissues were often colocalised resulting in sections of the midline where no *col2a* expression could be detected. Focused analysis of such regions revealed that a notochord-like structure was occasionally visible but appeared necrotic (Figure 6.10p). Analysis of the localisation of the no-tail (ntl) protein, which is found in undifferentiated notochord cells revealed the presence of a partially undifferentiated notochord in embryos injected with *pak2a$^{\Delta c}$YFP* or *pak2b$^{\Delta c}$YFP* which was very thin in places but did not have the large gaps revealed by *col2a* expression (data not shown).

6.6 Discussion.

Initial investigations into the role of Pak in early zebrafish development, using embryos injected with *pak2* morpholino or *pak2$^{\Delta C}$YFP* constructs, have begun to reveal a role for Pak2 in the development of midline tissues. In addition to Pak2 function in the maintenance of cell adhesion and tissue integrity, our results suggest that Pak2 may be involved in regulating myogenic cell fate.

A contribution from Pak to the myogenic pathway is indicated by the ectopic expression of *myoD* in embryos where levels of Pak have been abrogated. *MyoD* is a dominant regulator of the myogenesis pathway and ectopic expression of *myoD* is sufficient to convert non-somitic cells into muscle cells (Hopwood and Gurdon, 1990; Ludolph *et al.*, 1994). In zebrafish, *myoD* is expressed at high levels in the mesoderm prior to somitogenesis (Weinberg *et al.*, 1996), however, the exact nature of the signals that regulate this expression remains to be established. It is known that a number of factors, such as, *sonic hedgehog (shh)*, *wnt* and *BMP*, act to promote or inhibit the expression of myogenic genes in order to restrict their expression to the future muscle cells. Additionally the overexpression of muscle-promoting factors, *shh* and *wnt* results in ectopic expression of *myoD* (Blagden *et al.*, 1997; Christian and Moon, 1993). *Shh* has a specific role in inducing adaxial cell fate and ectopic expression of *shh*, either in wild-type or mutant embryos, leads to ectopic slow muscle and abrogation of the number of *engrailed* positive cells (Barresi *et al.*, 2000; Blagden *et al.*, 1997). However the number of MP Engrailed positive cells and slow muscle cells is not affected in *pak2a* or *pak2b* morpholino injected embryos. This raises the possibility that Pak acts on the wnt pathways to restrict the expression of *myoD*.

In *Xenopus laevis* Xwnt-8 activity has been shown to be necessary for expression of *myoD* during gastrula stages and overexpression of *Xwnt-8* leads to ectopic expansion of *XmyoD* across the dorsal midline, converting notochord progenitors to muscle (Fisher *et al.*, 2002). Furthermore injection of an Xwnt-8 construct into *Xenopus* embryos results in fusion of the lateral somite files along the ventral midline, however this may be secondary to the loss of the notochord in these embryos (Christian and Moon, 1993). Since overexpression of *wnt* leads to ectopic expression of *myoD* it is possible that zebrafish Pak2 normally functions to inhibit this signalling pathway. In mammals Wnt signalling can act through a classic pathway involving the receptor frizzled-1 and downstream proteins: Dishevelled, Glycogen synthase 3-kinase, β-catenin and the transcriptional regulator T-cell factor (Tcf) to activate transcription of myogenic genes (Cossu and Borello, 1999). The co-repressor

C-terminal binding protein (CtBP) interacts with Tcf and inhibits transcription of wnt responsive genes in the absence of wnt signalling (Chinnadurai, 2002). During *Xenopus* development the binding of XTcf-3 to Xmyf-5 restricts expression of this gene in the mesoderm of *Xenopus* embryos (Yang *et al.*, 2002). The N-terminus of active Pak1 has recently been demonstrated to interact with the CtBP and inhibit translocation of CtBP into the nucleus, where it acts to repress gene transcription (Barnes *et al.*, 2003). The Pak2a$^{\Delta c}$YFP and Pak2b$^{\Delta c}$YFP proteins may act to restrict CtBP to the cytosol and therefore prevent repression of *myoD* gene expression.

In contrast Wnt signalling can also influence myogenic gene transcription through a β-catenin-independent pathway and studies in *Xenopus* have demonstrated that this pathway involves Frizzled, G-proteins, Protein Kinase C and Pax3 (Cossu and Borello, 1999; Sheldahl *et al.*, 1999). During myogenesis in chick Pax3 activates the transcriptional activator *Eya2* and the homeodomain-containing transcription factor *Six1.* Eya2 is an intrinsically cytosolic protein and is translocated into the nucleus by interaction with Six, whereupon transcription of downstream genes is induced. Association of Eya with Six and translocation to the nucleus is inhibited by the activated G-protein subunit, $G\alpha_z$ (Fan *et al.*, 2000). As Pak is an important activator of $G\alpha_z$ (Fan *et al.*, 2000) the absence of prolonged activation of $G\alpha_z$ in morpholino injected embryos may result the Six-Eya complex freely translocating to the nucleus and ectopic expression of *myoD*. Thus it appears that pak2a and pak2b can affect levels of gene expression, either indirectly by regulating localsation of key transcription factors or directly by binding to a repressor of transcription.

Pak2 also appeared to influence the maintenance of cell adhesion and tissue integrity within the somites. We have already shown that Pak2 plays an important role in maintaining the integrity of the lateral line primordium during zebrafish development and decreased levels of focal adhesion proteins at the somite boundaries and the detachment and rounding up of somitic cells suggest a similar role in somitic tissue. Decreased levels of Vinculin at the somite boundaries in embryos injected with either *pak2a* or *pak2b* morpholino would appear to be inconsistent with the increased levels

of cytoskeleton associated Vinculin detected by Western blot analysis (Chapter Five). However this may simply be the result of using different methods to assay for Vinculin as Western blot analysis does not look specifically at levels within somitic tissue.

Preliminary investigations using Pak2a$^{\Delta c}$YFP and Pak2b$^{\Delta c}$YFP constructs have begun to shed more light on the role of Pak during zebrafish development. Although the precise mechanism of Pak2a$^{\Delta c}$YFP and Pak2b$^{\Delta c}$YFP action needs to be clarified, interesting similarities were observed in the phenotypes of morpholino and *pak2$^{\Delta c}$YFP* injected embryos. The phenotype of a short body axis observed in embryos injected with *pak2a* and particularly *pak2b* morpholino (Chapter Five) was also observed in embryos injected with *pak2a$^{\Delta c}$YFP* or *pak2b$^{\Delta c}$YFP*. This suggests a critical role for Pak in the convergent extension movements required to lengthen the body axis possibly through Pak-mediated regulation of the actin cytoskeleton to initiate the changes to cell shape during intercalculation and extension. Defects in formation of the eye were also a consistent factor in morpholino and *pak2$^{\Delta c}$YFP* injected embryos, insinuating a direct or indirect role for Pak2 in eye and forebrain development.

Confocal time-lapse imaging of Pak2a$^{\Delta c}$YFP and Pak2b$^{\Delta c}$YFP expressing cells revealed that cells remained able to actively extend lamellipodia. This confirms our work looking at migration of the lateral line primordium where the ability of cells to extend processes was not affected. These data are in agreement with previous work suggesting that Pak is not required for the formation of lamellipodia or membrane protrusions (Sells *et al.*, 1999).

Despite the preliminary nature of our investigations into the effect of Pak2a$^{\Delta c}$YFP and Pak2b$^{\Delta c}$YFP in zebrafish development these constructs and the Pak2a^{FL}YFP, Pak2b^{FL}YFP constructs nevertheless provide a foundation on which we can build on our understanding of Pak2 function in zebrafish development. The introduction of mutations within these constructs to alter the ability of Pak2 to interact with proteins such as Rac, Cdc42, PIX, or Nck will allow careful dissection of the precise roles of Pak2 during zebrafish development.

Chapter Seven

General Discussion

Cell migration is a highly regulated process that is important for many aspects of development. Cell migration requires the formation of membrane protrusions at the leading edge and these protrusions form attachment sites, known as focal adhesions, with the ECM. Actin-myosin filaments terminate at these focal adhesions allowing the cell to generate the necessary force to pull itself over the ECM. As the cell advances, focal adhesions are disassembled at the trailing end and the rear of the cell is pulled forward by contraction of myosin fibres (Christopher and Guan, 2000). Key regulators that control actin dynamics and adhesion formation are the Rho family of small GTPases and their effectors the p21 activated kinases.

We set out to investigate the migration of the posterior lateral line primordium in zebrafish development and the possible regulation of this migration by Pak2 proteins. Initially we used confocal time-lapse imaging of the posterior lateral line primordium to gain an understanding of the organisation and activity of cells within the primordium during its migration. Furthermore we investigated the distribution of Pak2 proteins, adhesion proteins and the actin cytoskeleton in the migrating lateral line primordium. The importance of the results and their implications on regulation of migration of the posterior lateral line primordium will be discussed here.

In order to investigate the role of Pak proteins in migration of the lateral line primordium we have identified and characterised two zebrafish genes, *pak2a* and *pak2b*, that are homologues of the h*pak2* gene. Using loss-of-function approaches, primarily through morpholino oligonucleotide-mediated knock down, we have analysed in detail, the defects in migration of the posterior lateral line primordium. We have

found that Pak2 function is important for dynamic regulation of cell adhesion, maintenance of tissue integrity and directed cell migration. We have also characterised other potential roles for Pak2a and Pak2b in early zebrafish development and found a possible role for Pak2a and Pak2b in regulation of myogenic cell fate.

7.1 Cell behaviour and cellular organisation in the migrating primordium.

7.1.1 Cells at the leading edge of the primordium explore the environment.

By using real time imaging of the migration of the lateral line primordium we have demonstrated that cell activity is dependant on the position within the primordium. Cells at the leading edge are highly active while cells away from the leading edge are tightly packed and less dynamic. Leading edge cells extended many membrane processes during migration along the trunk. This suggests that these cells actively explore their surrounding environment and that factors in the environment restrict migration to the correct pathway. This sensing activity fits with the evidence that migration of the posterior lateral line primordium is directed by chemotactic signalling and guided by inhibitory signals in the dorsal and ventral myotome (David *et al.*, 2002; Shoji *et al.*, 1998).

The attractive chemokine *sdf1a* is expressed along the horizontal myoseptum and its receptor (*cxcr4b*) is localised to the migrating cells of the lateral line primordium (David *et al.*, 2002). It is likely that multiple membrane protrusions at the leading edge of the migrating primordium detect where levels of Sdf1a are high and this is then interpreted by the cells to elicit a directional migration response by coordinated reorganisation of the cytoskeleton. The chemotactic nature of these cells suggested to us that Pak may be involved in regulating migration of the lateral line

primordium as Pak is known to mediate reorganisation of the actin cytoskeleton in response to chemotactic activation in neurophils (Dharmawardhane *et al.*, 1999).

7.1.2 The rosette-like structures within the primordium represent nascent neuromast precursors.

Work from our laboratory has shown that there is a morphological and molecular prepattern within the migrating primordium that presages deposition of neuromast precursors. By analysis of cellular organisation using membrane labels, we confirmed the presence of a morphological prepattern. Rosette-like structures were readily discernable within the migrating lateral line primordium and at different stages of development 2-4 rosette-like structures could be identified. It is interesting to note that the rosette-like structures were absent in the leading front of the primordium. Cells at the leading edge were generally less organised which may reflect that neuromast precursors were determined in an anterior to posterior manner, with anterior neuromasts determined first. Using time-lapse analysis we have been able to show that the rosette structure is maintained during deposition and is deposited as a unit. Our data clearly demonstrated that the rosette-like structures represent the nascent neuromast precursors.

During deposition the leading edge of the primordium maintains a constant speed of migration while cells of the depositing rosette slow their migration. As a result a boundary becomes apparent between the rosette and the remainder of the primordium. Cells at the boundary became highly elongated and returned to their normal rounded shape once deposition had been completed. These observations suggested that strong cell to cell adhesions were acting within the rosette and the primordium to maintain cell to cell contact during deposition.

7.1.3 Cell adhesions maintain the organisation of the primordium.

Maintenance of the rosette structure within the primordium and during deposition can be ascribed to strong clustering of adhesion molecules. Adherens

junctions play pivotal roles in cell and tissue organisation by mediating cell adhesion (Tepass, 2002). By interdigitation of stable adhesive elements between adjacent cells, adherens junctions maintain overall tissue architecture (Gumbiner, 1996). By analysing the localisation of components of adherens complexes; β-catenin, E-cadherin and F-actin we have shown that these proteins were localised at the focal point of the established rosettes and are likely to be important in maintaining the integrity of the lateral line primordium and nascent neuromasts. Consistent with this idea we found that there was no clustering of E-cadherin and β-catenin proteins at the leading edge where rosettes were not discernable. It is likely that formation and clustering of adherens junctions functions to draw cells together to form the nascent neuromast rosettes.

By studying the organisation and behaviour of cells within the lateral line primordium we have increased our understanding of how regulation of cell adhesion and the actin cytoskeleton may contribute to controlling the migration of the lateral line primordium. We have attested to the need for dynamic regulation of cell adhesion and the directed reorganisation of the actin cytoskeleton in response to chemotactic signals for migration of the lateral line primordium.

7.2 Characterisation of the zebrafish *pak2* genes.

We have identified and characterised two zebrafish homologues of *pak2* which represent the first *pak* family members to be characterised in zebrafish. The ubiquitous nature of *pak2a* and *pak2b* expression was in agreement with observations of *pak2* localisation in rat and *Xenopus* (Jaffer and Chernoff, 2002; Souopgui *et al.*, 2002). In addition, the enrichment of zebrafish *pak2a* and *pak2b* expression in the brain, eye and tailbud was mirrored by the expression of X*pak2* in Xenopus (Souopgui *et al.*, 2002). It is interesting to note that two homologues of *pak2* have been also been identified in Xenopus, although only one of these has been characterised (Cau *et al.*, 2000; Souopgui *et al.*, 2002). We also observed a high level of *pak2a*

expression in the cells surrounding the migrating primordium. This pattern of expression is very reminiscent of the localisation of focal adhesion proteins and may suggest a role for Pak2 in regulating the formation of focal adhesions.

By *in vitro* translation of *pak2a* and *pak2b* RNA we found the molecular weights of the Pak2a and Pak2b proteins were approximately 62kD and 67kD respectively. This is in agreement with published data for the group I Pak proteins (Pak1: 68kD, Pak2: 62kD, Pak3: 65kD) (Knaus and Bokoch, 1998). It is well known that many zebrafish genes are duplicated, probably as a result of a whole genome duplication event during the evolution of fish (Amores *et al.*, 1998). The presence of two zebrafish homologues of *pak2* many reflect this duplication. The protein sequences of the two zebrafish Pak2 homologues revealed that divergence has occurred between these two proteins after duplication. The similar lengths of vertebrate Pak2 and zebrafish Pak2a proteins suggests that this gene was duplicated to give rise to Pak2b and this was followed by an insertion event, resulting in a larger Pak2b protein. By looking at the conservation of protein interaction domains and detecting binding of known interacting proteins with Pak2a and Pak2b we are able to predict conserved and divergent roles for these proteins in zebrafish.

7.2.1 Zebrafish Pak2 protein domains are highly conserved.

By aligning the known homologues of Pak2 to the zebrafish homologues we have been able to ascertain the conservation of key features common to the Pak Group I family of proteins. The catalytic kinase domains of Pak2a and Pak2b were highly homologous to other Pak2 proteins and all the sites for autophosphorylation were conserved. Within the N-terminal regulatory domains of Pak2a and Pak2b the consensus binding site for Rac/Cdc42 (CRIB) was completely conserved and using pull down assays we were able to show that Pak2a and Pak2b were capable of binding both Rac and Cdc42. Interaction of Pak with Rac and Cdc42 has been shown to be important in regulating organisation of the actin cytoskeleton (Eby *et al.*, 1998)

therefore both Pak2a and Pak2b proteins could act as downstream mediators of Rac and Cdc42 in regulating the actin cytoskeleton.

We were also able to identify other known sites of protein interaction within Pak2a and Pak2b including the three proline rich SH3 domain binding sites and the Gβγ subunit binding site. We also show that not only is the atypical PIX SH3-domain binding site conserved in both Pak2a and Pak2b, but PIX isoforms were able to bind both these proteins, although some differences in the number and size of isoforms bound was observed. As the interaction of Pak and PIX leads to recruitment of Pak to focal adhesion sites (Brown *et al.*, 2002) we can predict that both Pak2a and Pak2b can be recruited to focal adhesions during zebrafish development.

7.2.2 Pak2a but not Pak2b binds to Nck.

The consensus binding site for the adaptor protein Nck was present in both Pak2a and Pak2b. However, pull down assays using Pak2$^{\Delta c}$YFP proteins and immunoprecipitation assays using Pak2$^{\Delta c}$GST proteins clearly revealed that only Pak2a could interact with Nck. Single amino acid differences around the Nck binding site (aa6,7, 25 and 29) in Pak2b may be responsible for preventing interaction with Nck. The inability of Pak2b to bind Nck may have important functional consequences. Nck-mediated recruitment of Pak to the cell membrane can increase the activation of Pak by colocalisation with its activator Rac (Lu *et al.*, 1997). Furthermore, in addition to PIX, Nck is also thought to play a role in recruiting Pak to sites of focal adhesion complexes (Zhao *et al.*, 2000a) Thus, the finding that Pak2a binds to Nck but Pak2b does not suggests differences in the function, protein localisation and activation of Pak2a and Pak2b.

7.3 Cell adhesion mediated by Pak2 is required for maintaining the integrity of the lateral line primordium.

We have presented detailed analysis of the organisation and behaviour of cells within the lateral line primordium during migration and have provided evidence that the cadherin-mediated adherens junctions may be important for maintaining the integrity of the lateral line primordium and nascent neuromast precursors. Using a morpholino-mediated loss-of-function approach we have been able to identify a role for Pak2 and particularly Pak2b in maintaining this integrity. By visualising the distribution of adherens junctions proteins and F-actin in the lateral line primordium of *pak2b* morpholino injected embryos we have found that knock down of Pak2b disrupts clustering of adherens junctions in the lateral line primordium.

Concomitant with disruptions to cell adhesion, time lapse analysis of primordium migration in embryos injected with *pak2b* morpholino revealed disruption to the cellular patterning of the lateral line primordium and loss of the rosette-like structures. These analyses have indicated a correlation between the localisation of adherens junctions proteins and the rosette-like pattern of the nascent neuromast precursors. This may suggest that adherens junctions play a role in forming or maintaining the rosette structure during migration of the primordium. Additionally the disruption to adherens protein localisation and rosette-structure following knock down of Pak, in particular Pak2b, may indicate that Pak2b plays a part in establishing and/or maintaining the nascent neuromast structure.

Despite disorganisation of adherens junction protein distribution, the analysis of levels of components associated with the cytoskeleton in embryos injected with *pak2a* or *pak2b* morpholino revealed no significant changes to adherens junction proteins. A direct action of Pak on adherens junctions has not been described, although it has been shown that *Drosophila* Mbt, a group II Pak protein, can be recruited to adherens junctions and associates with the *Drosophila* homologue of β-catenin, Armadillo (Schneeberger and Raabe, 2003). However, clustering of cadherin-

mediated adhesions is stabilised through the association of the transmembrane E-cadherin molecules with the actin cytoskeleton (Jamora and Fuchs, 2002) and Pak is known to regulate organisation of the actin cytoskeleton (Dharmawardhane *et al.*, 1997). Therefore the primary adhesion defects may lie with the loss of F-actin concentration at the apex of the rosettes. F-actin stabilises the membrane localisation of the E-cadherin-β-cateinin complex (Pokutta and Weis, 2002). Our time-lapse analyses showed the formation of adhesive focal points within the rosette is highly dynamic, therefore it is likely that dynamic regulation of F-actin by Pak2 is important for the dynamic formation of adherens junctions within the migrating primordium. The loss of adhesion within the primordium may result in disruption to the regular shape of the primordium.

Another possible mechanism by which Pak2 proteins could mediate regulation of the adherens junctions is through dissociation from the cytoskeleton. E-cadherin is actively internalised from the cell membrane by endocytosis and then recycled back to the cell surface (Le *et al.*, 1999). We have observed high levels of cytoplasmic and membrane associated E-cadherin within and surrounding the lateral line primordium by immunostaining, suggesting that cycling of E-cadherin is likely to occur and underlie the dynamic regulation of adherens junctions during migration of the lateral line primordium. It is known that active Rac can promote endocytosis of E-cadherin from the membrane and Pak can promote Rac activity by acting upstream of Rac (Obermeier *et al.*, 1998). Additionally, Pak can directly mediate endocytosis by phosphorylation of myosin and the activation of Pak increases endocytic uptake (Buss *et al.*, 1998; Dharmawardhane *et al.*, 2000). Therefore Pak2a may function to regulate internalisation of E-cadherin from the membrane and disassociation of adherens junction proteins.

7.4 Pak2b plays a role in migration of the lateral line primordium.

We have shown that morpholino-mediated knock down of Pak2a and in particular, Pak2b has striking effects on the migration of the lateral line primordium and on cell adhesion proteins within the primordium and throughout the whole embryo. Our results compare favourably with the defects that have been observed in cell culture systems following decrease in Pak activity. Such studies have shown that Pak activity has significant effects on cell adhesion, contractility (Kiosses *et al.*, 1999) and the polarisation of the actin cytoskeleton required for directed migration (Eby *et al.*, 1998; Sells *et al.*, 1999).

We have also begun to elucidate a role for Pak2b in controlling migration of the posterior lateral line primordium. Time-lapse analysis of embryos injected with *pak2b* morpholino revealed retarded migration of lateral line primordium. We have shown that while no directed migration was observed, the ability of lateral line primordium cells to extend processes was not affected. This is in agreement with previous work demonstrating that Pak is not required for the formation of lamellipodia or membrane protrusions (Sells *et al.*, 1999). However, Pak is known to regulate other aspects of cell migration control, for example Pak can regulate cell migration through myosin-dependant contraction of the cell rear. Pak has been shown to phosphorylate myosin light chain (MLC) and phosphorylation of MLC by Pak2 has been demonstrated to induce cell retraction (Kiosses *et al.*, 1999). In the lateral line primordium, knock down of Pak2b, may inhibit retraction of the trailing end accompanied by a failure to form new adhesions at the leading edge. This could result in a primordium that remains static. The ability of the lateral line primordium to migrate normally in embryos injected with *pak2a* morpholino may suggest some redundancy in Pak2a function or significant functional differences between Pak2a and Pak2b. Functional differences of the two zebrafish Pak2 proteins may be a reflection on differences in amino acid sequence and interacting proteins, as we have identified

significant differences in the binding of Nck and PIX isoforms between Pak2a and Pak2b.

7.4.1 Pak2 regulates focal adhesions to aid migration of the posterior lateral line primordium.

Dynamic cycling of focal adhesions resulting in formation of new adhesions at the leading edge and dissolution of adhesions at the trailing end is a key factor in regulating migration (Mitchison and Cramer, 1996). Since a component of the focal adhesions, Paxillin, is highly concentrated where the posterior lateral line primordium is in contact with the ECM we suggest that these adhesions are dynamically formed and disassembled as the primordium moves along the trunk to provide the traction forces for migration. The binding of Pak to the guanine nucleotide exchange factor, PIX, recruits Pak to focal adhesion sites through interaction with Paxillin (Brown *et al.*, 2002). Analysing the level of Paxillin proteins associated with the cytoskeleton in embryos injected with *pak2a* morpholino indicated a role for Pak2a in the dissolution of focal adhesions, as levels were increased in the absence of Pak2a. This idea is supported by previous studies which have shown that expression of activated Pak leads to a dramatic loss of focal adhesions (Frost *et al.*, 1998; Manser *et al.*, 1997; Qu *et al.*, 2001).

However in contrast to this, both immunocytochemical and Western blot analysis indicated a opposing role for Pak2b as levels of Paxillin are decreased in the absence of Pak2b. This suggested that endogenous zebrafish Pak2b functions to regulate formation or stabilisation of focal adhesions. In support of such a role the expression of a constitutively active mutant of Pak in endothelial cells has been shown to result in an increase in the number of focal adhesions (Kiosses *et al.*, 1999). These opposing roles for Pak suggest that Pak acts in a highly regulated way to control the dynamic formation and dissolution of focal adhesions.

A role for Pak in linking the dynamic regulation of focal adhesions and the migration of cells has been demonstrated in *Drosophila*. Dorsal closure involves migration of the lateral epidermal flanks to close a hole in the dorsal epidermis occupied by an epithelium called the amnioserosa (Harden, 2002). During dorsal closure the *Drosophila* homologue of Pak, DPak, is enriched in epidermal cells flanking the amnioserosa and is associated with the focal adhesions at the leading edge of these migrating cells (Harden *et al.*, 1996). Harden *et al* proposed that DPak may be regulating the cytoskeleton through its association with focal adhesions and may be participating with DRacA in a c-Jun amino-terminal kinase (JNK) signalling pathway required for dorsal closure. JNK phosphorylates the focal adhesion protein Paxillin and regulates cell migration in keratocytes and epithelial cells and it has been shown that JNK is required for dorsal closure in *Drosophila*. (Huang *et al.*, 2003; Riesgo-Escovar *et al.*, 1996). The retardation of migration of the lateral line primordium in embryos injected with *pak2b* morpholino indicates that Pak2b may be regulating the cytoskeleton during primordium migration through its association with focal adhesions.

In addition to the cell adhesion defects, knock down of Pak2b may be affecting cell migration through Pak-mediated regulation of the cytoskeleton. Cell motility requires polarized rearrangements of the actinomyosin cytoskeleton (Sells *et al.*, 1999) and Pak is known to colocalise with F-actin and regulate the cytoskeletal changes required for cell migration (Dharmawardhane *et al.*, 1997; Sells *et al.*, 1997). The action of Pak on the actin cytoskeleton is mediated though kinase-dependant activation of downstream molecules, such as LIM-kinase (LIMK) and myosin light chain kinase (MLCK) (Edwards *et al.*, 1999; Kiosses *et al.*, 1999; Sells *et al.*, 1999). LIMK inhibits the action of cofilin which dissociates F-actin and thereby allows the polymerisation of the actin cytoskeleton required for extension of lamellipodia and cell migration (Edwards *et al.*, 1999). However we have shown that following either morpholino-mediated knock down of Pak function or expression of dominant negative Pak protein, cells remain able to form lamellipodia. From this we interpret that Pak2 is

not required for the formation of lamellipodia and this is in agreement with cell culture work demonstrating a non-essential role for Pak in the formation of membrane protrusions such as lamellipodia (Sells *et al.*, 1999).

Although cells at the leading edge of the migratory lateral line primordium, in embryos injected with *pak2b* morpholino remain active, their movement is highly retarded. In addition to the extension of lamellipodia at the leading edge of the cell, migration requires that the trailing end of the cell is retracted. Active Pak increases MLC phosphorylation which then induces retraction at the trailing end of a migratory cell (Kiosses *et al.*, 1999). It remains to be investigated whether loss of Pak2b function results in decreased phosphorylation of MLC that in turn may lead to a failure in cell retraction.

7.4.2 Pak2a and Pak2b may regulate directed cell migration in response to chemotactic signals.

By looking at migration of both the lateral line primordium and the PGCs we have been able to propose a role for Pak2a and Pak2b in the regulation of migration in response to a chemotactic gradient. Migration of both the lateral line primordium and the primordial germ cells is dependant on Sdf-1-mediated chemotactic signalling (David *et al.*, 2002; Knaut *et al.*, 2003) and we have shown that injection of *pak2a* or *pak2b* morpholino disrupts migration of both cell types. In embryos injected with pak2b morpholino the lateral line primordium failed to migrate and did not reach the tail. Analysis of PGC migration in *pak2a* or *pak2b* morpholino injected embryos revealed that some PGCs failed to reach the final site of germ cell development. In addition, the presence of a chemotactic gradient exerts a polarising effect on responding cells such that lamellipodia are only extended in the direction of the chemotaxis signal (Zigmond, 1980). In embryos injected with *pak2b* morpholino we observed that cells in the lateral regions of the primordium extended lamellipodia in an aberrant direction away from the pathway of chemokine expression. Furthermore we observed increased protrusive

activity at the leading edge. Taken together these data suggest that cells of the lateral line primordium and PCG were unable to undergo polarised, directional migration in response to chemotaxis signals.

Chemoattractants are known to stimulate signaling pathways that involve Rho family GTPases and Pak has been proposed to act downstream of Rac and Cdc42 in regulating chemoattractant-driven cytoskeletal changes (Dharmawardhane *et al.*, 1999). Also, the *Dictyostelium discoideum* Pak homologue, PAKa, is required for maintaining directed cell migration, suppressing lateral pseudopod extension, and retraction of the posterior of chemotaxing cells (Chung and Firtel, 1999). Studies in *Dictyostelium* and neutrophils have also shown that Pak may be acting downstream of G-protein coupled receptors and Rac1B to regulate the actinomyosin cytoskeleton (Chung and Firtel, 1999; Xu *et al.*, 2003). Thus it appears likely that the defects observed in embryos injected with either *pak2a* or *pak2b* morpholino result from a defect in reorganisation of the actin cytoskeleton. The precise action of *pak2* on regulation of the actin cytoskeleton in response to chemotaxis is unclear. However, recent work has shown that chemotaxis utilises a signalling pathway involving αPIX-associated Pak, the Gβγ subunit and Cdc42 and this pathway is essential for the localization of F-actin, directional sensing, and the directional migration of chemotactic leukocytes (Li *et al.*, 2003). Abrogation of the formation of a polarised actin cytoskeleton can result in loss of motility, as is observed in macrophage expressing dominant negative Rac (Allen *et al.*, 1998). Loss of Pak2b activity downstream of Rac may explain the failure of the lateral line primordium to migrate in embryos injected with *pak2b* morpholino. A different role for Cdc42 in chemotaxis has emerged and macrophage expressing dominant negative Cdc42 remain able to migrate but lack directionality in response to the chemotactic cues (Allen *et al.*, 1998). Therefore the aberrant directional migration of PGCs in embryos injected with either *pak2a* or *pak2b* morpholino may indicate that Pak2a and Pak2b primarily act downstream of Cdc42, rather than Rac, during PGC migration. This divergence in signalling pathway between migration of the lateral line primordium and PGCs and the observation that

the lateral line primordium can migrate normally in embryos injected with *pak2a* morpholino suggests that Pak2a and Pak2b have different roles in regulating the response to chemotaxis in different cell types.

7.5 Pak2 may regulate gene expression in early development.

7.5.1 Possible regulation of *myoD* gene expression by Pak2.

In addition to regulation of cell adhesion and migration we have also begun to elucidate a role for Pak2 in the development of midline tissues through the regulation of myogenic gene expression and development of the notochord. This is evidenced by the ectopic expression of the master regulator of myogenesis, *myoD* in embryos injected with either the *pak2*a and *pak2b* targeted morpholinos or the Pak2a$^{\Delta c}$YFP and Pak2b$^{\Delta c}$YFP proteins.

In vertebrates the *Pax-Six-Eya-Dach* regulatory network is an important inducer of myogenic genes and myogenesis (Kawakami *et al.*, 2000). It is known that the active G-protein α_z subunit can inhibit Eya activity and that Pak is an important activator of Gα_z (Fan *et al.*, 2000). During myogenesis in chick, Pax3 activates the transcriptional activator *eya* and the homeodomain-containing transcription factor *six.* Eya and Six physically interact and synergise to induce expression of myogenic genes, including *myoD* (Heanue *et al.*, 1999). Eya is an intrinsically cytosolic protein and is translocated into the nucleus by interaction with Six, whereupon transcription of downstream genes is induced. Association of Eya with Six and translocation to the nucleus is inhibited by activated Gα_z (Fan *et al.*, 2000). The absence of prolonged activation of Gα_z by Pak in embryos injected with either *pak2a* or *pak2b* morpholino would result in unregulated nuclear translocation of the Six-Eya complex and therefore increased activation and ectopic expression of the *myoD* gene. However the possibility that Pak2 proteins regulate myogenic gene expression through activation of Gα_z in zebrafish embryos remains to be investigated.

Additionally, Pak may regulate myogenesis by activating transcriptional repressors of myogenic genes. CtBP, C-terminal binding protein, is a co-repressor regulating the transcription of many genes (Chinnadurai, 2002). Recently it has been reported that Pak1 can modulate CtBP activity through phosphorylation and that phosphorylated CtBP is retained in the cytoplasm preventing co-repressor function in the nucleus (Barnes *et al.*, 2003). Studies in *Xenopus* have indicated a role for CtBP in modulation of the wnt signalling pathway, which promotes myogenic cell fate (Chinnadurai, 2002; Cossu and Borello, 1999). In mammals Wnt signalling can act through a classic β-catenin pathway and the Wnt1-activated Tcf-β-catenin complex may directly activate transcription of target genes (including *MyoD*) (Cossu and Borello, 1999). In the absence of wnt signalling Tcf inhibits transcription of wnt responsive genes, binding of XTcf-3 to Xmyf-5 restricts expression of this gene in the mesoderm of *Xenopus* embryos (Chinnadurai, 2002; Yang *et al.*, 2002). CtBP interacts with Tcf and may contribute to the transcriptional repression activity of Tcf. The binding site for Pak interaction with CtBP is located in the N-terminal regulatory region, therefore expression of the Pak2$^{\Delta C}$YFP constructs may act to sequester CtBP in the cytoplasm and prevent its repressor function.

7.5.2 Pak2a$^{\Delta c}$YFP and Pak2b$^{\Delta c}$YFP affect notochord development.

An interesting role of CtBP as a context-dependant transcriptional activator has been described in mouse embryogenesis (Phippen *et al.*, 2000). In mouse ctbp-2 null mutants the expression of *brachyury (bra)* is reduced, suggesting that ctbp-2 can activate transcription of *bra* (Chinnadurai, 2003). In zebrafish embryos injected with Pak2a$^{\Delta c}$YFP or Pak2b$^{\Delta c}$YFP we find decreased expression of *no tail (ntl),* the zebrafish homologue of *bra,* and absence of a discernable notochord structure. This suggests that Pak2a$^{\Delta c}$YFP or Pak2b$^{\Delta c}$YFP proteins may sequester CtBP in the cytosol and prevent the activation of *ntl* expression by CtBP. This effect may result in the failure of

the notochord to express the later marker, *col2a*, in Pak2a$^{\Delta c}$YFP and Pak2b$^{\Delta c}$YFP injected embryos.

7.6 Perspectives

The work presented in this thesis begins to reveal important and diverse actions of Pak2 in many aspects of zebrafish development. Using loss-of-function analysis of Pak2a and Pak2b we have shown that Pak2b plays an important role in maintaining the architecture of the posterior lateral line primordium, possibly through an influence on dynamic regulation of adherens junctions. Pak2b is also likely to stabilise Paxillin-containing focal adhesions that provide the traction forces required for migration of the lateral line primordium. Analysis of the migration of PGCs in embryos injected with *pak2a* or *pak2b* morpholino has also alluded to a requirement for Pak2 activity in directed response to chemotactic signals. Finally we found that loss of active Pak2a or Pak2b through injection of morpholinos or expression of Pak2a$^{\Delta c}$YFP and Pak2b$^{\Delta c}$YFP proteins leads to ectopic expression of *myoD* in the ventral midline. Further experiments are required to determine the precise roles played by Pak2a and Pak2b in regulating cell adhesion, cell migration and gene transcription during zebrafish development.

7.6.1 Further characterisation of Pak2a and Pak2b and Pak2a$^{\Delta c}$YFP and Pak2b$^{\Delta c}$YFP.

In order to better interpret the results of the work described here and future experiments it will be helpful to have a better understanding of the behaviour of the both the endogenous and Pak2a$^{\Delta C}$YFP and Pak2b$^{\Delta C}$YFP proteins. Development of antibodies specific to Pak2a and Pak2b will be of significant help. By looking at the subcellular localisation of these proteins within the lateral line primordium we will be able to discover whether Pak2a and Pak2b are associated with adherens junction complexes at the apex of the nascent neuromast rosettes. Colocalisation of Pak2a

and Pak2b with proteins such as β-catenin would suggest that these proteins do play a role in regulating cadherin-mediated adhesions in the lateral line primordium. In the same way a link between Pak2a and Pak2b and the regulation of focal adhesions could be better established by investigating colocalisation with Paxillin.

Our work using Pak2$^{\Delta C}$YFP proteins is very preliminary and characterisation of the effect of these proteins is required. We expect that these constructs will act to sequester active Rac and Cdc42. As a result we would expect immunocyctochemical analysis using active Rac and Cdc42 antibodies to reveal colocalisation with the Pak2a$^{\Delta c}$YFP and Pak2b$^{\Delta c}$YFP proteins. Additionally we need to investigate how the Pak2$^{\Delta c}$YFP proteins affect activation of endogenous Pak2a and Pak2b.

A further method by which to understand the roles of zebrafish Pak is to dissect the function of specific domains of the proteins during development. The Pak family of proteins interact with and regulate a number of different proteins through their many protein binding sites and the kinase domain. Generation of constructs comprising the full length active Pak protein or the activity of specific Pak domains only, could be coinjected with the *pak2a* or *pak2b* morpholinos. Analysis of the resulting phenotypes, and any aspects that were rescued by injection of these constructs, would provide data to dissect the function of specific Pak activities.

7.6.2 Dissecting the role of zebrafish Pak2 in migration and chemotaxis.

It is of particular interest to us to extend our understanding of the role of *pak2a* and *pak2b* in the control of cell migration and in cellular response to chemotactic signals during lateral line primordium and PGC migration.

We have demonstrated that knock down of Pak2b leads to a failure of the lateral line primordium to migrate. We have discussed the possible mechanism by which knock down of Pak2 disrupts migration. In order to address the possibility that Pak2b may be having an effect on contraction of the trailing end of the cell through an action of MLC we could analyse levels of phosphorylated MLC in the lateral line

primordium. Pak2 is known to phosphorylate MLC (Sells *et al.*, 1999), therefore we might expect a decrease in the level of phosphorylation MLC in embryos injected with *pak2b* morpholino. A defect in MLC phosphorylation may contribute to the failure of the lateral line primordium to migrate.

Further work is required in order to distinguish whether Pak is specifically activated in response to a chemotactic signal or is simply mediating the effects of cell migration. This could be approached through *in vitro* assays looking at the response of cells to a chemotactic gradient when aspects of Pak activity have been abrogated, for example following transfection of kinase dead or CRIB domain mutants. Following chemotactic stimulation of these cells it would be of interest to visualise the localisation of components of the migratory machinery, in particular F-actin, as Pak is known to regulate the organisation of F-actin (Dharmawardhane *et al.*, 1997).

7.6.3 Understanding the role of zebrafish Pak2 in transcriptional regulation.

We have proposed that Pak2a and Pak2b inhibit the expression of the myogenic gene, *myoD* by preventing transcriptional regulators, Eya and CtBP from translocating from the cytoplasm to the nucleus. In order to confirm that zebrafish Pak2a and Pak2b can influence the localisation of these proteins we need to visualise the localisation of Eya and CtBP in normal embryos and in embryos injected with *pak2* morpholino or Pak2$^{\Delta c}$YFP proteins. Development of antibodies against Eya and CtBP or construction of fluorescent fusion proteins would allow us to confirm whether this was the case. In addition the development of an antibody specific to CtBP would enable us to determine whether Pak2a or Pak2b could bind to CtBP by GST pull down of Pak2a and Pak2b and Western blot analysis.

Our work has revealed that the zebrafish Pak2 proteins integrate many signalling pathways and as a result a great deal more work is required to fully understand the very interesting functions of Pak2 during zebrafish development.

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Appendix

i) DNA sequence of *pak2a*

pak2a coding sequence base pairs: 1 to 1554

```
        10         20         30         40         50
ATGTCTGACA ACGGAGAGCT GGAGGACAAA CCCCCTGCCC CCCCAGTCAG
TACAGACTGT TGCCTCTCGA CCTCCTGTTT GGGGGACGGG GGGGTCAGTC
 M  S  D   N  G  E  L   E  D  K   P  P  A   P  P  V  R>
__________a_TRANSLATION OF pak2a coding seq_a_________>

        60         70         80         90        100
AATGAGCAGC ACCTTCAGCA CCGGTATTAA AGACAGTATG TCAACAAACC
TTACTCGTCG TGGAAGTCGT GGCCATAATT TCTGTCATAC AGTTGTTTGG
  M  S  S   T  F  S   T  G  I  K   D  S  M   S  T  N>
__________a_TRANSLATION OF pak2a coding seq_a_________>

       110        120        130        140        150
CCAGCTCTAA ACCCCTGCCC TCCGTCCCCG AGGAGAAGAG GGACAAACCG
GGTCGAGATT TGGGGACGGG AGGCAGGGGC TCCTCTTCTC CCTGTTTGGC
P  S  S  K   P  L  P   S  V  P   E  E  K  R   D  K  P>
__________a_TRANSLATION OF pak2a coding seq_a_________>

       160        170        180        190        200
CGCAACAAGA TCATATCAAT ATTCTCAGCT GAGAAAGGAA GAAAAAAAGA
GCGTTGTTCT AGTATAGTTA TAAGAGTCGA CTCTTTCCTT CTTTTTTTCT
 R  N  K   I  I  S  I   F  S  A   E  K  G   R  K  K  D>
__________a_TRANSLATION OF pak2a coding seq_a_________>

       210        220        230        240        250
CAAGGATAAG GAGCGTCCTG AGATTTCTAA CCCTTCAGAT TTCGAGCACA
GTTCCTATTC CTCGCAGGAC TCTAAAGATT GGGAAGTCTA AAGCTCGTGT
  K  D  K   E  R  P   E  I  S  N   P  S  D   F  E  H>
__________a_TRANSLATION OF pak2a coding seq_a_________>

       260        270        280        290        300
CTATACATGT GGGCTTTGAT TCTGTCACAG GGGAGTTCAC GGGCATGCCA
GATATGTACA CCCGAAACTA AGACAGTGTC CCCTCAAGTG CCCGTACGGT
T  I  H  V   G  F  D   S  V  T   G  E  F  T   G  M  P>
__________a_TRANSLATION OF pak2a coding seq_a_________>

       310        320        330        340        350
GAGCAGTGGG CTCGGCTACT GCAGACCTCC AACATCACGA AATCTGAGCA
CTCGTCACCC GAGCCGATGA CGTCTGGAGG TTGTAGTGCT TTAGACTCGT
 E  Q  W   A  R  L  L   Q  T  S   N  I  T   K  S  E  Q>
__________a_TRANSLATION OF pak2a coding seq_a_________>
```

```
       360         370         380         390         400
GAAGAAAAAC CCTCAGGCTG TGTTGGACGT GCTCAAATTC TACGACTCCA
CTTCTTTTTG GGAGTCCGAC ACAACCTGCA CGAGTTTAAG ATGCTGAGGT
  K  K  N   P  Q  A   V  L  D  V   L  K  F   Y  D  S>
__________a_TRANSLATION OF pak2a coding seq_a_________>

       410         420         430         440         450
CAGGCAACAG CAGGCAGAAA TACCTCAGCT TCACAGATAA AGATGCACCA
GTCCGTTGTC GTCCGTCTTT ATGGAGTCGA AGTGTCTATT TCTACGTGGT
T  G  N  S   R  Q  K   Y  L  S   F  T  D  K   D  A  P>
__________a_TRANSLATION OF pak2a coding seq_a_________>

       460         470         480         490         500
CAAGCAAAAA AAGGCTCAGA GCAATCTCCT GTCAAAGACC CTGATGATGA
GTTCGTTTTT TTCCGAGTCT CGTTAGAGGA CAGTTTCTGG GACTACTACT
 Q  A  K   K  G  S  E   Q  S  P   V  K  D   P  D  D  D>
__________a_TRANSLATION OF pak2a coding seq_a_________>

       510         520         530         540         550
TGACGAGGAT GCTCCACCCC CTGTTGTAGC ACCACGCCCA CAGCACACCA
ACTGCTCCTA CGAGGTGGGG GACAACATCG TGGTGCGGGT GTCGTGTGGT
  D  E  D   A  P  P   P  V  V  A   P  R  P   Q  H  T>
__________a_TRANSLATION OF pak2a coding seq_a_________>

       560         570         580         590         600
TATCTGTATA CACTCGTTCT GTCATCGATC CTATTCCAGC ACCTGCTGCC
ATAGACATAT GTGAGCAAGA CAGTAGCTAG GATAAGGTCG TGGACGACGG
I  S  V  Y   T  R  S   V  I  D   P  I  P  A   P  A  A>
__________a_TRANSLATION OF pak2a coding seq_a_________>

       610         620         630         640         650
ATTGCAGACA CAGATGGCTC TAAAGCTGCA GACAAACAGA AGAAGGGCAA
TAACGTCTGT GTCTACCGAG ATTTCGACGT CTGTTTGTCT TCTTCCCGTT
 I  A  D   T  D  G  S   K  A  A   D  K  Q   K  K  G  K>
__________a_TRANSLATION OF pak2a coding seq_a_________>

       660         670         680         690         700
GGGAAAGATG ACCGATGAGG AGATTATGGA GAAACTTAGA ACCATTGTCA
CCCTTTCTAC TGGCTACTCC TCTAATACCT CTTTGAATCT TGGTAACAGT
  G  K  M   T  D  E   E  I  M  E   K  L  R   T  I  V>
__________a_TRANSLATION OF pak2a coding seq_a_________>

       710         720         730         740         750
GTATTGGAGA CCCCAAGAAG AAATACACAA GATACGAAAA AATTGGACAA
CATAACCTCT GGGGTTCTTC TTTATGTGTT CTATGCTTTT TTAACCTGTT
S  I  G  D   P  K  K   K  Y  T   R  Y  E  K   I  G  Q>
__________a_TRANSLATION OF pak2a coding seq_a_________>

       760         770         780         790         800
GGTGCGTCTG GTACAGTGTA CACAGCCATT GATGTTGCTA CTGGCCAAGA
CCACGCAGAC CATGTCACAT GTGTCGGTAA CTACAACGAT GACCGGTTCT
 G  A  S   G  T  V  Y   T  A  I   D  V  A   T  G  Q  E>
__________a_TRANSLATION OF pak2a coding seq_a_________>
```

```
      810        820        830        840        850
GGTTGCTATT AAGCAGATTA ACCTACAGAA GCAGCCCAAA AAAGAGCTGA
CCAACGATAA TTCGTCTAAT TGGATGTCTT CGTCGGGTTT TTTCTCGACT
  V  A  I   K  Q  I   N  L  Q  K   Q  P  K   K  E  L>
__________a_TRANSLATION OF pak2a coding seq_a_________>

      860        870        880        890        900
TCATCAATGA GATCCTGGTG ATGAAGGAGC TGAAGAATCC AAACATTGTC
AGTAGTTACT CTAGGACCAC TACTTCCTCG ACTTCTTAGG TTTGTAACAG
I  I  N  E   I  L  V   M  K  E   L  K  N  P   N  I  V>
__________a_TRANSLATION OF pak2a coding seq_a_________>

      910        920        930        940        950
AACTTCTTAG ACAGCTTCTT GGTTGGAGAT GAGCTCTTTG TGGTGATGGA
TTGAAGAATC TGTCGAAGAA CCAACCTCTA CTCGAGAAAC ACCACTACCT
 N  F  L   D  S  F  L   V  G  D   E  L  F   V  V  M  E>
__________a_TRANSLATION OF pak2a coding seq_a_________>

      960        970        980        990       1000
GTATCTTGCT GGAGGCTCTC TGACAGACGT AGTAACAGAA ACCTGCATGG
CATAGAACGA CCTCCGAGAG ACTGTCTGCA TCATTGTCTT TGGACGTACC
  Y  L  A   G  G  S   L  T  D  V   V  T  E   T  C  M>
__________a_TRANSLATION OF pak2a coding seq_a_________>

     1010       1020       1030       1040       1050
ATGAGGCTCA AATTGCTGCT GTCTGCAGAG AGTGTTTACA AGCACTGGAG
TACTCCGAGT TTAACGACGA CAGACGTCTC TCACAAATGT TCGTGACCTC
D  E  A  Q   I  A  A   V  C  R   E  C  L  Q   A  L  E>
__________a_TRANSLATION OF pak2a coding seq_a_________>

     1060       1070       1080       1090       1100
TTTCTGCATG CAAACCAGGT CATCCATCGA GACATCAAAA GTGACAACGT
AAAGACGTAC GTTTGGTCCA GTAGGTAGCT CTGTAGTTTT CACTGTTGCA
 F  L  H   A  N  Q  V   I  H  R   D  I  K   S  D  N  V>
__________a_TRANSLATION OF pak2a coding seq_a_________>

     1110       1120       1130       1140       1150
TCTTTTAGGA ATGGACGGAT CAGTCAAACT AACCGATTTT GGGTTCTGTG
AGAAAATCCT TACCTGCCTA GTCAGTTTGA TTGGCTAAAA CCCAAGACAC
  L  L  G   M  D  G   S  V  K  L   T  D  F   G  F  C>
__________a_TRANSLATION OF pak2a coding seq_a_________>

     1160       1170       1180       1190       1200
CTCAAATCAC TCCTGAGCAA AGCAAGAGGA GCACCATGGT GGGAACACCC
GAGTTTAGTG AGGACTCGTT TCGTTCTCCT CGTGGTACCA CCCTTGTGGG
A  Q  I  T   P  E  Q   S  K  R   S  T  M  V   G  T  P>
__________a_TRANSLATION OF pak2a coding seq_a_________>

     1210       1220       1230       1240       1250
TACTGGATGG CCCCTGAGGT GGTCACACGT AAAGCCTACG GGCCCAAAGT
ATGACCTACC GGGGACTCCA CCAGTGTGCA TTTCGGATGC CCGGGTTTCA
 Y  W  M   A  P  E  V   V  T  R   K  A  Y   G  P  K  V>
__________a_TRANSLATION OF pak2a coding seq_a_________>
```

```
      1260        1270        1280        1290        1300
GGACATTTGG TCTTTGGGTA TCATGGCCAT TGAAATGGTT GAGGGCGAAC
CCTGTAAACC AGAAACCCAT AGTACCGGTA ACTTTACCAA CTCCCGCTTG
  D  I  W   S  L  G   I  M  A  I   E  M  V   E  G  E>
__________a_TRANSLATION OF pak2a coding seq_a_________>

      1310        1320        1330        1340        1350
CTCCATATCT CAATGAGAAT CCTCTGAGGG CGTTGTACCT CATCGCTACT
GAGGTATAGA GTTACTCTTA GGAGACTCCC GCAACATGGA GTAGCGATGA
P  P  Y  L   N  E  N   P  L  R   A  L  Y  L   I  A  T>
__________a_TRANSLATION OF pak2a coding seq_a_________>

      1360        1370        1380        1390        1400
AATGGCACCC CGGAGCTCCA GAACCCAGAA AAGCTGTCTC CCATTTTTAG
TTACCGTGGG GCCTCGAGGT CTTGGGTCTT TTCGACAGAG GGTAAAAATC
 N  G  T   P  E  L  Q   N  P  E   K  L  S   P  I  F  R>
__________a_TRANSLATION OF pak2a coding seq_a_________>

      1410        1420        1430        1440        1450
AGACTTCCTA AACCGCTGCC TGGAGATGGA TGTGGAGAAG AGAGGAGGAG
TCTGAAGGAT TTGGCGACGG ACCTCTACCT ACACCTCTTC TCTCCTCCTC
  D  F  L   N  R  C   L  E  M  D   V  E  K   R  G  G>
__________a_TRANSLATION OF pak2a coding seq_a_________>

      1460        1470        1480        1490        1500
GAAAAGAGCT TCTGCAACAT CCTTTCCTCA AGCTGGCAAA GCCTCTTTCC
CTTTTCTCGA AGACGTTGTA GGAAAGGAGT TCGACCGTTT CGGAGAAAGG
G  K  E  L   L  Q  H   P  F  L   K  L  A  K   P  L  S>
__________a_TRANSLATION OF pak2a coding seq_a_________>

      1510        1520        1530        1540        1550
AGCCTCACTC CTCTTATACT TGCTGCCAAG GAGGCAATGA AGAGTAACCG
TCGGAGTGAG GAGAATATGA ACGACGGTTC CTCCGTTACT TCTCATTGGC
 S  L  T   P  L  I  L   A  A  K   E  A  M   K  S  N  R>
__________a_TRANSLATION OF pak2a coding seq_a_________>

TTAG
AATC
   *>
____>
```

ii) DNA sequence of *pak2b*

pak2b coding sequence base pairs: 1 to 1620

```
         10         20         30         40         50
 ATGTGTGATA ATGGCGATGT GGAGGACAAG CCGCCCGCTC CGCCTGTCAG
 TACACACTAT TACCGCTACA CCTCCTGTTC GGCGGGCGAG GCGGACAGTC
 M  C  D   N  G  D  V   E  D  K   P  P  A   P  P  V  R>
 _________a___TRANSLATION OF PAK2B [A]_____a__________>

         60         70         80         90        100
GATGAGCAGC ACCATCTTCA GCGGCGCCAA AGACCACGCC CTCACCGCCA
CTACTCGTCG TGGTAGAAGT CGCCGCGGTT TCTGGTGCGG GAGTGGCGGT
  M  S  S   T  I  F   S  G  A  K   D  H  A   L  T  A>
__________a___TRANSLATION OF PAK2B [A]_____a__________>

        110        120        130        140        150
ATCACAGCTC CAAGCCCCTC CCATCGGTCC CAGAGGAGCG CAAGCGCAAC
TAGTGTCGAG GTTCGGGGAG GGTAGCCAGG GTCTCCTCGC GTTCGCGTTG
N  H  S  S   K  P  L   P  S  V   P  E  E  R   K  R  N>
__________a___TRANSLATION OF PAK2B [A]_____a__________>

        160        170        180        190        200
AAGATCTACT CCATCTTCTC CGGCGCAGAG AAAGGTGGAC GCAGGAAGGA
TTCTAGATGA GGTAGAAGAG GCCGCGTCTC TTTCCACCTG CGTCCTTCCT
 K  I  Y   S  I  F  S   G  A  E   K  G  G   R  R  K  D>
__________a___TRANSLATION OF PAK2B [A]_____a__________>

        210        220        230        240        250
TCGTGATAAA GAGCGTCCTG AAATCTCTCC ACCGTCAGAC TTCGAACACA
AGCACTATTT CTCGCAGGAC TTTAGAGAGG TGGCAGTCTG AAGCTTGTGT
  R  D  K   E  R  P   E  I  S  P   P  S  D   F  E  H>
__________a___TRANSLATION OF PAK2B [A]_____a__________>

       260        270        280        290        300
CCATTCATGT GGGATTTGAC GCCGTTACTG GAGAGTTCAC TGGAATGCCG
GGTAAGTACA CCCTAAACTG CGGCAATGAC CTCTCAAGTG ACCTTACGGC
T  I  H  V   G  F  D   A  V  T   G  E  F  T   G  M  P>
__________a___TRANSLATION OF PAK2B [A]_____a__________>

       310        320        330        340        350
GAGCAGTGGG CTCGATTACT GCAGACCTCC AACATCACCA AGTCGGAGCA
CTCGTCACCC GAGCTAATGA CGTCTGGAGG TTGTAGTGGT TCAGCCTCGT
 E  Q  W   A  R  L  L   Q  T  S   N  I  T   K  S  E  Q>
__________a___TRANSLATION OF PAK2B [A]_____a__________>


       360        370        380        390        400
GAAGAAAAAC CCGCAGGCCG TTCTAGACGT GCTCAAGTTC TACGACTCCA
CTTCTTTTTG GGCGTCCGGC AAGATCTGCA CGAGTTCAAG ATGCTGAGGT
  K  K  N   P  Q  A   V  L  D  V   L  K  F   Y  D  S>
__________a___TRANSLATION OF PAK2B [A]_____a__________>
```

```
       410         420         430         440         450
CAGGCAACGG CAGACAGAAG TACCTCAGTT TCTCCTCAGA GAAGGACAGC
GTCCGTTGCC GTCTGTCTTC ATGGAGTCAA AGAGGAGTCT CTTCCTGTCG
T  G  N  G   R  Q  K   Y  L  S   F  S  S  E   K  D  S>
__________a___TRANSLATION OF PAK2B [A]_____a__________>

       460         470         480         490         500
TTTCCCTCCG GTGAACAGTC GCCTGCAAAG AAAACTCCAG AGCCGTCGTC
AAAGGGAGGC CACTTGTCAG CGGACGTTTC TTTTGAGGTC TCGGCAGCAG
 F  P  S   G  E  Q  S   P  A  K   K  T  P   E  P  S  S>
__________a___TRANSLATION OF PAK2B [A]_____a__________>

       510         520         530         540         550
TCCAGTCAAA GCAGTGGATG ATGAAGATAA TGGTGAAGAT GAAGACGATG
AGGTCAGTTT CGTCACCTAC TACTTCTATT ACCACTTCTA CTTCTGCTAC
  P  V  K   A  V  D   D  E  D  N   G  E  D   E  D  D>
__________a___TRANSLATION OF PAK2B [A]_____a__________>

       560         570         580         590         600
ATGATGATGA TGATGAAGAA ACGCCGCCGC CTGTCGTAGC ACCCCGGCCT
TACTACTACT ACTACTTCTT TGCGGCGGCG GACAGCATCG TGGGGCCGGA
D  D  D  D   D  E  E   T  P  P   P  V  V  A   P  R  P>
__________a___TRANSLATION OF PAK2B [A]_____a__________>

       610         620         630         640         650
GAACACACTA AGAGCGTATA CACTCGACCG TCGGTCATCG ACCCACTCCC
CTTGTGTGAT TCTCGCATAT GTGAGCTGGC AGCCAGTAGC TGGGTGAGGG
 E  H  T   K  S  V  Y   T  R  P   S  V  I   D  P  L  P>
__________a___TRANSLATION OF PAK2B [A]_____a__________>

       660         670         680         690         700
CCCGCCGGTG ACGTCTCCAG AAAGTGACGC AGCGTCGAAG GCCACTGACC
GGGCGGCCAC TGCAGAGGTC TTTCACTGCG TCGCAGCTTC CGGTGACTGG
  P  P  V   T  S  P   E  S  D  A   A  S  K   A  T  D>
__________a___TRANSLATION OF PAK2B [A]_____a__________>

       710         720         730         740         750
GACAGAGACC CAAAAAGGGC AAGATGACGG ACGAGGAGAT CATGGACAAG
CTGTCTCTGG GTTTTTCCCG TTCTACTGCC TGCTCCTCTA GTACCTGTTC
R  Q  R  P   K  K  G   K  M  T   D  E  E  I   M  D  K>
__________a___TRANSLATION OF PAK2B [A]_____a__________>

       760         770         780         790         800
CTCAGAACCA TAGTCAGCAT TGGAGACCCC AAAAAGAAAT ACACACGATA
GAGTCTTGGT ATCAGTCGTA ACCTCTGGGG TTTTTCTTTA TGTGTGCTAT
 L  R  T   I  V  S  I   G  D  P   K  K  K   Y  T  R  Y>
__________a___TRANSLATION OF PAK2B [A]_____a__________>

       810         820         830         840         850
CGAGAAGATC GGACAAGGAG CGTCAGGAAC CGTGTTCACC GCAATAGACG
GCTCTTCTAG CCTGTTCCTC GCAGTCCTTG GCACAAGTGG CGTTATCTGC
  E  K  I   G  Q  G   A  S  G  T   V  F  T   A  I  D>
__________a___TRANSLATION OF PAK2B [A]_____a__________>
```

```
       860        870        880        890        900
TCGCCACTGG ACAGGAGGTG GCCATCAAAC AGATCAACCT GCAGAAACAG
AGCGGTGACC TGTCCTCCAC CGGTAGTTTG TCTAGTTGGA CGTCTTTGTC
V  A  T  G   Q  E  V   A  I  K   Q  I  N  L   Q  K  Q>
__________a___TRANSLATION OF PAK2B [A]_____a__________>

       910        920        930        940        950
CCCAAGAAAG AGCTGATCAT CAACGAGATC CTCGTCATGA AGGAGCTGAA
GGGTTCTTTC TCGACTAGTA GTTGCTCTAG GAGCAGTACT TCCTCGACTT
 P  K  K   E  L  I  I   N  E  I   L  V  M   K  E  L  K>
__________a___TRANSLATION OF PAK2B [A]_____a__________>

       960        970        980        990       1000
GAATCCAAAC ATCGTCAACT TCTTAGACAG TTTTCTTGTA GGTGAGGAGC
CTTAGGTTTG TAGCAGTTGA AGAATCTGTC AAAAGAACAT CCACTCCTCG
  N  P  N   I  V  N   F  L  D  S   F  L  V   G  E  E>
__________a___TRANSLATION OF PAK2B [A]_____a__________>

      1010       1020       1030       1040       1050
TGTTTGTGGT GATGGAGTAT TTGGCCGGCG GGTCGCTCAC CGATGTCGTA
ACAAACACCA CTACCTCATA AACCGGCCGC CCAGCGAGTG GCTACAGCAT
L  F  V  V   M  E  Y   L  A  G   G  S  L  T   D  V  V>
__________a___TRANSLATION OF PAK2B [A]_____a__________>

      1060       1070       1080       1090       1100
ACTGAGACCT GCATGGATGA AGCTCAGATC GCCGCTGTGT GCCGAGAGTG
TGACTCTGGA CGTACCTACT TCGAGTCTAG CGGCGACACA CGGCTCTCAC
 T  E  T   C  M  D  E   A  Q  I   A  A  V   C  R  E  C>
__________a___TRANSLATION OF PAK2B [A]_____a__________>

      1110       1120       1130       1140       1150
TTTGCAGGCG CTGGAGTTTC TTCATGCTAA TCAGGTCATT CATCGAGACA
AAACGTCCGC GACCTCAAAG AAGTACGATT AGTCCAGTAA GTAGCTCTGT
  L  Q  A   L  E  F   L  H  A  N   Q  V  I   H  R  D>
__________a___TRANSLATION OF PAK2B [A]_____a__________>

      1160       1170       1180       1190       1200
TCAAGAGCGA CAACGTGCTT CTAGGAATGG ACGGTTCTGT CAAACTCACG
AGTTCTCGCT GTTGCACGAA GATCCTTACC TGCCAAGACA GTTTGAGTGC
I  K  S  D   N  V  L   L  G  M   D  G  S  V   K  L  T>
__________a___TRANSLATION OF PAK2B [A]_____a__________>

      1210       1220       1230       1240       1250
GACTTCGGTT TCTGTGCCCA GATCACTCCC GAACAGAGTA AGAGGAGCAC
CTGAAGCCAA AGACACGGGT CTAGTGAGGG CTTGTCTCAT TCTCCTCGTG
 D  F  G   F  C  A  Q   I  T  P   E  Q  S   K  R  S  T>
__________a___TRANSLATION OF PAK2B [A]_____a__________>

      1260       1270       1280       1290       1300
GATGGTGGGT ACGCCGTACT GGATGGCTCC GGAAGTGGTG ACGCGCAAAG
CTACCACCCA TGCGGCATGA CCTACCGAGG CCTTCACCAC TGCGCGTTTC
  M  V  G   T  P  Y   W  M  A  P   E  V  V   T  R  K>
__________a___TRANSLATION OF PAK2B [A]_____a__________>
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      1310        1320        1330        1340        1350
CCTACGGACC CAAAGTGGAC ATCTGGTCAT TGGGCATCAT GGCTATCGAG
GGATGCCTGG GTTTCACCTG TAGACCAGTA ACCCGTAGTA CCGATAGCTC
A  Y  G  P   K  V  D   I  W  S   L  G  I  M   A  I  E>
__________a___TRANSLATION OF PAK2B [A]_____a__________>

      1360        1370        1380        1390        1400
ATGGTGGAGG GAGAGCCGCC GTATCTGAAT GAAAATCCAC TCAGGGCGCT
TACCACCTCC CTCTCGGCGG CATAGACTTA CTTTTAGGTG AGTCCCGCGA
 M  V  E   G  E  P  P   Y  L  N   E  N  P   L  R  A  L>
__________a___TRANSLATION OF PAK2B [A]_____a__________>

      1410        1420        1430        1440        1450
GTACCTGATT GCAACCAATG GCACTCCAGA GTTGCAGAGT CCCGAAAAGT
CATGGACTAA CGTTGGTTAC CGTGAGGTCT CAACGTCTCA GGGCTTTTCA
  Y  L  I   A  T  N   G  T  P  E   L  Q  S   P  E  K>
__________a___TRANSLATION OF PAK2B [A]_____a__________>

      1460        1470        1480        1490        1500
TGTCGCCAAT CTTCCGAGAC TTCCTAGGTC GCTGTCTGGA AATGGACGTT
ACAGCGGTTA GAAGGCTCTG AAGGATCCAG CGACAGACCT TTACCTGCAA
L  S  P  I   F  R  D   F  L  G   R  C  L  E   M  D  V>
__________a___TRANSLATION OF PAK2B [A]_____a__________>

      1510        1520        1530        1540        1550
GAGAAGAGAG GTGGAAGCAA AGAGCTTTTG CAGCATCCCT TCCTGAAGTT
CTCTTCTCTC CACCTTCGTT TCTCGAAAAC GTCGTAGGGA AGGACTTCAA
 E  K  R   G  G  S  K   E  L  L   Q  H  P   F  L  K  L>
__________a___TRANSLATION OF PAK2B [A]_____a__________>

      1560        1570        1580        1590        1600
GGCCAAACCT CTCTCCAGTC TCACTCCTCT AATTCTGGCC GCCAAGGACG
CCGGTTTGGA GAGAGGTCAG AGTGAGGAGA TTAAGACCGG CGGTTCCTGC
  A  K  P   L  S  S   L  T  P  L   I  L  A   A  K  D>
__________a___TRANSLATION OF PAK2B [A]_____a__________>

      1610        1620
CCATGAAGAA CAACCGCTAG
GGTACTTCTT GTTGGCGATC
A  M  K  N   N  R  *>
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iii) Poster Abstract.

Presented at the Third European Meeting on Zebrafish and Medaka Development and Genetics, Paris 11-14 June 2003.

A Possible Role for p21 Activated Kinase in Midline Patterning.

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The p21 activated kinase (Pak) family of proteins are downstream effectors of the small GTPases Rac and Cdc42. Pak has been shown to play an important role in regulating the cytoskeleton and cell-cell adhesions as well as functioning in a number of signalling cascades. Here we present our investigations into the role of Pak in early zebrafish development.

We have identified two novel zebrafish *pak* genes with homology to h*pak2*, which we call z*pak2a* and z*pak2b*. Both genes are expressed maternally and ubiquitously throughout early embryogenesis. At later stages *pak2a* is expressed at higher levels in the notochord, epidermis and at the extremity of the embryo and *pak2b* expression is stronger in the intersomitic tissues.

To investigate the function of these *pak* genes during development we used a morpholino knock down approach. Both *pak2a* and *pak2b* morphant embryos showed "u" shaped somites, small heads and high levels of epidermal cell death. The somite phenotype observed in morphant embryos prompted us to look for a role for Pak in midline patterning. It is well established that induction of specific cell fates surrounding the midline requires graded signalling by factors secreted from the notochord. Changes to gene expression patterns in the midline of embryos injected with *pak* morpholino are consistent with disruptions to notochord derived signalling. Possible mechanisms by which zPak2 proteins pattern midline tissues will be presented.

iiii) Supplementary Data – Time-lapse movies.

Figure 3.3: Time-lapse series focusing on the leading edge of the migrating posterior lateral line primordium.

Figure 3.4: Time-lapse series showing posterior lateral line primordium migration and neuromast precursor deposition.

Figure 5.4: Time-lapse series showing of the leading edge of the migrating posterior lateral line primordium following injection of *pak2b* morpholino.