

**Control of microtubule dynamics by
Par3 is required for contact inhibition
of locomotion**

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I declare that the work presented in this thesis is my own, unless otherwise indicated.

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Abstract

Cell-cell adhesion junctions are essential for the maintenance of epithelial cell polarity and thus tissue integrity. However, the role of such proteins in collectively migrating mesenchymal cells has not yet been investigated. Here, I investigate the role of the polarity protein Par3 in the neural crest, a collectively migrating embryonic cell population. Par3 is localised to the adhesion complex and is important in the definition of apico-basal polarity in epithelial cells, but the localisation and function of Par3 in mesenchymal cells is not well characterised. Here, I show that Par3 is localised to the cell-cell contact in neural crest cells and is important for contact inhibition of locomotion, a phenomenon essential for appropriate migration of neural crest cells. Par3 promotes contact inhibition of locomotion by controlling microtubule dynamics at the site of cell-cell contact. A morpholino designed to inhibit Par3 reduces microtubule depolymerisation at the site of cell-cell contact and abrogates contact inhibition of locomotion. This can be rescued by a low concentration of the microtubule depolymerising drug nocodazole, by inhibiting Rac1, or by coinjection with a morpholino against the Rac1-GEF Trio. Further, Trio and Par3 interact, as shown by co-immunoprecipitation, and localise at the cell contact, as shown by immunofluorescence. I propose that Par3 sequesters Trio and prevents Rac1 activation at cell contacts, thus inhibiting Rac1 promotion of microtubule stability.

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List of abbreviations

+TIP	plus-tip tracking protein
ADAM	a disintegrin and metalloproteinase
AP	alkaline phosphatase
APC	adenomatous polyposis coli
aPKC	atypical protein kinase C
aPKCdn	aPKC dominant negative
AMOT	angiomotic
BCIP	5-bromo-4-chloro-3-indoyl-phosphate
BMP	bone morphogenetic protein
BSA	bovine serum albumin
C3aR	C3a receptor
CIL	contact inhibition of locomotion
CRMP	collapsin response mediator protein
Cxcl	C-X-C chemokine ligand
Cxcr	C-X-C chemokine receptor
DCC	deleted in colorectal cancer
DEPC	diethylpyrocarbonate
DFA	Danilchick's solution
Dlg1	Disks large homologue 1
DLMZ	dorsolateral marginal zone
DMSO	dimethyl sulfoxide
EB	end binding
ECM	extracellular matrix

EMT	epithelial-to-mesenchymal transition
FDX	fluorescein-dextran
FGF	fibroblast growth factor
FRAP	fluorescence recovery after photobleaching
FRET	Förster/fluorescence resonance energy transfer
GAP	guanine activating protein
GEF	guanine-nucleotide exchange factor
GFP	green fluorescent protein
GSK3 β	glycogen synthase kinase 3 β
IM	intermediate mesoderm
JAM	junctional adhesion molecule
LB	lysogeny broth
Lgl	lethal giant larvae
MAP	microtubule-associated protein
MCAK	mitotic centromere-associated kinesin
MEM	minimum essential medium
MEMFA	minimum essential medium with formaldehyde
MMP	matrix metalloproteinase
MMR	Marc's modified Ringer's
MO	morpholino
mRNA	messenger ribonucleic acid
NAM	normal amphibian medium
NBT	4-nitro blue-tetrazolium-chloride
NTP	nucleoside triphosphate
Par	partitioning-defective
PATJ	Pals1-associated tight junction protein

PBS	phosphate buffered solution
PBT	phosphate buffered solution with Triton-X
PCNS	protocadherin in neural crest and somites
PCP	planar cell polarity
PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor
PDZ	postsynaptic density protein, Dlg1, ZO1
PI3K	phosphoinositide 3-kinase
PLEKH	pleckstrin homology domain-containing protein
PSMG	pregnant serum mare gonadotropin
PTEN	phosphatase and tensin homolog
RA	retinoic acid
RDX	rhodamine-dextran
ROCK	Rho-associated protein kinase
rpm	rotations per minute
RNP	ribonucleoprotein
Sdf1	stromal-cell-derived factor 1
SH	Src-homology
SSC	saline-sodium citrate
TBS	tris-buffered saline
TGF	transforming growth factor
VEGF	vascular endothelial growth factor
XMAP215	<i>X. laevis</i> microtubule-associated protein
ZO	zona occludens

I Introduction

1. The neural crest

The neural crest is a transient, migratory cell population that is found in all vertebrate embryos. It is induced at the neural plate border and, as neurulation progresses, becomes located within the elevating neural folds. Neural crest cells delaminate from the dorsal neural tube and migrate extensively throughout the embryo, then differentiate into a wide variety of cell types (Fig. 1.1; reviewed in Theveneau and Mayor, 2012).

Due to the wide variety of tissues to which the neural crest contributes, its aberrant development can lead to many different congenital diseases. Investigation of the neural crest naturally contributes to an understanding of these conditions, termed neurocristopathies, and to potential therapies to treat them. However, since the neural crest was first described by Wilhelm His (1868), embryologists have also used the neural crest as a model for studying tissue induction, fate determination and cell migration. More recently, the neural crest has caught the attention of those interested in stem-cell-based therapies due to its multipotency and ability to regenerate following ablation (reviewed in Crane and Trainor, 2006). Furthermore, the development of neural networks has been studied using subpopulations of neural crest cells (for example, Landman et al., 2011). Research into neural crest cell delamination, collective cell migration and invasion of the embryo has also been used to develop an understanding of cancer metastasis (reviewed in Theveneau and Mayor, 2012). Thus, the study of neural crest migration provides knowledge that can be applied across disciplines.

The neural crest population can be divided based on position along the anterior-posterior axis into the cranial, cardiac, vagal, trunk and sacral neural crest

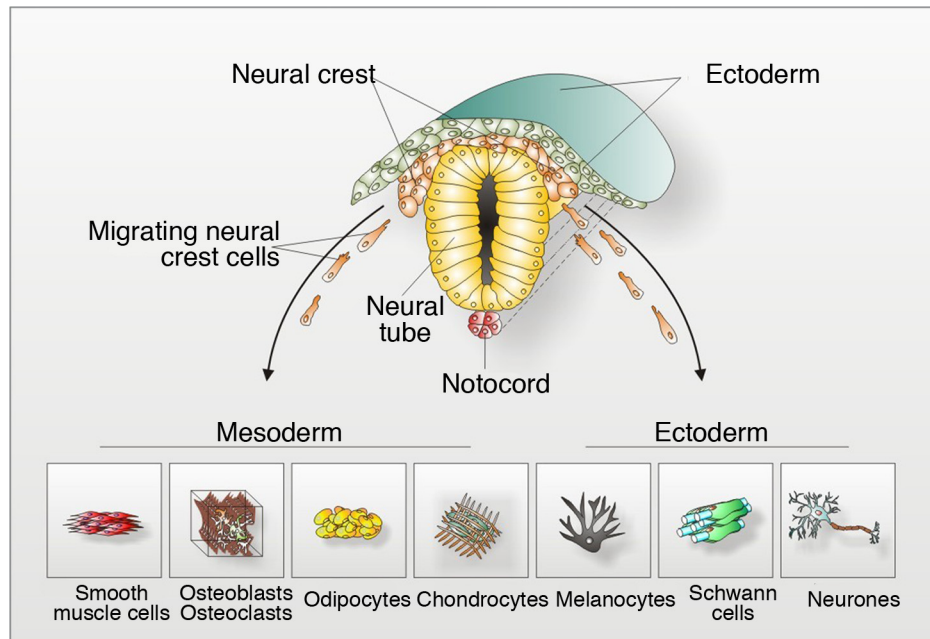


Figure 1.1. Neural crest migration and derivatives

Neural crest cells migrate from the dorsal aspect of the neural tube to many parts of the developing embryo. Once they have reached their targets, they differentiate to form smooth muscle, bone and cartilage, connective tissue, pigment cells and components of the nervous system.

(From <http://web.biologie.uni-bielefeld.de/cellbiology/index.php/research/neural-crest-derived-stem-cells>)

populations. The focus of this thesis is the cranial neural crest in *X. laevis*, which arises at the anterior neural plate border and differentiates into craniofacial structures including the cranial nerves and ganglia, smooth muscle and connective tissue, bone and cartilage, and dermis (reviewed in Theveneau and Mayor, 2011). As such, neurocristopathies associated with the cranial neural crest involve cleft lip, cleft palate, ear defects and eye defects. The cardiac neural crest contributes to the arteries and heart and the trunk neural crest forms the parasympathetic nervous system, while the vagal and sacral neural crest populations give rise to the enteric nervous system. Much of this introduction regards concepts relevant to all neural crest populations; nonetheless, relevant differences will be noted.

1. 1. Formation of the neural crest

The complex process of neural crest formation from naïve ectoderm has been described using a two-step model of neural crest induction followed by neural crest maintenance (Sauka-Spengler and Bronner-Fraser, 2008; Steventon et al., 2009; Steventon et al., 2005). This leads to an epithelial-to-mesenchymal transition (EMT), the delamination of neural crest cells and their subsequent migration.

1. 1. 1. Neural crest induction

Patterning of the embryonic ectoderm at the late blastula stage involves differentiation of neural from non-neural ectoderm. During gastrulation, high levels of retinoic acid (RA) and fibroblast growth factor (FGF) and low levels of Wnt pattern the embryonic ectoderm and lead to the formation of the neural plate border, which demarcates the boundary between neural and non-neural ectoderm (Mancilla and Mayor, 1996; Moury and Jacobson, 1990; Selleck and Bronner-Fraser, 1995). Neural crest

induction subsequently occurs at the neural plate border, and requires an increase in Wnt activity. It is the inhibition of Wnt signalling that restricts neural crest formation in the anterior neural fold (Carmona-Fontaine et al., 2007). Neural crest induction also requires inhibition of bone morphogenetic protein (BMP) signalling. Before gastrulation, BMP signalling occurs throughout the ectoderm. During neural crest induction, a dorso-ventral gradient of BMP signalling forms, with intermediate levels of BMPs at the presumptive neural crest between high levels in the epidermis and low levels in the future neuroectoderm. This is required - but not sufficient - to induce neural crest formation (Delaune et al., 2005; Garcia-Castro et al., 2002; LaBonne and Bronner-Fraser, 1998; Linker and Stern, 2004; Marchant et al., 1998).

Both of these requirements - Wnt activity and BMP inhibition - are facilitated by the dorsolateral marginal zone (DLMZ), which is located adjacent to the presumptive neural crest at gastrula stages (Fig. 1.2). The DLMZ expresses Wnt8 and chordin (a BMP inhibitor) and is both required and sufficient for neural crest induction (Steventon et al., 2009). FGF and RA are also required for neural crest induction, although it has been shown that FGF activity is mediated via Wnt8 (Begemann et al., 2001; Kengaku and Okamoto, 1993; Mayor et al., 1995; Villanueva et al., 2002). Another important signalling pathway is Notch-Delta, which has been suggested to control BMP expression in *D. rerio*, *G. gallus* and *X. laevis* and may promote neural crest development by repressing neurogenesis (Cornell and Eisen, 2000; Endo et al., 2002; Glavic et al., 2004).

This combination of neural crest inducers - intermediate levels of BMP signalling and the activity of Wnt, FGF, RA and Notch - sets in motion a genetic programme in the targeted ectodermal cells to express transcription factors that control cell specification. Three of the first genes activated in the presumptive neural crest and required for its specification in *M. musculus*, *D. rerio* and *X. laevis* are *Msx1/2*, *Dlx5*

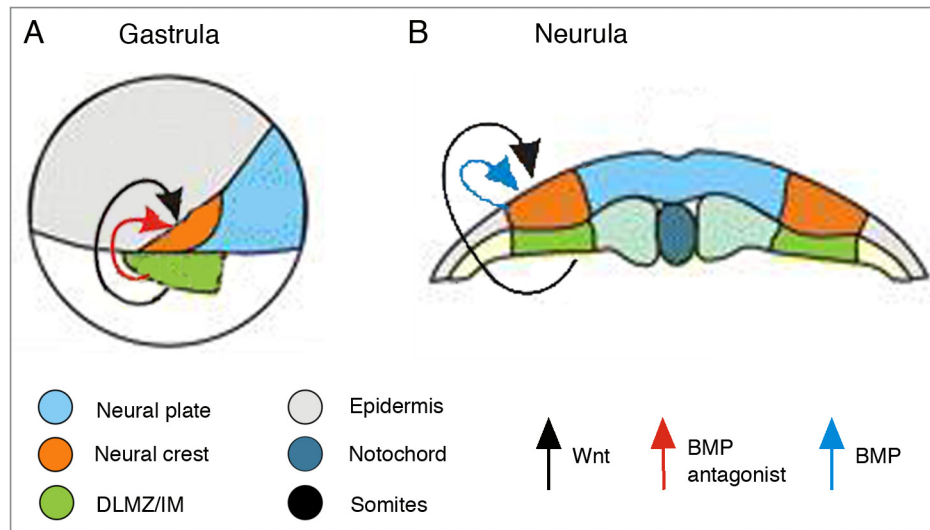


Figure 1.2. Neural crest induction

A diagramme summarising the temporal requirement for Wnt and BMP pathways during development. (A) At gastrula stage, the DLMZ is found adjacent to the presumptive neural crest and releases Wnt and chordin, a BMP antagonist. (B) At neurula stage, the intermediate zone is found adjacent to the neural crest and releases Wnt and BMP.

(From Steventon et al., 2009)

and *AP2* (Luo et al., 2002; Tribulo et al., 2003; Woda et al., 2003). These genes are initially expressed in a broad area and then restricted to the neural folds. This pattern corresponds with the assumed BMP gradient, and cis-regulatory elements in these genes suggest that they may be targets of BMP (Alvarez Martinez et al., 2002; Park and Morasso, 2002; Takebayashi-Suzuki et al., 2007). Further neural crest specifiers include *Zic1/3* (Nakata et al., 1997; Nakata et al., 1998), *Pax3* (Bang et al., 1997; Monsoro-Burq et al., 2005), *Pax7* (Mansouri et al., 1996) and *c-Myc* (Bellmeyer et al., 2003). *Zic1/3*, *Pax3*, *Pax7* and *c-Myc* are expressed in a more restricted domain than *Msx1/2*, *Dlx5* and *AP2* but in a wider area than the premigratory neural crest cells.

1. 1. 2. Neural crest maintenance

The second step of neural crest development is maintenance, which occurs during neurulation. Wnt activity is required for neural crest maintenance (Carmona-Fontaine et al., 2007) and *Wnt8* is expressed by the intermediate mesoderm (IM) in *X. laevis* (Steventon et al., 2009). In contrast to the induction step, BMP activity also is required for neural crest maintenance (Steventon et al., 2009). Chordin is no longer expressed adjacent to the neural crest at this stage, thus BMP activity increases (Sasai et al., 1994). Other survival signals are also upregulated during this time, such as FGF and RA. Interestingly, the IM is derived primarily from the DLMZ and, due to embryonic morphogenesis, becomes located directly underneath the neural crest at the neurula stage (Steventon et al., 2009). That the appropriate tissue changes both its position relative to the neural crest and the release of signals required by the neural crest during development is an illustration of the complex nature of embryogenesis and the importance of spatial and temporal regulation.

A gene network becomes activated to maintain the neural crest precursors. In *X. laevis*, *Snail2* responds directly to Wnt signalling (Vallin et al., 2001). *Snail1* is expressed slightly earlier than *Snail2* and appears to work upstream of it (Aybar et al., 2003) but both are required and sufficient for neural crest development (del Barrio and Nieto, 2002; Labonne and Bronner-Fraser, 2000; Mayor et al., 2000). *Snail2* promotes expression of other maintenance genes, including *FoxD3*, *Sox9*, *Sox10*, *Id3* and *Twist* (Aybar et al., 2003; Honore et al., 2003; Labonne and Bronner-Fraser, 2000; Sasai et al., 2001). These genes ensure maintenance of the population by inhibiting apoptosis and reducing proliferation (Tribulo et al., 2004; Vega et al., 2004). For example, *Snail1* stops cells in the transition between G1 to S phases of mitosis. Interestingly, trunk neural crest cells must enter S phase synchronously as they delaminate, suggesting that this control of the cell cycle may be important for trunk neural crest cell delamination (Burstyn-Cohen et al., 2004).

Following the maintenance phase, neural crest cells undergo an epithelial-to-mesenchymal transition (EMT) and delaminate from the neuroepithelium. These processes will be discussed in more detail later in this Introduction. Briefly, EMT refers to the transition from a fully polarised epithelial tissue to a mesenchymal cell population, whereas delamination describes the movement of the neural crest cells away from the neuroepithelium. Some cell maintenance genes, such as *Sox10*, *Sox9*, *Snail2* and *FoxD3*, are involved in these processes. For example, *Sox10* and *Sox9* promote delamination; in fact, *Sox9*, *Snail2* and *FoxD3* can induce ectopic generation and delamination of neural crest cells when overexpressed together (Cheung et al., 2005).

Neural crest specification, EMT and delamination may be all coordinated by maintenance genes, but they are separate processes. Ectopic *Sox9* expression, for example, can induce neural crest but not EMT (Cheung and Briscoe, 2003). Further,

inhibiting trunk neural crest cells from entering S-phase as they delaminate aborts delamination but does not affect the induction of genes involved in EMT, such as *Snail2* (Burstyn-Cohen et al., 2004), indicating that EMT and delamination are two separate events, at least in the trunk. Nonetheless, in some cases the roles do overlap: *Sox9* is important in specification and survival, but also coordinates with *Snail2* to trigger EMT (Cheung et al., 2005).

By stages 19-20 in the *X. laevis* embryo, the cranial neural crest has been defined to a specific region, adjacent to the neural tube. It has converted from a fully polarised epithelial tissue to a migratory, mesenchymal cell population. Migration commences shortly before closure of the anterior neural tube. Cranial neural crest cells migrate between the basal lamina beneath the epidermis and the developing dermomyotome, remaining close to the surface ectoderm. The population migrates ventrally down the side of the head and splits in the mandibular, hyoid and branchial streams, which enter the branchial arches and migrate extensively and invasively throughout the embryo towards their many targets.

1. 2. Neural crest migration

This section will present an overview of cell migration and then focus on cranial neural crest migration. Cranial neural crest migration is facilitated by a number of mechanisms that can be separated into three broad groups: restriction, co-attraction and contact inhibition of locomotion (CIL). Both cell-cell and cell-environment interactions are important and cranial neural crest cells integrate these multiple inputs to achieve directional migration.

1. 2. 1. Cell migration

Cell migration is essential for many aspects of embryonic development and it also plays a key role in other processes such as wound healing and immune system function. Consequently, it has been intensely studied. Cell polarity and directional migration are intertwined concepts, as directional migration requires establishment and maintenance of polarity by the cell. Indeed, the first step of directional migration is the polarisation of the cell at both the morphological and molecular levels, leading to a cell with distinct front and rear ends. This can be in response to an external signal such as growth factors or the extracellular matrix (ECM), but can also occur spontaneously (reviewed in Sohrmann and Peter, 2003; see also Altschuler et al., 2008). Thus, the mechanism controlling spontaneous polarisation needs to be integrated into the cellular response to external cues. To do this, small differences in the concentration of signalling molecules in the cytoplasm are amplified; this is sufficient to polarise cells (Altschuler et al., 2008). Common molecular pathways controlling cell polarity include the localisation of PI3K at the front of the cell and PTEN at the back (reviewed in Kay et al., 2008), polarised distribution of members of the Wnt planar cell polarity (PCP) pathway (for example, Carmona-Fontaine et al., 2008), small GTPases (reviewed in Etienne-Manneville and Hall, 2002) and partitioning-defective (Par) proteins (reviewed in Goldstein and Macara, 2007). These pathways control cell structures and processes including the cytoskeleton, and will be discussed in more detail within this Introduction.

Rho GTPases

To polarise, a protrusion is extended at the front of the cell and protrusive activity is reduced elsewhere. A protrusion could be a large, flat lamellipodium or thin, rod-shaped, actin-based filopodia. Typical Rho GTPases - RhoA, Rac1 and Cdc42 - control cell polarity by regulating different aspects of cytoskeletal dynamics, so are

important for migration (Fig. 1.3). Rac1 promotes the formation of lamellipodia by regulating actin polymerisation (Ridley et al., 1992), and Cdc42 controls the actin cytoskeleton in filopodia (Kozma et al., 1995). RhoA is generally localised at the back of the cell, particularly as Rac1 and RhoA mutually inhibit each other (Rottner et al., 1999). However, all three GTPases can be activated at the front of migrating cells, where RhoA initiates the formation of a protrusion, which Rac1 and Cdc42 subsequently reinforce and stabilise (MacHacek et al., 2009; Pertz et al., 2006).

Rho GTPases are thus major regulators of cell polarity and migration. Cdc42 is active at the front of migrating cells and has been suggested to be required for cell polarity (Etienne-Manneville and Hall, 2002). However, in cranial neural crest cells, disrupting cell polarity by inhibiting PCP has no effect on the activity of Cdc42 (Matthews et al., 2008). These results cannot rule out a contribution by Cdc42 in influencing polarity; nonetheless, Cdc42 activity appears not to be sufficient to regulate polarisation and protrusion formation in all cell types.

Rac1, on the other hand, has been consistently implicated in protrusion extension and thus migration. *D. melanogaster* oocyte border cells are unable to migrate when they express dominant negative Rac (Murphy and Montell, 1996), and their motility and protrusion by leader cells can be induced by polarised activation of a photoactivatable Rac1 (Wang et al., 2010). Rac1 inhibition in *D. rerio* germ cells inhibits protrusion formation and migration (Kardash et al., 2010). Although *M. musculus* neural crest cells do not require Rac1 for homing (Fuchs et al., 2009), *D. rerio* neural crest cell chemotaxis is regulated by Rac1 (Theveneau et al., 2010). Thus, Rac1 appears to be an important controller of migration *in vivo*, including in the neural crest of some species.

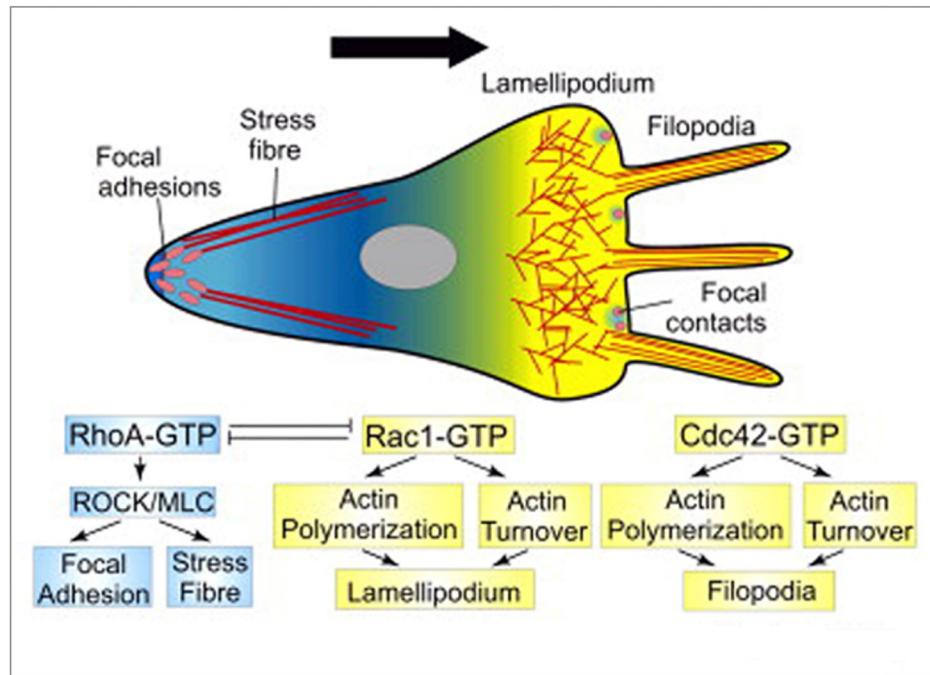


Figure 1.3. Rho GTPases in cell migration

Cell polarity, and thus directional migration, is influenced by polarised activity of small GTPases. Rho GTPases control actin dynamics and cell-substrate adhesion as well as microtubule dynamics. At the front of the cell, Cdc42 activity promotes filopodia formation, and Rac1 activity promotes lamellipodia formation. RhoA promotes retraction at the back of the cell.

(From Mayor and Carmona-Fontaine, 2010).

Cell-substrate interactions

The protrusion is stabilised by cell-substrate adhesions called focal complexes that, at least *in vitro*, have a punctate distribution. Rac1 is required for focal complex assembly at the front of the cell (Rottner et al., 1999). Most of these quickly turnover as the lamellipodium moves over them, but some are induced by RhoA to mature into larger, more stable focal adhesions (Rottner et al., 1999). Focal adhesions at the front of the cell are mostly stationary relative to the substrate, whereas those at the rear and sides of the cell can move inwards with the retracting edge before disassembling (Kaverina et al., 2000; Smilenov et al., 1999). Focal complexes and adhesions are made up of integrins and a range of adaptor proteins, through which they are linked to the actin cytoskeleton. Actin filaments associate with myosin motors to form stress fibres, which can be induced by RhoA and which pull on focal adhesions to provide traction and contraction forces (Ridley and Hall, 1992). Traction pulls the cell along the substrate, extending the cell. RhoA acts via Rho-associated protein kinase (ROCK) to phosphorylate myosin and inhibit its phosphatase, promoting actomyosin contractility at the back of the cell, where focal adhesions are disassembled and the rear is retracted.

Upon delamination from the dorsal neural tube, neural crest cells express the appropriate integrins to attach and migrate along the ECM. ECM proteins fibronectin, collagen and laminin are involved in trunk neural crest cell migration in *G. gallo* (Bilozur and Hay, 1988), whereas in *X. laevis*, fibronectin is located in the cranial neural crest migratory streams (Epperlein et al., 1988), and acts as a permissive substrate. Integrin- $\alpha5\beta1$ upregulated in migrating *X. laevis* neural crest cells, possibly due to the activity of neural crest specifiers FoxD3 and Sox10 (Cheung

et al., 2005), allowing them to migrate on fibronectin rather than vitronectin, laminin or collagen (Alfandari et al., 2003).

Neural crest cells also interact with fibronectin through Syndecan-4. Syndecan-4 binds fibronectin and negatively regulates Rac1 in cranial neural crest cells. It is necessary for directional migration (Matthews et al., 2008) and appears to be required for the formation of focal adhesions (Woods et al., 2000). It may be involved in reducing Rac1 activation at lateral and trailing edges; indeed, loss of Syndecan-4 increases Rac1 activation and lamellipodia protrusion around the whole cell (Matthews et al., 2008). However, the effect of the syndecan-4—fibronectin interaction cannot be explained solely by Rac1 inactivation, so Syndecan-4 might also be involved in the PCP pathway (Matthews et al., 2008).

Neural crest cells not only attach to the ECM to facilitate their migration, but also degrade it in order to form a path along which to migrate. Remodelling of the ECM is achieved by the expression of a number of matrix metalloproteinases (MMPs) and the related a-distintegrin-and-metalloproteinases (ADAMs). MMPs are endopeptidases that degrade ECM proteins and are involved in processing molecules such as cell surface receptors. ADAMs are cell-surface-bound glycoproteins that both remodel the ECM and participate in cell-cell adhesion by cleaving transmembrane proteins. A number of ADAMs are expressed in *X. laevis* cranial neural crest cells, of which only ADAM13 is known to be required for neural crest migration (Alfandari et al., 2001). As both integrins and Syndecan-4 - which are both involved in cell-fibronectin interactions - are able to interact with ADAMs, it has been suggested that proteinases may facilitate neural crest migration by degrading the ECM to reveal cryptic cell-binding sites (Theveneau and Mayor, 2011).

Degradation of the ECM can also release otherwise trapped growth factors and signalling molecules. Indeed, positive signals involved in cranial neural crest migration and expressed along the neural crest migratory pathway, including Sdf1 and members of the FGF, VEGF and PDGF families, interact directly with fibronectin (Martino and Hubbell, 2010; Pelletier et al., 2000). MMPs and ADAMs could help to shape gradients from homogenous expression patterns; for example, MMP2, which is expressed by *X. laevis* cranial neural crest cells (Tomlinson et al., 2009), can cut and inactivate the chemokine molecule stromal-cell derived factor (Sdf) 1 (or C-X-C chemokine ligand (Cxcl) 12; McQuibban et al., 2001). So, theoretically, neural crest cells could digest Sdf1 as they migrate to create a gradient in front of themselves. It is interesting to note that some molecules relevant to cranial neural crest cell migration, such as PDGF, stimulate expression of MMPs (Robbins et al., 1999). This might form a feedback loop in which MMPs and ADAMs access and degrade ligands trapped within the ECM in order to form a gradient, in turn eliminating the signals that regulate proteinase expression (Theveneau and Mayor, 2011).

Cell-cell interactions

Thus, neural crest cells interact with the ECM as they migrate. However, as cranial neural crest cells delaminate almost simultaneously and migrate as a group, they also interact with neighbouring neural crest cells through transmembrane glycoproteins, cadherins. N-cadherin is localised to cell-cell contacts, where it indirectly inhibits Rac1 and promotes protrusion extension in the opposite direction (Theveneau et al., 2010). Downregulating N-cadherin expression increases in ectopic protrusions between cells and promotes dispersion of cells (Theveneau et al., 2010). Another cadherin, cadherin-11, is expressed in cranial neural crest cells before and during migration in *X. laevis* (Vallin et al., 1998). It localises to cell-cell contacts like N-cadherin, but also to protrusions and the cell-substrate interface *in vitro* (Kashef et al.,

2009). Knocking down cadherin-11 prevents migration *in vivo* and cells *in vitro* have reduced filopodia and cell spreading, but overexpression of cadherin-11 also prevents neural crest migration (Borchers et al., 2001; Kashef et al., 2009). The binding partners of cadherin-11 and its precise role have not yet been elucidated. However, two interactions through intracellular domains are important for migration: one is with the cell-cell adhesion molecule β -catenin and the other is with the Rac1 and RhoA guanine-nucleotide exchange factor (GEF) Trio (Kashef et al., 2009). The interaction with Trio is presumed to regulate small GTPase activity, as Trio, RhoA, Rac1 or Cdc42 are sufficient to rescue cell spreading and migration in cadherin-11 knockdown cells and embryos (Kashef et al., 2009). As such, it may be that cadherin-11 promotes the activation of, or otherwise controls, small GTPases to regulate protrusion. Interestingly, ADAM13 is able to cleave cadherin-11 extracellularly to separate the extracellular domain from the transmembrane-cytoplasmic domains (McCusker et al., 2009). In doing so, it decreases cadherin-11-dependent adhesion without affecting cell signalling (McCusker et al., 2009). As MMP2 can digest N-cadherin, it seems that, as well as ECM structure and cell-ECM adhesions, MMPs and ADAMs can influence cell-cell adhesions (McCusker et al., 2009; Monsonego-Ornan et al., 2012). Another cadherin, the protocadherin PCNS, is also expressed in *X. laevis* cranial neural crest and is required for migration *in vivo* (Rangarajan et al., 2006); however, the exact function has not yet been elucidated.

1. 2. 2. Restriction of the neural crest streams

Shortly after delamination from the neuroepithelium, the cranial neural crest cell population splits into three distinct streams, leaving two neural crest-free areas between them. The migratory route of each stream is well defined: it may be, in part, that neural crest cells migrate through permissive spaces between structures such as the branchial pouches. However, although this could be an important factor, it is not

sufficient to restrict migration to the pathways. Rather, neural crest cells express proteins whose complementary, restrictive factors are expressed by the surrounding tissue. These include semaphorin/neuropilin, Eph/ephrin and Slit/Robo interactions. Restrictive cues define the migratory streams by lining corridors within which neural crest cells migrate. They are expressed differently along the body axis and the receptors expressed by neural crest cells seem to change accordingly. Thus, these molecules may also be involved in directing different neural crest subpopulations to their respective targets.

Semaphorins/neuropilins

Members of the class 3-semaphorin family - 3A, 3F and 3G - are expressed by tissues surrounding the cranial neural crest streams and their associated neuropilin receptors - Npn1 and Npn2 - are expressed by cranial neural crest cells (Eickholt et al., 1999; Gammill et al., 2007; Koestner et al., 2008; Osborne et al., 2005; Yu and Moens, 2005). Semaphorins are inhibitory to cranial neural crest migration; as such, this juxtaposition keeps neural crest cells in their appropriate streams. Indeed, neural crest cells preferentially avoid semaphorin-containing areas and inhibition of semaphorin signalling causes ectopic migration of cranial neural crest cells between the normal streams in *M. musculus* (Gammill et al., 2007). However, this signalling pathway is not restricted to the cranial neural crest. In *M. musculus*, Npn2^{-/-} and Sema3^{-/-} embryos show that SEMA3F-NPL2 signalling also guides migration of trunk neural crest cells through the anterior half of the somite (Gammill et al., 2006). *X. laevis* cranial neural crest cells have also been shown to express neuropilins (Koestner et al., 2008), although the role of semaphorin-neuropilin signalling in *X. laevis* has not yet been elucidated.

Ephs/ephrins

Bidirectional signalling occurs between the receptor tyrosine kinase Ephs and their ephrin ligands. Eph-ephrin interactions are promiscuous and there is variation in the combinations of ephrins and Eph receptors expressed by the cranial neural crest and the surrounding mesoderm in animal models (Mellott and Burke, 2008; Smith et al., 1997; Winning and Sargent, 1994). Nonetheless, their functions appear to be conserved. Ephs and ephrins are involved in sorting cells into a specific stream according to their origin along the anterior-posterior axis and provide repulsive cues to restrict neural crest migration into specific areas of the embryo. This is illustrated by experiments showing that interrupting normal Eph or ephrin expression can lead to either invasion of the surrounding tissue by neural crest cells or the mislocalisation of neural crest cells into the wrong stream without perturbing neural crest-free areas (Smith et al., 1997). For example, ephrin-B2 is expressed in the second branchial arch and acts as a repellent for neural crest cells expressing EphA4 and EphB1, such as those that migrate to the third and fourth branchial arches (Smith et al., 1997). This signalling pattern appears to prevent intermingling between streams. However, as well as Ephs, *M. musculus* neural crest cells also express ephrin-B1, which is required cell-autonomously in cranial neural crest cells and controls directional migration towards the target tissue (Davy et al., 2004).

Eph-ephrin signalling is also important in the migration of trunk neural crest cells. Ephrin-B1 and -B2 are expressed in the posterior half of each somite in *G. gallo* and *M. musculus* and restrict the migration of neural crest cells, which express cognate Eph receptors, to the anterior somite half (Krull et al., 1997; Santiago and Erickson, 2002; Wang and Anderson, 1997). Eph-ephrin signalling in *G. gallo* also repels those trunk neural crest cells that delaminate first, which express EphB, from the dorsolateral pathway, which contains the opposing ephrin (Santiago and Erickson,

2002). Thus, early migrating trunk neural crest cells take the ventromedial pathway and differentiate into dorsal root and sympathetic ganglia and neural crest cells that delaminate later migrate along the dorsolateral pathway to become melanoblasts (Santiago and Erickson, 2002).

Slit/Robo

Slit ligands and their Robo receptors are involved in the migration of trunk neural crest, but they have not yet been investigated in cranial neural crest cell migration. In the trunk, Slit2 is expressed in the dorsolateral pathway and restricts early migrating trunk neural crest cells, which express Robo1 and 2, to the ventromedial migratory pathway (Giovannone et al., 2012; Jia et al., 2005). As with Eph/ephrin signalling, this prevents premature migration into the dorsolateral pathway.

1. 2. 3. Chemoattraction

A number of molecules are involved in the positive regulation of neural crest cell migration; nonetheless, the existence of a chemoattractant for neural crest cells is still controversial. *G. gallo* cranial neural crest cells migrate towards a VEGF source *in vitro* and to an endogenous source, the second branchial arch, *in vivo* (McLennan et al., 2010). Nonetheless, it is not necessarily clear that VEGF acts as a chemoattractant, as an ectopic source of VEGF *in vivo* slightly changes migration but does not induce ectopic migration. Likewise, PDGF receptor (PDGFR) α is expressed by cranial neural crest cells in *X. laevis* and *M. musculus* (Ho et al., 1994; Schatteman et al., 1992; Takakura et al., 1997) and its ligands, PDGFA and PDGFC, are expressed in the branchial arches and oral and nasal cavities (Ding et al., 2000; Tallquist et al., 2000). Mutations in PDGFR α in *M. musculus* lead to defects associated with a disruption in neural crest development, such as cleft palate and cranial bone defects (Tallquist and Soriano, 2003). However, neural crest cells are

still able to colonise the face in these mice, suggesting that the role of PDGFR α is either to regulate differentiation or to promote migration of only a small subset of neural crest cells (Tallquist and Soriano, 2003). Thus, the precise influence of PDGF on cell migration, if there is one, is yet to be elucidated. Later in development, netrins expressed by the submucosal ganglia and pancreas may act as chemoattractants for enteric neural crest cells, which express the netrin receptor DCC (Jiang et al., 2003). Enteric neural crest cells are attracted towards these tissues or pure netrin. However, netrin also has an effect on cell survival (Jiang et al., 2003) and migratory ability is linked to population size (Simpson et al., 2007). This makes it difficult to determine definitively the contribution of chemoattraction to neural crest migration

Another factor that has been studied as a potential neural crest attractant is Sdf1. Neural crest cells express the Sdf1 receptor, C-X-C chemokine receptor (Cxcr) 4. Sdf1 signalling is required for the positioning of a neural crest derivative, the dorsal root ganglia, in *M. musculus* (Belmadani et al., 2005). Craniofacial development and melanophore patterning are dependent on Sdf1 in *D. rerio* (Olesnick Killian et al., 2009; Svetic et al., 2007) and *D. rerio* melanocytes will migrate to an ectopic Sdf1 source (Svetic et al., 2007). Sdf1 also promotes migration of trunk neural crest cells as they invade the hair follicles (Belmadani et al., 2009). Sdf1 attracts *X. laevis* cranial neural crest cells *in vitro* (Theveneau et al., 2010). Cxcr4 is activated by Sdf1 and promotes Rac1 activation at the leading edge of the cell, stabilising lamellipodia and promoting directional migration. Unsurprisingly, its expression is also required for normal neural crest migration *in vivo* in *X. laevis* and *D. rerio* embryos and misexpression can lead to either ectopic cranial neural crest cell migration or the early halt of migration as the cells cluster around an Sdf1 source (Olesnick Killian et al., 2009; Theveneau et al., 2010).

Nonetheless, the question as to whether or not these proteins act as chemoattractants *in vivo* remains unresolved. Some evidence suggests the contrary: for example VEGF, PDGFA, PDGFC and FGF ligands appear to be expressed homogeneously along the migratory pathways rather in a gradient. This is also true of Sdf1 mRNA, which appears to be distributed homogeneously in the ectoderm surrounding the neural crest (Theveneau et al., 2010), and along the path of melanocytes migrating into hair follicles (Belmadani et al., 2009). However, it is possible that there is a protein gradient and, in *X. laevis* embryos, Sdf1 expression alters as development progresses, moving ventrally to remain in front of neural crest cells as they migrate and appearing between them as they split into streams. Nonetheless, individual neural crest cells *in vivo* can migrate in the opposite direction to the main population of neural crest cells, which would be against the supposed gradient. Thus, it is not yet determined whether these signals are chemokinetic or chemotactic cues.

A novel method of interaction between neural crest cells has recently been established. The complement factor C3a is released by neural crest cells and the same cells also express the C3a receptor (C3aR; Carmona-Fontaine et al., 2011). These two molecules facilitate the co-attraction of neural crest cells to each other: neural crest cells both release the chemoattractant and express its receptor. Activation of the C3a receptor leads to Rac1 activation and so promotes migration towards the C3a source. This appears to be a mechanism to maintain neural crest cells in a group as they migrate. Interestingly, these molecules have, until recently, been described only in the immune system, where they form part of the complement cascade of proteins released upon recognition of a foreign cell. Such a mechanism of co-attraction has not previously been reported in animal cells, making it particularly novel.

1. 2. 4. Contact inhibition of locomotion

The molecular interactions described above provide mechanisms for cellular migration itself and for the restriction of neural crest cells within their appropriate migratory paths. However, this is not sufficient to drive directional migration. A phenomenon known as contact inhibition of locomotion (CIL) is also required (Fig. 1.4).

CIL was first described by Abercrombie and Heaysman (1953). They cultured two embryonic *G. gallo* hearts close together and analysed collisions between fibroblasts emerging from the opposing explants. They observed that fibroblasts lower their speed in proportion to the number of cells that they encounter; illustrating that contact with other cells reduces cell motility. They also noticed that fibroblasts coming into contact with each rarely migrate on top of each other, but instead stop migrating or disperse elsewhere. They concluded that a fibroblast would preferentially adhere to the substrate than to a neighbouring cell and named this contact inhibition of locomotion. They also noticed that neighbouring cells tended to align with each other and that explants tended to form a monolayer and suggested that this was also due to CIL. CIL was suggested to facilitate wound healing, in that constraints on fibroblast migration at cell-cell contacts were released upon wounding, and that fibroblast migration into the free space after wounding would lead to wound closure (Abercrombie, 1970). In further work, Abercrombie and Ambrose (1958) observed that cell protrusions were retracted upon contact and that a protrusion was then extended in another part of the cell. These details were added to the previous observations to lead to the more complete definition of CIL as: “*the phenomenon of a cell ceasing to move in the same direction after contact with another cell*”.

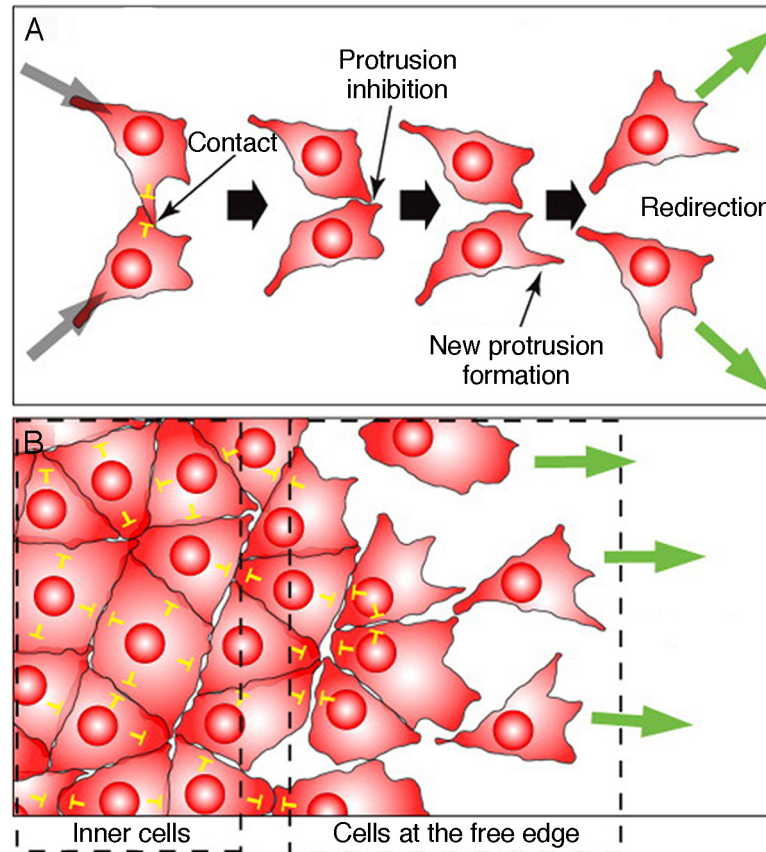


Figure 1.4. Contact inhibition of locomotion

(A) When two individual neural crest cells come into contact, contact inhibition of locomotion leads them to stop migrating, repolarise and migrate away from each other in another direction. This diagramme illustrates the process of contact inhibition of locomotion as occurring in distinct steps for simplicity, but in actuality this is not necessarily the case. (B) In a group of neural crest cells, contact inhibition of locomotion prevents cells within the group from extending protrusions in any direction. Only cells at the group edge are able to extend protrusions, and they are only able to do so away from the group. In this way, contact inhibition of locomotion can promote cell dispersion.

(From Mayor and Carmona-Fontaine, 2010).

Neural crest cells also exhibit CIL (Carmona-Fontaine et al., 2008; Erickson, 1985). Indeed, the first description of CIL *in vivo* was by Carmona-Fontaine and colleagues (2008), who observed CIL between cranial neural crest cells of *X. laevis* and *D. rerio*. Cranial neural crest cells from *X. laevis* also exhibit CIL when cultured on a fibronectin matrix *in vitro* (Carmona-Fontaine et al., 2008). In the context of neural crest cells, CIL is described as leading to two individual colliding cells stopping migration upon contact and then migrating in another direction. This has been proposed to correlate with the collapse of protrusion at the site of contact and the subsequent extension of a protrusion in another direction (Carmona-Fontaine et al., 2008).

At the molecular level, the Wnt-PCP pathway appears to mediate CIL in neural crest cells (Carmona-Fontaine et al., 2008). Wnt signalling is complicated as it involves a number of different pathways that each involve many different proteins. However, in CIL in neural crest cells, the non-canonical PCP pathway is required (Carmona-Fontaine et al., 2008; De Calisto et al., 2005), and will thus be the only pathway explained here. When two neural crest cells contact each other, Wnt11 from each cell appears to bind to Frizzled7 in its neighbour. This leads to the recruitment of Dishevelled to the membrane of both cells at the site of cell-cell contact. Dishevelled activates a number of other proteins including RhoA (Matthews et al., 2008). RhoA activation reorganises the actin cytoskeleton, leading to the collapse of protrusions at the site of cell-cell contact, and inhibits Rac1. Asymmetrical activation of Rac1, with higher activation of Rac1 away from the contact, promotes protrusion away from the site of cell-cell contact and leads to a change in cell polarity and in the direction of migration.

Disturbing the activity of Wnt-PCP pathway components in *X. laevis* or *D. rerio* inhibits neural crest migration *in vivo* and *in vitro* (Carmona-Fontaine et al., 2008; De Calisto et al., 2005). This was first illustrated by De Calisto et al (2005), who showed that downregulating PCP signalling in *X. laevis* embryos inhibits neural crest migration. The authors concluded that the PCP pathway was necessary for correct polarisation of neural crest cells. Wnt signalling between neural crest cells is thought to activate the PCP pathway in both cells at the site of contact (Carmona-Fontaine et al., 2008).

As well as being able to interact with fibronectin in neural crest cells, Syndecan-4 is a modulator of PCP signalling during convergent extension in *X. laevis* gastrulation, perhaps through direct interaction with Dishevelled and Frizzled7 (Munoz et al., 2006). Syndecan-4 has been shown to activate a pathway that, in parallel with PCP signalling, also leads to Rac inhibition (Matthews et al., 2008). Levels of Rac signalling are thus tightly regulated in neural crest cells, perhaps reflecting that front-back polarity of small GTPase activity is vital for the initiation and maintenance of cell polarity. Indeed, the importance of regulating small GTPase activity may explain why both inhibition and overexpression of Syndecan-4 or PCP signalling inhibit neural crest cell migration (De Calisto et al., 2005; Matthews et al., 2008). N-cadherin is also required for CIL at cell-cell contact (Theveneau et al., 2010). Inhibition of N-cadherin leads to an increase in Rac activity at the site of cell-cell contacts. It is interesting to note that PCP, Syndecan-4 and N-cadherin signalling all converge to regulate small GTPase activity at the cell-cell contact, by either promoting RhoA or inhibiting Rac1 at this site. Nonetheless, direct interactions between N-cadherin, Syndecan-4 and PCP signalling have not yet been elucidated.

Importantly, CIL does not occur between neural crest cells and other cell types (Carmona-Fontaine et al., 2008). This presumably allows neural crest cells to invade

the surrounding tissue as they migrate towards their targets. This is reminiscent of malignant mesenchymal cells: sarcoma cells do not exhibit CIL upon contact with fibroblasts but continue to extend processes and are able to move on top of them (Abercrombie and Ambrose, 1958). As such, it has been suggested that CIL could be a control mechanism that fails in the case of metastatic cancer (Abercrombie, 1979; Vesely and Weiss, 1973). That is, if a cell is usually constrained to a certain location by contact with the cells surrounding it, a loss of CIL could allow it to migrate away from this location and into other tissues as is seen in metastasis.

Some (Paddock and Dunn, 1986), but not all (Abercrombie and Ambrose, 1958), malignant cells maintain the ability to undergo CIL when contacting one another but do not exhibit CIL upon contact with other cell types. When a group of malignant cells migrate together, this might enhance metastasis by promoting cancer cell dissemination (by encouraging the malignant cells to move away from each other) without impeding invasion (by allowing the cells to move through tissues consisting of other cell types). The role of CIL in collective migration will be discussed in more detail in the following section. As a brief aside, it is interesting to note that Eph-ephrin signalling has also been associated with CIL in tumourigenic cells (Astin et al., 2010), considering the role of Eph-ephrin signalling in neural crest migration (see Restriction of neural crest migration, page 33). However, a possible role for Eph-ephrin signalling in CIL has not been investigated in neural crest cells.

1. 2. 5. Collective migration

The work of Abercrombie and colleagues was ultimately the study of how the behaviour of one cell is influenced by others. Nonetheless, the appreciation that most motile cells do not move in isolation *in vivo* but interact with their neighbours is a relatively new concept and exactly how cells interact with each other during migration is not yet fully understood.

In the multicellular organism, cells can move in groups rather than individually. The term ‘collective cell migration’ describes groups of cells that move together and in contact with each other, and that influence each other while migrating (reviewed in Rorth, 2009). Variations of collective migration have been described: sheets of mesenchymal cells, as found in the developing head mesoderm in amphibians (Winklbauer and Selchow, 1992); clusters of epithelial cells, such as the lateral line in *D. rerio* embryos and border cells in *D. melanogaster* embryos (Haas and Gilmour, 2006; Inaki et al., 2012); chains, as observed in endothelial cells in angiogenesis (Wimmer et al., 2012); and streaming, in mammalian endoderm (Migeotte et al., 2010). There is an element of diversity between the different modes of collective migration, but there are also common themes. They often migrate directionally and all exhibit protrusions at the leading edge. Although it has not been definitively determined, it may be that aspects of CIL, such as protrusion inhibition, occur within cell clusters during collective cell migration (Theveneau and Mayor, 2011).

It is the last form of collective cell migration mentioned above - streaming - that has been described for cranial neural crest cells. Cranial neural cells delaminate continuously and in large numbers; as such, they experience maintain contact with each other as they commence migration. The cells move together in a loose cluster, within which individual cells can be identified but are constantly interacting with each other. CIL means that cells in the middle of the group – surrounded on all sides by other neural crest cells - are inhibited from extending protrusions on all sides and only cells at the edge of the group are able to extend protrusions (Fig. 1.4B). Once one takes into account the repulsive factors on each side of the migrating neural crest streams, which prevent sideways migration of cells into these areas, only the “leader” cells at the front of the stream are able to effectively extend protrusions. Furthermore, leader cells are only able to extend protrusions away from the group due to CIL

preventing extension towards the cells contacting them from behind. As leader cells extend protrusions and migrate forward, they leave a gap between themselves and the cells directly behind them. The second row of cells can now extend a protrusion in this direction and so migrate into and close the gap, making contact again with the leader cells. This again promotes forward movement of the leader cells, and so on. Inert beads inserted into the neural crest region ‘migrate’ in a manner similar to neural crest cells, suggesting that at least some of the mechanisms driving neural crest cell migration are passive and that non-motile cells, such as those undergoing mitosis, can be carried by their motile neighbours (Jesuthasan, 1997).

External cues including both repulsive (Sema/Npn, Eph/ephrin) and attractive factors (such as VEGF and Sdf1; McLennan et al., 2010; Theveneau et al., 2010), play an important role in directing neural crest migration. However, neural crest cells can also migrate directionally *in vitro*, where these signals – and, presumably, most others influencing neural crest migration - are absent. Thus, directional collective migration does not require external signals, but perhaps allows the cells to better integrate them. Indeed, chemotaxis is dependent on cell-cell interactions as it requires high cell density and is lost when cells are dispersed (Theveneau et al., 2010). But, groups of as few as three cells are able to migrate towards an Sdf1 source as effectively as large groups of hundreds of cells (Theveneau et al., 2010). Even increasing the density of individual cells (causing instances of cell-cell contact and so CIL to occur more frequently) increases the effectiveness of chemotaxis (Theveneau et al., 2010). Individual cells exhibit a number of small, short-lived protrusions in all directions, whereas cells in contact with other cells do not have protrusions at the cell contact, but rather have larger, stable protrusions oriented away from the site of cell-cell contact (Theveneau et al., 2010). As such, cells in contact with other cells are more polarised than individual cells. Sdf1 stabilises protrusions but does not induce the formation of

protrusions (Theveneau et al., 2010); it is, therefore, not surprising that cell-cell interaction leading to protrusion extension is required for collective chemotaxis. Thus, the intrinsic polarity of these cells, caused by CIL and mediated by co-attraction, is required for the adequate response to extrinsic signals. Indeed, balances between repulsion (CIL) and attraction (co-attraction) have also been used to explain swarming behaviour in collective animal migration (Buhl et al., 2006; Romanczuk et al., 2009).

A number of factors allow neural crest cells to migrate in a collective manner (Fig. 1.5; Theveneau and Mayor, 2011). As discussed above, neural crest migration is facilitated by the restriction of streams by repulsive factors, keeping the neural crest within certain areas of the embryo. Co-attraction exhibited between neural crest cells keeps them together in a group. CIL is essential for neural crest migration (Carmona-Fontaine et al., 2008), but CIL alone leads to cell dispersion and thus decreases the frequency of cell-cell contacts, with the consequence of more randomised cell polarity and inefficient directional migration. Cell density is essential for the efficient migration of neural crest cell populations (Carmona-Fontaine et al., 2011; Landman et al., 2011). One way of maintaining high cell density is to maintain strong cell-cell adhesion; however, this would also interfere with collective migration. So, coattraction counterbalances dispersion by maintaining neural crest cells at a density that allows interactions. Once neural crest cells make new contacts, cell adhesion could play a transient role in maintaining high cell density and cell-cell interactions, thus leading to cell polarisation and a full response to chemoattractants. Further, chemokinesis due to the presence of Sdf1 enhances neural crest cell migration. The sum of these signals and interactions drive the directional, collective migration of the cranial neural crest cells. Indeed, computer models have confirmed the results of wet

lab studies, that all of these factors are required for neural crest cell migration (Carmona-Fontaine et al., 2011).

2. Epithelial cell-cell interactions

Cell-cell adhesions are essential for the organisation of cells into tissues during embryonic development, as well as in cell growth, migration and differentiation (reviewed in Takeichi, 1991). Epithelial sheets consist of fully polarised epithelial cells with a basolateral cell surface alongside the basement membrane and an opposing apical edge. Full development of apicobasal polarity requires organisational cues from cell-cell and cell-ECM adhesions (reviewed in Yeaman et al., 1999). The boundary between the apical and basolateral sections is identified and reinforced by the apical complex, which forms asymmetrically at the apical region of lateral cell-cell contacts and is composed of three major adhesive structures: the tight junction, the adherens junction and desmosomes (Fig. 2.1; St Johnston and Ahringer, 2010). Each structure comprises at least one membrane-bound adhesion molecule linking neighbouring cells and bound to cytoplasmic scaffold proteins, each of which has multiple protein-protein binding motifs. Scaffolding proteins can link various sub-complexes, organise signalling complexes and link to the cytoskeleton (for example Ligon et al., 2001; also reviewed in Fukata et al., 2002). Some proteins at the apical junctional complex also act as transcriptional factors of gene expression (Balda and Matter, 2003). Together, they maintain cell polarity and tissue structure.

Desmosomes consist of transmembrane desmogleins, which anchor intermediate filaments such as vimentin to the cortex through desmoplakins. Desmosomes are required for a fully polarised epithelial phenotype (Perez-Moreno et al., 2003), but their formation is not well understood and they are not relevant to this thesis. Thus, they will not be discussed further in this Introduction.

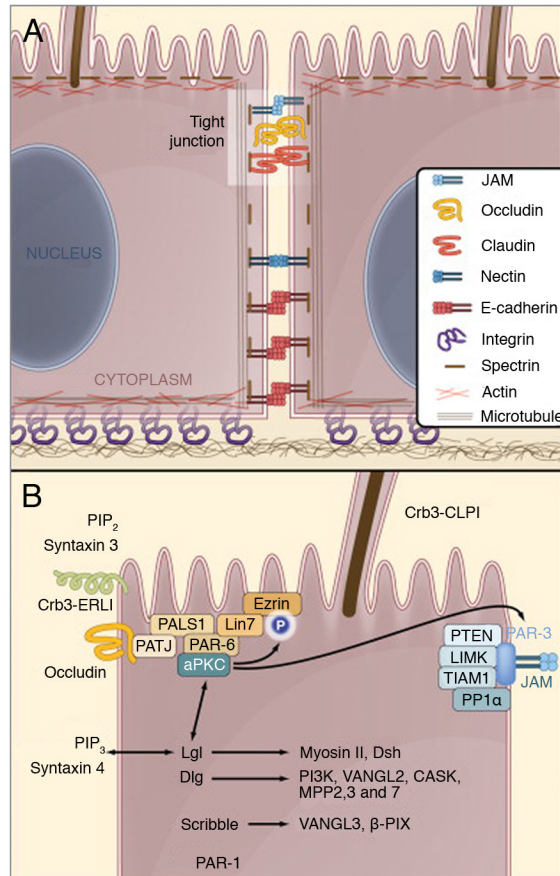


Figure 2.1. The epithelial adhesion complex

The vertebrate epithelial adhesion complex includes the adhesion junction and tight junction and is found at the apical region of lateral cell-cell contacts. (A) The tight junction includes proteins such as JAMs, occludins and claudins. Adherens junctions are found laterally to tight junctions and include cadherins and nectins. (B) A network of polarity proteins is required to establish and maintain the adhesion complex in the epithelial cell.

(Adapted from St Johnston and Ahringer, 2010).

2. 1. Adherens junctions

The formation of cell-cell contacts and the development of intercellular junctions begins with actin filaments from adjacent cells meeting and forming multiple transient contacts, which are subsequently stabilised (McNeill et al., 1993). These are thought to serve as a landmark, providing positional cues for membrane growth and the recruitment of other proteins (Yeaman et al., 1999). The first spot-like cell-cell contacts are primordial adherens junctions and contain typical adherens junction proteins such as E-cadherin, α -catenin and β -catenin, but also typical tight junction proteins including zona occludens (ZO)-1 and junctional adhesion molecule (JAM)-A (Ebnet et al., 2001; Suzuki et al., 2002; Yonemura et al., 1995). Multiple primordial adherens junctions at early contact sites fuse as the cells start to polarise, following which the primordial junctions separate into adherens and tight junctions as they mature (Ando-Akatsuka et al., 1999).

2. 1. 1. Components of the adherens junction

Adherens junctions anchor neighbouring cells to one another through Ca^{2+} -dependent, homophilic interactions between cadherins. Cadherins are absolutely required for cell-cell adhesion formation: depletion of Ca^{2+} results in a loss of junctions despite the fact that other adhesion molecules such as catenins are not Ca^{2+} -dependent (Takeichi, 1977). Further, transfection of non-polarised fibroblasts with a construct encoding E-cadherin induces polarisation into apical and basolateral domains (McNeill et al., 1990). The cytoplasmic tail of classical cadherins (such as E-cadherin) binds to intracellular catenins such as α -catenin, β -catenin, γ -catenin and p120-catenin (reviewed in Kemler, 1993). The protein regions involved in this interaction are highly conserved, highlighting the importance of interaction between these proteins. Indeed, deletion of β -catenin in *D. melanogaster* embryos inhibits the formation of adherens

junctions and thus development of an epithelium and cellular structures (Cox et al., 1996). The adhesive activity of E-cadherin is dependent on its association with β -catenin (Ozawa et al., 1990), which is interesting as it implies that intracellular interactions affect extracellular adhesions. This association is also required to protect β -catenin from degradation. There is also a reciprocal regulation of localisation of E-cadherin and p120-catenin, although in this case p120-catenin regulates E-cadherin turnover (Davis et al., 2003).

Catenins are known to link cadherins to the actin cytoskeleton; however, it appears that α -catenin forms a heterodimer with β -catenin but can interact with F-actin only as a homodimer (Drees et al., 2005; Yamada et al., 2005). It has also been reported that cadherins can interact directly with actin (Ehrlich et al., 2002). The Ca^{2+} -independent cell adhesion molecules nectins are also found at the adherens junction, where they undergo homophilic or heterophilic interactions with other nectins or nectin-like molecules. One of these, afadin, may directly link nectins with F-actin, although it also interacts with other adherens junction proteins, including α -catenin (Tachibana et al., 2000; Takahashi et al., 1999). So, the link between adherens junctions and the actin cytoskeleton is not clear. Nonetheless, formation of cadherin-mediated cell-cell contacts triggers reorganisation of the actin cytoskeleton. The ring of cell-cell junctions at the same level all around a cell within a monolayer form an apical belt called the zonula adherens. The actin filaments in many cells can thus form a network across the tissue that is mediated by cadherin-catenin complexes and allows mechanical force to be applied across the whole cellular sheet. This is important for morphogenetic events such as neural tube formation (Morita et al., 2010).

2. 1. 2. Adherens junction signalling

Protein complexes are dynamic in order to disassemble and reform quickly enough to maintain epithelial integrity, and their composition is subject to regulation depending on junctional maturation and integrity. p120-catenin influences Rho GTPases by interacting with the Rac GEF Vav2 (Noren et al., 2000), which may be important in the formation of cell-cell contacts, as Cdc42 and Rac are usually upregulated and RhoA inhibited during this time (Noren et al., 2001). Nectins can also activate Cdc42 and Rac1 through FGD-1-related Cdc42 GEF and Vav2, respectively (Fukuhara et al., 2004; Kawakatsu et al., 2005). As well as affecting the actin cytoskeleton, this might regulate the separation of tight and adherens junctions during adhesion maturation.

As well as the actin cytoskeleton, adherens junctions also interact with microtubules. During the formation of an epithelium, the radial microtubule array of the cell is reorganised so that microtubules that run from the apical surface or cell-cell contacts to the basolateral surface. The microtubule-anchoring protein ninein is involved in this process (Mogensen et al., 2000). In mammalian cells p120-catenin, which can bind cadherins, recruits PLEKHA7 and Nezha to the zonula adherens (Meng et al., 2008). Nezha binds microtubules and provides a site for microtubule anchoring and outgrowth independent of ninein (Meng et al., 2008). The microtubule-associated motor protein kinesin-14 is also localised to adherens junctions, in a manner dependent on Nezha and PLEKHA7 (Meng et al., 2008). Further, microtubule depolymerisation leads to the loss of kinesin-14 and Nezha and disruption of the zonula adherens, supporting the idea that the zonula adherens is a dynamic structure and must be actively maintained (Meng et al., 2008).

2. 2. Tight junctions

Tight junctions are a part of the epithelial junctional complex and situated apically to the adherens junction. The formation of tight and adherens junctions are linked. Tight junction proteins JAM-A and ZO-1 are associated with primordial adherens junctions during epithelialisation (Ando-Akatsuka et al., 1999; Ebnet et al., 2001; Suzuki et al., 2002). Other tight junction proteins appear later, including occludin, claudin and Par3.

Tight junctions have two functions. The first is a tissue-specific function, which is to regulate the paracellular permeability of the epithelial sheet for ions and small solutes (reviewed in Furuse and Tsukita, 2006). In a more generic manner, the direct contact between membranes, which forms a belt-like structure around each cell within a monolayer, physically separates the apical and basolateral membranes and is necessary for maintenance of asymmetric distribution of membrane components and so the development of membrane polarity (Tsukita et al., 2001).

2. 2. 1. Tight junction membrane proteins

There are three classes of integral membrane proteins at tight junctions. One is comprised of occludin, claudins and tricellulin, all of which contain four transmembrane domains with two extracellular loops. Claudins provide the molecular basis of the tight junction strands and can induce the formation of strands upon ectopic expression in fibroblasts (Furuse et al., 1998). Within strands, hetero- and homophilic interactions between a variety of claudins form size- and charge-selective aqueous pores that are responsible for the selective permeability barrier (Furuse et al., 1998; Furuse and Tsukita, 2006; Tsukita et al., 2001). Occludins are also incorporated into strands and may modulate some functions of tight junctions (Furuse

et al., 1998; Yu et al., 2005). Tricellulin is specifically localised to tri-cellular contact sites (Ikenouchi et al., 2005).

The second class of integral membrane proteins are members of the immunoglobulin superfamily, including JAM-A, -B and -C. Apart for JAM-A, there is very little known about these proteins. JAM-A localises early during cell–cell contact formation (Ebnet et al., 2001; Suzuki et al., 2002), probably recruited by the scaffolding protein ZO-1 (Ebnet et al., 2000). JAM-A in turn recruits the scaffolding protein Par3 to the tight junctions (Ebnet et al., 2001; Itoh et al., 2001). Downregulation of JAM-A or expression of a JAM-A dominant-negative leads to mislocalisation of Par3 and a general defect in tight junction formation, such as increased paracellular permeability and defects in the development of membrane asymmetry (Mandell et al., 2005; Rehder et al., 2006).

The third membrane protein involved in tight junctions is Crumbs3, a homologue of the *D. melanogaster* Crumbs protein. It interacts directly with two peripheral membrane proteins, Pals1 (Roh et al., 2002) and Par6 (Lemmers et al., 2004), both of which are components of polarity protein complexes associated with tight junctions. Overexpression of Crumbs3 delays tight junction formation (Lemmers et al., 2004) and ectopic expression in a mammalian cell line that does not otherwise express Crumbs3 or form tight junctions can result in the development of functional tight junctions (Fogg et al., 2005). It may be that these effects are due to the association of Crumbs3 with cell polarity proteins and the regulation of their localisation.

2. 2. 2. Tight junction protein complexes

Along with integral membrane proteins, tight junctions include scaffolding and adapter proteins, small GTPases, G-proteins, kinases and phosphatases, transcription factors and factors regulating RNA processing: thus, tight junctions are a signalling

focal point. The organisation of signalling networks is regulated by proteins containing multiple protein–protein interaction domains such as PDZ, SH2 or SH3 domains. Although there is increasing recognition that tight junction proteins interact interdependently as a network, for the purposes of this Introduction they can be considered as forming three major tight junction protein complexes: the ZO protein complex, the Crumbs—Stardust–PATJ complex, and the Par3–atypical protein kinase C (aPKC)–Par6 complexes.

ZO protein complex

ZO-1 is a classical scaffolding protein that contains three PDZ domains, an SH3 domain and a guanylate kinase domain (Funke et al., 2005). It can directly associate with the integral membrane proteins occludin, claudins and JAMs and may cluster them at the tight junctions. ZO-2 and ZO-3 can also interact with occludin and claudin (Itoh et al., 1999a; Itoh et al., 1999b). Between the individual ZO proteins, some redundancy might exist. Loss of ZO-1 delays tight junction formation (Umeda et al., 2004), but blocking tight junction formation requires loss of all three ZO proteins (Umeda et al., 2006). The exact composition of the ZO complex is not certain, but ZO-1 forms independent complexes with ZO-2 and ZO-3 and can interact with other cytoplasmic proteins, including F-actin (Itoh et al., 1997).

ZO proteins are involved in receiving and delivering signals. ZO-1, for example, binds and sequesters regulatory molecules at tight junctions, influencing gene expression (reviewed in Matter and Balda, 2003). In this way, ZO-1 promotes proliferation of epithelial cells by indirectly regulating expression of proliferation genes and other cell cycle proteins (Balda et al., 2003; Balda and Matter, 2000). ZO-2 contains nuclear localisation and nuclear export signals and can interact with transcription factors and other nuclear-associated proteins, and ZO-2 may restrict transcription factors to the tight junctions in confluent cells and relocate with them to

the nucleus in subconfluent cells (Betanzos et al., 2004). Thus, ZO-1 and -2 regulation of proliferation is dependent on cell polarity.

Par3-aPKC-Par6 complex

The Par3-aPKC-Par6 complex is a highly conserved protein complex that is involved in the formation and maintenance of polarity in many cell types, including those without tight junctions. The Par complex appears to be more important in the formation of tight junctions rather than their maintenance, as dominant negative mutants of Par3, Par6 or aPKC affect tight junctions when expressed during the formation of cell-cell contacts, but not in cells that are already fully polarised (Chen and Macara, 2005; Gao et al., 2002; Nagai-Tamai et al., 2002; Suzuki et al., 2001; Yamanaka et al., 2001).

During tight junction maturation Par3 appears to be recruited before, and serve as a scaffold for, aPKC and Par6 (Suzuki et al., 2002). Par3 binds specifically to JAM-A, -B and -C (Ebnet et al., 2003; Ebnet et al., 2001), and it may be the interaction with JAM-A that recruits Par3 to the tight junctions (Itoh et al., 2001). Thus, Par3-aPKC-Par6 is not required for the positioning of primordial adhesion sites in mammalian epithelia, but for their subsequent reorganisation. Once the Par3-aPKC-Par6 complex is localised at primordial contacts, the kinase activity of aPKC is required for their maturation into belt-like adherens and tight junctions (Suzuki et al., 2002; Suzuki et al., 2001). Activation of aPKC could be facilitated by the binding of active Cdc42 or Rac1 to Par6 and the subsequent conformational change of Par6, which allows aPKC to become activated (Garrard et al., 2003; Yamanaka et al., 2001). Both E-cadherin or nectin-2 can activate Cdc42 and Rac1 and so could potentiate activation of aPKC and so the Par3-aPKC-Par6 complex (Fukuhara et al., 2003; Kawakatsu et al., 2002; Nakagawa et al., 2001). Par3 can interact with the Rac1 GEF Tiam1 (Chen and

Macara, 2005), but the role of this in tight junction development is not clear. One study showed that knockdown of Par3 in MDCK cells increased Tiam1-Rac1 signalling and inhibited tight junction formation (Chen and Macara, 2005). Another study, in keratinocytes, knocked down Tiam1 or Rac1 to demonstrate that the activity of these proteins is required for tight junction formation and act upstream of Par3 (Mertens et al., 2005). The discrepancy between these studies may reflect differences in cell type, but it is more likely to illustrate the fine spatiotemporal balance of polarity proteins and GTPases required for adhesion formation. Interestingly, aPKC expression in Tiam1 knockout keratinocytes rescues actomyosin reorganisation and tight junction formation (Mertens et al., 2005), indicating that aPKC acts downstream of Tiam1 and Rac1 in this situation. Indeed, Tiam1 associates with Par3 and aPKC in an interaction that leads to phosphorylation (activation) of aPKC in single keratinocytes (Pegtel et al., 2007).

The activity of aPKC can then promote the maturation of cell–cell contacts and the development of tight junctions from nascent adhesions. The mechanism behind this is not clear. aPKC could phosphorylate tight junction proteins and regulate their localisation or function; indeed, it can phosphorylate occludin, claudin-1 and ZO-1 (Nunbhakdi-Craig et al., 2002), but this has been only demonstrated *in vitro*. Alternatively, aPKC could indirectly influence tight junction formation by regulating cell polarity. In polarised epithelial cells, another serine-threonine kinase, Par1, localises to the basolateral membrane (Bohm et al., 1997). aPKC phosphorylates Par1, leading to dissociation from the membrane into the cytoplasm (Hurov et al., 2004). In *D. melanogaster* epithelial cells, Par1 phosphorylates Par3/Bazooka, inhibiting its dimerisation and blocking its interaction with aPKC and Par6 (Benton and St Johnston, 2003). This reciprocal inhibition thus generates two distinct membrane domains - Par1 at the basolateral domains and Par3-aPKC-Par6 at

apicolateral sites - and it is possible that aPKC could indirectly promote tight junction formation in this way. However, it is not clear whether the involvement of Par3-aPKC-Par6 in tight junction formation and cell polarity are actually linked.

Rich-Amot complex

In contrast to the Par3-aPKC-Par6 complex, the Rich1-Amot protein complex appears to regulate the maintenance rather than the formation of tight junctions (Wells et al., 2006). Rich1 is a Cdc42 and Rac1 GAP, but appears to selectively activate Cdc42 in epithelial cells (Richnau and Aspenström, 2001; Wells et al., 2006). Amot is a scaffolding protein that localises the complex to tight junctions by interacting with PATJ (Bratt et al., 2002). However, Rich1-Amot can also interact with Pals1, Par3 and aPKC (Wells et al., 2006). Two other Amot-like proteins are also components of the tight junction (Nishimura et al., 2002; Patrie, 2005). However, the precise function of these proteins and their interactions are not yet clear.

2. 3. Gap junctions

Gap junctions are specialised cell junctions that allow passive diffusion of ions and small molecules between cells through intercellular channels (reviewed in Bukauskas and Verselis, 2004; Wei et al., 2004a). Each hemichannel is a hexamer of connexins; connexins are conserved proteins comprising of four transmembrane domains and a cytoplasmic C-terminus that carries out regulatory functions (Bukauskas and Verselis, 2004; Wei et al., 2004a). Connexins are widely expressed and analysis of knockout mice suggests that different connexins may function differently in specific cells and tissues (reviewed in Wei et al., 2004a).

Hemichannels can localise at specific areas of the cell membrane to form plaques, often at adherens junctions (Angst et al., 1997). Connexin-43 can bind ZO-1 through its C-terminus, which modulates the trafficking and turnover of connexin-43 (Giepmans et al., 2001). Connexin-43 also affects tight junction integrity, as connexin-43 knockout in epicardial cells leads to redistribution of ZO-1 to the cytoplasm and a disruption in epithelial sheet organisation (Rhee et al., 2009). Also, mislocalisation of connexin-43 plaques to the lateral surface can be associated with mislocalised adherens junction proteins (Matsushita et al., 1999).

2. 4. Epithelial-to-mesenchymal transition

Epithelial cells are motile; for example, they are able to swap neighbours. Nonetheless, they do so while remaining within an organised epithelial layer and associated with the basal lamina, maintaining their apico-basal polarity. Mesenchymal cells contact their homotypic neighbours through looser cell-cell contacts, and this is reflected in their different organisation of junctional proteins and cytoskeleton when compared to epithelial cells. EMT refers to a series of changes encompassing the loss of apico-basal polarity and the transition to a mesenchymal cell type (reviewed in Nieto, 2012).

EMTs are important for a range of embryological processes. In the context of neural crest cells, the transition from a fully polarised epithelial tissue, with relatively stable cell-cell adhesions, to a mesenchymal cell population, marks the change from a pre-migratory to a migratory population. EMT of the neural crest is accompanied by delamination. Delamination can have varying definitions, depending on the context and on the author, and is sometimes considered to be a component of neural crest EMT. However, delamination is defined here as the splitting of a tissue into two

populations (Theveneau and Mayor, 2012). In the cranial neural crest in *X. laevis*, neural crest cells delaminate as a group from the surrounding neuroectoderm before undergoing EMT to become a migratory cell population (Theveneau and Mayor, 2012). Nonetheless, it is important to note that the temporal relationship between EMT and delamination differs between neural crest cell populations in different species and at varying anterior-posterior locations.

EMT is a dynamic process that requires complex alterations to cellular architecture. Overall, there is a change in cell-cell junctions and a disruption of apico-basal polarity, accompanied by changes in cytoskeletal organisation and regulation of a variety of targets, such as small GTPases. Proteins associated with the epithelial phenotype are downregulated and those required for cell migration upregulated. Neural crest maintenance genes are important for EMT of the neural crest and genes from the Snail family play a particularly important role (see Neural crest maintenance, page 24).

As epithelial cells, premigratory neural crest cells have well-established adherens and tight junctions that must be downregulated. This is a key feature of EMT - indeed, EMT of neural crest cells can be stimulated by disruption of junctional complexes with collagenase treatment or Ca^{2+} -free medium *in vitro* (Newgreen and Gooday, 1985). Neural crest adhesion molecules are altered during EMT. For example, classical cadherins can be separated into type I, usually expressed in epithelial cell populations, and type II, usually expressed by mesenchymal cells. As such, type-I cadherins such as E-cadherin are generally downregulated during EMT and type-II cadherins such as cadherin-11 are often upregulated during EMT.

Many signalling pathways that control EMT converge on the control of the type I cadherin, E-cadherin. Repression of E-cadherin by transcription factors Snail1 and

Snail2 leads to loss of E-cadherin-dependent junctional complexes (Bolos et al., 2003; Cano et al., 2000). Snail1 can also induce EMT in cultured *M. musculus* epithelial cells by suppressing claudin and occludin expression (Ikenouchi et al., 2003). Although E-cadherin is downregulated during EMT, adhesion molecules are essential for neural crest migration, as illustrated by the fact that some type-II cadherins are upregulated at this time. Type-II cadherins required for neural crest cell migration include cadherin-7, which is possibly upregulated by FoxD3 and Sox10, and cadherin-11 (Cheung et al., 2005; Hadeball et al., 1998; Nakagawa and Takeichi, 1995). Although type-I classical cadherins are typically considered to be downregulated during EMT this is not always the case, as the type-I cadherin N-cadherin is expressed in migrating cranial neural crest cells (Bronner-Fraser et al., 1992; Theveneau et al., 2010; Xu et al., 2001).

Stable cell-cell junctions being dissolved leads to lower adhesiveness and an increase in motility. However, cells are still able to form cell-cell contacts, allowing CIL and collective migration. Interestingly, connexin-43 is also upregulated during neural crest EMT (Lo et al., 1997), and is important for invasion into the embryonic heart (Rhee et al., 2009). Whether migration requires the gap junction channel is unknown; however, analysis of transgenic and knockout *M. musculus* lines have found no correlation between the level of gap junctional coupling and cell motility (Francis et al., 2011).

EMT can be triggered by extracellular signals such as secreted ligands of the TGF β and FGF families. For example, in epithelial cell cultures TGF β receptors phosphorylate Par6, leading to Par6 association with the E3 ubiquitin ligase Smurf1 (Ozdamar et al., 2005). Smurf1 controls local RhoA degradation by ubiquitinating it; thus, TGF β receptor activation promotes tight junction dissolution (Ozdamar et al., 2005). Further, tyrosine kinase signalling leads to sequestering of aPKC-Par6 away

from Par3 by Numb during EMT (Wang et al., 2009). Thus, polarity protein activity and relocalisation appears to be important in EMT.

Cross-regulation occurs between cell-cell and cell-matrix adhesions. For example, endocytosis of E-cadherin activates the small GTPase Rap1, which in turn promotes focal-adhesion formation (Balzac et al., 2005). This sets in motion a positive-feedback loop, as integrin-linked kinase, which is activated by integrin- β 1 and - β 3 interactions with ECM and growth factors, downregulates E-cadherin expression (Oloumi et al., 2004). Integrin- β 1 can disrupt cadherin-mediated adhesions through activation of RhoA and Rac1 (Gimond et al., 1999). Rac activation is associated with lamella extension and constitutively activated Rac1 does not allow the establishment of contacts, while RhoA promotes actomyosin-mediated contraction, in turn strengthening focal adhesion association with the ECM and retraction of the cell rear (Chu et al., 2004). Thus, RhoA needs to be downregulated at sites of cell-cell contact (Ozdamar et al., 2005) but activated at focal adhesions (Gimond et al., 1999). Thus, the temporal and spatial activity of small Rho GTPases must be tightly regulated.

These processes do not follow a strict series of checkpoints. Time-lapse imaging of trunk neural crest cells migrating away from the neural tube in *G. gallo* shows that, for example, α -catenin is usually downregulated before the cell rear is retracted, but that it sometimes remains at the tip of the tail during retraction (Ahlstrom and Erickson, 2009). In fact, the cell tail can tear distal to the α -catenin spot during retraction, illustrating that neither the adherens junction nor apicobasal polarity needs to be completely disrupted before commencement of neural crest cell migration (Ahlstrom and Erickson, 2009).

EMTs occur normally in development but ectopic EMTs also play a central role in tumour invasion. Acquisition of mesenchymal characteristics changes adhesive and

proteolytic properties, which allows invasion and thus the establishment of secondary tumours (reviewed in Theveneau and Mayor, 2012).

As noted above, there are important differences in the EMT, delamination and migration of neural crest cell populations in various species and at various anterior-posterior positions. Similarly, there are also variations between the many different types of cancer. For example, cancer cells can migrate collectively or as individual cells. Further, in cancer cells the acquisition of a mesenchymal phenotype can be stable or transient (reviewed in Hanahan and Weinberg, 2011). Nonetheless, the mesenchymal gene expression patterns acquired through EMT and involved in tumour progression are strikingly similar to those that regulate developmental EMT. Indeed, Snail genes, which were first described in the neural crest, have subsequently been found to be essential for EMT of cancer cells (reviewed in De Craene et al., 2005). For example, Snail1 regulates the expression of EMT-associated genes in colorectal carcinoma cells.

3. Par3

3.1. Par proteins and the *C. elegans* zygote

Of the three major tight junction proteins complexes, the Par3-aPKC-Par6 complex is the most ancient in evolutionary terms. The Par proteins are a group of structurally unrelated proteins that were first observed to establish the initial anteroposterior axis in the *C. elegans* zygote (Kemphues et al., 1988; Morton et al., 2002; Watts et al., 1996). The embryos identified during the maternal-effect mutant screens in which the Par proteins were discovered had unusually synchronous and equal cell divisions (Kemphues et al., 1988). The term ‘Par’ represents ‘partitioning-defective’, as the embryos did not correctly partition cytoplasmic P granules into appropriate daughter cells during the first few mitotic events (Kemphues et al., 1988). Par proteins have turned out to be highly conserved, and homologues of all except Par2 have subsequently been shown to be key regulators in determination and maintenance of cell polarisation throughout the metazoa (Fig. 3.1). There are seven Par proteins in total: Par1, Par4 and aPKC are serine threonine kinases, Par2 is a ring-finger domain protein similar to E3 ubiquitin ligases, Par3 and Par6 are PDZ domain-containing scaffold proteins and Par5 is a member of the 14-3-3 family of signalling proteins (reviewed in Goldstein and Macara, 2007).

In the *C. elegans* zygote, the sperm centrosome or its associated microtubules contact the cortex (Goldstein and Macara, 2007), providing a cue that identifies the posterior pole of the embryo and initiates the development of polarised cortical domains (Cowan and Hyman, 2004). This as-yet-unidentified signal appears to weaken the actomyosin network locally and transiently, which leads to asymmetrical contraction (Munro et al., 2004). The resulting flow of cortical actin transports Par3-aPKC-Par6

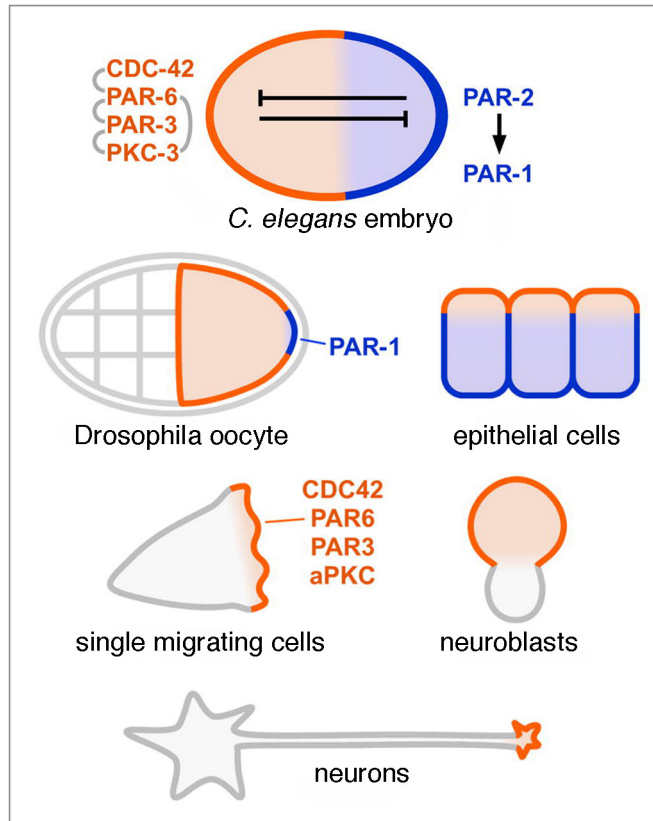


Figure 3.1. Par3 localisation in various cell types

Par3 localisation is consistently polarised in various cell types. Par3, alone or in conjunction with Par6, aPKC, Cdc42 (or a combination thereof) is found at the areas shown in orange. Par1 is found at the areas indicated in blue.

(Adapted from Goldstein and Macara, 2007).

towards the future anterior pole of the zygote; meanwhile, Par2 is recruited to the cortex close to the sperm centrosome (Etemad-Moghadam et al., 1995; Munro et al., 2004; Tabuse et al., 1998). Interestingly, it seems that the Par3 complex is able to promote cortical flows and its own transport, whereas Par2 is able to inhibit its own recruitment to cortical myosin by an unknown mechanism.

aPKC can phosphorylate Par1, inhibiting its cortical association and affecting its activity (Hurov et al., 2004; Kusakabe and Nishida, 2004); thus, the presence of Par2 and absence of the Par3 complex allows Par1 to become active at the *C. elegans* zygote posterior cortex (Cheeks et al., 2004; Cowan and Hyman, 2004; Munro et al., 2004). Par1 in turn is able to phosphorylate Par3, blocking its interaction with aPKC and the association of the Par3 complex to the cortex (Benton and St Johnston, 2003; Hurd et al., 2003). The competitive exclusion between Par1 and the Par complex is important not only in the development of polarity, but also for its maintenance. Par4 and Par5 remain symmetrically localised, but Par5 facilitates cell polarity by binding the phosphorylated forms of both Par1 and Par3 and removing them from the cortex (Morton et al., 2002). Par4 is required for the asymmetry of other Par proteins, but the actual mechanism behind this is not clear.

Interaction with microtubules is a common theme throughout studies into the effect of Par3 (and the Par complex) on polarity and migration. Even during symmetry breaking in the *C. elegans* zygote, microtubules and Par proteins interact. In the absence of cortical flows, embryos depend solely on Par2 for symmetry breaking, and Par2 localisation to the prospective posterior domain is dependent on microtubules (Zonies et al., 2010). Par2-microtubule interaction inhibits aPKC phosphorylation of Par2 *in vitro*. This might allow Par2 localisation to the cortex and establish polarised cortical domains, which can then be maintained by reciprocal inhibition between Par3

and Par1 (Zonies et al., 2010). Thus, in wildtype embryos Par2-microtubule binding may work in parallel with cortical flows to quickly load Par2 to - and displace Par complex proteins from - the future posterior cortex (Zonies et al., 2010).

As well as being affected by microtubules, Par proteins are able to influence microtubules themselves, organising the microtubule array to regulate cell polarity (reviewed in Goldstein and Macara, 2007). Par3 stabilises microtubules at the anterior pole (Labbe et al., 2003), and spindle-severing experiments illustrate that Par3 regulates pulling forces on microtubules (Etemad-Moghadam et al., 1995; Grill et al., 2001). Asymmetry in the stabilisation of microtubules along the anterior-posterior axis correctly positions of the mitotic spindle, which is essential for correct location of subsequent mitotic events.

3.2. The Par complex(es)

In the Par complex, Par3 and Par6 undergo direct interactions with aPKC and are thought to regulate the localisation and activity of aPKC, respectively (Ohno, 2001). Par3-aPKC-Par6 can bind Cdc42 to regulate cell polarity, cytoskeletal rearrangement and tight junction assembly (Gao et al., 2002; Lin et al., 2000). These proteins often act as a complex (Macara, 2004), but Par6 and aPKC can also form a complex with each other separate to Par3 (Chen and Macara, 2005; Harris and Peifer, 2005; Schmoranzer et al., 2009; Tabuse et al., 1998; Zhang and Macara, 2008). In NIH 3T3 cells Par3, but not Par6 and only occasionally aPKC, localise to the cell-cell contact (Schmoranzer et al., 2009). This is in contrast to epithelial cells, where the three proteins strongly colocalise at cell-cell contacts (Macara, 2004). Even when they are localised close together, they may not always interact with each other. In *D.*

melanogaster embryos, Par6-aPKC can interact with marginal zone¹ members Crumbs and Stardust (a homologue of Pals1), while Par3 localises separately to the adherens junction (Harris and Peifer, 2005; Hurd et al., 2003; Lemmers et al., 2004). Par3 and Par6-aPKC can affect the same pathways in different ways. For example, in epithelial cells, Par3 is required for apico-basal polarity and Par6-aPKC bind and inhibit the basolateral membrane protein, Lgl (Betschinger et al., 2005; Yamanaka et al., 2003), which reciprocally prevents movement of Par6-aPKC into the lateral cortex (Chalmers et al., 2005; Hutterer et al., 2004). In endothelial cells, two Par complexes have been identified: one containing Par3-aPKC-Par6, and another where Par3 and Par6 associate with VE-cadherin but not aPKC (Iden et al., 2006). In other cell types, Par complex proteins are localised to opposing domains. For example, although Par3 is able to form a complex with Par6-aPKC and interact directly with JAM-C (Ebnet et al., 2003), Par3 is localised in a different area to JAM-C, Par6 and aPKC in polarised mice spermatids and JAM-C deficiency mislocalises Par6 and aPKC but not Par3 (Gliki et al., 2004). Thus, it appears that there are differences in protein interaction depending on the requirements of specific cell types.

As in the *C. elegans* embryo, Par complex proteins and Par1 tend to be located in different, if not opposing, locations in many situations. In polarised epithelial cells in vertebrates, Par1 localises to the basolateral membrane domain opposite the Par complex at the apicolateral junction (Bohm et al., 1997). In *D. melanogaster* epithelial cells, the Par complex is found in the marginal zone, whereas Par1 again localises to the basolateral domain (Benton and St Johnston, 2003). The reciprocal inhibition of the Par complex and Par1 leads competitive exclusion between them and

¹ The marginal zone is the most apical region of cell-cell contact. It is equivalent to the vertebrate epithelial cell tight junction.

so the establishment and maintenance of cell polarity through the formation of two distinct membrane domains.

3. 3. Par3 and cell polarity

Exogenous Par3 expression does not affect the epithelial polarity of confluent cells, but transforms the morphology of cells cultured at low density into a fibroblastic form with high motility and protrusions that continues when the cells contact each other again, even though E-cadherin and ZO-1 localisation are not affected (Mishima et al., 2002). Low concentration of nocodazole, which depolymerises microtubules, also inhibits the effect of Par overexpression. This suggests that the effect of Par3 is in some way linked to the regulation of microtubule dynamics. Interestingly, both dominant negative and constitutively active forms of Rac1 also suppress the effect of Par3 overexpression (Mishima et al., 2002). These observations suggest that Par3 overexpression causes abnormal activation of Rac1, perhaps by perturbing the balance of small GTPase activation, and thus induces cell migration even after forming extensive cell-cell adhesions. Such seemingly contradictory results, along with a link to Rac1 and the microtubule array, are common themes in studies of Par3. The role of Par3 in epithelial cells has been discussed previously in this Introduction (see Tight junction protein complexes, page 55). This sub-section will investigate Par3 in other situations.

3. 3. 1. Par3 and polarity in migrating cells

During wound healing, the apicobasal polarity of epithelial cells is disrupted and the polarity proteins are redistributed. The tight junction protein occludin accumulates at the leading edge of migrating MDCK cells during wound healing (Du et al., 2010). Occludin can directly interact with aPKC, which may be the mechanism with which it

also recruits Par3-aPKC and PATJ to the leading edge and promotes directional migration (Du et al., 2010). Par3-aPKC localisation at the leading edge is required for centrosome polarisation and leads to the perpendicular orientation of microtubules with respect to the wound (Du et al., 2010; Shin et al., 2007). Par3 also regulates centrosome positioning in the other cell types, such as the developing *D. rerio* neural tube (Hong et al., 2010).

However, in wound-edge fibroblasts, Par3 localises to cell-cell contacts, along with microtubule ends and dynein (Schmoranzer et al., 2009). LPA stimulates actomyosin contraction and cortical flow to push the nucleus towards the back of the cell, resulting in reorientation of the nucleus-centrosome axis and polarisation of the cell (Gomes et al., 2005). Knockdown of Par3 or dynein disrupts positioning of the centrosome; it moved rearward with the nucleus (Schmoranzer et al., 2009). LPA stimulation does not affect microtubules at the leading edge, but causes microtubules at cell-cell contacts to become less dynamic (increase pausing) in a Par3-dependent manner (Schmoranzer et al., 2009). In starved cells, microtubules spend the same amount of time pausing at cell-cell contact and at the leading edge. These results suggest that Par3 interacts with dynein to tether microtubules to the cell-cell contact, perhaps generating tension on microtubule ends to allow them to resist forces (such as actin retrograde flow) that would otherwise displace the centrosome. Par3 and dynein are also required for wound closure and migration (Schmoranzer et al., 2009). Interestingly, Par6 was not observed at the cell-cell contact and aPKC was rarely found at the cell-cell contact (Schmoranzer et al., 2009), but aPKC-Par6 has been shown to be important for centrosome orientation in a previous study in the same cell types (Gomes et al., 2005). Thus, cytoplasmic aPKC-Par6 also appears to have an as-yet-unidentified role in centrosome localisation.

In wounded astrocyte monolayers, endogenous Par3 localises to the cell-cell contacts, while Cdc42 binds Par6 and activates aPKC at the leading edge (Etienne-Manneville and Hall, 2001). But, in this case, Par3 does not appear to be involved in cell polarity at all (Etienne-Manneville and Hall, 2001). Instead, aPKC-Par6 promotes protrusion by recruiting Rac1 (Etienne-Manneville and Hall, 2001). aPKC-Par6 controls centrosome translocation through inactivation of the kinase glycogen synthase kinase (GSK) 3 β (Etienne-Manneville and Hall, 2003), and/or by recruiting Disks large homologue (Dlg1) to the leading edge (Etienne-Manneville et al., 2005), which both promote the interaction of the microtubule-associated protein adenomatous polyposis coli (APC) with microtubule ends. APC-microtubule interaction has been suggested to anchor microtubule ends to the cortex and allow them to recruit dynein-dynactin (Etienne-Manneville and Hall, 2003; Etienne-Manneville et al., 2005), although this has not yet been tested.

Par3 localises to the leading edge in keratinocytes and recruits the Rac GEF Tiam1 (Pegtel et al., 2007). These two proteins, along with aPKC, are required for directional migration in single keratinocytes and MDCK cells (Pegtel et al., 2007). It is important to note that this affects directional migration but not migration *per se* (Pegtel et al., 2007), illustrating a role in polarity determination. Thus, Par3 seems to regulate two different polarity states: the relatively permanent apico-basal polarity and the more transient front-rear polarity during directional migration. Tiam1 knockout in keratinocyte monolayers also inhibits polarisation after wounding and decreases healing, but the localisation of neither Par3 nor Tiam1 have been analysed (Pegtel et al., 2007).

3. 3. 2. Par3 and the microtubule array

In single cells, Par3 and Tiam1 regulate keratinocyte polarity by controlling microtubule dynamics. Single migrating keratinocytes treated with nocodazole lose front-rear polarity and lose directional migration similar to Par3 or Tiam1 knockout cells (Pegtel et al., 2007). Loss of Par3, Tiam1 or aPKC does not seem to affect the number or length of microtubules, but does reduce the amount of stable microtubules (Pegtel et al., 2007). Rac activity was decreased in Tiam1-depleted cells (Pegtel et al., 2007), suggesting that Par3-Tiam1 may control directional migration by activating Rac1 at the leading edge. Constitutively active Rac1 could not rescue the Tiam1-depleted phenotype (Pegtel et al., 2007), suggesting that Rac1 may need to be activated in a polarised manner in order to promote directional migration. Par3 can also bind the Rac GEF Tiam2 in cultured neuroblastoma cells and hippocampal neurons, where they are localised to the axonal tip and act downstream of Cdc42 to activate Rac1 and so promote lamellipodial activity, axon outgrowth and neuronal polarisation (Nishimura et al., 2005). In turn, Cdc42 can be recruited to the Par complex, binding Par6 and locally promoting the kinase activity of aPKC. This illustrates that interaction between Par proteins and small GTPases can be bidirectional, allowing Par proteins to regulate their own (or each others) activity. Par3 itself can also be affected by Rho GTPase signalling. ROCK can phosphorylate Par3 to disrupt interactions between the two complexes of aPKC-Par6-Cdc42 and Par3-Tiam1 and so disrupt activation of Rac1 (Nakayama et al., 2008). This impairs cell polarity and directional migration. As ROCK is most active at the back of migrating cells (Nakayama et al., 2008), this may promote directional migration by inhibiting Rac activation and so lamellipodia extension away from the leading edge.

Par complex proteins and the microtubule array also affect cell polarity by regulating vesicle trafficking. Two types of motor proteins are associated with microtubules:

kinesins, which move away from the centrosome, and dyneins, which move towards the centrosome. Both carry cargo along microtubules. Par proteins can directly interact with motor proteins or indirectly affect their destination by regulating the microtubule array. In *D. melanogaster* embryos, Cdc42, Par3, Par6 and aPKC regulate junctional integrity by preventing endocytotic uptake of apical proteins from the plasma membrane and promoting the processing of apical proteins from the early to late endosomal compartments (Georgiou et al., 2008; Harris and Tepass, 2008; Leibfried et al., 2008). Again, there are variations in different cell types, even within the same animal model: in the *D. melanogaster* dorsal thorax, Cdc42, Par6 and aPKC directly regulate endocytosis at adherens junctions but Par3 is not involved in trafficking (Georgiou et al., 2008; Leibfried et al., 2008). Par3 and/or the Par complex determine polarity of many neuronal cell types but, in developing neurones in *D. melanogaster* embryos *in vivo*, Par3/Par6/aPKC complex is not asymmetrically localised and is not required for their polarisation (Rolls and Doe, 2004).

Conversely, motor proteins can regulate localisation of polarity proteins by transporting them along microtubules. For example, Par3-aPKC-Par6 can associate with the kinesin Kif3a to be localised to cilia in MDCK cells (Fan et al., 2004). Kif3a and the microtubule-associated protein APC mediate polarity of developing hippocampal neurons by transporting Par3-aPKC-Par6 to the tip of one immature neurite, which subsequently promotes growth cone extension by activating Rac1 through Tiam2 (Shi et al., 2004a). However, the situation may actually be more complicated, as aPKC could promote the transport of Par3-aPKC-Par6 itself by facilitating the loading of Kif3a onto microtubules (discussed in Wiggin et al., 2005). Dynein-based transport also contributes to establishment and maintenance of apical Par3 localisation and thus epithelial polarity in *D. melanogaster* embryos (Harris and Peifer, 2005). In *D. melanogaster* epithelial cells, Par3 localises to the apicolateral

boundary independently of cadherins and is then required for cadherin and β -catenin recruitment (Harris and Peifer, 2004; Wei et al., 2005). Thus, dynein may be required for Par3 localisation in contexts where cadherins do not recruit Par3 to the cell-cell contact.

4. Microtubules

Microtubules are one of the three main types of cytoskeletal networks in cells, along with actin filaments and intermediate filaments. Microtubules provide shape and structure to the cell and form the mitotic spindle, segregating chromosomes during division. They are important in many dynamic cellular processes. For example, microtubules interact with and regulate cell-cell and cell-substrate adhesion machinery and contribute to cell polarisation and migration.

4.1. Microtubule structure

Microtubules are a combination of α - and β -tubulin monomers (Fig. 4.1). These monomers form α -tubulin and β -tubulin dimers, which polymerise head-to-tail in linear protofilaments. Protofilaments associate laterally with each other to form hollow tubes, but do not quite align parallel with each other. As α -tubulin- β -tubulin dimers are asymmetrical, the resulting microtubules are also asymmetrical, with so-called 'plus' and 'minus' ends. The α -tubulin of each protofilament is at the minus end. The minus end has much slower dynamics than the plus end and is often anchored to the centrosome or other microtubule-organising centre. The centrosome is formed by two centrioles and surrounded by proteins necessary for microtubule nucleation and anchoring (Bornens et al., 1987).

Interfering with the delivery of centrosomal proteins to the centrosome disrupts its integrity and the radial organisation of the microtubule array (Dammermann and Merdes, 2002; Quintyne et al., 1999). Nonetheless, the centrosome can be disrupted without affecting microtubule nucleation (Dammermann and Merdes, 2002;

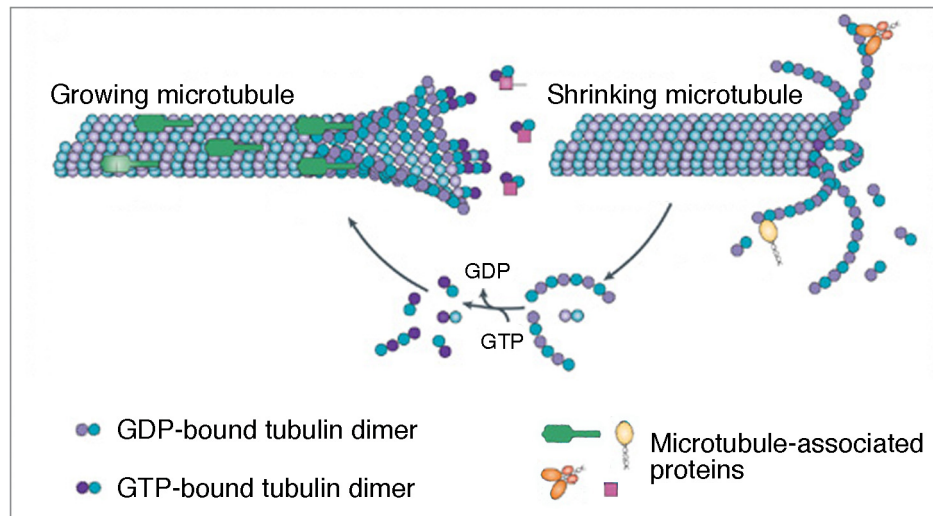


Figure 4.1. Microtubule dynamics

Microtubule plus-ends are in a state of dynamic instability. Tubulin dimers are added to the end of microtubule plus-ends during growth phases and GTP is hydrolysed to GDP. Release of tubulin dimers from a microtubule during catastrophe leads to loss of the GTP cap and protofilaments to peel off. Microtubules undergoing catastrophe can be rescued and recommence the growth phase. Microtubule-associated proteins can be found on growing and shrinking plus-ends, on tubulin dimers and on the microtubule shaft.

(Adapted from Conde and Caceres, 2009)

Quintyne et al., 1999). In some cell types, minus ends are not associated with the centrosome. This can be caused by microtubule severing (Odde et al., 1999; Waterman-Storer and Salmon, 1997), release from the centrosome (Keating et al., 1997), or assembly in other parts of the cell, such as the Golgi complex (Efimov et al., 2007). For example, in migrating newt lung cells up to 90% of the microtubules are not bound to the centrosome (Waterman-Storer and Salmon, 1997), and in epithelial cells microtubules form bundles parallel to the apico-basal axis with the minus ends localised to the adherens junctions (Bacallao et al., 1989). Also, axonal microtubules in neurones are not anchored to the centrosome (Baas and Ahmad, 1992). In these cells, minus-ends remain essentially static as they are capped by specialised factors such as γ -tubulin and Nezhha (Meng et al., 2008). Nonetheless, this capping is stochastic, and microtubules can lose the cap and quickly depolymerise from the minus end (Keating et al., 1997).

Plus ends grow from the microtubule-organising centre into the cytoplasm. Microtubule plus ends can exist in one of three states: growth, shrinkage or pause (Fig. 4.1). The growth phase occurs when the microtubule is being polymerised by tubulin dimers. Shrinkage is a synonym for depolymerisation. At times when the microtubule end is neither growing nor shrinking, the microtubule is referred to as being paused. A change from growth to shrinkage is known as catastrophe, while from shrinkage to growth is termed rescue. Plus ends can change stochastically between the states of growth, pause and shrinkage, in a non-equilibrium process that is known as dynamic instability (Mitchison and Kirschner, 1984). Plus end dynamics can be described by five parameters: the rates of growth and shortening, frequency of catastrophes, frequency of rescues, and time spent in pauses. It must be noted that it is as yet unclear whether pausing microtubules are completely stable or whether they are

actually undergoing phases of growth and shrinkage at either low speed or high transition frequency that cannot be resolved with current imaging techniques. Nonetheless, within any cell, one would expect to find coincident growing, shrinking and stable microtubules. Dynamic instability allows the microtubule array to continuously probe the cell's three-dimensional space and adapt quickly to changing stimuli (Kirschner and Mitchison, 1986).

Tubulin is a nucleotide triphosphatase and, in the cytoplasm, each α -tubulin- β -tubulin dimer binds two GTP molecules (reviewed in Galjart, 2010). One GTP binds α -tubulin and is non-exchangeable; the other binds an exchangeable site of β -tubulin. Incorporation of the tubulin dimer into the growing microtubule is thought to trigger hydrolysis of β -tubulin-bound GTP to GDP. Therefore, β -tubulin along the length of the microtubule is bound to GDP, while a growing plus end is capped by the most recently polymerised dimers containing GTP-bound β -tubulin (Carlier and Pantaloni, 1982). GTP-bound tubulin is straight and allows polymerisation, whereas it is more energetically favourable for GDP-bound tubulin to adopt a curved conformation. So, the 'cap' of straight, GTP-bound tubulin, which also forms strong lateral bonds, is important in preventing microtubule depolymerisation. Without this cap, protofilaments tend to curve and 'peel off' from the microtubule, breaking lateral bonds and causing catastrophe. There is as yet no consensus regarding the size or indeed precise function of the cap; however, it is interesting that individual protofilaments within a microtubule can contain a varying amount of GTP-bound tubulin subunits at the plus-end cap, and that individual protofilaments can shorten when the microtubule is in growth phase (Schek Iii et al., 2007). Nonetheless, the GTP cap appears to be important in regulating dynamic instability of the microtubules.

4. 2. **Microtubule dynamics**

Microtubules form a highly dynamic and ever-changing array that reaches throughout the entire cell and can react quickly to stimuli. Dynamic instability is particularly well suited to restructuring and self-assembly of the microtubule network, but the high rigidity of microtubules would tend to limit such network plasticity (Brangwynne et al., 2007). In order for the microtubule array to explore the cell, microtubules must be able to grow along a variety of paths rather than repeatedly growing along the same straight path. Indeed, fixed microtubules show bends (Brangwynne et al., 2007), and the growing ends of microtubules are more flexible than the central region (Janson and Dogterom, 2004; Kurachi et al., 1995). During microtubule growth, intracellular forces cause fluctuations in the orientation of the growing microtubule tip, curving its growth trajectory (Brangwynne et al., 2007). Microtubules frequently change their direction of growth, suggesting a persistent random walk trajectory (Brangwynne et al., 2007). Once the microtubule has grown, the resulting bend is stabilised by the surrounding elastic cellular network (Brangwynne et al., 2007). Thus, microtubules are rigid and reinforced by the surrounding network but at the same time are deformable during growth (Brangwynne et al., 2007).

The dynamics of microtubules are central to their biological functions and are different in different cell types: for example, microtubules are less dynamic in epithelial cells than fibroblasts (Wadsworth and McGrail, 1990). Microtubule dynamics can be altered by various situations. Differences in epithelial and fibroblast microtubule dynamics may reflect the relative stability of differentiated, polarised cells. Cultured human endothelial cells within a monolayer have a lower average microtubule growth rate than single cells because single cells have a subgroup of microtubules that grow particularly quickly (Alieva et al., 2010), illustrating that microtubule dynamics can be affected by the extracellular environment. Epithelial

cells and fibroblasts migrating directionally into a wound have a greater number of growing microtubules per unit area at sites of cell-cell contact than at the leading edge (Salaycik et al., 2005), showing that microtubule dynamics can be regulated differently in different parts of the cell and that this may also be influenced by extracellular factors such as the cell-cell contacts and/or cell polarity.

A number of post-translational modifications are known to occur to tubulin, usually when it is polymerised. The causes of these modifications are not well understood, although the consequences have been somewhat clarified. Stable microtubules in particular appear to accumulate a variety of post-translational modifications. Microtubules can be covalently modified by a carboxypeptidase, which removes the C-terminal Tyr residue of α -tubulin, and a tubulin Tyr ligase that subsequently ligates it again (Hammond et al., 2008). Only α -tubulin that has been incorporated into a microtubule lattice can undergo detyrosination, but Tyr ligation occurs on tubulin heterodimers after depolymerisation. Detyrosinated microtubules are considered to be more stable than tyrosinated microtubules (Schulze et al., 1987). Tubulin detyrosination in fibroblasts inhibits microtubule disassembly (Peris et al., 2009), and detyrosinated microtubules have been observed in the presence of low concentrations of nocodazole, a compound that promotes microtubule depolymerisation (Conde and Caceres, 2009). However, microtubule stabilisation appears to induce detyrosination, which is thus considered a consequence rather than a cause of microtubule stabilisation (Khawaja et al., 1988).

Acetylation of a lysine on α -tubulin within microtubules is also associated with stability. Microtubule acetylation correlates well with a longer half-life but, as with detyrosination, it is thought that acetylation is a consequence rather than a cause of stability (Hammond et al., 2008). However, because deacetylation can occur on both polymerised tubulin or heterodimers, whereas tyrosination can only occur on tubulin

heterodimers, detyrosinated and acetylated microtubule populations may not necessarily overlap (Hammond et al., 2008). That is, microtubules of equivalent stability do not necessarily exhibit the same post-translational modifications. Indeed, both acetylated and detyrosinated microtubules more prevalent in axons than in dendrites, despite the fact that microtubules in axons and dendrites have similar stability (Hammond et al., 2010). This might be due to differences in the localisation of enzymes responsible for post-translational modifications and illustrates that post-translational modifications may give rise to some type of subcellular cytoskeletal differentiation. Although it is thought that post-translational modifications are the consequence rather than the cause of microtubule stability, it is conceivable that they do have some function. Indeed, this is supported by findings that alterations in the balance between tyrosinated and detyrosinated tubulin can affect tumour growth (Mialhe et al., 2001), disorganise neuronal circuits (Erck et al., 2005) and cause abnormalities in morphogenesis and polarity (Peris et al., 2006).

There are many isotypes of tubulin. These do have some function, as improper isotype expression leads to the loss of some axoneme function in *D. melanogaster* sperm (Raff et al., 1997). Different isotypes have different levels of stability, but dimer and microtubule polymer stability do not necessarily correlate (Panda et al., 1994). Nonetheless, it is interesting to note that cell lines exposed to taxol, a microtubule stabilising drug, can alter the distribution of isotypes (Downing and Nogales, 1998). The mechanism behind this is not understood, but it may be an adaptation to compensate for the change in microtubule stability induced by the drug.

4. 3. Microtubule-associated proteins

Microtubule dynamics are a fundamental component of the interaction between microtubules and the rest of the cell. This is underscored by experiments showing that even mild suppression of microtubule dynamics by low doses of microtubule stabilising or destabilising drugs can have a disproportionate effect on the organisation of the mitotic spindle and directional migration, as well as specific processes such as synaptogenesis (Jaworski et al., 2009; Liao et al., 1995). It is thus understandable that microtubule dynamics are tightly regulated and that control is exerted at a number of levels. Increasing or decreasing the pool of free tubulin can change microtubule growth rate and catastrophe frequency. Microtubule dynamics are also influenced through interactions with a large number of microtubule-associating proteins (MAPs), including proteins that promote microtubule assembly, stabilise or destabilise microtubules, cut microtubules and microtubule-based motors. MAPs can thus regulate microtubule dynamics spatially and temporally by influencing tubulin dimers, the microtubule wall lattice and/or the microtubule ends.

4. 3. 1. Plus-end tracking proteins

A specialised subgroup of MAPs is plus-end tracking proteins (+TIPs), which affect microtubule dynamics when they accumulate at the plus ends of growing microtubules. Since the first description of +TIPs, several theories have been proposed regarding the mechanisms leading to microtubule plus-end accumulation. The first was termed a ‘treadmilling’ mechanism, whereby the +TIP binds the growing microtubule and then dissociates after a couple of seconds due to modifications or conformational changes of the +TIP or a conformational change of the microtubule (Perez et al., 1999). An alternative explanation was that +TIPs co-polymerise with tubulin heterodimers. Subsequent experiments illustrated rapid association and disassociation of +TIPs with microtubule ends. This was shown by looking at the

single molecule level *in vitro* (Bieling et al., 2007) and by fast FRAP measurements in living cells (Dragestein et al., 2008). Increasing tubulin concentration *in vitro* increases microtubule growth rates without increasing association of +TIPs, and +TIP diffusion appears to be the rate-limiting factor for their binding to microtubule plus ends.

These results, which support neither treadmilling nor co-polymerisation ideas, led to the development of the ‘fast exchange’ model. In this model, microtubule polymerisation provides a large number of binding sites for +TIPs at the microtubule plus end. Each binding site would bind and dissociate a number of times with +TIPs, and then the binding sites would disappear exponentially as the microtubule continued to polymerise. A likely explanation for the presence and then disappearance of binding sites at the end of the microtubule is a structural difference between the end and the remainder of the polymer. The most obvious candidate for this is the GTP cap; however, +TIPs accumulate on up to 2 μm of the microtubule (Komarova et al., 2005). This is much longer than the supposed GTP cap length of 320 nm (Zovko et al., 2008), and also indicates that +TIPs interact laterally with microtubules as well as at the very tip; indeed, lateral interactions are more likely to occur.

Plus-tip tracking

There are a many different +TIPs that bind growing microtubule plus-ends. They have varying rates of turnover at microtubule ends, microtubule binding domains and mechanisms of recognising microtubule ends, based on which they have been loosely classified. Fluorescently labelled +TIPs are used to track the growing ends of microtubules, a process known as plus-end tracking (Perez et al., 1999; Salaycik et al., 2005; Stepanova et al., 2003). The first +TIP used for this purpose was a green-fluorescent protein (GFP)-labelled form of CLIP-170 (Perez et al., 1999). In time-lapse

imaging experiments, the fluorescent protein was observed as “comet-like” fluorescent dashes moving throughout the cell. It is important to note that individual +TIPs bind and dissociate very quickly from the microtubule plus ends. As the microtubule plus end moves through the cytoplasm, +TIPs are transiently immobilised at the growing microtubule end and do not actually move. Thus, the comet is just an optical illusion.

Previously, microtubule dynamics were observed using fluorescently labelled tubulin, but the density of microtubules in the cell body only allowed analysis at the cell periphery. Thus, imaging of +TIPs has the advantage of providing much more information about microtubule growth within the cell body. However, an inherent disadvantage of tracking microtubules using plus-tip tracking is that +TIPs bind only to growing microtubules. Nonetheless, details of phases of pause and shrinkage can be recovered from the growth trajectories: for example, two microtubule growth events that run along the same track and are separated by a short period of time are likely to be the same microtubule (Applegate et al., 2011). By linking such information from growth tracks, details about shrinkage and pause can be inferred (Applegate et al., 2011; Matov et al., 2010). Software (plusTipTracker) that provides this analysis and automatically tracks the +TIP comets has been recently developed, thus removing human error (Applegate et al., 2011).

EB proteins

Subsequent plus end tracking has also used fluorescently tagged end binding (EB) proteins. Like CLIP-170, three EB proteins (EB1, EB2 and EB3) are small dimers and each monomer contains conserved amino-terminal calponin homology (CH) and carboxy-terminal coiled-coil domains. Interestingly, CH domains, which are necessary and sufficient to recognise growing microtubule ends (Honnappa et al., 2005; Slep et

al., 2005), are typically found in actin-binding and signalling proteins (Gimona et al., 2002), indicating some other proteins that might also interact with microtubules. EB3 is upregulated in neurones and myocytes and is important in myotube differentiation and neuronal development (Jaworski et al., 2009; Straube and Merdes, 2007).

Like other +TIPs, EB proteins have been shown to control microtubule behaviour, influencing microtubule dynamics, stability and nucleating microtubules. EB1 family members are conserved in plants, yeasts and mammals (Tirnauer and Bierer, 2000). There is conflicting evidence regarding the role of EB proteins in regulating microtubule dynamics. Fission yeast Mal3p and vertebrate EB1 decrease catastrophe frequency; however, *D. melanogaster* EB1 and the budding yeast homologue Bim1p increase catastrophe frequency (Rogers et al., 2002; Tirnauer et al., 1999). *In vitro* studies show that addition of EB proteins promotes both growth rate and catastrophe frequency (Komarova et al., 2009; Vitre et al., 2008). As EB proteins associate autonomously with growing microtubule-regulating factors and also interact with other +TIPs (reviewed in Bieling et al., 2007; Lansbergen and Akhmanova, 2006), it may be that changing the amount of EB protein *in vitro* only affects their primary roles in controlling microtubule dynamics, whereas changing the amount of EB protein in cells also affects their association with other microtubule-regulating factors, thus complicating attempts to define the exact function of EB proteins. Nonetheless, ectopic expression of fluorescent EB proteins (or CLIP-170) at levels that allow observation and tracking of microtubule plus ends do not appear to affect microtubule dynamics, at least not compared to the microtubule dynamics in cells expressing fluorescent tubulin (Komarova et al., 2002).

+TIPs and microtubule dynamics

Although most +TIPs can bind directly to the microtubule, interactions with other +TIPs can influence their localisation and many 'hitchhike' on EB proteins, (reviewed in Lansbergen and Akhmanova, 2006). Alternatively, they can bind EB proteins at microtubule tip in order to be retained there, after arriving using another method. For example, the +TIP dynactin has a microtubule-binding domain that can move along microtubules (Culver-Hanlon et al., 2006). One-dimensional diffusion may cause plus end accumulation of at least two +TIPs, mitotic centromere-associated kinesin (MCAK) and *X. laevis* microtubule associated protein (XMAP) 215 (Brouhard et al., 2008; Helenius et al., 2006). One dimensional diffusion was first proposed to help explain how a DNA-binding protein may find a particular target sequence, as movement in only one dimension - along the DNA molecule - decreases the time taken for a protein to reach its target. XMAP215 regulates catastrophe rate and dramatically increases the growth rate (Tournebize et al., 2000). MCAK is a member of the kinesin-13 family, non-motile kinesins that are able to induce catastrophe by destabilising microtubule ends in an ATP-dependent manner (Desai et al., 1999). The catastrophe levels in XMAP215-depleted extracts could be rescued to control levels by the inhibition of MCAK in *X. laevis* egg extracts (Tournebize et al., 2000). This suggests that XMAP215 antagonises the microtubule destabilising activity of MCAK, and that they could oppose each other to regulate catastrophes without any extra input. They can both also bind EB1 (Jiang et al., 2009; Kronja et al., 2009), perhaps competitively, even though XMAP215 is the only protein apart from EB proteins that can autonomously track microtubules (reviewed in Bieling et al., 2007). So, MCAK may reach microtubule ends through diffusion and then bind EB1 (and other +TIPs) in order to stay there, while XMAP215 is able to associate with microtubule plus ends

by itself and perhaps inhibit the localisation of MCAK to the plus ends by inhibiting its interaction with EB1.

Another type of +TIP is the collapsin response mediator protein (CRMP) family. There are five, highly related CRMPs (1-5). CRMP2 is the best studied. It self-oligomerises (Wang and Strittmatter, 1997), but partly dissociates when it binds directly to tubulin heterodimers, leading to heterotrimeric complexes of CRMP2 and a tubulin heterodimer (Fukata et al., 2002). CRMP2 binds tubulin heterodimers with a much higher affinity than the polymer, so may copolymerise with tubulin dimers into the microtubules (Fukata et al., 2002). It is expressed exclusively in the developing nervous system, and is crucial for axonal growth and determination of axon-dendrite fate, establishing and maintaining neuronal polarity: dominant-negative mutants of CRMP2 inhibit formation of the primary axon, whereas overexpression in cultured hippocampal neurones promotes neurite formation (Inagaki et al., 2001). In *G. Gallo* and *M. musculus*, the CRMP2 homologue is a mediator of semaphorin-induced growth-cone collapse, and in rodents and CRMP proteins are important in neurite pathfinding using neuropilin signalling (Deo et al., 2004; Goshima et al., 1995; Shapovalova et al., 2007).

4. 3. 2. Search and capture

An important model of microtubule dynamic instability was proposed several decades ago. Entitled ‘search and capture’, dynamic microtubules were suggested to explore the cytoplasmic space to be ‘selectively stabilised’ at certain sites within the cell, for example at the cortex (Gundersen, 2002; Gundersen and Bretscher, 2003). They would then be momentarily stabilised in order to, for example, facilitate the directed transport of vesicles or the separation of chromosomes to daughter cells. Direct evidence for microtubule capture, as required for the search-and-capture model, has been obtained by observing changes in the dynamic behaviour of microtubules at the

cortex (Gundersen et al., 2004). The minus end directed motor protein dynein and its complex partner dynactin accumulate at microtubule plus ends, so that minus-end-directed transport can be initiated when they encounter vesicles or other organelles (Vaughan et al., 2002; Watson et al., 2005). Localisation of +TIPs at plus ends means that they are in a strategic position to regulate interactions between microtubules and other intracellular structures.

Once at the cortex, pushing or pulling forces can be generated by microtubule polymerisation and depolymerisation respectively (Inoue and Salmon, 1995), which are capable of pushing vesicles, cell membranes and centrosomes (Dogterom et al., 2005). Accumulation of the minus end-directed microtubule-associated motor dynein at microtubule ends can exert a pulling force on the microtubule network if the dynein-associated membrane structure is large enough. Certain details, such as the link between dynein and the cortex, are not yet clear. Nonetheless, dynein can pull on the ends of cortically anchored microtubules to position the centrosome or mitotic spindle (reviewed in Dujardin and Vallee, 2002). CLIP-170 regulates dynein association with microtubule tips (Goodson et al., 2003; Lansbergen et al., 2004).

APC, which can be transported by kinesin to the microtubule plus-ends where it can promote polymerisation, can also anchor microtubules to the cell cortex (Nakamura et al., 2001; Reilein and Nelson, 2005; Zumbunn et al., 2001) and can do so by interacting with actin-binding proteins (Wen et al., 2004). CLASPs, on the other hand, bind the cortex independently from microtubules, then attach microtubule plus ends to the cortex and stabilise them by rescuing them from depolymerisation (Mimori-Kiyosue et al., 2005). EB3 can also bind the F-actin-binding protein debrin while associated with the microtubule plus end in an interaction that is important for growth cone formation and neuritogenesis (Geraldo et al., 2008). In *D. melanogaster*, at least one actin cytoskeletal regulator, RhoGEF2, associates specifically with

growing microtubule ends (Rogers et al., 2004), and in other organisms +TIPs can link microtubule ends directly to actin. In vertebrate cells, cytoskeletal linking proteins such as spectraplakins can bind growing microtubules end as well as actin filaments, organising directional growth of microtubules along actin fibres and stabilising microtubules (Kodama et al., 2003; Slep et al., 2005). CLIP-170 can also interact with actin-binding proteins to target microtubule plus-ends to the leading edge (Fukata et al., 2002).

+TIPs also link microtubule plus ends to actin and intermediate filaments. Microtubule plus ends can also bind the cortex and intercellular junctions, focal adhesions, vesicles and intracellular membranes through +TIPs. This could establish the targeted delivery of vesicles to certain sections of the cell. For example, kinesins are required for the modulation of focal adhesion site dynamics in fibroblasts by dynamic microtubules plus ends, even though the kinesin does not affect the targeting of microtubules to adhesion sites or microtubule distribution or dynamics (Krylyshkina et al., 2002). This suggests that kinesin is required for the delivery of components that control adhesion site turnover (Krylyshkina et al., 2002).

Not all +TIPs appear to directly influence microtubule dynamics, indicating that growing microtubule ends might serve more purpose than to randomly explore the cytoplasm. One interesting - albeit poorly explored - suggestion is the 'raft' model, in which the concentration of proteins at microtubule plus ends provides a platform for spatial organisation of protein interactions, allowing a cascade of signalling processes as they travel through the cell (Galjart and Perez, 2003). In this model, the microtubule plus end has been compared to having the organising capabilities of lipid rafts. The idea has been taken further with the suggestion that microtubule plus ends not only concentrate +TIPs but also that the regular pattern of the microtubule surface could orient them in a specific manner, enhancing the possibility of signalling

processes occurring (Galjart and Perez, 2003). For example, EB1 and CLIP-170 may unfold as they bind the microtubule end. One unknown feature of this model is whether the protein interactions persist after dissociation from the microtubule plus end. If so, this may explain how, for example, some +TIPs interact with microtubule plus ends but can promote rescue events: they may colocalise with other proteins at the growing microtubule end to form a relatively stable complex and dissociate from the microtubule, then function as a rescue complex when the microtubule shrinks back past the same place (Galjart and Perez, 2003).

4. 4. Microtubules, polarity and migration

The functional organisation and thus polarity of a cell is reflected in the organisation of its microtubule array. In fibroblasts, for example, microtubules are usually arranged in a radial array, with minus-ends anchored at the centrosome and the plus-ends extending towards the edge of the cell (Kellogg et al., 1994). This is also true of non-polarised epithelial cells, but the microtubules of polarised epithelial cells have minus ends at the adherens junction and plus ends at the basal surface (Bacallao et al., 1989). Thus, establishment of epithelial polarity is accompanied by a dramatic change in microtubule organisation.

Actin polymerisation and contractility are often required for migration (Etienne-Manneville and Hall, 2002; Raftopoulou and Hall, 2004), but microtubules are the cytoskeletal element that provide the framework for polarisation itself (Watanabe et al., 2005): disruption of microtubules perturbs the polarity of the actin cytoskeleton and cell, but actin depolymerisation often does not affect the polarity of the microtubule array (Omelchenko et al., 2002). Inhibition of microtubule dynamics through either global stabilisation or depolymerisation of the microtubule array

inhibits cellular polarity and directional migration (Redd et al., 2006; Takesono et al., 2010; Xu et al., 2005). Microtubule orientation supports polarised vesicle trafficking and sorting of membrane components (reviewed in Kamal and Goldstein, 2000). The role of microtubules in cell polarity may also be due to other functions, such as morphogenesis and signalling to adhesions (Kaverina et al., 1998; Kirschner and Mitchison, 1986). However, microtubule array dynamics are regulated by MAPs, post-translational modifications and interactions with the actin cytoskeleton. The polarised organisation, dynamics and functions of the microtubule cytoskeleton are likely to reflect the temporal and spatial activity of MAPs. However, the precise cytoskeletal rearrangements that permit a change in front-rear polarity, and how these are regulated, are as yet unknown.

4. 4. 1. Centrosome reorientation

In several cell types, the centrosome and Golgi complex are reoriented during polarisation. During polarised migration these structures are often reoriented to the front of the cell (Etienne-Manneville, 2004; Gomes et al., 2005), although this is not always the case (Pouthas et al., 2008; Ratner et al., 1997). During EMT of trunk neural crest cells in *G. gallo*, the centrosome stays at the apical end of the cell tail as the tail detaches from the neuroepithelium and is retracted by the cell (Ahlstrom and Erickson, 2009). In wound-healing assays of migrating astrocytes, where Par6 and aPKC orient the centrosome and Golgi close to the wound edge, it appears that centrosome orientation and protrusion, although both coordinated by Cdc42, are independent events (Etienne-Manneville and Hall, 2001). Thus, centrosome positioning may be a consequence of polarity rather than a requirement in this situation. However, in some cases, centrosome reorientation serves as a mechanism to establish a polarised microtubule network and thus directional and polarised flow of membrane and polarity proteins and cell polarisation. Dynein-dynactin centre the

centrosome in interphase cells and position the mitotic spindle by pulling on plus ends of cortically attached microtubules (Dujardin and Vallee, 2002). CLIP-170 may also contribute to pulling on plus ends, because it regulates dynactin association with microtubule tips (Goodson et al., 2003; Lansbergen et al., 2004), although the nature of the actual link between dynein and the cortex is not yet entirely clear. In mitotic keratinocytes, astral microtubules are positioned by dynein–dynactin, acting in concert with a number of polarity factors including Par3 (Lechler and Fuchs, 2005). In migrating cells, APC is localised to the leading edge and can anchor microtubules to the cortex through polarity proteins such as Dlg1 (Etienne-Manneville et al., 2005; Nakamura et al., 2001; Reilein and Nelson, 2005; Wen et al., 2004; Zumbrunn et al., 2001). The ability of APC to stabilise microtubules in protrusions is reliant on it anchoring them to the cortex (Zaoui et al., 2010). In *M. musculus* embryonic fibroblasts, connexin-43 controls the orientation of the centrosome and Golgi complex during directional migration by stabilising microtubules in the direction of migration (Rhee et al., 2009; Xu et al., 2006). Microtubules can bind connexin-43 and extend preferentially to connexin-43 gap junction plaques, remaining there longer than at other sites (Shaw et al., 2007). Microtubules are important in trafficking connexin-43 to the cell membrane (Thomas et al., 2005) and loss of connexin-43 interferes with wound closure (Francis et al., 2011). Interestingly, this does not appear to be linked to channel activity, as cell-cell contact is not required for this interaction (Francis et al., 2011).

The nucleation of microtubules can occur in a polarised manner. The centrosome itself nucleates microtubules symmetrically (Salaycik et al., 2005), but microtubules can also be nucleated at other parts of the cell. The Golgi complex acts as a microtubule-organising centre in migrating epithelial cells in a γ -tubulin-dependent manner (Efimov et al., 2007). However, microtubules nucleated at the Golgi complex

differ from centrosomal microtubules in that they are only nucleated from the trans-Golgi network and so are asymmetrically organised (Efimov et al., 2007). As the Golgi complex is often reoriented towards the leading edge in cells migrating on 2D surfaces (Etienne-Manneville and Hall, 2001), this has the effect of orienting microtubules nucleated at the Golgi complex towards the leading edge. These microtubules appear to be particularly stable as they are acetylated quickly (Chabin-Brion et al., 2001). This polarisation of the microtubule array is important, as a loss of Golgi-nucleated microtubules inhibits migration in a wound-healing assay; however, the cells are still able to reorient their centrosome and Golgi complex towards the leading edge (Rivero et al., 2009), suggesting that Golgi-nucleated microtubules affect migration independently of centrosome and Golgi complex polarity. Indeed, centrosome localisation may influence microtubule (and so cellular) polarity indirectly by asymmetrically positioning the Golgi complex (Efimov et al., 2007).

In the "search and capture" model, a population of dynamic microtubules searching the cellular space are captured by various microtubule-binding proteins at the cortex, leading to cell asymmetry. However, there may be more regulation of microtubules that this model allows. APC is transported by kinesin to plus ends where it binds actin-binding proteins such as β -catenin or the formin mDia and facilitates microtubule transport along actin to the cortex (Wen et al., 2004). Movement and organisation of F-actin affects dynamic organisation of microtubules in migrating cells and it appears that microtubules and F-actin associate with one another *in vivo*, although whether this is a direct interaction or mediated by MAPs is as yet unclear (Salmon et al., 2002). Thus, microtubules may be guided towards the cortex to be captured, rather than relying on chance to lead to their localisation there. Despite the focus on the anchoring of microtubules to the cortex in localising the centrosome,

centring mechanisms can function without microtubules contacting the cortex (Wuhr et al., 2010). Further, to centre the nucleus in response to changes in cell shape, microtubules pull on the nucleus with a force proportional to microtubule length (Minc et al., 2011). This suggests that longer microtubules accumulate more motors, either because of their increased length, or because longer microtubules have accumulated more posttranscriptional modifications that can affect the affinity to MAPs to microtubules (Minc et al., 2011).

4. 4. 2. Microtubules and cell-cell contacts

It is well established that microtubule populations within different areas of the same cell can have different dynamics and that +TIPs themselves can regulate microtubule dynamics. Microtubules adjacent to cell–cell contacts spend much more time in pause than microtubule plus-ends near non-contacted cell edges in squamous epithelial cells (Waterman-Storer et al., 2000). This is reminiscent of MDCK cells, where the microtubule half-life doubles upon contact (Pepperkok et al., 1990). However, cell-cell contact seems to elicit a cell-type-dependent effect on the microtubule array; for example, cell-cell interactions leading to the formation of an epithelium stabilise microtubules but cell-cell contacts between migrating fibroblasts appear to depolymerise microtubules (Nagasaki et al., 1992). However, in individual cells there are also differences in microtubule dynamics in different parts of the cell, with microtubule ends close to the back and sides exhibiting shorter growth periods and more frequent catastrophes, whereas longer growth phases and fewer catastrophes occur in protruding regions (Wadsworth, 1999). This suggests that cell polarity can affect microtubule dynamics, but that cell-cell contacts define this polarity when present. Further, differences in microtubule dynamics have been illustrated between cells within a monolayer (that is, completely in surrounded by cell-cell contacts) and those that are at the edge of a monolayer (that is, have a cell-cell contact on one side

but also have a free edge). For cells at the edge of a monolayer, microtubules at the free edge have a unimodal distribution but microtubules at the cell-cell contact have a bimodal distribution: a subset have similar dynamics to microtubules at the free edge but a subset are more dynamic than those at the free edge (Waterman-Storer et al., 2000). Interestingly, microtubules at the edge of cells within a monolayer have a unimodal distribution of dynamics (Waterman-Storer et al., 2000). This illustrates the complexity of microtubule dynamics and again suggests that cell polarity affects microtubule dynamics.

The microtubule array can be polarised by its post-translational modifications. Cells at the leading edge of a wounded fibroblast monolayer orient their centrosomes and detyrosinated microtubules towards the leading edge, suggesting that cell-cell contact influences microtubule array polarity (Gundersen and Bulinski, 1988; Nagasaki et al., 1992). Cells within the monolayer have detyrosinated microtubules, but they are coiled around the nucleus in a non-polarised manner (Nagasaki et al., 1992). In contrast, tyrosinated microtubules are found throughout the cells irrelevant as to their position in a wounded monolayer (Nagasaki et al., 1992). Following wounding, cells migrate forward to close the gap. As they touch cells on the other side of the wound, stable detyrosinated microtubules are lost in the vicinity of the cell-cell contact (Gundersen and Bulinski, 1988; Nagasaki et al., 1992). As such, polarisation of detyrosinated microtubules is lost and perinuclear localisation of detyrosinated microtubules increases coincidentally (Nagasaki et al., 1992). Tyrosinated microtubules are also reduced at new contact sites in a low number of cells; however, whether this is due to inconsistency in the response to cell-cell contact or to a transient effect is not clear (Nagasaki et al., 1992).

Microtubules also affect cell-cell contacts and are required for the maintenance of adherens junctions (Waterman-Storer et al., 2000). In rat embryonic fibroblasts,

microtubules and kinesin are required for N-cadherin localisation to adherens junctions but cell-cell contact promotes N-cadherin transport, suggesting that contact formation may in some way regulate kinesin activity (Mary et al., 2002). In centrosome-free cytoplasts, N-cadherin signalling at one part of the cell boundary appears to stabilise microtubule minus ends throughout the cell (Chausovsky et al., 2000). Thus, the impact of cell-cell contacts on microtubule dynamics is not necessarily local, and the interaction goes in both directions. Nocodazole application leads to loss of cell-cell adhesions but, interestingly, this occurs even in low concentrations of nocodazole that inhibit microtubule polymerisation without causing microtubule depolymerisation. This suggests that the effect of nocodazole on cell-cell adhesions is not due to microtubule depolymerisation, but is instead due to the lack of microtubule polymerisation that occurs at both high and low concentrations of nocodazole (Waterman-Storer et al., 2000). Depolymerisation of microtubules or suppression of microtubule growth releases APC from the microtubule ends into the cytoplasm. This free APC then could compete with α -catenin to bind β -catenin, removing β -catenin from junctions and thus inducing the breakdown of adherens junctions (Waterman-Storer et al., 2000).

It is also possible that adherens junctions are disrupted by microtubule mediation of small GTPases (Waterman-Storer et al., 2000). Small GTPase activity affects microtubule dynamics in a number of ways. Considering the polarised activity of these molecules, they can have a dramatic impact on the polarity of microtubule dynamics, of the microtubule array and of the cell. Rac1 can promote microtubule growth at the leading edge of migrating cells through Pak1, which phosphorylates and inhibits the microtubule-destabilising protein stathmin (Wittmann et al., 2003). Inhibiting RhoA signalling increases microtubule stability (Takesono et al., 2010) and ROCK can phosphorylate Tau and MAP2 in neurones, dissociating them from

microtubules and so promoting microtubule destabilisation (Amano et al., 2003). However, RhoA can also selectively stabilise microtubules through its effector mDia1 at the leading edge by inducing the alignment of microtubules along stress fibres (Ishizaki et al., 2001), and increasing the number of detyrosinated microtubules (Palazzo et al., 2001).

4. 4. 3. **Microtubules and Rho GTPases**

Considering the links between Rho GTPases, microtubules, cell-cell contacts and polarity, it is not surprising that RhoA and microtubules have been implicated in CIL. In *G. gallo* embryonic fibroblasts, microtubules increase the frequency of catastrophe and the rates of shrinkage and growth at the site of cell-cell contact (Kadir et al., 2011). This reorganisation of the microtubule network is necessary to mediate successful CIL and is dependent on Rho and ROCK signalling. ROCK inhibition leads to an increase of detyrosinated or acetylated microtubules, increases microtubule persistence at cell-cell contacts and inhibits CIL (Kadir et al., 2011). ROCK inhibition also reduces actomyosin contractility and CIL, but the authors show that interfering with myosin contractility does not significantly affect CIL (Kadir et al., 2011). However, nocodazole could partially rescue the loss of microtubule dynamics and CIL by ROCK inhibition (Kadir et al., 2011), suggesting that RhoA activation at cell-cell contacts leads to depolymerisation of microtubules and so a change in the polarity of the microtubule array. RhoA is also activated at cell-cell contacts during CIL in neural crest cells (Carmona-Fontaine et al., 2008). In *D. melanogaster* hemocytes, the rapid repolarisation observed upon cell collisions is preceded by and requires the alignment of stable microtubule bundles in the colliding cells (Stramer et al., 2010). Although the microtubule array has not been investigated in this situation, Rho mutant hemocytes clump together and maintain cell-cell

contacts during migration (Stramer et al., 2005), again suggesting that RhoA may be important in CIL through the affect on the microtubule array.

Microtubules can also affect Rac1. One theme is that Rac1 is activated at the front of the cell by penetration of microtubules into advancing regions. As GTP-bound Rac1 binds tubulin dimers but not microtubules (Best et al., 1996), microtubule polymerisation could release active Rac1. Indeed, microtubule polymerisation leads to an increase in Rac1 activity (Waterman-Storer et al., 1999), which has lead to the suggestion that microtubules are directly involved in actin polymerisation at the front of the cell, inducing lamellipodial protrusion. Microtubule catastrophe leads to local release of a large group of MAPs, particularly +TIPs. These include APC, which can bind members of the Rac- and Cdc42-regulatory pathways (Kawasaki et al., 2000; Watanabe et al., 2004). Release of APC can induce degradation of β -catenin, silencing Wnt signalling (reviewed in Polakis, 2007), or potentially affecting adherens junctions (Waterman-Storer et al., 2000). Further, Rac1 may be activated by the Rac-GEF ASEF (Kawasaki et al., 2000). The GEF activity of ASEF is regulated by its binding to APC (Nathke et al., 1996). IQGAP, a Cdc42 and Rac effector, may also be transported to the leading edge via binding to the +TIP CLIP170 (Fukata et al., 2002). Again, this regulation may depend on cell types, as microtubules can lag behind protruding regions, and in rapidly migrating cells they may not enter them at all (Ballestrem et al., 2000). Regarding RhoA, microtubule depolymerisation leads to its upregulation (Bershadsky et al., 1996). Nocodazole disrupts the polarity of macrophages as they migrate towards wounds in *D. rerio* larvae, and this can be rescued by ROCK inhibition (Redd et al., 2006). Nocodazole perturbs migration and polarity in T cells, which can again be restored by ROCK inhibition (Takesono et al., 2010), or GEF-H1 depletion (Chang et al., 2008). The activity of GEF-H1 towards

RhoA is suppressed when it binds to microtubules and microtubule catastrophe leads to its local release (Krendel et al., 2002).

4. 4. 4. Microtubules and focal adhesions

The effects of microtubules on Rac1 and RhoA may be a mechanism by which microtubules influence lamellipodia and filopodia organisation, cell migration and cell contractility: microtubule polymerisation at the leading edge promotes Rac-dependent lamellipodia protrusion, whereas microtubule depolymerisation promotes RhoA-dependent actomyosin contraction at the cell rear. Indeed, microtubule polymerisation favours dissolution of focal adhesions, whereas disruption of microtubules leads to a general increase in cell contractility and to the formation of stress fibres and focal adhesions (Bershadsky et al., 1996). Direct targeting of microtubules to focal contacts followed by the downregulation of the same focal contacts has been demonstrated in fibroblasts and it was hypothesised that microtubules deliver a relaxing impulse to cell-substrate contacts, facilitating their turnover (Kaverina et al., 1999). Microtubule-promoted turnover of adhesions has been suggested to be mediated by kinesin delivery of a signal (or signals), such as a myosin inhibitor (Kaverina et al., 1999; Kaverina et al., 1998; Krylyshkina et al., 2002). Controlling the number of targeting events would have varying relaxation effects on different adhesion sites, thus affecting polarity and migration. If microtubules can exert such relaxing effects in an asymmetric manner in a cell they could, by this route, influence the polarity of the actin cytoskeleton. Indeed, targeting of focal adhesions by microtubules occurs more often at the retracting edge than at the leading edge (Kaverina et al., 1999). Fibroblasts that are rendered apolar and immotile by microtubule depolymerisation can be induced to polarise and move by the asymmetric application of actomyosin inhibitors (Kaverina et al., 2000). Interestingly, kinesin preferentially binds to acetylated microtubules, (Reed et al.,

2006), suggesting that controlling the post-translational acetylation of microtubules could mediate processes such as contractility by affecting the delivery of kinesin-bound cargo.

Focal adhesion-microtubule interactions may help to explain the varying influence of microtubules on different cell types. The migration of fish keratocytes, which exhibit focal complexes that do not mature into focal adhesions (Anderson and Cross, 2000), is unaffected by microtubule disassembly (Euteneuer and Schliwa, 1984). Microtubules might be required for adhesion release only when stress at the adhesion sites exceeds a certain threshold, and perhaps this threshold is not reached in keratocytes (Small et al., 2002). Microtubules do in fact enter lateral adhesions in keratocytes and also lamellipodia regions when stress is increased by mechanical manipulation (Kaverina et al., 2002), suggesting that microtubule responses to tension could assist in establishing cell polarity. Fibroblasts depend on microtubule dynamics for polarisation and migration and are strongly adherent (Bershadsky et al., 1996; Liao et al., 1995). Melanoma cells can extend protrusions and migrate without microtubules but tail retraction is incomplete, which fits with observations that few microtubules grow to the front of the cell but many invade the rear (Ballestrem et al., 2000). Leukocytes are less adherent and can move even faster after losing microtubules, polarising spontaneously (Keller et al., 1984). The increase in chemokinesis and polarisation is apparently due to increased RhoA activation, as it can be suppressed by ROCK inhibition (Niggli, 2003). That a requirement for microtubules is related to adhesive properties is illustrated by strongly and weakly adherent sublines of Walker carcinosarcoma cells, which respond to microtubule disruption in a way that is similar to fibroblasts and leukocytes, respectively (Sroka et al., 2002).

Nonetheless, this explanation does not hold true for changes in cell substrate. Fibroblasts cultured on a cover slip do not require microtubules for lamellipodial extension but cannot polarise without them, whereas on a 3D matrix fibroblasts lack stress fibres and focal adhesions but without microtubules are unable to form any protrusion (Rhee et al., 2007). Thus, in the absence of stress fibres or focal adhesions, fibroblasts require microtubules to form dendritic protrusions, but when there is high tension between cell-matrix adhesions, fibroblasts can spread using focal adhesions and stress fibres and microtubules are required for polarisation (Rhee et al., 2007). However, this is not the case for all cells migrating in 3D environments. Treatment of *D. rerio* embryos with nocodazole interferes with the directional migration of macrophages and thus their recruitment to wounds (Redd et al., 2006). ROCK inhibitor Y-27632 is capable of suppressing the effects of nocodazole on macrophage recruitment to wounds *in vivo* (Redd et al., 2006), again highlighting that microtubule depolymerisation can activate RhoA.

On the other hand, focal adhesions can also influence microtubules. Following microtubule targeting to focal adhesions, the microtubules undergo rapid catastrophe and rescue (Efimov et al., 2008; Kaverina et al., 1998). Catastrophe appears to be dependent on paxillin (Efimov et al., 2008), and allows them to target the focal adhesions a number of times in short succession (Kaverina et al., 1998). However, microtubule binding to focal adhesions can also transiently stabilise the microtubule to nocodazole (Kaverina et al., 1998), suggesting that a brief stabilisation phase occurs before paxillin-dependent catastrophe. This illustrates the complexity of the system: cell-cell contacts can affect the stability of cell-substrate adhesions by affecting microtubule stability, and yet microtubules are important in cell-cell adhesion maintenance and cell-substrate adhesions can affect microtubule dynamics.

The role of microtubules in transport delivery makes the polarised transport of cargo an obvious method through which microtubules could influence cell polarity. An intact microtubule network is also required for proper cell repair, as it mediates localised transport of vesicles to the wound site (Abreu-Blanco et al., 2011). In the *D. melanogaster* embryo, a subset of mRNA particles is transported by the dynein-dynactin complex to the apical cytoplasm and modulating the number of motor proteins changes the frequency of dynein-mediated localisation (Wilkie and Davis, 2001). Bias has also been described for oskar mRNA in *D. melanogaster* oocytes through a kinesin motor (Zimyanin et al., 2008), allowing spatial and temporal regulation of gene expression.

4. 4. 5. Microtubules in neuronal development

The initiation and maintenance of neuronal polarity has been intensely studied and thus provides a convenient example of how various aspects of the microtubule cytoskeleton interconnect to influence cell polarity. The microtubule array is particularly important in initiating neuronal polarity. Local microtubule assembly and stabilisation in one neurite is at least one way of specifying neuronal polarisation; indeed, changing microtubule dynamics is sufficient to alter axon and dendrite specification (Witte et al., 2008). In cultured hippocampal pyramidal neurons, APC localises to the tips of all neurites but then becomes enriched only in the future axon (Votin et al., 2005). This occurs before morphological polarity is established and is required for the targeting of Par3 to the axonal growth cone, where Par3-aPKC-Par6 is essential for neuronal polarisation (Shi et al., 2004b). It seems that an APC-EB1 interaction leads to microtubule capture at the cortex and that Par3 is carried to the growth cone via kinesin transport. Inhibition of the Rho-ROCK pathway is also required for Par3 accumulation at the growth cone, because otherwise ROCK phosphorylates Par3; this prevents Par3 interaction with Tiam1 and Tiam2, which is

important for axon formation (Kunda et al., 2001; Nakayama et al., 2008; Nishimura et al., 2005; Takefuji et al., 2007). Thus, a feedback loop involving the reciprocal inhibition of RhoA and Rac1 and the regulation of microtubule dynamics is crucial during axon specification and so neuronal polarisation. However, it should be noted that APC is not required for polarisation in all neurons: complete knockdown of APC in *D. melanogaster* neurons does not affect axon outgrowth or targeting (Rusan et al., 2008).

The kinesin1 motor domain also accumulates in the future axon before morphological polarisation (Jacobson et al., 2006). A complex of kinesin and CRMP2 transports tubulin heterodimers and oligomers to the tip of growing axons (Kimura et al., 2005), where CRMP2 then promotes microtubule assembly (Fukata et al., 2002). The kinesin KIF13 transports PI3K, whose signalling plays a role in neuronal polarisation (Shi et al., 2003), to accumulate at the tip of the future axon (Horiguchi et al., 2006). As some kinesin family members preferentially bind acetylated microtubules (Reed et al., 2006), it may be that stable microtubules provide a landmark that maintains axonal identity (Gomis-Ruth et al., 2008). Interestingly, the transport of neurofilaments into and along axons requires not only an intact microtubule array but also both kinesin and dynein motor proteins (Lee et al., 2011).

Microtubules are severed from the centrosome and transported to axons and neurites: in axons they are oriented with their plus end towards the growth cone and in dendrites they have a mixed orientation (Baas et al., 1988). Microtubules are bundled in the axon and then splay upon entering the growth cone, where individual dynamic microtubules display splaying, looping, bending and bundling. Within an axon, the proximal end of an individual microtubule tends to be detyrosinated and acetylated while close to the growth cone it is high in tyrosinated tubulin and is thus much more dynamic (Baas et al., 1993; Brown et al., 1992). Dynamic microtubules are captured

at stable actin filaments within filopodia, and, as a consequence, are protected against catastrophe. Remodelling and organisation of individual dynamic axonal microtubules in the growth cone is required for persistent growth cone advance, axonal elongation and recognition of guidance cues (Tanaka and Kirschner, 1995; Tanaka and Kirschner, 1991). Low doses of vinblastin that decrease microtubule dynamics rather than affecting integrity of the axonal microtubule array abolish axonal elongation (Tanaka and Kirschner, 1995). In *X. laevis* spinal cord neurones in culture, a growth cone turning at borders between permissive and nonpermissive molecules will reorient its microtubules in the direction of the turn. Inhibiting microtubule dynamics blocks growth cone turning at substrate borders (Challacombe et al., 1997), and also block turning in response to focally applied guidance cues (Buck and Zheng, 2002). In addition, stabilising microtubules on one side of the growth cone by locally uncaging caged taxol within the growth cone or by asymmetric delivering of taxol or a chemotactic cue, turns the growth cone toward that side (Buck and Zheng, 2002; Zumbunn et al., 2001). Conversely, if microtubules are locally depolymerised on one side of the growth cone, the growth cone steers away (Buck and Zheng, 2002).

Once the polarity is established, microtubules in different neuronal sections differ in their complement of MAPs: for example, MAP2 is found mostly in dendrites and tau is found mainly in axons. The MAP Futsch, which organises synaptic microtubules and is itself dependent on homologues of Wnt and GSK3 (Packard et al., 2002), localises aPKC to synapses where it recruits Par3 and Par6 and is required for synaptic activity (Ruiz-Canada et al., 2004). Thus, polarisation of the microtubule array on a number of levels controls cellular polarity throughout neuronal development.

5. Hypothesis

The importance of cell-cell contacts between neural crest cells is evident from the importance of CIL in migration and cell polarisation. However, little is known about the composition or effect of cell-cell junctions in neural crest cells. Par3 is important in cell contact formation in epithelial cells; through these and other mechanisms it influences epithelial cell polarity. Par3 is also important in mesenchymal cell polarity and the directionality of cell migration. It achieves this through influencing the stability of the microtubule array. Neural crest cells are not epithelial, but neither do they migrate singly like the mesenchymal cells in which Par3 has been studied; indeed, contacts between neural crest cells affect their polarity and are crucial to collective migration. Thus, I propose the following hypothesis (Fig. 5.1):

That Par3 is localised to transient cell contacts formed during CIL, where it influences microtubule dynamics and so promotes cell polarity and directional migration.

To test this hypothesis, I will address the following questions:

1. Is Par3 required for neural crest cell migration?
2. Is Par3 required for CIL?
3. Is Par3 localised to cell-cell contacts during CIL?
4. Are microtubule dynamics required for neural crest cell migration?
5. Are microtubule dynamics required for CIL?

6. Does Par3 affect microtubule dynamics during CIL?

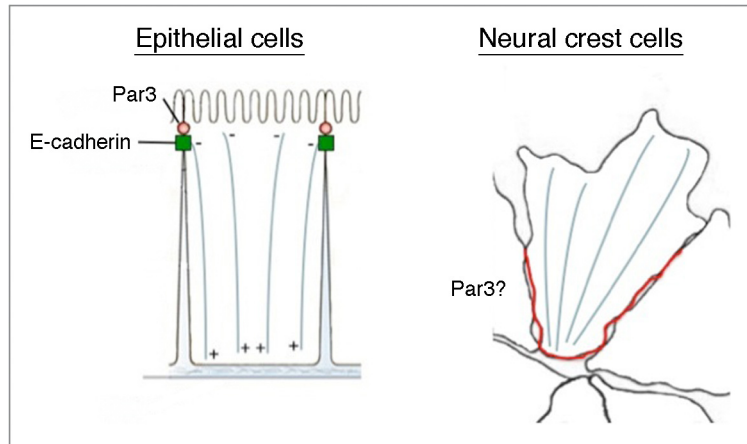


Figure 5.1. Hypothesis

Par3 is localised with other adhesion proteins (for example, E-cadherin) to the adhesion complex in epithelial cells, where it is required for contact formation and influences cell polarity. The microtubule array is important for maintenance of epithelial cell polarity and is influenced by cell contacts. Neural crest cells are mesenchymal but migrate in a group. The hypothesis proposed in this thesis is that Par3 is also localised to contacts between neural crest cells and that Par3 controls contact inhibition of locomotion by influencing microtubule dynamics.

II Materials and Methods

6. Solutions

6.1. Solutions for molecular biology and general use

DEPC water

0.1% diethylpyrocarbonate (DEPC)

In water

Sterilise twice by autoclaving

PBS and PBT

137 mM NaCl

2.7 mM KCl

4.3 mM Na₂HPO₄

1.4 mM H₂PO₄

Adjust pH to 7.3

Add 0.1% Tween-20 to make PBT

mRNA NTP-mix (10 x)

10 mM GFP

10 mM ATP

10 mM UTP

10 mM CTP

Labelled NTP-mix (10 x)

10 mM GTP

10 mM ATP

10 mM CTP

6.5 mM UTP

3.5 mM Dig-UTP

MEM salts (10 x)

1 M MOPS

10 mM MgSO₄

20 mM EGTA

In DEPC water

MEMFA

1 vol MEM salts

1 vol 37% formaldehyde

8 vol DEPC water

SSC (20 x)

3 M NaCl

0.3 M Tri-sodium citrate

Adjust pH to 7.0

LB broth

10 g LB agar (Sigma)

In 500 ml water

Sterilise by autoclaving

Added 500 uL antibiotic

6. 2. Solutions for embryonic experiments

Modified Ringer Solution (MMR; 10 x)

1 M NaCl

20 mM KCl

10 mM MgSO₄

20 mM CaCl₂

50 mM HEPES

1 mM disodium-EDTA

Adjust pH to 7.8, lowers to 7.6 when diluted to 1 x

Normal Amphibian Medium (NAM) A

1.1 M NaCa

20 mM KCl

10 mM Ca(NO₃)₂

10 mM MgSO₄

1 mM EDTA

Sterilise by autoclaving

NAM B

20 mM NaH₂PO₄

Adjust pH to 7.5

Sterilise by autoclaving

NAM C

100 mM NaHCO₃

Sterilise by millipore filtration

Working concentrations for NAM

	NAM A	NAM B	NAM C
NAM1/10	10 mL	10 mL	1 mL
NAM3/8	37 mL	37 mL	1 mL
NAM3/4	75 mL	75 mL	1 mL

Add 1 mL streptomycin (50 mg/ml) and water up to 1 L

Tricaine solution

5 g Ethyl 3-aminobenzoate methanesulfonate

Add water up to 1 L

Ficoll

3% Polysucrose

in NAM3/8

Fish water

60 mg “Instant Ocean”

Add water up to 1 L

Danilchick’s Solution (DFA)

53 mM NaCl

5 mM Na₂CO₃

4.5 mM K-Gluconate

32 mM Na-Gluconate

1 mM MgSO₄

1 mM CaCl₂

0.1% BSA

Adjust pH to 8.3 with 1 M Bicine

Add 50 ug/ml streptomycin before use

6. 3. Solutions for *in situ* hybridisation**Bleaching solution**

20% H₂O₂

5% Formamide

2.5% 20 x SSC

In DEPC water

Hybridisation buffer

50% Formamide

5 x SSC

1 x	Denhardt's Solution
1 mg/ml	Ribonucleic acid
100 ug/ml	Heparin
0.1%	CHAPS
10 mM	EDTA
0.1%	Tween-20

Adjust pH to 5.5

Probe washing solutions

Solution	Formamide	SSC 20 x
1	50 mL	10 mL
2	25 mL	10 mL
3	12.5 mL	10 mL
4	-	10 mL
5	-	1 mL

Add 0.1 mL Tween-20

Add DEPC water up to 100 mL

AP Buffer

0.1 M	NaCl
0.1 M	TrisHCl, pH 9.5
50 μ M	MgCl ₂
1%	Tween

In water

6. 4. Solutions for Western blot and coimmunoprecipitation

Homogenisation buffer

2 mL	1 M Tris-HCl (pH 7.6)
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10 mL 1 M NaCl

1 mL 1% TritonX-100

Add distilled water up to 100 mL

Protein buffer (10 x; 1 L)

250 mM Tris

1921 mM Glycine

Adjust pH to 8.3

Running buffer

10% Protein buffer

0.1%/v SDS

In water

Transfer buffer

10% Protein buffer (to 1 x)

20% Methanol

0.03%/v SDS

In water

Tris-buffered saline (10 x)

4 g NaCl

0.2 g KCl

12.5 ml TrisHCl, pH7.5

Add distilled water up to 50 mL

Peroxidase detection stock solutions

Solution 1

0.5 mL Luminol

0.22 mL Coumeric acid

5 mL Tris-HCl

Add distilled water up to 50 mL

Solution 2

32 μ L Hydrogen peroxide

5 mL Tris-HCl

Add distilled water up to 50 mL

NOP buffer

10 mM HEPES (pH 7.4)

150 mM NaCl

2 mM EDTA

1% NP-40

7. Experimental procedures

7. 1. Embryological procedures

7. 1. 1. *X. laevis* embryo collection

Adult *X. laevis* were kept in standard conditions (Portsmouth Animal Facility, UK or Nasco, USA). Females were injected subcutaneously with 100 units of pregnant serum mare gonadotropin (PSMG; Intervert) 4 to 7 days before use and with 500 units of chorion gonadotropin (Intervert) approximately 16 hours before the planned fertilisation to induce ovulation. While laying, females were kept in MMR solution at approximately 17°C in order to maintain the possibility of the oocytes to be fertilised after laying. Males were killed in 0.5% Tricaine solution (Sigma, E10521). The testes were removed and stored in Leibovitz L-15 medium (Invitrogen, #11415) with added streptomycin (5 µg/mL, Sigma, #85886) at 4°C. For fertilisation, a section of testes was crushed in MMR and mixed with oocytes in a petri dish. The oocytes were activated 15-30 minutes after fertilisation by flooding with NAM1/10. At the 2-8 cell stage, embryos were left for 15 minutes in 2% L-cysteine (pH 8.2, Sigma) to remove the jelly. Embryos were raised in NAM1/10 and staged according to Nieuwkoop and Faber (1967).

7. 1. 2. *D. rerio* embryo collection

D. rerio strains were maintained and bred according to standard procedures (Westerfield, 2000). Wildtype embryos were obtained following natural spawning of an AB wild-type colony. Transgenic *sox10:egfp* embryos (Carney et al., 2006) or *sox10:mGFPnRFP* (not published) were obtained from crosses between heterozygous *sox10:egfp* or *sox10:mGFPnRFP* carriers respectively. Embryos were raised in fish water at 28.5°C and staged according to Kimmel et al (1995).

7. 1. 3. *X. laevis* embryo injection

X. laevis embryos were injected using a Narishige IM300 microinjector under a Leica MZ6 or Nikon SMZ645 dissecting microscope. Needles were made from borosilicate glass capillaries (Intracel, #01-001-06) with an internal diameter of 0.5 mm, using a Narishige PC-10 needle puller. The puller was set to the two-step mode with both steps adjusted to 69.5% capacity. Each needle was filled with mRNA, DNA or morpholino (or combination thereof) and calibrated using an eyepiece graticule to inject 5 nl or 10 nl per injection. Fluorescein-dextran (FDX; Invitrogen, D1821, 3 µg) or rhodamine-dextran (RDX; Invitrogen, D1824, 5 µg) were used as tracers. Embryos were injected in a dish of Ficoll at the 2- to 16-cell stage; to target the neural crest specifically, embryos were injected into the animal ventral blastomeres at the 8- or 16-cell stage on one side. They were cultured in Ficoll for approximately 16 hours at 14.5°C and were then transferred to NAM1/10 before the onset of gastrulation. However, the temperature was sometimes altered to change the speed of development.

7. 1. 4. *D. rerio* embryo injection

D. rerio embryos were injected into the yolk with 4 nl per injection. Embryos were injected dry at the 1-cell stage. Embryos were then developed in fish water at 28.5°C, although the temperature was occasionally altered to change the speed of development.

7. 1. 5. Neural crest transplant

Neural crest transplants performed as described by (De Calisto et al., 2005). Briefly, the procedure was carried out in NAM3/4 when the embryos were at stages 18-20. The neural crest was dissected from embryos that had previously been injected, and thus labelled, with FDX. Neural crest was removed from uninjected host embryos and

the fluorescent neural crest from injected embryos transferred into their place. Embryos were cultured in NAM3/8 to heal and develop.

7. 1. 6. Neural crest dissection and *in vitro* culture

Fibronectin-coated plastic or glass dishes were prepared by incubating the dish for one hour at 37°C with 10 µg/mL or 50 µg/mL fibronectin (Sigma, F1141) in PBS respectively. The dish was washed three times in PBS and then blocked with 0.1% bovine serum albumin (BSA)/PBS for 30 minutes at 37°C, washed again with PBS and filled with DFA. Neural crest dissection was carried out in NAM3/8. This was performed at stage 17-20 using a standard technique, described by Alfandari et al (2003). Once removed from the embryo, explants were transferred to the pre-prepared fibronectin-coated dish. They were left still for 30 minutes to allow for neural crest attachment to the fibronectin. If required, paclitaxel (taxol; Sigma, T7402; final concentration 0.2 µM), nocodazole (Sigma, M1404; final concentration 13-65 nM), sodium orthovanadate (Sigma, S6508; final concentration 1-100 µM), NSC23766 (Tocris, #2161; final concentration 20 µM), cytochalasin D (Sigma, C8273; final concentration 0.2 µM) or dimethyl sulfoxide (DMSO; vehicle) were added to the DFA.

7. 1. 7. Cell reaggregation assay

Embryos were injected with a tracer (FDX or RDX) and a morpholino (ControlMO, Par3MO or N-CadherinMO). Neural crest cells were dissected as described above from two groups of embryos, one marked with FDX and one marked with RDX. The explants were put in Ca²⁺- and Mg²⁺-free DFA and agitated with an eyebrow knife in order to dissociate the cells. The cells were then put into a eppendorf tube in standard DFA overnight at 4°C to allow reaggregation.

7. 2. Molecular biology

7. 2. 1. Obtaining DNA clones

DNA clones were obtained either suspended in water or spotted onto filter paper. See Table 7.1 for a list of clones used for this thesis. If obtained on paper, the spotted region was cut out of the paper and soaked in water to resuspend the plasmid. Competent DH5- α bacteria were thawed on ice for 5 minutes and 1-5 μ l plasmid was added. Bacteria were kept on ice for 20 minutes, heat-shocked at 42°C for 45 seconds and put back on ice. After a further 2 minutes, 600 μ l of nuclease-free water was added and then 100 μ l of bacteria mixture plated on LB-ampicillin or LB-kanamycin plates as appropriate. Plates were incubated at 37°C for 16 hours, by which time colonies were present, then left at 4°C. A sterile micro-pipette tip was used to pick one colony from the plate and grown in 100 ml of LB-ampicillin or LB-kanamycin medium at 37°C with agitation for 16 hours. The bacteria were pelleted by centrifugation (15 minutes, 5000 rpm) at 4°C and the supernatant discarded. The amplified DNA was then extracted from the bacteria using the Plasmid midiprep kit (Qiagen, #12143). DNA concentration was measured using a Nanodrop spectrophotometer (ND-1000).

7. 2. 2. Synthesis of mRNA for injection

Restriction digest

Plasmid DNA was linearised by cutting at a 3' restriction site with an appropriate restriction endonuclease. Enzymes, buffers and BSA were obtained from Promega. A 100 μ L reaction of 10 μ g plasmid DNA, 10 μ L of appropriate 10x buffer, 1 μ L of BSA and 1 μ L of enzyme in water was incubated at 37°C for two hours.

Table 7.1. List of constructs

mRNA	Abbreviation	Purpose	Plasmid	Restriction Enzyme	Transcription Enzyme	Amount injected (per embryo)
membrane GFP	mbGFP	expression	pCS2+	Not1	Sp6	300 pg
membrane RFP	mbRFP	expression	pCS2+	Not1	Sp6	300 pg
nuclear (H2B) GFP	nGFP	expression	pCS2+	Not1	Sp6	300 pg
nuclear (H2B) RFP	nRFP	expression	pCS2+	Not1	Sp6	300 pg
end binding protein 3 GFP	EB3GFP	expression	pCS2+	Not1	Sp6	300 pg
Par3 GFP	Par3GFP	expression	pCS2+	Not1	Sp6	500 pg
Par3 RFP	Par3RFP	expression	pCS2+	Not1	Sp6	500 pg
Trio GFP	TrioGFP	expression	pEGFC2+	-	-	300 pg
aPKC dominant negative HA	aPKCdnHA	dominant negative	pCS2+	Not1	Sp6	300 pg
p120-catenin GFP	p120GFP	expression	pCS2+	Not1	Sp6	120 pg
β -catenin GFP	β cateninGFP	expression	pCS2+	Not1	Sp6	300 pg

Digested DNA was purified using chloroform extraction and precipitated with ethanol. To do this, 100 μ L of DEPC-water and 200 μ L of Phenol:Chloroform:Isoamyl (25:24:1) added. This was vortexed to mix and centrifuged at 4°C for 10 minutes at 13,000 rpm. The top layer was carefully removed and placed in a new 1.5 mL eppendorf tube. Once this volume was measured, twice the amount of 100% ethanol and 1/10 the volume of 3 M NaAc were added, and the mixture incubated at -20°C overnight. The samples were centrifuged at 4°C for 30 minutes at 13,000 rpm. The supernatant was removed, the samples washed with 350 μ L of 70% ethanol and centrifuged again at 4°C for 15 minutes at 13,000 rpm. Again, the supernatant was removed and the samples were air dried and resuspended in 20 μ L of TrisEDTA.

Transcription

Two μ L of 3' linearised and purified plasmid DNA were mixed in with 10 μ L NTP cap, 2 μ L of 10x buffer, 2 μ L of enzyme mix, 1 μ L of GTP and 3 μ L of water and incubated for two hours at 37°C. Synthetic capped mRNA was thus transcribed using the SP6 or T7 Ambion mMessage mMachine kit (Invitrogen, AM1340 and AM 1344, respectively). One μ L of the reaction mixture was replaced with 1 μ L of DNAase and the mixture incubated for a further hour at 37°C in order to remove the DNA. The mRNA was precipitated by adding 100 μ L of TrisEDTA, 10 μ L of 4 M LiCl, 300 μ L of 100% ethanol and leaving on ice for 30 minutes. Samples were centrifuged for 10 minutes at 4°C at 13,000 rpm, the supernatant removed and 300 μ L of 70% ethanol added, then the centrifugation step repeated. The supernatant was again removed and the pellet resuspended in 100 μ L nuclease-free water. The concentration was measured using a Nanodrop spectrophotometer (ND-1000).

7. 2. 3. **Synthesis of antisense RNA probes for *in situ* hybridisation**

Plasmids were synthesised, purified and linearised as described above. *In vitro* transcription of antisense RNA was carried out by mixing together 2 µL of linearised DNA, 4 µL of 5 x buffer, 2 µL of 10 x DTT, 2 µL of NTP-Dig, 0.5 µL of RNase inhibitor and 1 µL of RNA polymerase and making the solution up to 20 µL with water. The NTP mix contains digoxigenin-labelled UTP, so the resulting RNA was labelled with digoxigenin. This mixture was incubated at 37°C for two hours. From this, 1 µL was replaced with 1 µL of DNAase and the mixture incubated at 37°C for an hour to degrade the DNA template. The mRNA was precipitated by adding 100 µL of TrisEDTA, 10 µL of 4 M LiCl, 300 µL of 100% ethanol and leaving on ice for 30 minutes. Samples were centrifuged for 10 minutes at 4°C at 13,000 rpm, the supernatant removed and 300 µL of 70% ethanol added, then the centrifugation step repeated. The supernatant was again removed and the pellet resuspended in 100 µL nuclease-free water. The concentration was measured using a Nanodrop spectrophotometer (ND-1000).

7. 2. 4. **Oligomorpholinos**

Oligomorpholinos were designed as recommended by GeneTools or using previously reported morpholinos. Three morpholinos were used for this thesis: *X. laevis* Par3 (8 ng, 3' - TACTTCCACTGCCACTCGAAACCCT - 5'), *D. rerio* Par3 (1.4 ng, Wei et al., 2004b) and *X. laevis* Trio (8 ng, Kashef et al., 2009), 3' - TGCGCATAGCTACAGCTGAAAAAAA - 5'). Equimolar concentrations of standard control morpholinos (ControlMO; 3' - ATATTTAACATTGACTCCATTCTCC - 5') were used in both species.

7. 2. 5. *In situ* hybridisation

Embryos were fixed in MEMFA for one hour at room temperature, washed in 100% methanol and kept in 100% methanol for storage at -20°C . Embryos were rehydrated with successive washes of 75% methanol/PBT, 50% methanol/PBT, 25% methanol/PBT and PBT. *X. laevis* embryos but not *D. rerio* embryos were bleached for 20 to 30 minutes and washed in PBT. All embryos were then fixed for 30 minutes in 3.7% formaldehyde/PBS, washed again in PBT and transferred to hybridisation buffer, in which they were incubated at room temperature for at least one hour then stored at -20°C . They were then left overnight in hybridisation buffer containing digoxigenin-labelled probe at 62°C . The probes used in this thesis were against *Snail2* (Mayor et al., 1995) and *Twist* (Hopwood et al., 1989) in *X. laevis* and *FoxD3* (Odenthal and Nüsslein-Volhard, 1998) in *D. rerio*. The post-hybridisation washes using Washing Solutions 1-4 were performed at 62°C for ten minutes each. A 30-minute post-hybridisation wash of Washing Solution 5 was carried out at 62°C . All solutions used up to this point were made with DEPC-treated water to prevent RNAase contamination, which could otherwise degrade the RNA probe. Embryos were rinsed with TBS, blocked in TBS/2% serum for 2 hours at room temperature and incubated in TBS/2% serum containing an antibody against digoxigenin labelled with AP (Roche, #11093274910) overnight at 4°C . They were then washed for 30 minutes in TBS/2% serum five times at room temperature, washed for 15 minutes in AP buffer three times at room temperature and developed using 4-nitro blue-tetrazolium-chloride (NBT; Roche, #11383213001) and 5-bromo-4-chloro-3-indoyl-phosphate (BCIP; Roche, #11585002001) diluted in AP buffer (1:4000) at room temperature. The reaction was stopped by washing in PBT and background staining was removed by a 20 minute wash in 100% methanol. Embryos were then fixed in 3.7% formaldehyde and kept at 4°C .

7. 2. 6. Cryosection of *X. laevis* embryos

Embryos were fixed in MEMFA at room temperature for one hour. They were washed in PBS and incubated in 15% sucrose (Sigma, S0389)/PBS overnight at 4°C. The following day they were incubated in 7% porcine gelatin (Sigma, G2500)/15% sucrose/PBS at 42°C for at least two hours then mounted in the same. Each embryo was cut out of the gelatin in a block, which was frozen in isopentane at -80°C and then kept at -80°C. Cryosections of 10 nm were collected on Superfrost glass slides and allowed to dry at 4°C. Gelatin was removed by incubating the slides in PBS at 37°C for 30 minutes and sections were processed for immunostaining.

7. 2. 7. Immunostaining

Immuostaining was performed on neural crest explants or cryosections. For most epitopes, neural crest explants were fixed in 3.7% formaldehyde/PBS for 30 minutes at room temperature. Cells were washed in PBS 3 times for 10 minutes per wash and permeabilised by incubation in ice-cold 0.2% Triton-X 100 in PBS twice for 5 minutes each time. Samples were again washed in PBS 3 times for 10 minutes each time and then blocked with 1% BSA/PBS for 30 minutes at room temperature. The primary antibody was diluted in 1% BSA/PBS and applied for 2 hours at room temperature or overnight at 4°C. Samples were washed 3 times in PBS, for 10 minutes each wash. The secondary antibody was diluted in 1% BSA/PBS and applied for one hour at room temperature. Samples were washed thoroughly in PBS and mounted in Mowiol. See Tables 7.2 and 7.3 for details of all antibodies.

For immunostaining against α -tubulin and acetylated tubulin, explants were fixed in 4% paraformaldehyde/0.2% gluteraldehyde/PBS for 15 minutes at room temperature then washed in ice cold 100% methanol for 10 seconds. They were then washed in

Table 7.2. List of primary antibodies

Target	Species	Source	Identification	Concentration
α -tubulin	Mouse IgG	DSHB	12G10	1:25
acetylated tubulin	Mouse IgG	Sigma	611B1	1:100
β -catenin	Rabbit IgG	AbCam	ab6302	1:500
Par3	Rabbit IgG	Millipore	07-330	1:200 (immuno), or 1:1000 (WB)
p42/44 MAPK	Rabbit IgG	Cell Signalling	9102S	1:1000
HA	Rabbit IgG	Abcam		1:2000
GFP	Rabbit IgG	Abcam		1:3000

Table 7.3. List of secondary antibodies

Target	Tag	Source	Identification	Concentration
Rabbit IgG	Alexa 488	Invitrogen	A11034	1:100
Mouse IgG	Alexa 488	Invitrogen	A11017	1:200
Rabbit IgG	HRP	ECK	NA934	1:25000
Rabbit IgG	AP	Dianova		1:2000
Chicken IgG	AP	Abcam		1:2000

PBS 3 times for 10 minutes each time and processed for immunostaining from the blocking step as above.

7. 2. 8. Coimmunoprecipitation

Cos7 cells were transfected with Par3GFP or TrioHA using TransPass (New England Biolabs), harvested after 48 hours and lysed with NOP buffer supplemented with Complete Protease Inhibitor (Roche). Trio was pulled down with an antibody against HA for 1 hour at 4°C, followed by a 1 hour incubation with protein A sepharose beads at 4°C. Immunoprecipitations were washed three times with NOP buffer, once with 0.5 M LiCl and three times with NOP buffer, then resolved by SDS-polyacrylamide gel electrophoresis. Western blotting was performed using NBT/BCIP to develop. See Tables 2.2 and 2.3 for details of all antibodies.

7. 2. 9. Western blots

Whole embryos were added to 100 µL of homogenisation buffer with 1 µL phosphatase inhibitor, homogenised with the tip of a micropipette and spun several times replacing the tube in each cycle. Then the samples were centrifuged for 10 minutes at 13,000 rpm at 4°C. The supernatants were recovered and placed in new tubes and stored at -80°C if required. Protein concentration was measured and made uniform among different samples. The gel was made by filling a mould with running gel solution and adding water to the top in order to keep the top level of the gel flat. Once the gel had solidified, the water was removed, the stacking gel solution used to fill up the rest of the mould and a comb inserted. To run the gel, the sample was diluted to the desired concentration, mixed with NuPAGE LDS sample buffer (Invitrogen, NP0007) and boiled at 95°C for three minutes. The electrophoretic chamber was filled with running buffer, the comb removed from the gel and the samples loaded. To estimate the weight of the bands obtained, a standard protein

ladder (Biorad, #161-0377) was run through the gel at the same time. Electrophoresis was performed at 200 V for 40 minutes. The protein was transferred to a PDVF membrane (Amersham Hybond, RPNFL/02/10) using a semi-dry blotter at 15 V for 45 minutes. The membrane was blocked overnight in 5% Marvel/TBST at 4°C and then cut in order to perform separate experiments on the sections of the gel where Par3 or MAPK was expected to be found. Membrane sections were incubated for 3 hours at room temperature in the appropriate primary antibody diluted in 5% Marvel/TBST. The membrane was washed well in TBST and incubated for 2 hours at room temperature in the secondary antibody tagged with horseradish peroxidase and diluted in 5% Marvel/TBST. After washes, equal volumes of two peroxidase solutions were mixed and applied to the membrane for five minutes in the dark. The excess peroxidase solutions mixture was removed from the membrane. The membrane wrapped in cling film to avoid the membrane drying and placed in a signal amplification cassette. In the dark, an X-ray film (Super RX, Fuji) was introduced into the cassette and developed in a X-OGraph machine. Band intensity was measured using ImageJ. See Tables 2.2 and 2.3 for details of all antibodies.

7.3. Imaging

X. laevis or *D. rerio* embryos were immobilised in a dish coated with 1% agarose (Sigma, A9414) to be photographed. Time-lapse imaging of *X. laevis* neural crest was carried out at low magnification to look at whole neural crest cell explants or at high magnification to look at individual or small groups of cells. Neural crest explants were observed at low magnification using 10 x or 20 x dry objectives on a Leica DM5500 compound microscope. When imaging one experimental condition, fibronectin-coated petri dishes containing cultured neural crest cells were filled with

DFA and closed so that no air bubbles were present. When more than one experimental condition was required, neural crest cells were cultured in up to four wells of a four-well dish. These were filled with DFA and sealed with 22 mm x 22 mm cover slips smeared with high-vacuum grease (Dow Corning), over which the dish lid was fixed. These were inverted and the cells imaged through the plastic. Images were collected every 5 minutes for a period of 8 hours. For high magnification, neural crest cells cultured in a fibronectin-coated glass dish filled with DFA were imaged using a 60 x or 100 x oil objective in an inverted spinning disk microscope. Images were collected every 2 seconds for a period of 15 to 30 minutes using Volocity software and edited using the same software. Fixed cells or sections were imaged using a Leica SPE confocal microscope and 40 x or 63 x objectives. Cell reaggregation experiments were also imaged on a Leica SPE confocal microscope, but in this case a 20 x objective was used. *Sox10:mGFP/nRFP* transgenic *D. rerio* embryos were used to observe neural crest migration *in vivo*. Embryos were dechorionated and mounted in low melting point agarose in a glass dish, and covered with fish water to prevent dehydration. They were imaged on a spinning disk microscope, with frames taken every 5 minutes. Colocalisation was analysed using the ImageJ Colocalisation Finder plugin. Pixel intensity was measured using ImageJ.

7. 4. Methods of analysis

7. 4. 1. Analysis of cell migration

Analysis of neural crest migration *in vivo* was performed by measuring distance from the top of the head to the most ventral neural crest cells and expressing this as a proportion of the distance between the top and bottom of the head. This was repeated at three locations for each embryo and the average proportion calculated.

From low-magnification time-lapse movies, individual cells were tracked using the ImageJ Manual Tracking plug-in. This method requires manual identification of each cell at each time point. The ImageJ Chemotaxis plug-in was then used to analyse speed and persistence of cell migration. Persistence is calculated as the distance between the start and end of migration divided by the actual distance migrated. Tracking of single cells began at least 30 minutes after previous cell-cell contact (if applicable), in order to limit any potential effect that cell-cell contact might have on single cell migration, and lasted for two hours. Any cells that contacted another cell during this time were not included in the analysis.

Separately, cells were tracked during the dispersion of explants; that is, tracking commenced when the cells were still in the explant and followed them as they moved out of the explant and interacted with other cells. In this case, 10 cells were tracked per explant and ten explants were analysed per condition. Tracking lasted for 8 hours unless the cell died or left the field of reference during this time, in which case tracking was stopped. The mean speed and persistence was calculated per cell, from which the mean speed and persistence of the population was calculated.

The dispersion of neural crest cell explants was analysed using the ImageJ Delaunay Triangulation plugin. For each explant at each time-point, Delaunay triangulation connects each cell with its closest neighbours in such a way that a network of triangles covers the entire explant and the area of the triangles are measured and averaged.

7. 4. 2. Analysis of single cell collisions

The x,y co-ordinates of the cell were manually identified using the ImageJ Manual Tracking plug-in at three points: before, during and after collision. From the measurements, the velocity and acceleration vectors were calculated and statistically

analysed using Matlab script as described previously (Carmona-Fontaine et al., 2008). To illustrate these results, the vectors were aligned by using the initial movement (between the points "before migration" and "during migration") as the x-axis.

7. 4. 3. Analysis of explant confrontation assay

Two neural crest explants, each labelled with a different coloured tracer, were cultured close to each other on fibronectin-coated dish and time-lapse imaging performed at low magnification. The resulting movies were observed and the frame at which the explants overlapped the most was determined. The image of this frame was set to threshold for each fluorescent channel and the result compared to the corresponding bright field image to ensure that the thresholded image was representative. The thresholded images of the two fluorescent channels were then merged and the total explant area and the overlap area measured. The overlap area was expressed as a percentage of total explant area.

7. 4. 4. Analysis of EB3GFP movies

Movies of EB3GFP-labelled cells were cropped to include only the cell of interest. If necessary, other cells were blocked out with the background colour. The images were processed using the translation transformation of the ImageJ StackReg plugin. They were then analysed manually and automatically. The ImageJ Manual Tracking plugin was used to track microtubule growth phases manually. To analyse microtubule catastrophe, a $5\mu\text{m} \times 5\mu\text{m}$ square was selected at the front and back of a cell. The total number of EB3GFP points that entered the square was counted, apart from those that were still in the square when analysis concluded, and compared to the total number of EB3GFP points that disappeared within the square within a 2.5 minute period.

Automatic analysis was performed using plusTipTracker. The general tracking algorithm used in the software package has been validated (Jaqaman et al., 2008). Subsequently, a sub-track linking concept was then applied by Matov et al. (2010), in order to infer microtubule pause and shrinkage events based on +TIP growth tracks. This method was then updated to plusTipTracker, which employs a more robust method that is self-adaptive and free of control parameters (Applegate et al., 2011). To analyse microtubule dynamics automatically using plusTipTracker, optimal tracking parameters were determined using plusTipSweepGUI. This allows a range of values to be tested for each parameter, keeping all others constant. From this, the following parameters were determined to be the most accurate and were used for all analyses: maximum gap length 30 frames, minimum track length 3 frames, search radius range 2-25 pixels; maximum forward angle 35°; maximum backward angle 10°; maximum shrinkage factor 1.5; fluctuation radius 2 pixels.

7. 4. 5. Analysis of centrosome position

Analysis of centrosome position was carried out using ImageJ. The position of the cell centroid was determined and the distance between the centrosome and the centroid was measured. The centroid position was designated 0,0 and the image rotated so that the protrusion extended along the y-axis. From this, the angle between the direction of protrusion and the position of the centrosome could be measured.

7. 4. 6. Statistical analysis

Comparison of percentages was performed using contingency tables as described in (Taillard et al., 2008). Two data sets were considered significantly different if $T > 3.841$ ($\alpha=0.05$, *), $T > 6.635$ ($\alpha=0.01$, **) or $T > 10.83$ ($\alpha=0.001$, ***). Normality of data sets was tested using Kolmogorov-Smirnov's test, d'Agostino and Pearson's test and Shapiro-Wilk's test using Prism4 (GraphPad). A data set was considered normal

if found normal by all three tests. Datasets following normal distribution were compared with Student T-test (two tailed, unequal variances) or a one-way ANOVA with a Dunnett's multiple comparisons post-test using Excel or Prism4 (GraphPad). Datasets that did not follow a normal distribution were compared using Mann Whitney's test or a non-parametric ANOVA (Kruskal-Wallis with Dunn's multiple comparisons post-test) using Excel or Prism4 (GraphPad). Cross-comparisons were performed only if the overall P value of the ANOVA was <0.05 .

III Results

8. Par3 is required for neural crest migration

8. 1. Par3 is required for neural crest cell dispersion

Par3 is required for the migration of a number of cell types, including wound healing of MDCK cells and fibroblasts and migration of single keratinocytes (Du et al., 2010; Pegtel et al., 2007; Schmoranzner et al., 2009). To investigate the role of Par3 in neural crest migration, I performed a loss-of-function experiment using an anti-sense morpholino against Par3, which efficiently decreases the level of Par3 protein in *X. laevis* embryos (Par3MO; Fig. 8.1A). Each embryo was injected in two dorsal blastomeres late in the 8-cell stage, in order to target the neural crest but minimise disturbance of other tissues. Injection of Par3MO did not affect neural crest induction, as analysed by *in situ* hybridisation against *Slug*, *FoxD3*, *Sox9* or *Sox10* all of which are known markers for pre-migratory neural crest (Fig. 8.1B,C; Mayor et al., 1995). However, Par3MO injection had a dramatic effect on neural crest migration, as analysed by *in situ* hybridisation against *Twist*, which marks migrating neural crest (Fig. 8.2A,B,D; Hopwood et al., 1989). Importantly, this effect could be rescued by coinjection of *D. rerio* Par3 mRNA, which does not contain the sequence targeted by the morpholino against *X. laevis* Par3 (Fig. 8.2C,D), showing specificity for the Par3MO.

Injection of Par3MO into two dorsal blastomeres of an 8-cell embryo targets the neural crest, but could also indirectly affect neural crest migration by affecting the tissue through which the neural crest migrates. To test whether the effect of Par3MO was cell-autonomous, a grafting experiment was performed. Neural crest cells were taken from embryos injected with a control morpholino (ControlMO) or Par3MO, and grafted into wildtype embryos. Neural crest injected with ControlMO showed no effect on neural crest migration (Fig. 8.2E), whereas neural crest injected with

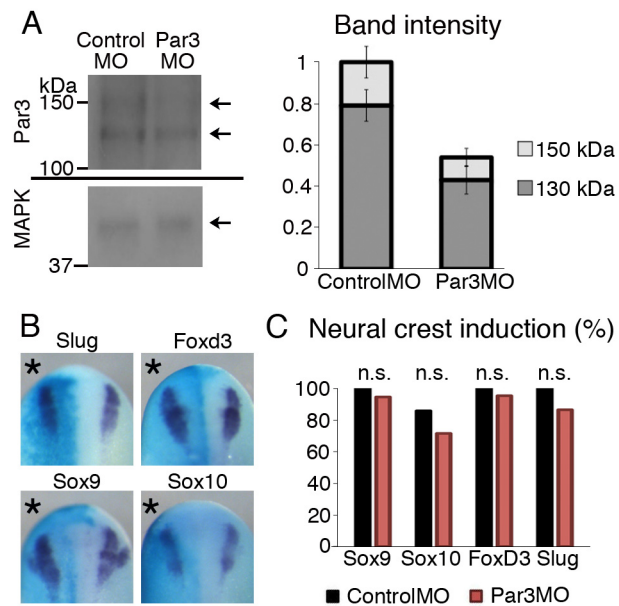


Figure 8.1. Par3 is not required for neural crest induction in *X. laevis*

(A) Western analysis of Par3 on protein extracts from *X. laevis* embryos injected with ControlMO or Par3MO. MAPK: loading control. Arrows: individual bands. Band intensity of both isoforms is shown relative to total Par3 protein level in ControlMO-injected embryos and normalised to loading control. (B) Dorsal view of Stage 16 *X. laevis* embryos injected unilaterally with Par3MO and processed for *in situ* hybridisation against *Slug*, *FoxD3*, *Sox9* or *Sox10*. Asterisk: injected side. (C) Par3MO does not affect neural crest induction (Par3MO: *Sox9* n=18; *Sox10* n=21; *FoxD3* n=22; *Slug* n=21; ControlMO (not shown): *Sox9* n=9; *Sox10* n=7; *FoxD3* n=6; *Slug* n=6; $p > 0.05$ for all genes).

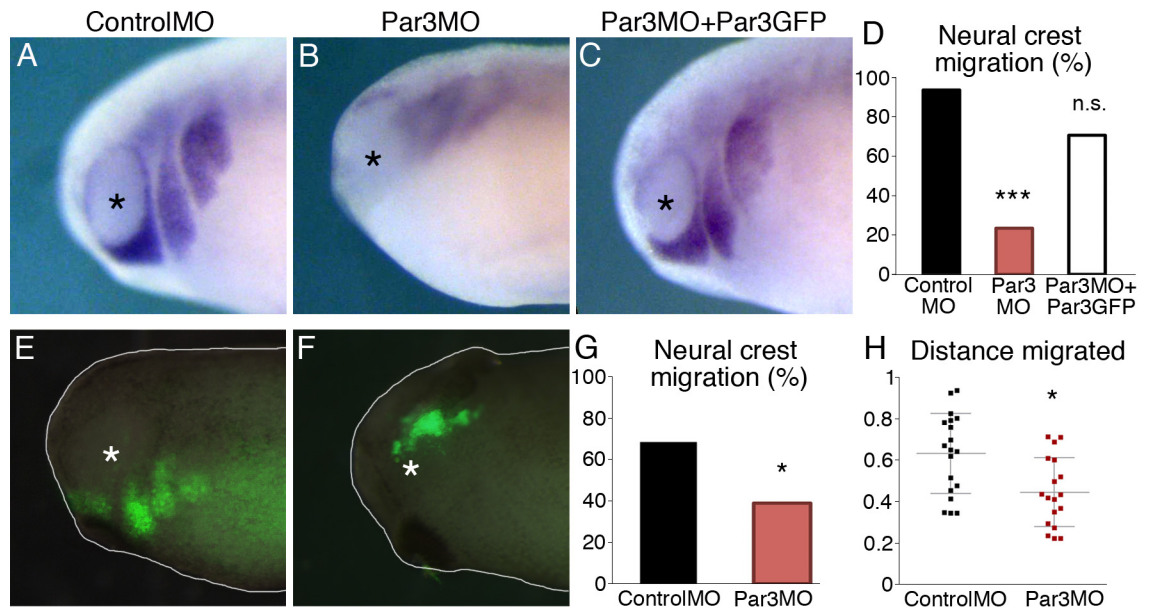


Figure 8.2. Par3 is required for neural crest migration in *X. laevis*

(A-D) Par3MO inhibits neural crest migration (B) but can be rescued by coinjection with mRNA for *D. rerio* Par3GFP (C). (A-C) Lateral view of ControlMO- (A), Par3MO- (B) or Par3MO+ParGFP-injected embryos (C) processed for *in situ* hybridisation against *Twist*. Asterisk: eye. (D) Percentage of embryos with normal neural crest migration is reduced in Par3MO-injected embryos (n=45) compared to ControlMO-injected embryos (n=24; $p < 0.001$). This can be rescued by coinjection with Par3GFP (n=34; $p < 0.001$). (E-H) Par3MO inhibition of neural crest migration is cell autonomous. (E-F) Lateral view of embryos following transplant of ControlMO NC (E) or Par3MO NC (F). Green (Fluorescein-Dextran): NC transplant. Asterisk: eye. (G) Percentage of embryos with normal neural crest migration following neural crest transplant is reduced in Par3MO-injected neural crest (n=18) compared to ControlMO-injected neural crest (n=19; $p < 0.05$). (H) Compared to ControlMO-injected neural crest (n=19), distance migrated by Par3MO-injected neural crest as a proportion of head size is reduced (n=18; $p = 0.0035$, error bars show mean and s.d).

Par3MO showed a strong impairment in neural crest migration (Fig. 8.2F-H). These results show that Par3 is required for neural crest migration in a cell-autonomous manner.

Par3 can act in concert with a number of other proteins such as aPKC, with which it forms a Par complex (reviewed in Goldstein and Macara, 2007). A loss-of-function experiment was performed by injecting *X. laevis* embryos with mRNA for a dominant-negative form of aPKC (aPKCdn; Fig. 8.3). *In situ* hybridisation against *Twist* showed that neural crest migration in aPKCdn-injected embryos was not significantly different to neural crest migration of control embryos (Fig. 8.3B,D). Despite this, it was interesting to note a mild phenotype consisting of minor disturbance of neural crest streams in a small number of aPKCdn-injected embryos. This disturbance involved the merging of neural crest streams and curvature of the streams (Fig. 8.3C). Nonetheless, even in these embryos that exhibited a minor disturbance of neural crest streams, neural crest migration was still apparent and, as such, this phenotype was very different to the phenotype observed following Par3MO-injection (refer to Fig. 8.2B). These results suggest that Par3 is not acting with aPKC in its role in neural crest migration and aPKC was not investigated further.

To further assess the requirement for Par3 in neural crest migration, explants were cultured on fibronectin and imaged using time-lapse cinematography. ControlMO-injected explants tended to disperse after few hours of cell culture (Fig. 8.4A) but explants injected with Par3MO failed to disperse (Fig. 8.4B; Supplementary Movie 01). Cell dispersion was quantified by measuring the area of triangles formed between the nuclei of closest neighbours, using Delaunay triangulation, as described previously (Carmona-Fontaine et al., 2011). A dramatic increase in cell dispersion starts at around 6 hours in the ControlMO-injected explants, but this is very much reduced in

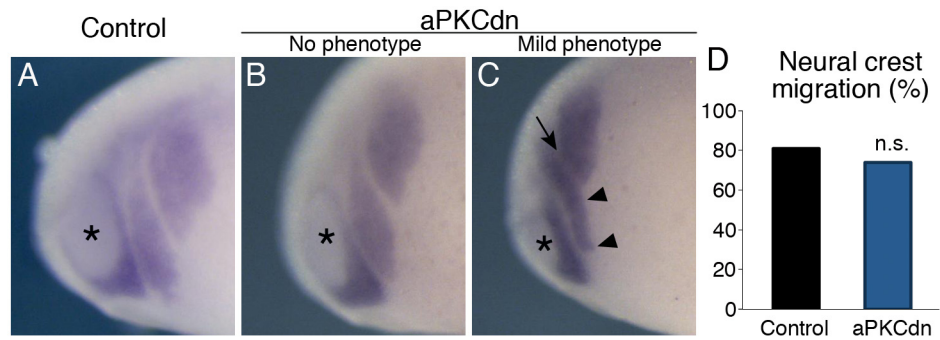


Figure 8.3. aPKC does not act with Par3 in controlling neural crest migration

(A-C) Lateral view of control embryo (A), aPKCdn-injected embryo with no phenotype (B) and aPKCdn-injected embryo with mild phenotype (C) processed for *in situ* hybridisation against *Twist*. The mild phenotype illustrated in (C) includes merged neural crest streams and curvature of the streams. Arrow: merged neural crest streams. Arrowhead: curved neural crest stream. Asterisk: eye. (D) Compared to control embryos (n=16), aPKCdn-injection does not affect neural crest migration (n=35; $p>0.05$).

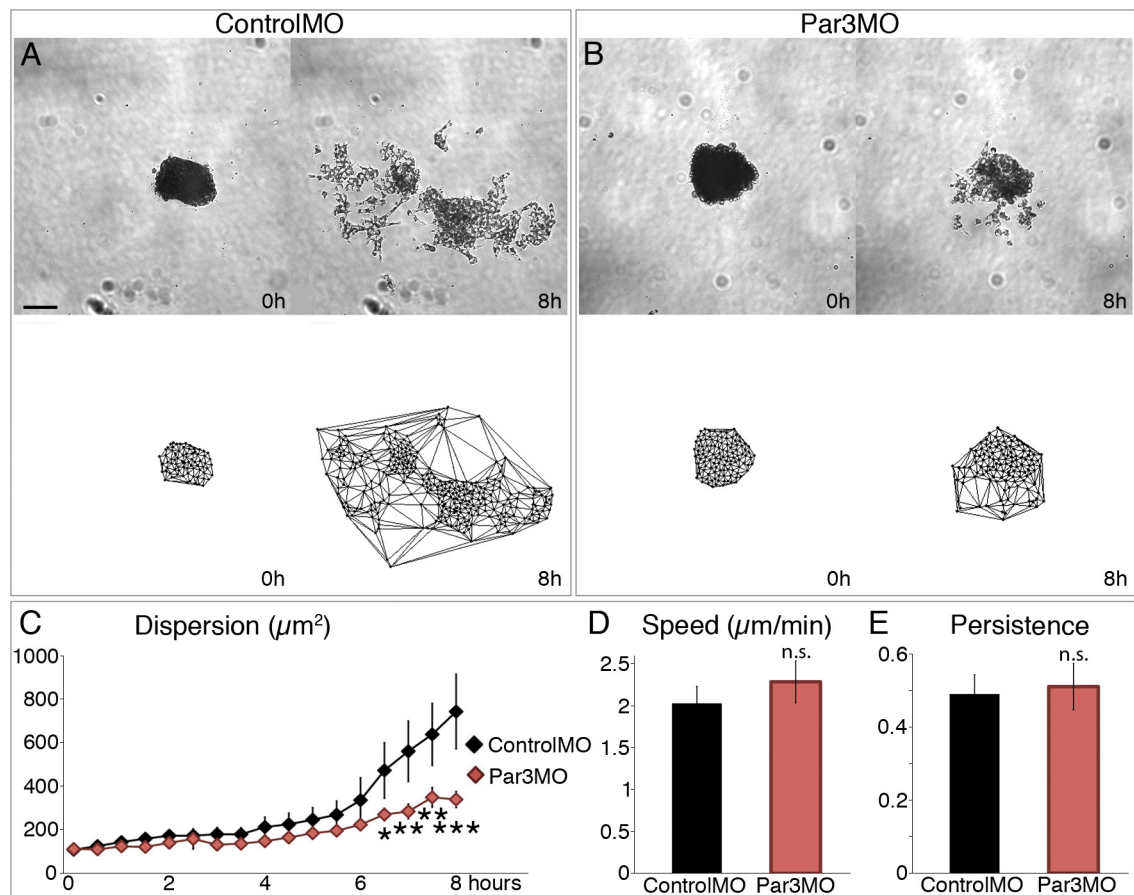


Figure 8.4. Par3 is required for neural crest dispersion *in vitro*

(A,B) Single frames from time-lapse movies showing control (A) and Par3MO-injected (B) neural crest explants and Delaunay triangulation at 0 hours and 8 hours. Scale bar: 100 μm . (C) Dispersion between cells increases over time in control explants (n=8 explants; compared to 0 hours: $p < 0.05$ at 7 hours, $p < 0.01$ at 7.5 hours, $p < 0.001$ at 8 hours). However, dispersion is reduced in Par3MO explants over time compared to controls (n=8 explants; $p < 0.05$ for all time points). There is less dispersion between Par3MO cells than control cells at 6.5 hours ($p < 0.05$), 7-7.5 hours ($p < 0.01$) and 8 hours ($p < 0.001$). (D) Speed of single cells is not affected by Par3MO (n=20; $p = 0.2146$). (E) Persistence of single cells is not affected by Par3MO (n=20; $p = 0.4021$).

Par3MO-injected explants (Fig. 8.4C). This could simply be due to an inability of Par3MO-injected cells to migrate. However, ControlMO- and Par3MO-injected single cells exhibited similar speed during migration (Fig. 8.4D). The persistence of migration was measured as the distance between the start and end coordinates of migration divided by the actual distance migrated. ControlMO- and Par3MO-injected cells also exhibited similar persistence during migration of single cells (Fig. 8.4E). These results demonstrate that Par3 is not required for cell motility, but is required for neural crest dispersion.

These results show an effect of Par3MO on *X. laevis* neural crest migration *in vivo* and *in vitro*. As live *in vivo* imaging is difficult to perform in *X. laevis* embryos, *D. rerio* embryos were used to perform live imaging of neural crest migration. First, I confirmed that a similar neural crest phenotype was achieved by using a previously characterised *D. rerio* Par3MO (Wei et al., 2004b). Indeed, injection of Par3MO does not affect neural crest induction in *D. rerio* (Fig. 8.5A), but leads to a clear inhibition of migration in trunk neural crest, as analysed by *in situ* hybridisation against *FoxD3* (Fig. 8.5B-D). Par3MO-injection also inhibits cephalic neural crest migration in *D. rerio* embryos (Fig. 8.5B,E,F).

To analyse cell behaviour *in vivo*, a transgenic cell line that expresses membrane-GFP and nuclear-RFP under the specific neural crest promoter of Sox10 was used. In accordance with *in vitro* observations using *X. laevis* neural crest, Par3MO-injection reduced neural crest dispersion *in vivo* (Fig. 8.6A,B; Supplementary Movie 02). Neural crest cell dispersion starts at around 4 hours in ControlMO-injected embryos, but this is very much reduced in Par3MO-injected embryos. Consequently, cell density is increased in Par3MO-injected embryos (Fig. 8.6C), as cell dispersion is

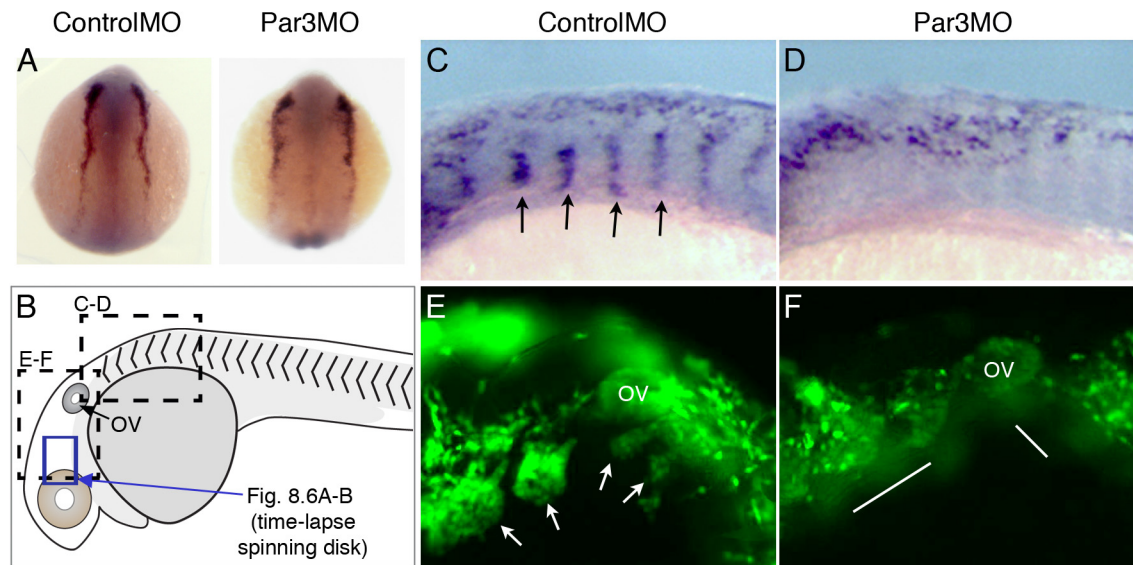


Figure 8.5. Par3 is required for neural crest migration in *D. rerio*

(A) Dorsal view of *D. rerio* embryos injected with ControlMO or Par3MO and processed for *in situ* hybridisation against *FoxD3*. (B) Schematic representation showing the location of trunk (C-D) and cranial (E-F) neural crest imaging of fixed *D. rerio* embryos and the location of live time-lapse imaging of *D. rerio* cranial neural crest (Fig. 8.6A,B). OV: otic vesicle. (C,D) Lateral view showing the trunk of ControlMO- (C) or Par3MO-injected (D) embryos processed for *in situ* hybridisation against *FoxD3* at the 20-somite stage. Arrows: distinct trunk neural crest streams. (E,F) Lateral views showing the cranial region of 24-hour *Sox10:egfp* embryos injected with ControlMO (E) or Par3MO (F). OV: otic vesicle. Arrows: distinct cranial neural crest streams. Lines: no distinct streams observed.

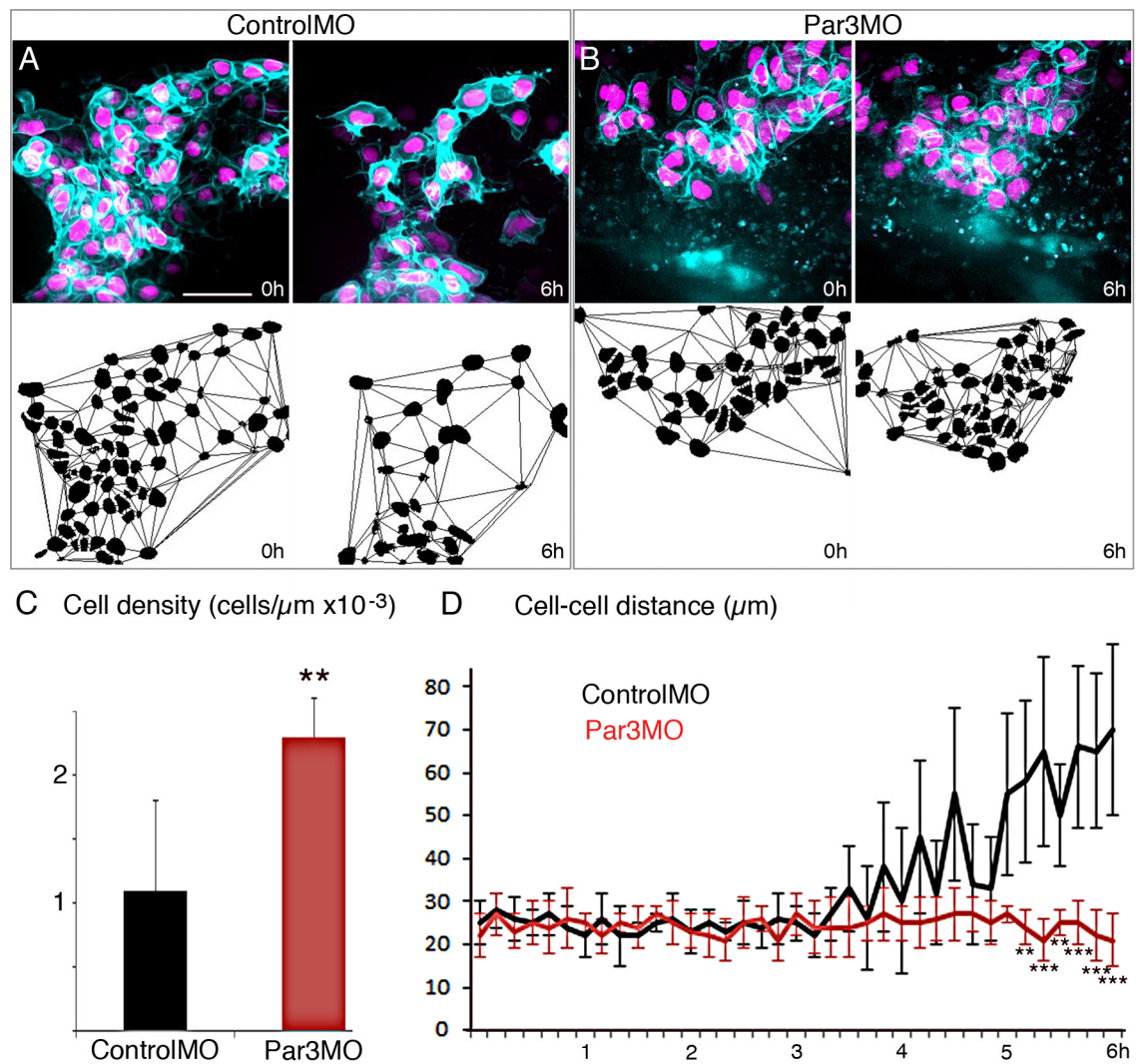


Figure 8.6. Par3 is required for neural crest dispersion *in vivo*

(A-B) Single frames from time-lapse movies showing one cranial neural crest stream and corresponding Delaunay triangulation at 0 hours (15-somites) and 6 hours from ControlMO-injected (A) and Par3MO-injected (B) embryos. Scale bars: 40 μm . (C) Cranial neural crest cell density at 6 hours is increased in Par3MO-injected embryos compared to ControlMO-injected embryos ($n=15$; $p<0.01$). (D) Distance between cranial neural crest cells increases over time in ControlMO embryos ($n=15$), but not Par3MO embryos ($n=15$). There is less distance between cranial neural crest cells in Par3MO embryos than in ControlMO embryos at 5.10 hours ($p<0.01$), 5.20 hours ($p<0.001$), 5.30 hours ($p<0.01$) and 5.40-6 hours ($p<0.001$).

impaired (Fig. 8.6D). Along with results from *X. laevis*, this confirms that Par3 is required to promote neural crest dispersion.

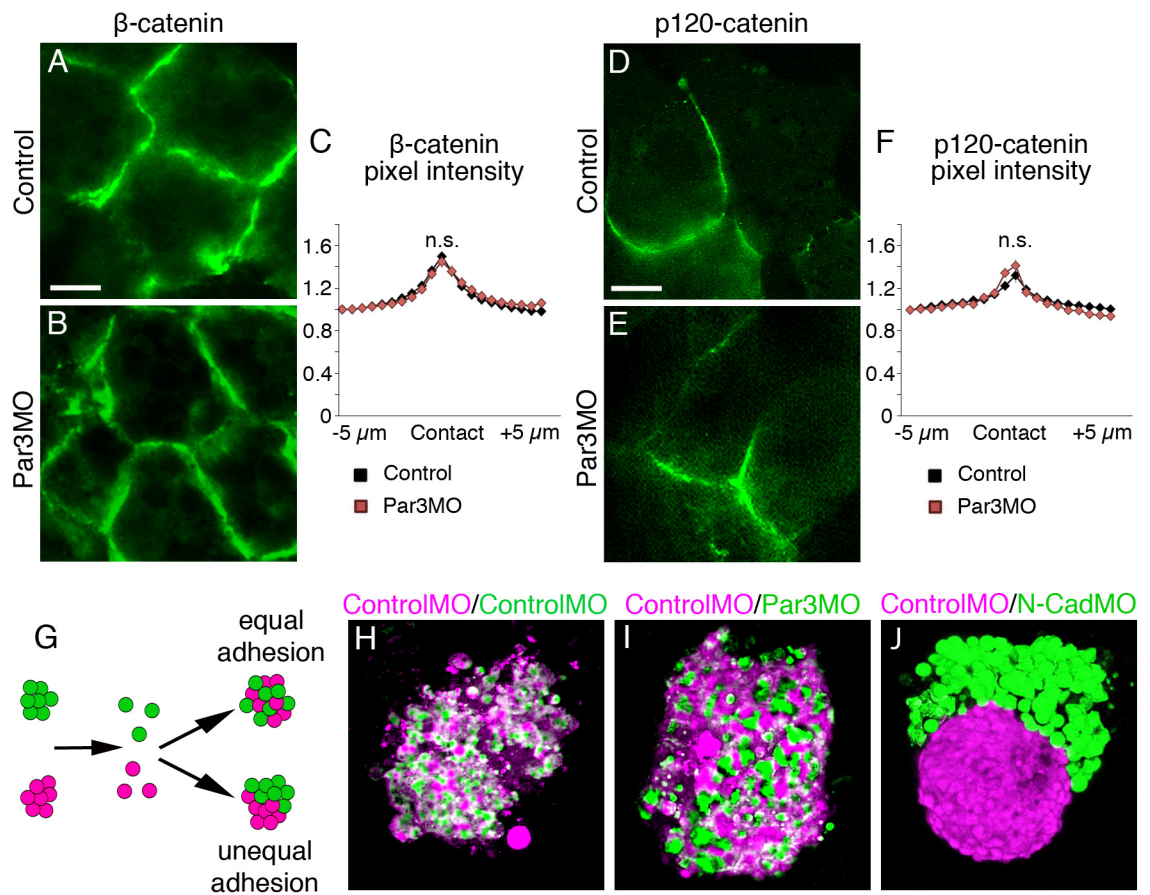
8. 2. Par3 is not required for cell-cell adhesion

One way in which Par3 could affect dispersion is through controlling cell-cell adhesion, as has been shown in other cell types (Chen and Macara, 2005; Mertens et al., 2005; Mishima et al., 2002). Neural crest adhesion molecules include β -catenin and p120-catenin, which are localised to the cell-cell contact and required for junction formation and maintenance. The effect of Par3MO on the levels and localisations of different cell adhesion proteins was measured. No effect was found on the localisation of β -catenin at the cell-cell junction in Par3MO-injected cells compared to control *X. laevis* neural crest cells (Fig. 8.7A-C). There was also no effect observed on the localisation of p120-catenin in Par3MO-injected cells compared to control cells (Fig. 8.7D-F).

Furthermore, I performed a cell-sorting assay to evaluate whether Par3MO affected cell adhesion. When two cell populations have the same adhesion properties, dissociation and reaggregation leads to a randomly mixed group of cells. However, if the two cell populations exhibit different cell adhesion the cells sort out, organising into two distinct groups (Fig. 8.7G; Friedlander et al., 1989). Dissociating ControlMO- and Par3MO-injected cells, mixing them together and allowing them to reaggregate resulted in a mixed cell population with no difference between ControlMO and Par3MO-injected cells (Fig. 8.7H,I). This is in stark contrast to a positive control experiment: mixing ControlMO neural crest cells with neural crest cells injected with a morpholino that downregulates N-cadherin (N-cadherinMO)

Figure 8.7. Par3 inhibition does not affect cell-cell adhesion properties

(A-C) β -catenin localisation is not affected by Par3MO. Scale bar: 10 μ m. (A) Immunostaining against β -catenin in control neural crest cells. (B) Immunostaining against β -catenin in neural crest cells from Par3MO-injected embryos. (C) Pixel intensity of β -catenin immunostaining was measured across the contact and normalised to the average value at -5μ m for each image. There is no difference in pixel intensity of β -catenin immunostaining between neural crest cells from control (n=84 contacts) and Par3MO-injected embryos (n=44 contacts; $p>0.05$ at all distances from contact). (D-F) p120-catenin localisation is not affected by Par3MO. Scale bar: 10 μ m. (D) Control neural crest cells expressing p120-cateninGFP. (E) Neural crest cells from Par3MO-injected embryos expressing p120-cateninGFP. (F) Pixel intensity of p120-cateninGFP was measured across the contact and normalised to the average value at -5μ m for each image. There is no difference in pixel intensity of p120-catenin immunostaining between neural crest cells from control (n=24 contacts) and Par3MO-injected embryos (n=41 contacts; $p>0.05$ at all distances from contact). (G-J) Cell adhesion is not affected by Par3MO. (G) Schematic representation of cell reaggregation assay. (H) Reaggregation of two neural crest cell populations from ControlMO-injected embryos leads to random mixing. (I) Reaggregation of neural crest cells from ControlMO- and Par3MO-injected embryos leads to random mixing, indicating no difference in cell adhesion between the two populations. (J) Reaggregation of ControlMO neural crest cells and N-cadherinMO neural crest cells leads to segregation of the two neural crest populations.



lead to their self-organisation into two distinct groups (Fig. 8.7J). Together, these results did not support a role for Par3 in regulating cell adhesion between neural crest cells and an alternative mechanism for the effect of Par3 inhibition on neural crest migration and dispersion needed to be explored.

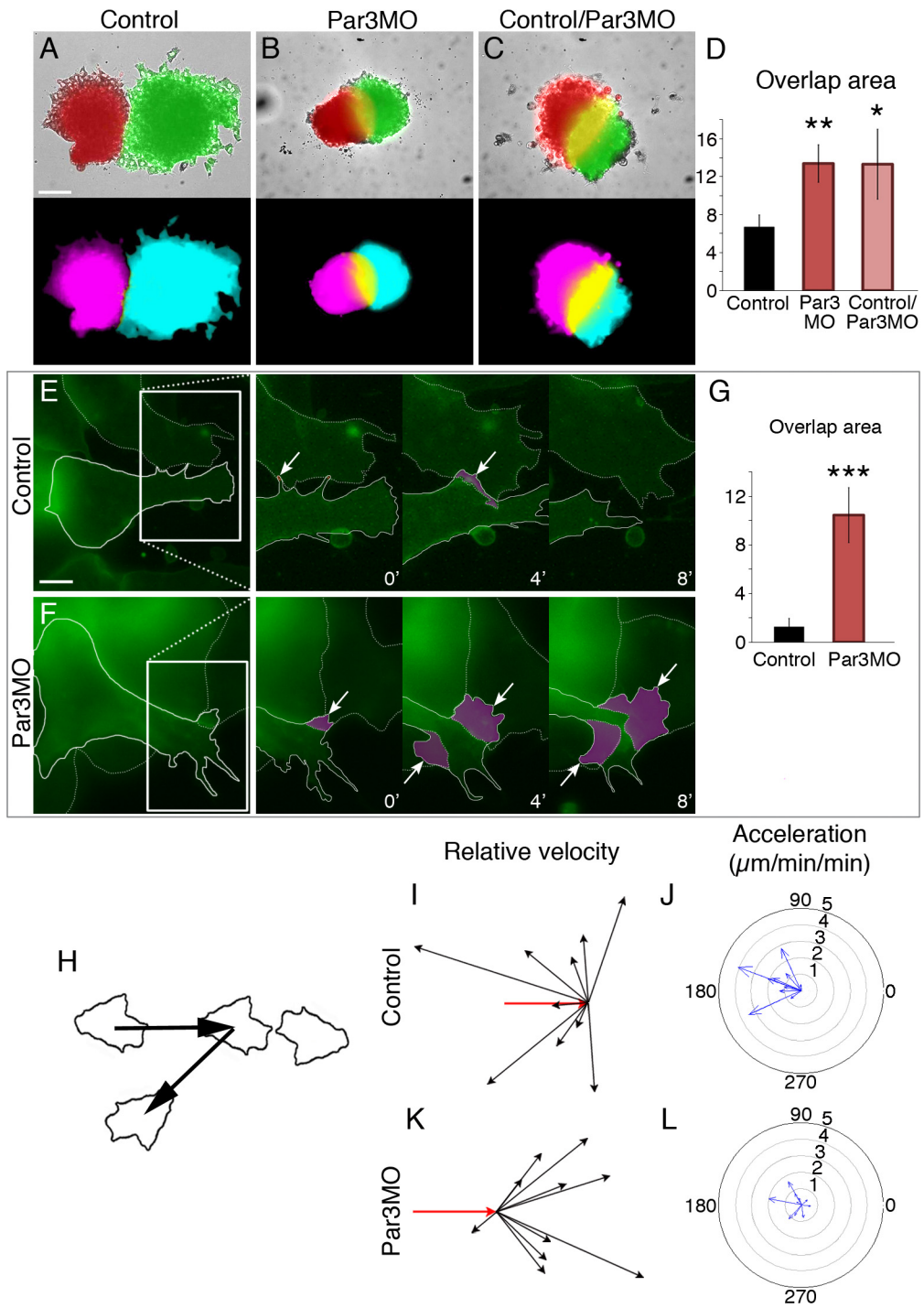
8. 3. Par3 is required for contact inhibition of locomotion

An alternative way in which Par3 could affect neural crest dispersion is through controlling CIL (Mayor and Carmona-Fontaine, 2010). CIL promotes dispersion by encouraging the migration of cells away from each other, so a loss of CIL results in the maintenance of cells as a cluster. Further, Par3 can affect cell polarity and directional migration, which are aspects of CIL in neural crest cells, in other migrating cells (Du et al., 2010; Pegtel et al., 2007; Schmoranzner et al., 2009).

Three different assays were used to analyse CIL, all of which have been used previously (Abercrombie and Heaysman, 1953; Carmona-Fontaine et al., 2008; Theveneau et al., 2010). First, two neural crest explants confronted with each other do not overlap if they exhibit CIL (Fig. 8.8A). The overlapping area can be analysed by using explants labelled with FDX or RDX and comparing the yellow area of overlap with the total fluorescent area. Par3MO-injected explants widely overlapped, as did pairs of one control and one Par3MO-injected explant, indicating a failure in CIL (Fig. 8.8B-D). Second, CIL dictates that two adjacent cells do not make protrusions on top of one another (Fig. 8.8E; Abercrombie and Heaysman, 1953); however, Par3MO-injected neural crest cells extended protrusions on top of their neighbours (Fig. 8.8F,G; Supplementary Movie 03). Third, the hallmark of CIL is that collision between two single cells will cause them to change velocity if CIL occurs (Fig. 8.8H; Abercrombie and Heaysman, 1953). However, colliding Par3MO-

Figure 8.8. Par3 is required for contact inhibition of locomotion *in vitro*

(A-D) Explant confrontation assay. (A-C) Single frames from time-lapse movies showing maximum overlap between two control explants (A), two Par3MO-injected explants (B) or one control and one Par3MO-injected explant (C). Scale bar: 100 μm . (D) Overlap area as a percentage of total explant area is greater between Par3MO/Par3MO (n=16; p=0.0026) or control/Par3MO explants (n=9; p=0.0252) than between control/control explants (n=27). (E-G) Protrusion overlap analysis. Scale bar: 10 μm . (E-F) Control (E) or Par3MO-injected (F) cells expressing mbGFP. White line: outline of individual cells. Purple overlay and arrows: protrusion overlap. (G) Protrusion overlap area as a percentage of cell area is greater in Par3MO-injected cells (n=40) than control cells (n=50; p=0.0002). (H-L) Cell collision assay. (H) Schematic representation of cell collision. (I-L) Cell position was analysed at 15-minute intervals to measure velocity (I,J) and acceleration (K,L) following collision between control or Par3MO-injected cells. Velocity change was clustered in control cells (n=17; p<0.001) but was not observed in Par3MO-injected cells (n=37; p<0.001). Red arrows: initial velocity vector.



injected cells showed a significant reduction in the change in velocity upon collision compared to control cells (Fig. 8.8I-L; Supplementary Movie 04). Together these results indicate that Par3 is required for CIL between neural crest cells in *X. laevis* embryos.

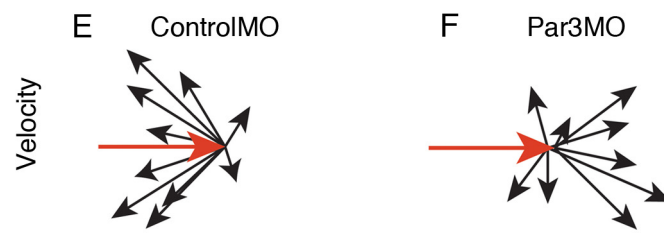
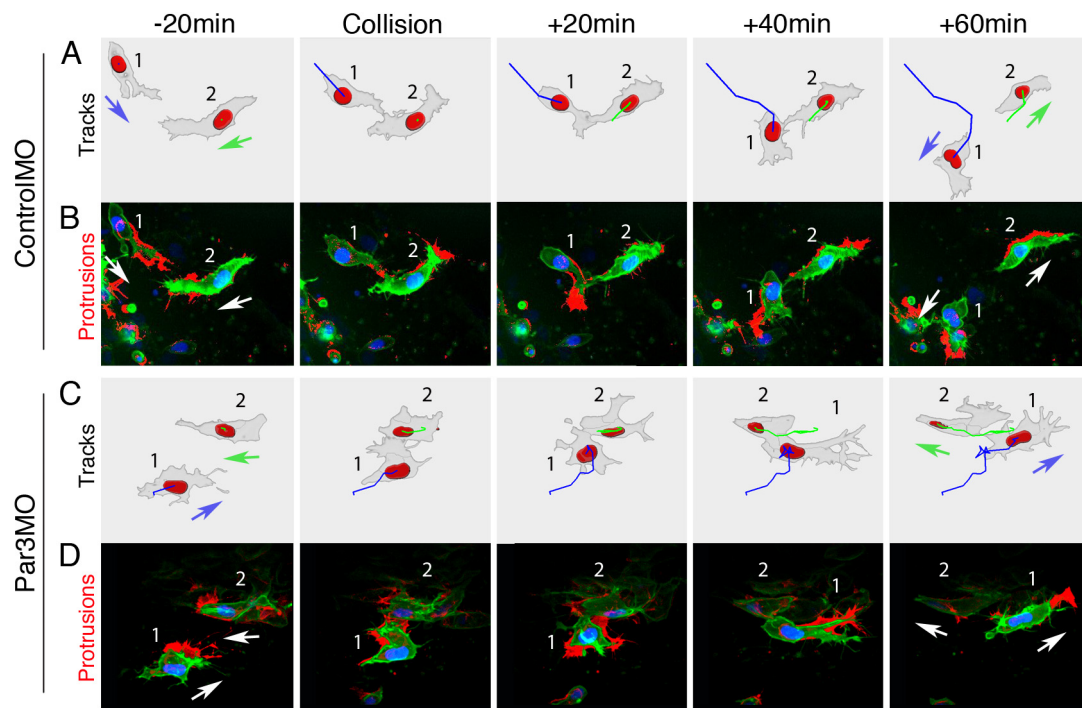
Next, the *Sox10:mGFPnRFP D. rerio* line was used to analyse CIL *in vivo*. Collisions between neural crest cells were analysed after time-lapse imaging of migrating neural crest cells. Neural crest cells in ControlMO-injected embryos exhibited CIL by increasing velocity following cell-cell collision, accompanied by a typical neural crest response to collision in the form of a change in the direction of migration (Fig. 8.9A, B, E; Supplementary Movie 05). In contrast, neural crest cell collisions in Par3MO-injected embryos lead to neither an increase in velocity nor a change in the direction of migration (Fig. 8.9C, D, F; Supplementary Movie 06). These experiments confirm that Par3 is required for CIL between neural crest cells both *in vitro* and *in vivo*.

8. 4. Par3 is localised to the cell-cell contact

In the cranial neural crest, CIL causes protrusion retraction upon cell-cell contact (Carmona-Fontaine et al., 2008). It is also considered to lead to the subsequent repolarisation and extension of a new protrusion (Carmona-Fontaine et al., 2008). Considering these distinct steps in neural crest response to cell-cell collision, it was important to assess Par3 localisation in order to determine its precise role during CIL. For example, Par3 could be required at cell-cell contact, or could be localised to a new protrusion to control repolarisation. To analyse the dynamics of Par3 localisation, *X. laevis* embryos were injected with Par3GFP mRNA and time-lapse imaging was performed on cultured neural crest explants and single cells. Par3GFP

Figure 8.9. Par3 is required for contact inhibition of locomotion *in vivo*

(A,B) Single frames from time-lapse movies illustrating a collision between two cranial neural crest cells in a *D. rerio* embryo injected with ControlMO. Images are overlapped with tracks of migratory paths (A) or the difference in protrusion between current and previous frames (B). Red area: new protrusions. (C,D) Single frames from time-lapse movies illustrating a collision between two cranial neural crest cells in a *D. rerio* embryo injected with Par3MO. Images are overlapped with tracks of migratory paths (C) or the difference in protrusion between current and previous frames (D). Red area: new protrusions. (E) Velocity vectors of colliding cranial neural crest cells in ControlMO-injected embryos. Cell position was recorded at 5-minute intervals. Velocity change was clustered in cells in ControlMO-injected embryos (n=10; p<0.05). Red arrow: initial velocity vector. (F) Velocity vectors of colliding cranial neural crest cells in Par3MO-injected embryos. Cell position was recorded at 5-minute intervals. No change in velocity was observed in collisions in Par3MO-injected embryos (n=9; p>0.1). Red arrow: initial velocity vector.



was localised to cell junctions both in clusters of neural crest cells that were already in contact (Fig. 8.10A), and during collisions of individual colliding cells (Fig. 8.10B; Supplementary Movie 07). To confirm that endogenous Par3 was expressed in *X. laevis* neural crest and localised to cell-cell contacts, membraneRFP (mbRFP)-labelled neural crest cells were transplanted into wildtype host embryos to identify the migrating neural crest cells, and immunostaining against Par3 was performed on cryosections. These results showed that endogenous Par3 was present in the neural crest and colocalised with mbRFP at cell-cell contacts (Fig. 8.10C-F).

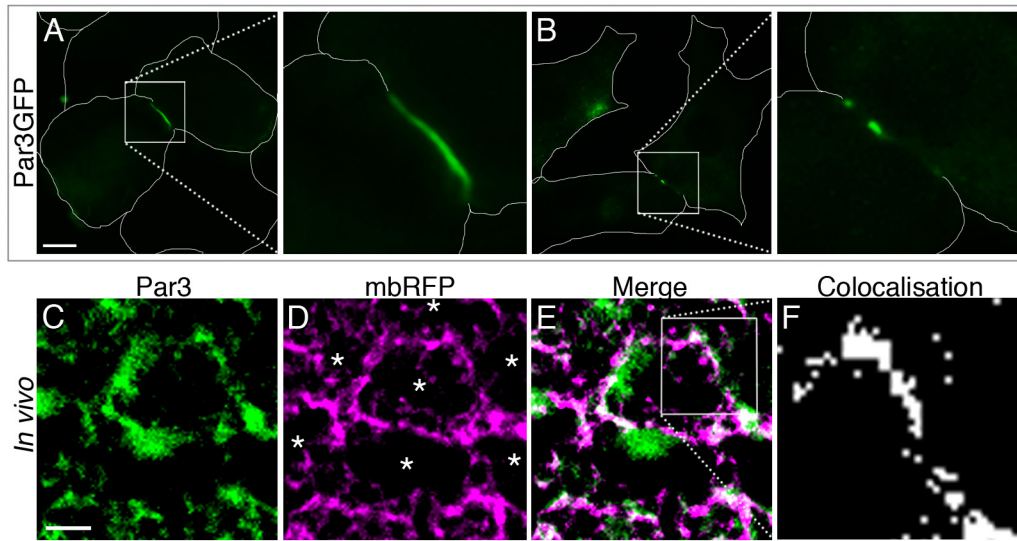


Figure 8.10. Par3 is localised to the cell-cell contact

(A,B) Par3GFP localises to contacts between *X. laevis* neural crest cells in clusters (A) and between colliding single cells (B). Scale bar: 10 μm . (C-F) Z-projection of confocal images showing immunostaining against endogenous Par3 in cryosections of *X. laevis* embryos. Scale bar: 20 μm . (C) Par3 is located to contacts between neural crest cells. (D) mbRFP identifies neural crest cells and cell contacts. (E) Merge of (C,D). (F) Colocalisation mask of (C,D) shows localisation of Par3 to the membrane at contacts between neural crest cells.

9. Par3 promotes contact inhibition of locomotion by controlling microtubule dynamics

How does Par3 regulate CIL in neural crest cells? Par3 has been shown to control microtubule array polarisation in other cell types by affecting microtubule dynamics or centrosome positioning (Pegtel et al., 2007; Schmoranzer et al., 2009). Further, microtubules are influenced by cell-cell contacts and are involved in CIL (Kadir et al., 2011; Stramer et al., 2010). Thus, I next investigated a possible interaction between Par3 and microtubules, in order to determine whether Par3 could regulate CIL in this way.

9.1. Par3 does not affect the microtubule array at a cellular level

The microtubule array has not yet been investigated in *X. laevis* neural crest cells. To characterise it, I first analysed the entire microtubule array using immunostaining against α -tubulin. In control neural crest cells cultured on fibronectin, microtubules were densely packed in the centre of the cell, close to the nucleus, and were sparser towards the edge of the cell (Fig. 9.1A). In cells with a protrusion, microtubules extended well into the lamellipodia (Fig. 9.1A). Par3MO-injected cells appeared to have a similar microtubule array to control cells (Fig. 9.1C). This was the case both for cells within the neural crest explant and those at the edge of the explant (Fig. 9.1C). These results suggested that Par3 inhibition had no effect on the entire microtubule array. Immunostaining was also performed against acetylated tubulin, which is incorporated into stable microtubules (Hammond et al., 2008). In control cells, the acetylated microtubule subpopulation was sparse and present close to the

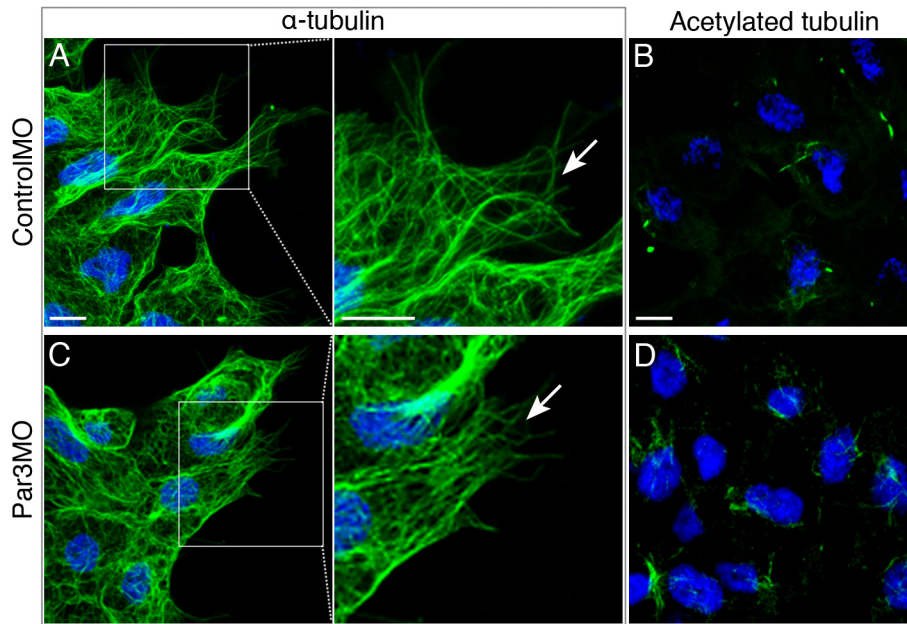


Figure 9.1. Par3 does not affect the overall microtubule array

(A,C) Immunostaining against α -tubulin (green) shows the microtubule array in ControlMO- (A) and Par3MO-injected (C) cells. Nuclei are stained with DAPI. Microtubules extend into the lamellopodia. Scale bar: 10 μ m. Arrows: lamellopodia.

(B,D) Immunostaining against acetylated tubulin (green) stains a stable subpopulation of microtubules in ControlMO- (B) and Par3MO-injected (D) cells. Nuclei are stained with DAPI. Scale bar: 10 μ m.

nucleus (Fig. 9.1B). This was also the case in Par3MO-injected cells (Fig. 9.1D), indicating that Par3 inhibition had no effect on acetylated microtubules.

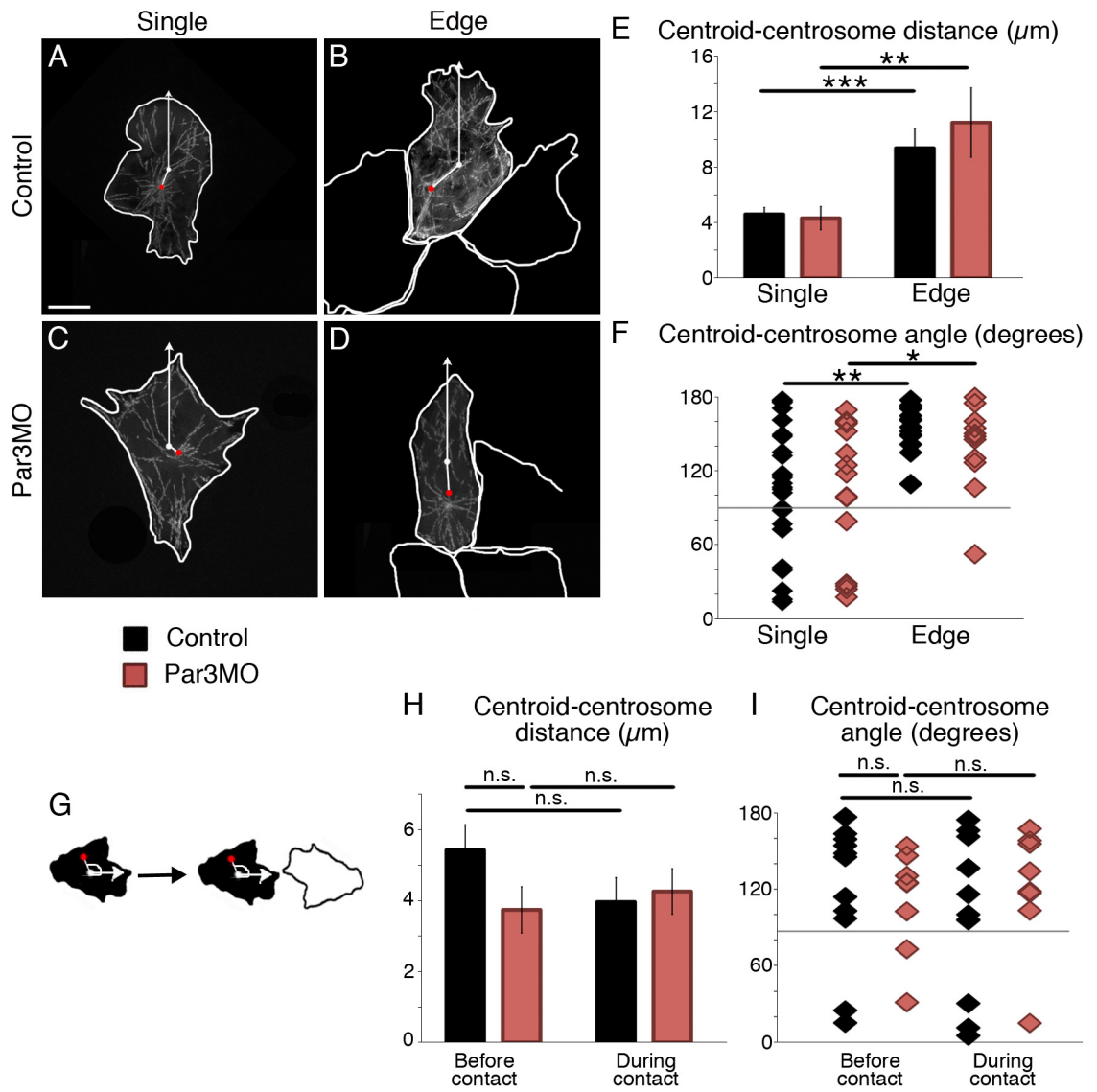
As Par3 has been shown to control centrosome orientation in other cell types (Schmoranzer et al., 2009), I next investigated a potential role for Par3 in controlling centrosome position. The centrosome was observed by labelling neural crest cells with EB3GFP. These cells exhibited green fluorescent “comets” corresponding to the tips of growing microtubules, as well as one considerably larger, motile, bright green fluorescent spot that corresponded to the centrosome (Alieva et al., 2010). To assess the polarity of the centrosome position, the position of the centrosome was analysed with respect to the centroid of EB3GFP-labelled cells in two separate ways.

For control neural crest cells, the centrosome was further from the cell centroid in cells at the edge of an explant compared to single cells (Fig. 9.2A,B,E). Edge cells tended to be more elongated than single cells due to the extension of a large protrusion away from the contact. However, edge cells also had a significantly greater centroid-centrosome distance as a proportion of cell length when compared to single cells (not shown). Centrosome position was analysed further in relation to cell polarity. The centrosome was located behind the centroid with respect to the direction of migration in edge cells but this was not the case in single cells (Fig. 9.2A,B,F). These two results imply that cell-cell contacts polarise the microtubule array.

In wound-edge fibroblasts, Par3 localises to cell-cell contacts and influences cell polarity by orienting the centrosome (Schmoranzer et al., 2009). It does this through an interaction with dynein leading to the tethering microtubule ends to the cell-cell contact (Schmoranzer et al., 2009). However, in cranial neural crest cells Par3 inhibition did not affect centroid-centrosome distance or centrosome position in single or edge cells (Fig. 9.2C-F). These results were supported by experiments showing that

Figure 9.2. Par3 does not affect centrosome position

(A-F) Centrosome position was analysed in EB3GFP-labelled single (A) and edge (B) control cells and single (C) and edge (D) Par3MO-injected cells. Scale bar: 10 μm . Arrow: direction of migration. White dot: cell centroid. Red dot: centrosome. White line: centroid-centrosome distance. (E) Centroid-centrosome distance is greater in control edge cells ($n=15$) than single cells ($n=32$; $p=0.0001$). There is also a difference in centroid-centrosome distance between Par3MO-injected single ($n=21$) and edge cells ($n=12$; $p=0.0017$). There was no difference between control and Par3MO-injected single ($p=0.7582$) or edge cells ($p=0.5086$). (F) The angle between the direction of migration, the cell centroid and the centrosome is biased towards 180° (i.e.: towards the cell rear) in control edge cells ($p<0.001$) and this is significantly different to control single cells ($p=0.0016$), which have no bias in centrosome position. The angle between the direction of migration, centroid and centrosome is also biased towards 180° in Par3MO-injected edge cells ($p<0.001$) and this is also significantly different to Par3MO-injected single cells ($p=0.0231$), which have no bias in centrosome position. There is no difference between control and Par3MO-injected edge cells ($p=0.2056$) or single cells ($p=0.7417$). Grey line: 90° . (G-I) Centrosome position was analysed in EB3GFP-labelled cells before and during collision. (G) Schematic representation of centrosome analysis before and during collision. (H) There was no difference in centroid-centrosome distance before contact and during contact in control ($p=0.1058$) or Par3MO-injected cells ($p=0.5849$). There was also no difference between control and Par3MO-injected cells before contact ($p=0.1126$). (I) The angle between the direction of migration, the centroid and the centrosome before contact was not different to the angle during contact in control ($p=0.2324$) or Par3MO-injected cells ($p=0.6524$). There was also no difference between control and Par3MO-injected cells before contact ($p=0.4147$).



culture of neural crest explants in sodium orthovanadate (1-100 μM), which inhibits dynein, has no effect on neural crest cell migration (not shown). In addition, centrosome position was not affected by the formation of a transient new cell contact during collisions of control or Par3MO-injected cells (Fig. 9.2G-I). Altogether, these results suggest that Par3 does not affect the microtubule array on a gross level or control centrosome positioning in clusters, single cells or colliding cells.

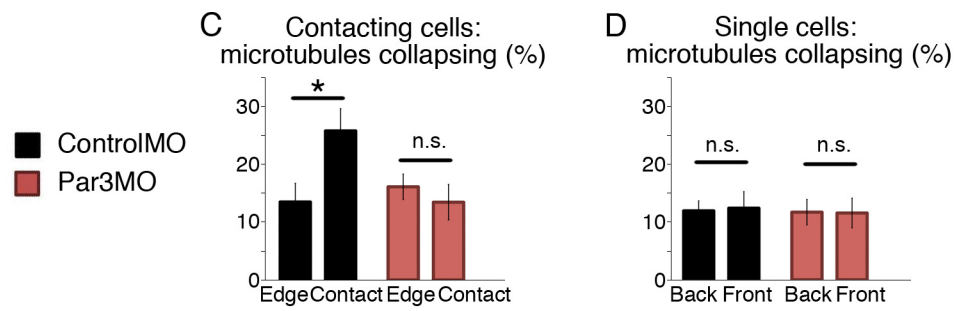
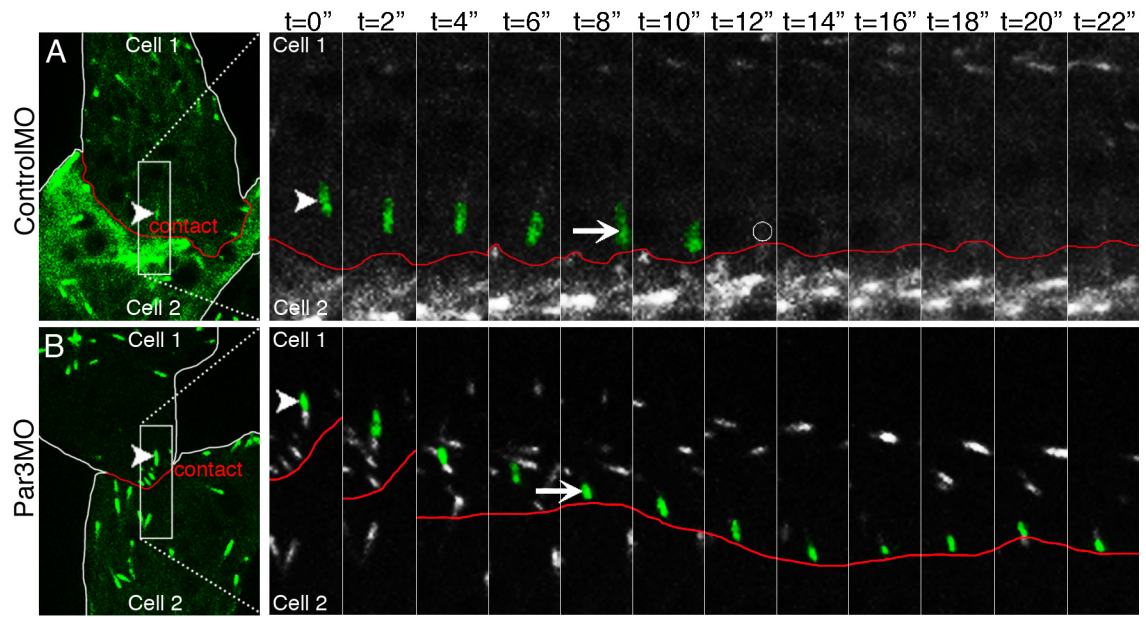
9. 2. Par3 promotes microtubule collapse at the cell-cell contact

Finally, microtubule catastrophe was analysed. To do this, a 5 μm x 5 μm square was selected at the contact and at the free edge of a cell undergoing collision. All microtubules that entered the square were analysed and the percentage that collapsed within the confines of the square was recorded. In control cells, a greater percentage of microtubules experienced catastrophe at the contact compared to the free edge (Fig. 9.3A,C). However, inhibiting Par3 abolished the increase in microtubule catastrophe at the contact (Fig. 9.3B,C; Supplementary Movie 09). This suggests that Par3 is required at the cell-cell contact to promote microtubule catastrophe.

An important control was performed by analysing the microtubule catastrophe at the back and front of single migrating cells. In these cells there was no difference in microtubule catastrophe between the front and back of the cell, and the rates of microtubule catastrophe were similar to that of the free edge of control cells during collision (Fig. 9.3D). There was also no significant difference between single migrating control and Par3MO-injected cells. As Par3 was localised to cell-cell contacts, this gives weight to the idea that Par3 promotes the microtubule collapse at the cell-cell contact.

Figure 9.3. Par3 promotes microtubule catastrophe at the cell-cell contact: manual analysis

EB3GFP-labelled cells were used to analyse microtubule catastrophe. (A-B) Low magnification images of ControlMO- (A) and Par3MO-injected (B) cells during collision, along with high magnification consecutive frames of time-lapse movies highlighting one microtubule of interest (green). White box: area of high magnification. Arrowhead: highlighted microtubule. White line: cell outlines. Red line: cell-cell contact. Arrow: microtubule contacts the membrane. Circle: site and time point of microtubule collapse. (C) A greater percentage of microtubules collapse at the cell-cell contact than at the free edge in ControlMO-injected cells ($n=15$; $p=0.0167$). However, there is no difference between the percentage of collapsing microtubules at the free edge and at the cell-cell contact in Par3MO-injected cells ($p=0.3$). (D) In ControlMO-injected single cells, there is no difference in the percentage of microtubules that collapse at the front of the cell than at the back ($n=25$; $p=0.8940$). There is also difference between the percentage of collapsing microtubules at the front and back of the cell in single Par3MO-injected cells ($n=18$; $p=0.9716$).



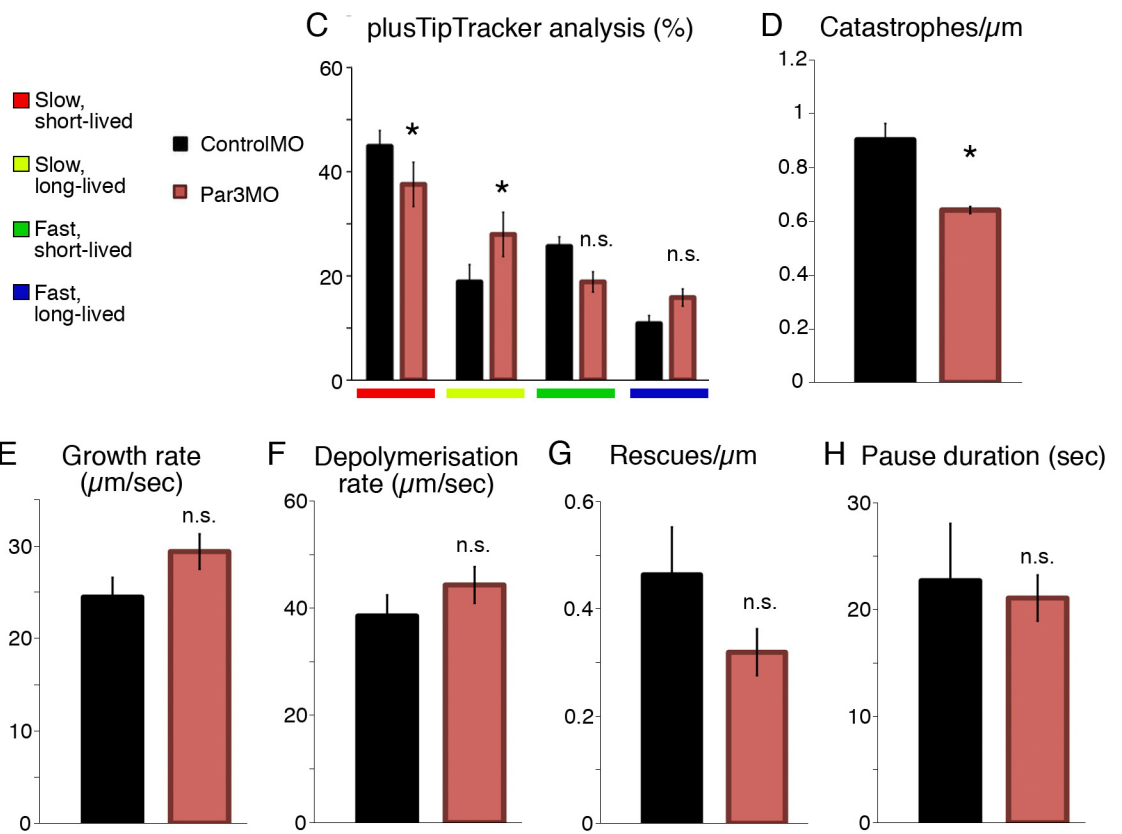
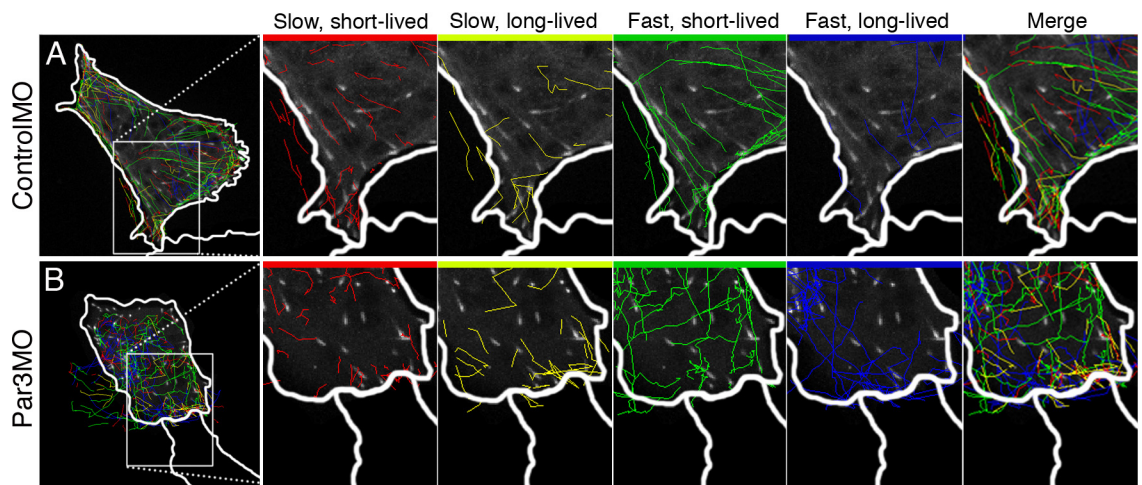
A caveat of using EB3GFP-labelled cells is that collapsing and pausing microtubules are not easy to distinguish or quantify manually. Therefore, I used the automatic tracking software plusTipTracker (Applegate et al., 2011) to perform an exhaustive analysis of microtubule dynamics. Importantly, plusTipTracker calculates which tracks belong to the same microtubule that may be cycling between phases of growth, pause and catastrophe (Applegate et al., 2011). In this way, it provides much more information about microtubule dynamics than manual analysis of EB3GFP tracks alone. Movies of EB3GFP-labelled cells were analysed at cell-cell contacts using plusTipTracker during CIL of *X. laevis* neural crest cells. Microtubule tracks were classified as being slow or fast and short-lived or long-lived based on the mean growth speed (26 $\mu\text{m}/\text{minute}$) and mean lifetime (20 seconds) of the data set, respectively. Par3 inhibition reduced the proportion of slow, short-lived microtubules in favour of slow, long-lived microtubules (Fig. 9.4A-C). This implies that slow microtubules live longer in Par3MO-injected cells; slow microtubules must therefore be undergoing catastrophe less frequently. Statistical analysis showed no effect of Par3 inhibition on the majority of factors routinely used for measuring microtubule dynamics, including growth rate, depolymerisation rate, rescue rate or pause duration (Fig. 9.4E-H). However, Par3 inhibition decreased the rate of microtubule catastrophe (Fig. 9.4D). Overall, the microtubule tracking analysis indicates that Par3 promotes microtubule catastrophe at the cell contact during CIL.

9.3 Dynamic microtubules are required for neural crest migration

Par3 promotion of microtubule catastrophe at the cell-cell contact could explain the requirement of Par3 for neural crest migration. If this were the case, one would expect

**Figure 9.4. Par3 promotes microtubule catastrophe at the cell-cell contact:
plusTipTracker analysis**

(A-C) Microtubule tracks at the cell-cell contact in control (A) and Par3MO-injected (B) cells were separated into four groups based on growth speed (slow < 26 $\mu\text{m}/\text{minute}$ < fast) and lifetime (short-lived < 20 seconds < long-lived). (C) Par3MO-injected cells have a smaller percentage of slow, short-lived microtubules than control cells (n=10 cells; p=0.0276) and a greater percentage of slow, long-lived microtubules (p=0.0238). (D-H) Par3MO-injected cells exhibited a lower catastrophe rate compared to control cells (D; p=0.035). However, no difference was found between control and Par3MO-injected cells in terms of growth rate (E; p>0.05), depolymerisation rate (F; p>0.05), rescue rate (G; p>0.05), or pause duration (H; p>0.05).



microtubule dynamics to be important in neural crest migration as well. To investigate this, neural crest explants were cultured in the presence of taxol or nocodazole. Taxol inhibits depolymerisation to stabilise microtubules, whereas nocodazole promotes microtubule depolymerisation.

9. 3. 1. Dynamic microtubules are required for neural crest dispersion

Explants cultured in the presence of the vehicle DMSO dispersed over time as described previously (Fig. 9.5A; Alfandari et al., 2003), whereas cells cultured in the presence of taxol failed to disperse (Fig. 9.5B,F; Supplementary Movie 10). These results show that microtubule dynamics are required for cell dispersion. However, explants cultured in the presence of nocodazole were able to disperse (Fig. 9.5C,F). Speed and persistence of cell migration was also reduced in the presence of both taxol and nocodazole (Fig. 9.5D,E). Cells in explants tend to migrate with lower speed but higher persistence than single cells (Carmona-Fontaine et al., 2008). Thus, a reduction in speed in the presence of taxol may be partly due to a failure of the explant to disperse. However, as both speed and persistence are reduced in both conditions, microtubule dynamics appear to play a role in neural crest cell motility.

9. 3. 2. The microtubule and actin cytoskeletons are required for centrosome positioning

Although it had been determined that Par3 was not involved in centrosome positioning (see Fig. 9.2, page 159), it was of interest to assess the role of microtubule dynamics in centrosome position in neural crest cells. In some cells, centrosome position is determined by the microtubule array itself but in other cell types the actomyosin network is responsible (reviewed in Luxton and Gundersen, 2011). Centrosome position was analysed in EB3GFP-labelled edge cells cultured in taxol or nocodazole. Both taxol and nocodazole decreased the distance between the centroid

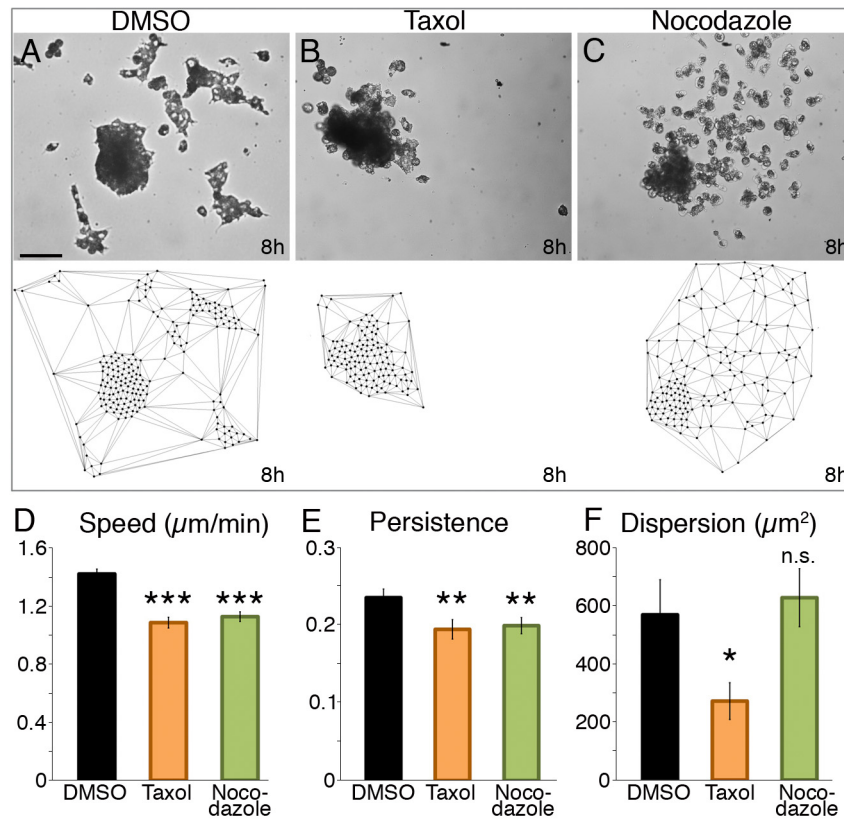


Figure 9.5. Dynamic microtubules are required for neural crest dispersion

(A-C) Single frames from time-lapse movies showing explants cultured in DMSO (A), taxol (B; $0.2 \mu\text{M}$) or nocodazole (C; 65 nM) and the corresponding Delaunay triangulation at 8 hours. Scale bar: $100 \mu\text{m}$. (D) Compared to explants cultured in DMSO ($n=110$ cells), migration speed is reduced in the presence of taxol ($n=100$ cells; $p=1.7043 \cdot 10^{-10}$) and nocodazole ($n=100$ cells; $p=3.1441 \cdot 10^{-9}$). (E) Compared to explants cultured in DMSO ($n=110$ cells), persistence is reduced in the presence of taxol ($n=100$ cells; $p=0.0152$) and nocodazole ($n=100$ cells; $p=0.0197$). (F) Compared to explants cultured in DMSO ($n=6$ explants), explant dispersion after 8 hours is reduced in the presence of taxol ($n=10$ explants; $p=0.0306$). However, nocodazole does not significantly affect neural crest explant dispersion ($n=11$ explants; $p=0.7222$).

and the centrosome (Fig. 9.6A-C,E) but did not affect the position of the centrosome with respect to cell polarity (Fig. 9.6F). In contrast, application of Cytochalasin D, which inhibits actin polymerisation, significantly reduced the distance between the centroid and centrosome (Fig. 9.6D,E) and also abolished the position of the centrosome with respect to the direction of migration (Fig. 9.6F). These results show that both the microtubule and actomyosin arrays are required for the localisation of the centrosome away from the cell centroid, but that dynamic microtubules are dispensable for the polarisation of centrosome position towards the cell-cell contact. The distance between the centroid and the centrosome was also calculated as a proportion of cell length for each condition and compared to control. This gave a similar result (not shown), indicating that this is not simply a consequence of changes in cell shape or elongation.

9. 3. 3. Dynamic microtubules are required for contact inhibition of locomotion

One way in which microtubules could affect neural crest dispersion is through controlling CIL, as this promotes dispersion (Mayor and Carmona-Fontaine, 2010). The effect of taxol and nocodazole on CIL was measured using two different assays (Carmona-Fontaine et al., 2008; Theveneau et al., 2010). First, two neural crest explants confronted with each other do not overlap if they exhibit CIL (Fig. 9.7A), but explants cultured in the presence of taxol or nocodazole widely overlapped, indicating a failure of CIL (Fig. 9.7B-D).

Secondly, collisions between single neural crest cells were analysed. In contrast to neural crest cells cultured in DMSO, velocity vectors of single colliding neural crest cells cultured in taxol or nocodazole were not clustered (Fig. 9.7E-J; Supplementary Movie 11), indicating that CIL is impaired in the presence of these drugs. Together,

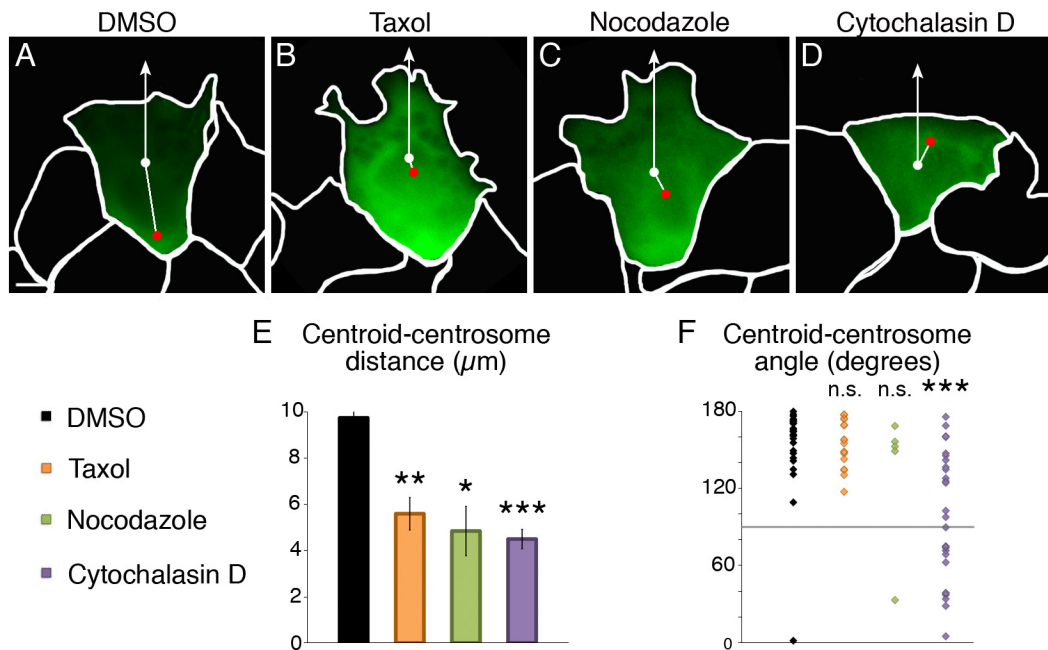
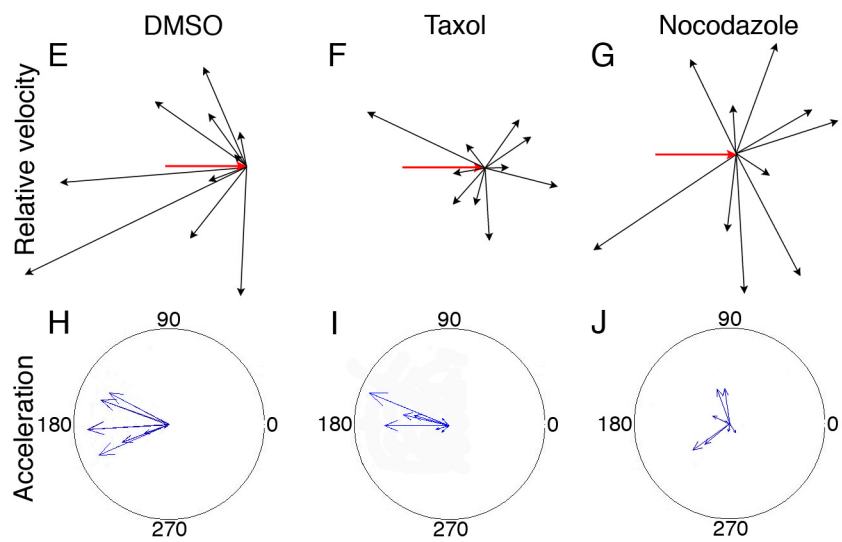
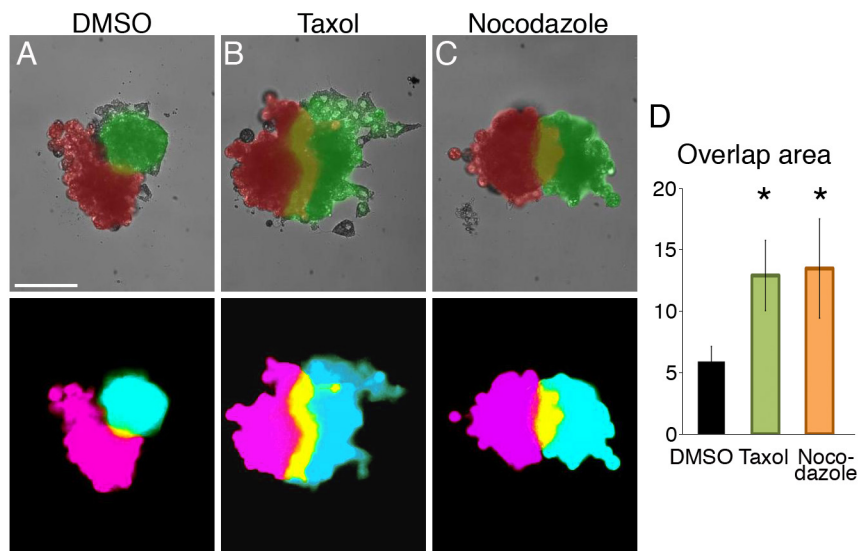


Figure 9.6. Microtubule and actin cytoskeletons are required for centrosome positioning

(A-D) Centrosome position was analysed in EB3GFP-labelled cells at the edge of neural crest explants cultured in DMSO (A), taxol (B; 0.2 μM), nocodazole (C; 65 nM) or cytochalasin D (D; 0.2 μM). Scale bar: 10 μm . Arrow: direction of migration. White dot: cell centroid. Red dot: centrosome. White line: centroid-centrosome distance. (E) Centroid-centrosome distance is greater in cells cultured in DMSO ($n=27$) than in cells cultured in taxol ($n=17$; $p=0.0032$), nocodazole ($n=10$; $p=0.0403$) or cytochalasin D ($n=28$; $p=4.6734 \cdot 10^{-6}$). (F) The angle between the direction of migration, the cell centroid and the centrosome is biased towards 180° (i.e.: towards the cell rear) in cells cultured in DMSO ($n=27$; $p<0.001$), taxol ($n=17$; $p<0.001$) and nocodazole ($n=10$; $p<0.001$) but not cells cultured in cytochalasin D ($n=28$, $p>0.05$). There is no significant difference in centrosome angle between cells cultured in DMSO and taxol ($p=0.9932$) or nocodazole ($p=2388$). However, there is a significant difference in the angle between the direction of migration, centroid and centrosome between cells cultured in DMSO and cytochalasin D ($p=8.8806 \cdot 10^{-6}$).

Figure 9.7. Microtubules and their dynamics are required for contact inhibition of locomotion

(A-D) Explant confrontation assay. (A-C) Single frames from time-lapse movies showing maximum overlap between two explants cultured in DMSO (A), taxol (B; 0.2 μ M) or nocodazole (C; 65 nM). Scale bar: 100 μ m. (D) Overlap area as a percentage of total explant area is greater in explants cultured in taxol (n=13; p=0.0197) or nocodazole (n=12; p=0.0443) than in explants cultured in DMSO (n=12). (E-J) Cell collision assay. Cell position was analysed at 15-minute intervals to measure velocity (E-G) and acceleration (H-J) following collision between cells cultured in DMSO (E,H), taxol (F, I; 0.2 μ M) or nocodazole (G, J; 65 nM). Velocity change was clustered in cells cultured in DMSO (n=10; p<0.001) but was not observed in cells cultured in taxol (n=17; p<0.005) or nocodazole (n=16; p<0.05). Red arrows: initial velocity vector.



these results show that microtubules and, specifically, microtubule dynamics, are required for CIL.

9. 3. 4. The effect of Par3MO on contact inhibition of locomotion can be rescued by nocodazole

Par3 may control CIL by promoting microtubule catastrophe to initiate a change in cell polarity. Indeed, previous results show that microtubule dynamics are required for CIL. To test whether the effect of Par3 on CIL was dependent on microtubule catastrophe, I attempted to rescue the Par3MO phenotype using a low concentration of nocodazole that does not affect cell dispersion when applied to control cells (13 nM; Fig. 9.8). As described above, Par3MO blocks neural crest dispersion (Fig. 9.9A,B,F). Although a low concentration of nocodazole (13 nM) produced a reduction in the motility of Par3MO-injected cells (Fig. 9.9D,E), a significant rescue in cell dispersion was observed (Fig. 9.9F). The explant confrontation assay showed that the increased overlap area induced by Par3MO (Fig. 9.10A,B,D) is reduced by nocodazole treatment (Fig. 9.10C,D), indicating that CIL behaviour is restored. Further, analysis of CIL using single cell collisions showed that the loss of CIL in Par3MO-injected cells (Fig. 9.10E,F,H,I) was rescued by nocodazole treatment (Fig. 9.10G,J; Supplementary Movie 13). This is particularly significant as previous experiments determined that nocodazole disrupts CIL in control cells (see Fig. 9.7, page 171). These results support the notion that Par3 controls CIL by promoting microtubule collapse at the cell-cell contact.

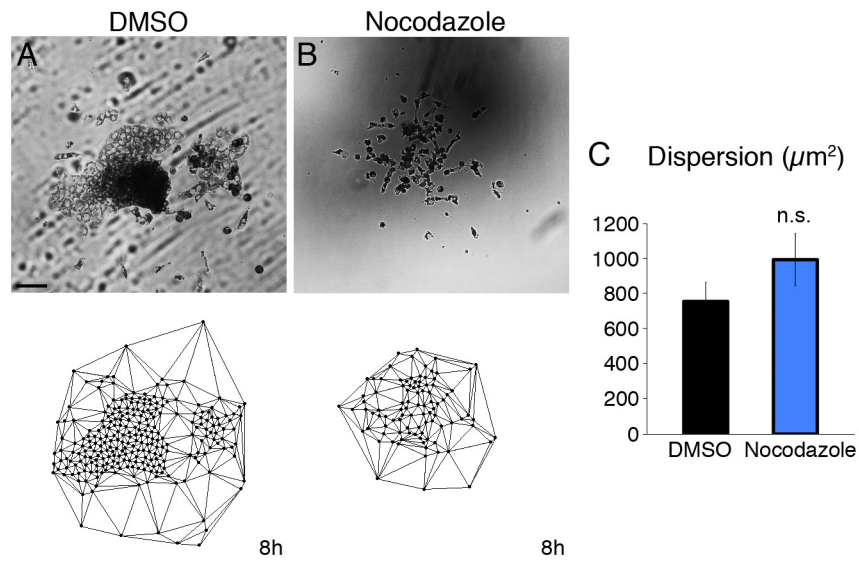
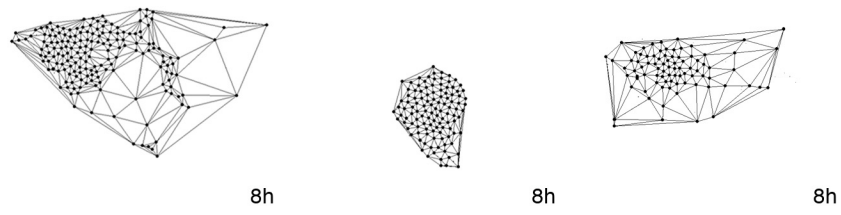
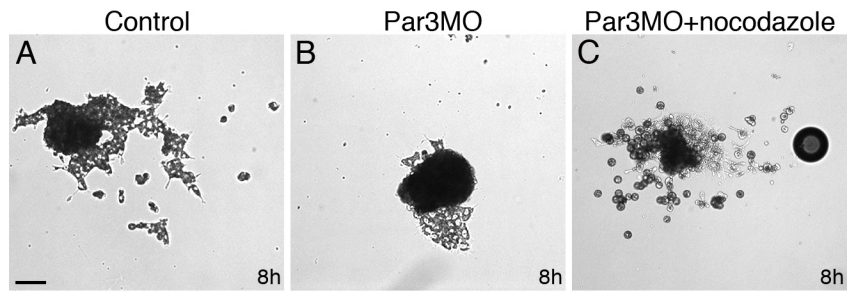


Figure 9.8. A low concentration of nocodazole does not affect explant dispersion

(A, B) Single frames from time-lapse movies showing an explant cultured in DMSO (A) and an explant cultured in a low concentration of nocodazole (B; 13 nM), and corresponding Delaunay triangulation after 8 hours. Scale bar: 100 μm . (C) There is no difference in dispersion of explants after 8 hours of culture between explants cultured in DMSO (n=8 explants) and explants cultured in a low concentration of nocodazole (n=11; p=0.248).

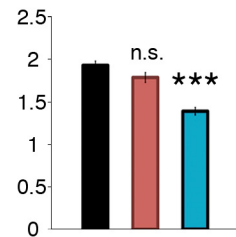
Figure 9.9. Par3MO inhibition of explant dispersion can be rescued by culture in nocodazole

(A-C) Single frames from time-lapse movies showing a control explant (A), Par3MO-injected explant (B), Par3MO-injected explant cultured in nocodazole (C; 13 nM), and corresponding Delaunay triangulation 8 hours after culture. Scale bar: 100 μ m. (D) Speed of cell migration is not affected in Par3MO-injected cells (n=70 for control cells; n=70 for Par3MO-injected cells; p=0.2138) but is reduced in Par3MO-injected cells in nocodazole (n=70; p=3.7508E-11). (E) Persistence is not affected in Par3MO-injected cells (n=60 for control cells; n=70 for Par3MO-injected cells; p=0.3131) but is reduced in Par3MO-injected cells in nocodazole (n=70; p=0.0064). (F) Dispersion between cells increases over time in control explants (n=11 explants; compared to 0 hours: p<0.01 at 6.5 hours, p<0.001 at 7-8hours). There is no change in dispersion of Par3MO-injected explants over time (n=6 explants; compared to 0 hours: p>0.05 at all time points). There is less dispersion between Par3MO-injected cells than control cells at 5-5.5 hours (p<0.05), 6 hours (p<0.01) and 6.5-8 hours (p<0.001). Dispersion between cells increases over time in Par3MO-injected explants in nocodazole (n=7 explants; compared to 0 hours p<0.05 at 6.5-8 hours). There was no difference between control explants and Par3MO-injected explants in nocodazole (p>0.05 at all time points).

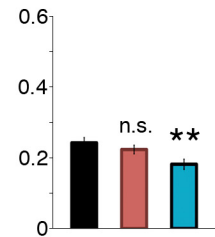


■ Control
 ■ Par3MO
 ■ Par3MO + nocodazole

D Speed ($\mu\text{m}/\text{min}$)



E Persistence



F Dispersion (μm^2)

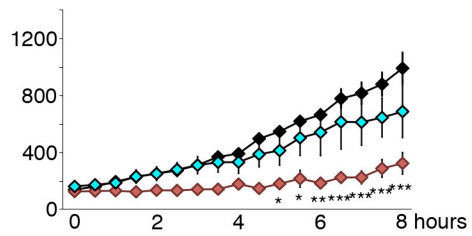
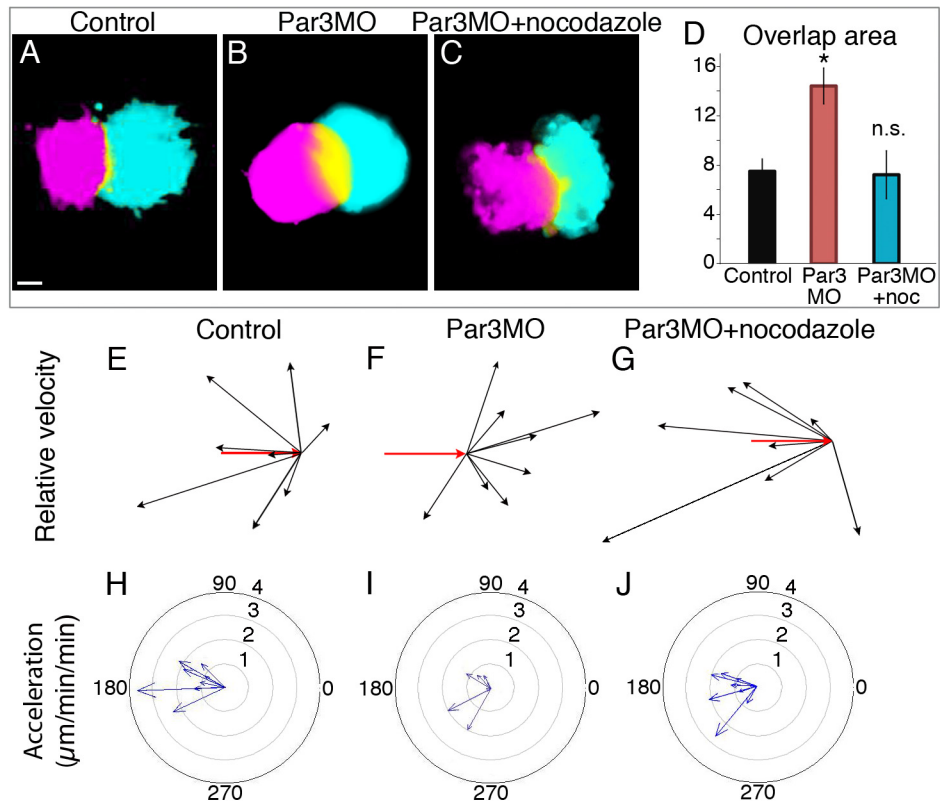


Figure 9.10. Par3MO inhibition of contact inhibition of locomotion can be rescued by culture in nocodazole

(A-D) Explant confrontation assay. (A-C) Single frames from time-lapse movies showing maximum overlap between control explants (A), Par3MO-injected explants (B) and Par3MO-injected explants in nocodazole (C ; 13 nM). Scale bar: 100 μ m. (D) Overlap area as a percentage of total explant area is greater between Par3MO explants (n=16) than between control explants (n=16; $p < 0.05$). There is no difference between control explants and Par3MO explants cultured in nocodazole (n=8; $p > 0.05$). (E-J) Cell collision assay. Cell positions were analysed at 15-minute intervals to measure velocity (E-G) and acceleration (H-J) following collision. Velocity change was clustered in control cells (E; n=22; $p < 0.001$) but no velocity change was observed in Par3MO-injected cells (F; n=30; $p < 0.005$). However, velocity vectors were clustered away from the cell contact in Par3MO-injected cells cultured in nocodazole (G; n=10; $p < 0.001$). Red arrows: initial velocity vector.



10. Par3 promotes contact inhibition of locomotion by controlling Trio

10.1. Rac1 inhibition rescues Par3MO phenotype

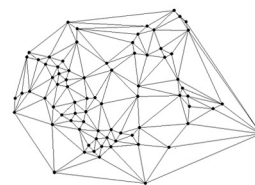
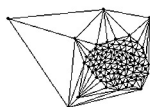
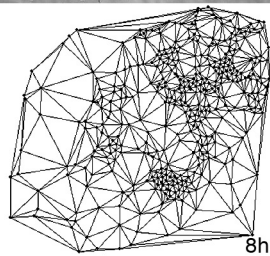
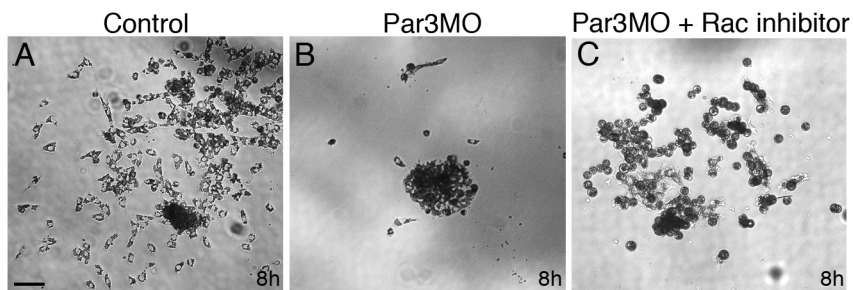
How can Par3 control microtubule dynamics? It is known that Rac1 can influence cell polarity through stabilising microtubules (Pegtel et al., 2007; Wittmann et al., 2003). Thus, inhibition of Rac1 activity is a potential mechanism through which Par3 could affect microtubules. Indeed, a low concentration of the Rac1 inhibitor NSC23766 was able to rescue the inhibition of cell dispersion induced by Par3MO in neural crest explants (Fig. 10.1A-C,F; Supplementary Movie 12), without affecting speed or persistence of migration (Fig. 10.1D,E). This is despite the fact that culture of control cells in the Rac inhibitor led to a decrease in explant dispersion (Fig. 10.2). That these two treatments (Par3 inhibition and Rac inhibition) both reduce explant dispersion alone but lead to dispersion equivalent to control explants supports the idea that these two factors are a part of the same pathway. Importantly, NSC23766 was also able to rescue CIL between Par3MO-injected explants (Fig. 10.3A-D) and Par3MO-injected colliding cells (Fig. 10.3E-J; Supplementary Movie 13).

10.2. Par3 inhibits Trio to promote contact inhibition of locomotion

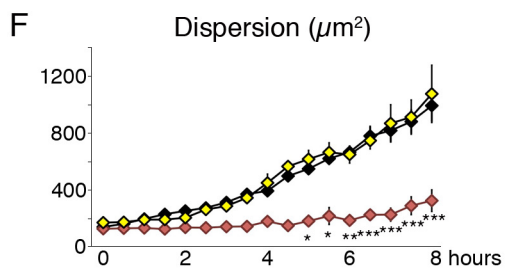
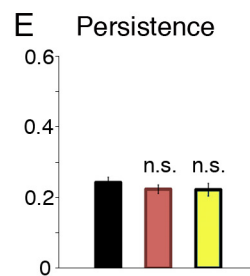
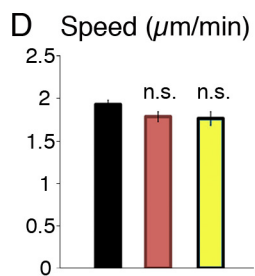
As Rac1 inhibition can rescue CIL in Par3MO-injected neural crest cells, Par3 may inhibit Rac1 during CIL. In other cell types, Par3 interacts with Rac-GEFs to influence Rac1 activity (Pegtel et al., 2007). It has been shown that the Rac-GEF Trio is an important regulator of neural crest migration (Kashef et al., 2009), and is therefore a possible candidate to be regulated by Par3. Indeed, Par3 and Trio

Figure 10.1. Par3MO inhibition of explant dispersion is rescued by culture in a Rac inhibitor

(A-C) Single frames from time-lapse movies showing a control explant (A), Par3MO-injected explant (B), Par3MO-injected explant cultured in Rac inhibitor (C ; 20 μ M) and corresponding Delaunay triangulation 8 hours after culture. Scale bar: 100 μ m. (D) Speed of cell migration is not affected in Par3MO-injected cells (n=40 for control cells; n=50 for Par3MO-injected cells; p=0.1527) or Par3MO-injected cells cultured in Rac inhibitor (n=50; p=0.0660). (E) Persistence is not affected in Par3MO-injected cells (n=40 for control cells; n=40 for Par3MO-injected cells; p=0.2578) or Par3MO-injected cells cultured in Rac inhibitor (n=50; p=0.7696). (F) Dispersion between cells increases over time in control explants (n=11 explants; compared to 0 hours: p<0.01 at 6.5 hours, p<0.001 at 7-8hours). There is no change in dispersion of Par3MO-injected explants over time (n=6 explants; compared to 0 hours: p>0.05 at all time points). There is less dispersion between Par3MO-injected cells than control cells at 5-5.5 hours (p<0.05), 6 hours (p<0.01) and 6.5-8 hours (p<0.001). Dispersion between cells increases over time in Par3MO-injected explants cultured in Rac inhibitor (n=5 explants; compared to 0 hours: p<0.05 at 7.5 hours, p<0.01 at 8 hours). There was no difference between control and Par3MO-injected explants cultured in Rac inhibitor (p>0.05 at all time points).



■ Control
 ■ Par3MO
 ■ Par3MO + Rac inhibitor



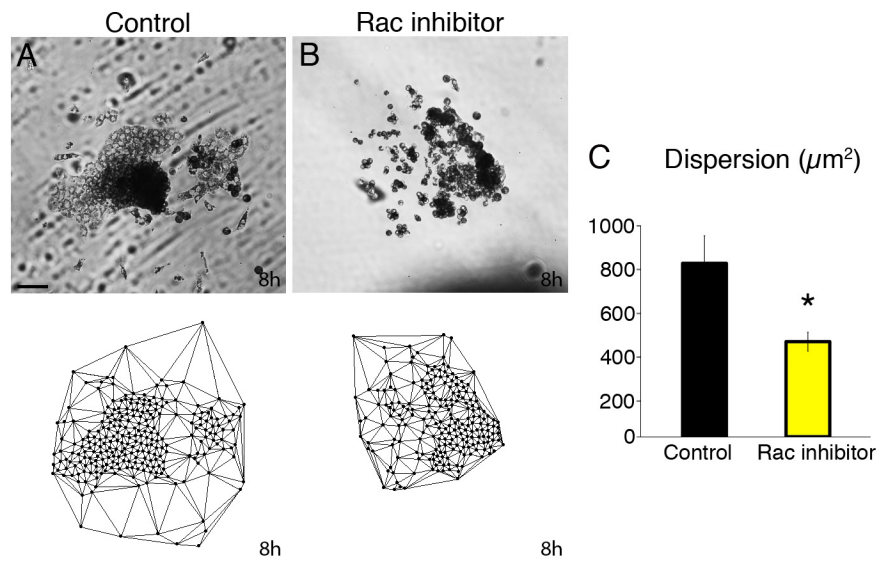
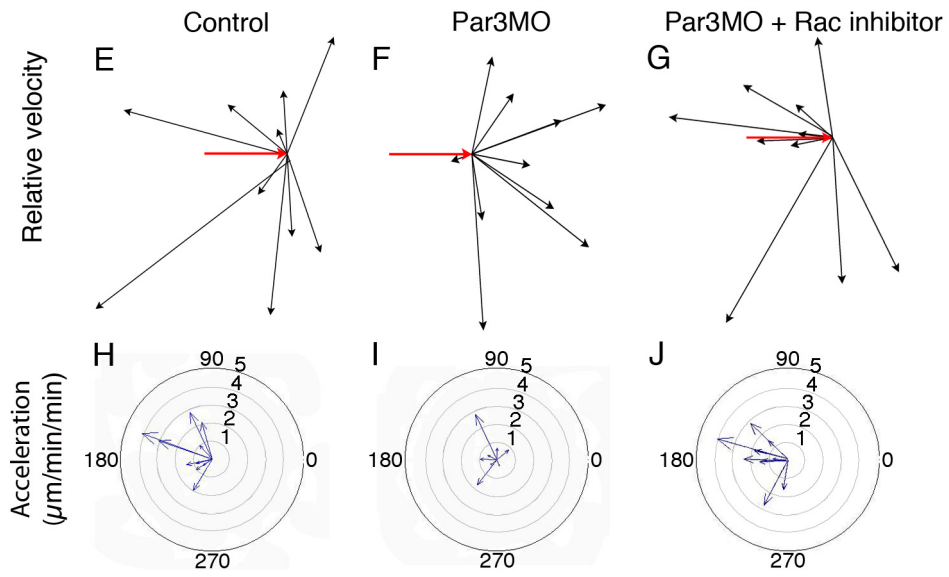
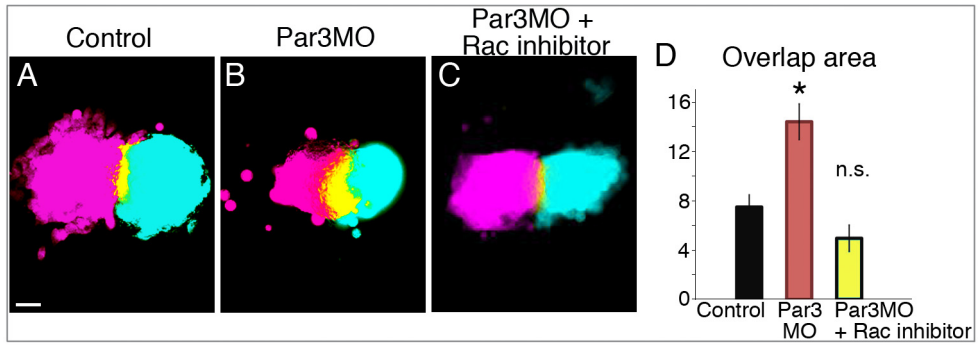


Figure 10.2. Rac inhibition reduces dispersion

(A, B) Single frames from time-lapse movies showing a control explant (A) and an explant cultured in a Rac inhibitor (B; NSC23766; 20 μM), and corresponding Delaunay triangulation after 8 hours. Scale bar: 100 μm. (C) Compared to control explants (n=7 explants), explants cultured in a Rac inhibitor showed reduced dispersion after 8 hours (n=6; p=0.029).

Figure 10.3. Par3 inhibition of contact inhibition of locomotion can be rescued by culture in a Rac inhibitor

(A-D) Explant confrontation assay. (A-C) Single frames from time-lapse movies showing maximum overlap between control explants (A), Par3MO-injected explants (B) and Par3MO-injected explants cultured in Rac inhibitor (C; 20 μ M). Scale bar: 100 μ m. (D) Overlap area as a percentage of total explant area is greater between Par3MO explants than between control explants (n=23; $p < 0.05$). There is no difference between control explants and Par3MO explants cultured in Rac inhibitor (n=8; $p > 0.05$). (E-J) Cell collision assay. Cell positions were analysed at 15-minute intervals to measure velocity (E-G) and acceleration (H-J) following collision. Velocity change was clustered in control cells (E; n=22; $p < 0.001$) but no velocity change was observed in Par3MO-injected cells (F; n=30; $p < 0.005$). However, velocity vectors were clustered away from the cell contact in Par3MO-injected cells cultured in Rac inhibitor (G; n=22; $p < 0.001$). Red arrows: initial velocity vector.



immunoprecipitate together (Fig. 10.4A) and colocalise at neural crest cell contacts (Fig. 10.4B-E).

I next investigated whether inhibiting Trio could rescue phenotypes induced by the Par3MO. An antisense morpholino against Trio was developed (TrioMO) that efficiently reduced GFP fluorescence when coinjected with Trio-GFP (Fig. 10.5). Also, the TrioMO phenotype on neural crest migration could be rescued by human Trio that does not hybridise with TrioMO (Fig. 10.6), showing specificity for the TrioMO. TrioMO was coinjected together with the Par3MO to perform a double loss-of-function experiment. TrioMO alone does not affect explant dispersion (Fig. 10.7) but Trio inhibition rescued the Par3MO phenotype by promoting explant dispersion to ControlMO levels (Fig. 10.8A-C,F; Supplementary Movie 14), without affecting speed or persistence of migration (Fig. 10.8D,E). Coinjection of TrioMO also rescued Par3MO-induced CIL deficiency, as analysed by the change in velocity after single cell collisions (Fig. 10.9; Supplementary Movie 15).

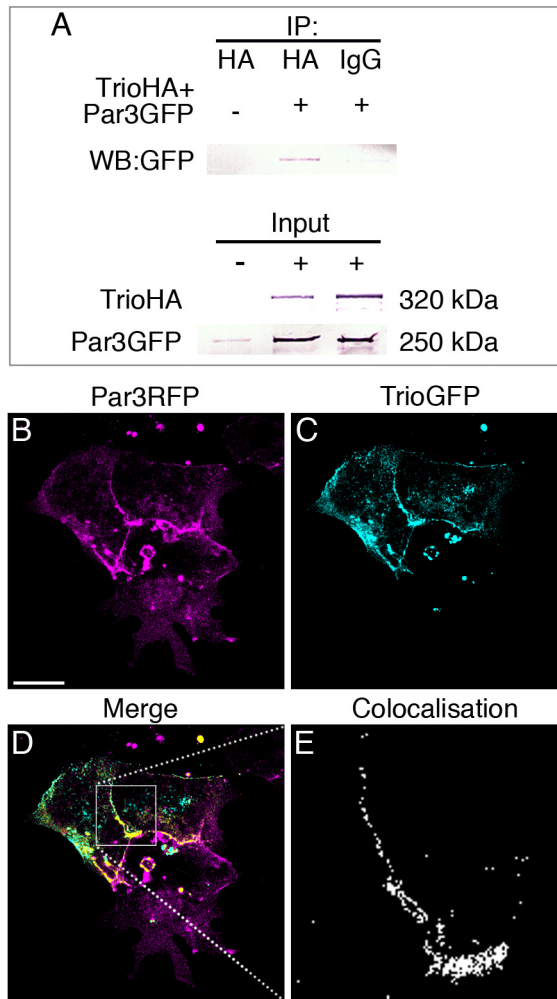
Finally, I analysed whether inhibition of neural crest migration by Par3MO *in vivo* could be rescued by inhibiting Trio. I found that coinjection of *X. laevis* embryos with TrioMO along with Par3MO efficiently restored neural crest migration *in vivo* (Fig. 10.10). TrioMO rescue of the Par3MO phenotype demonstrates that Par3 inhibits Trio in neural crest cells.

10. 3. Par3 inhibits Trio to promote microtubule collapse

As Rac1 has been shown to stabilise microtubules (Wittmann et al., 2003), Trio activation of Rac1 could promote microtubule stability. Thus, Par3 could promote microtubule catastrophe by inhibiting Trio. I analysed microtubule dynamics by

Figure 10.4. Par3 and Trio colocalise at cell-cell contacts

(A) Coimmunoprecipitation of Trio and Par3 was performed using Cos7 cells transfected with TrioHA and Par3GFP. Following pull-down with HA tag, western blotting against HA showed a band at 320 kDa corresponding to TrioHA and against GFP showed a band at 250 kDa corresponding to Par3GFP. Controls using no input or no antibody showed no HA or GFP staining. Input lanes showed that TrioHA and Par3GFP were present and ran to 320 kDa and 250 kDa respectively. (B-E) Par3RFP (B) and TrioGFP (C) are both found at contacts. (D) Merge of Par3RFP (purple) and TrioGFP (blue), which colocalise (yellow) at cell-cell contacts. (E) Magnification and colocalisation mask of area indicated in (D). Scale bar: 20 μ m.



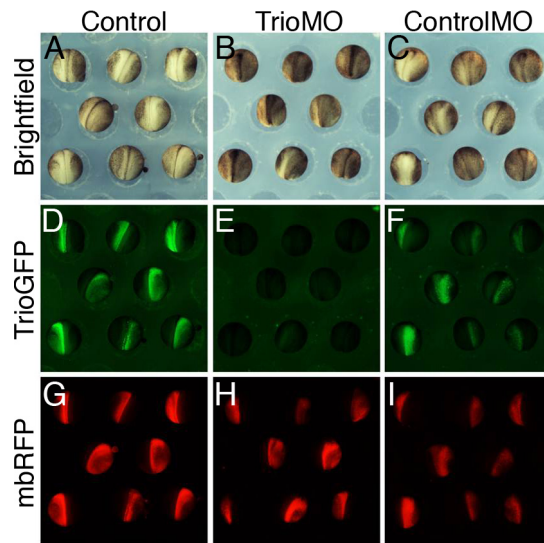


Figure 10.5. TrioMO inhibits TrioGFP in *X. laevis* embryos

Dorsal view of neurula *X. laevis* embryos that had been injected into two unilateral animal blastomeres at the 8-cell stage with TrioGFP and mbRFP alone (A, D, G), with TrioMO, TrioGFP and mbRFP (B, E, H) or with ControlMO, TrioGFP and mbRFP (C, F, I). While both TrioGFP (D) and mbRFP (G) are expressed in control embryos, TrioMO inhibits expression of TrioGFP (E) but not mbRFP (H). In contrast, ControlMO inhibits the expression of neither TrioGFP (F) nor mbRFP (I). Amounts injected per embryo were: TrioGFP 300 pg; mbRFP 300 pg; TrioMO 8 ng; ControlMO 8 ng.

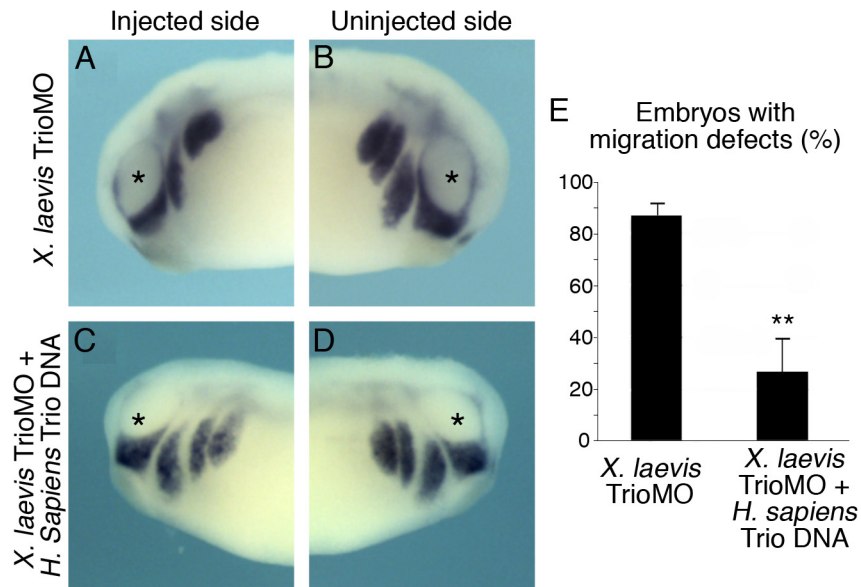


Figure 10.6. TrioMO-induced migration defects can be rescued by coinjection with *H. sapiens* Trio DNA

(A-D) Lateral view of the injected (A) and uninjected (B) sides of embryo injected with TrioMO and the injected (C) and uninjected (D) sides of an embryo injected with TrioMO and Trio DNA processed for *in situ* hybridisation. (E) **Injection of *X. laevis* TrioMO leads to migration defects in the majority of embryos, but the percentage of embryos with migration defects is reduced when *X. laevis* TrioMO is coinjected with *H. sapiens* Trio DNA (p=0.007).**

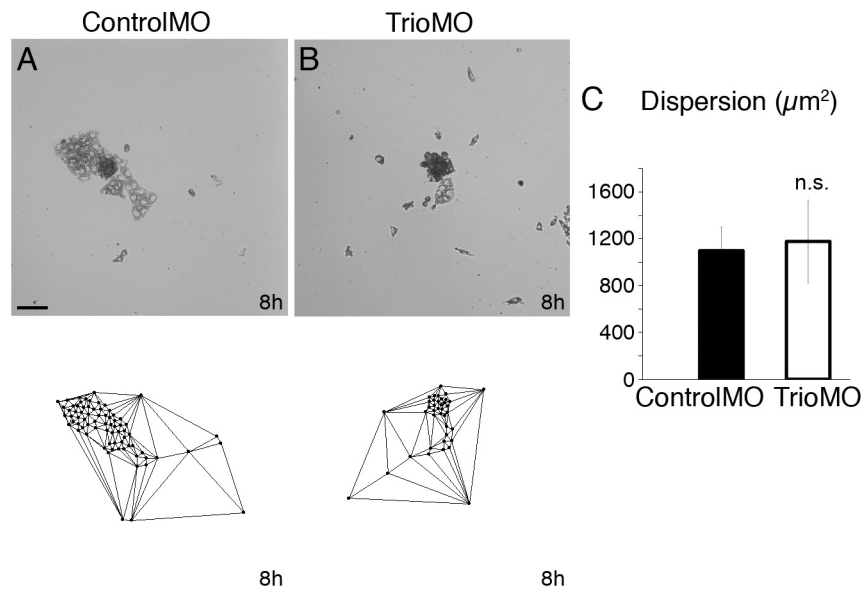
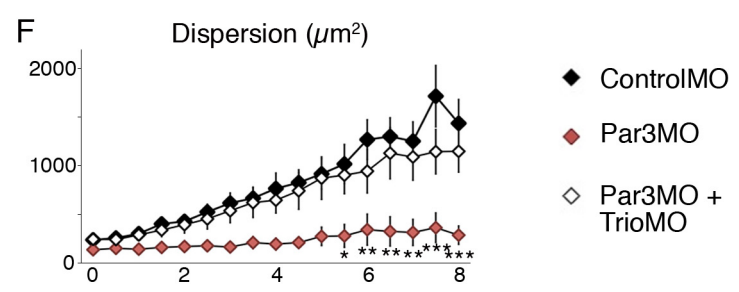
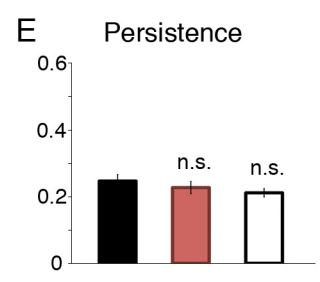
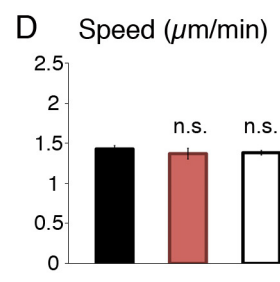
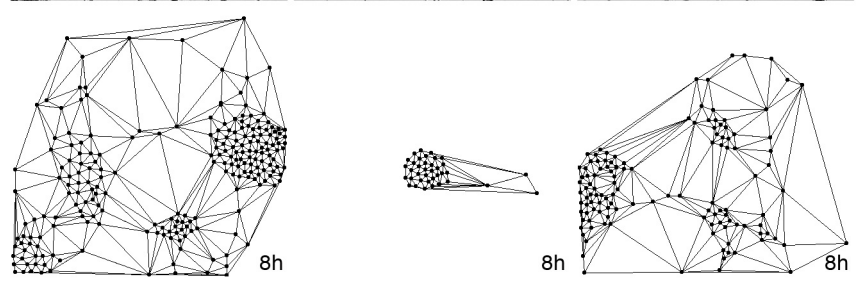
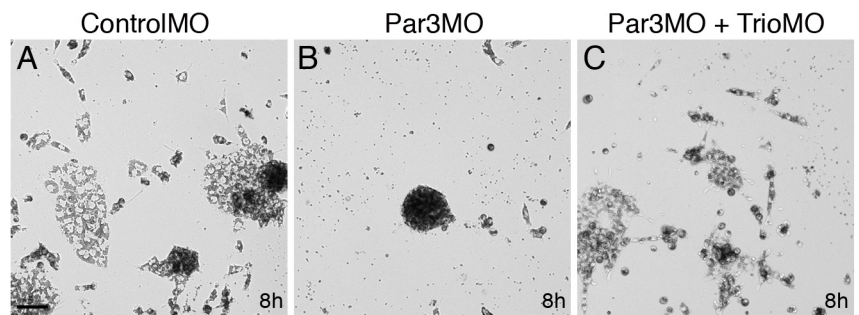


Figure 10.8. TrioMO does not affect explant dispersion

(A, B) Single frames from time-lapse movies showing ControlMO- (A) and TrioMO-injected explants (B), and corresponding Delaunay triangulation after 8 hours. Scale bar: 100 μm . (C) There was no difference in dispersion between ControlMO- (n=10 explants) and TrioMO-injected explants (n=7; $p=0.837$) after 8 hours of culture.

Figure 10.8. Inhibition of Trio rescues dispersion of Par3MO-injected explants

(A-C) Single frames of time-lapse movies showing ControlMO-injected (A), Par3MO-injected (B) and Par3MO+TrioMO-injected (C) explants and corresponding Delaunay triangulation at 8 hours. Scale bar: 100 μm . (D) Speed is not affected in Par3MO-injected cells ($n=70$; $p=0.4642$) or Par3MO+TrioMO-injected cells ($n=90$; $p=0.2713$) compared to ControlMO-injected cells ($n=70$). (E) Persistence is also not affected in Par3MO-injected cells ($n=70$; $p=0.4637$) or Par3MO+TrioMO cells ($n=90$; $p=0.1126$) compared to ControlMO-injected cells ($n=70$). (F) There is less dispersion of Par3MO-injected explants ($n=6$ explants) than control explants ($n=7$ explants) at 5.5 hours ($p<0.05$), 6-7 hours ($p<0.01$) and 7.5-8 hours ($p<0.001$). There was a difference between control and Par3MO+TrioMO explants only at 7.5 hours ($n=12$; $p<0.05$).



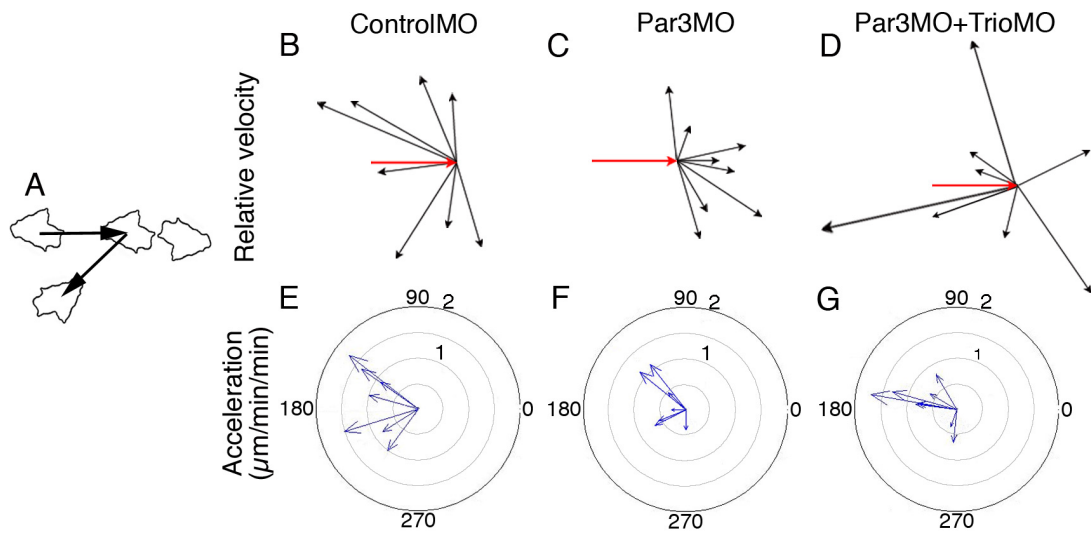


Figure 10.9. Inhibition of Trio rescues contact inhibition of locomotion in Par3MO-injected cells

(A) Schematic representation of cell collision. (B-G). Cells positions were analysed at 15-minute intervals to measure velocity (B-D) and acceleration (E-G) following collision. Velocity change was clustered in ControlMO-injected cells (B; $n=12$; $p<0.001$) but no velocity change was observed in Par3MO-injected cells (C; $n=10$; $p<0.005$). However, change of velocity was clustered away from the collision in Par3MO+TrioMO-injected cells (D; $n=12$; $p<0.05$). Red arrows: initial velocity vector.

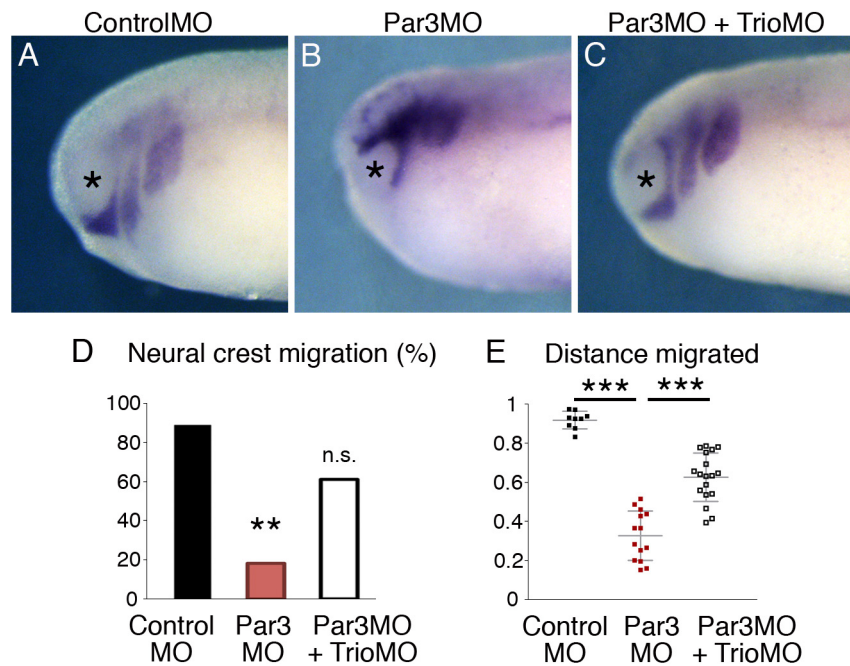


Figure 10.10. Par3MO inhibition of neural crest migration *in vivo* can be rescued by TrioMO

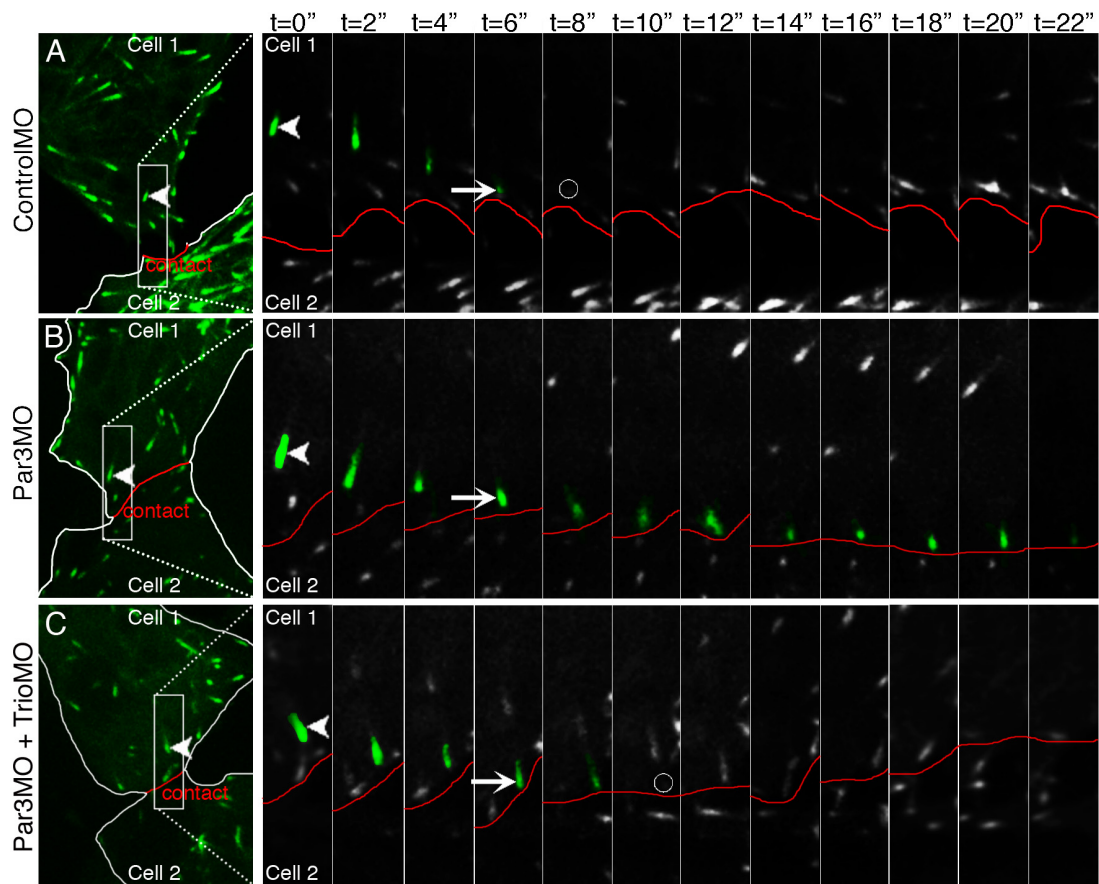
(A-C) Lateral view of ControlMO-injected (A), Par3MO-injected (B) and Par3MO+TrioMO-injected (C) embryos processed for *in situ* hybridisation against *Twist*. Asterisk: eye. (D) Percentage of embryos with normal neural crest migration is reduced from 89% in control embryos (n=9) to 18% in Par3MO-injected embryos (n=13; $p<0.01$) but this is rescued in Par3MO+TrioMO-injected embryos to 61% (n=18; $p<0.05$). (E) The distance migrated by neural crest cells relative to mean migration in ControlMO-injected embryos. Migration is reduced in Par3MO-injected embryos ($p<0.001$) but coinjection of TrioMO with Par3MO increases the distance of neural crest migration ($p<0.001$).

performing time-lapse imaging of EB3GFP-labelled neural crest cells. I found that Trio inhibition increased microtubule catastrophe in Par3MO-injected cells at the cell-cell contact to a level comparable to control cells (Fig. 10.11A-D). These results were supported by plusTipTracker analysis, which showed that inhibition of both Trio and Par3 increased the microtubule catastrophe rate to control levels (Fig. 10.11E). This supports the notion that Par3 is required at contacts between neural crest cells to inhibit Trio-dependent activation of Rac1, thus inhibiting Rac1 promotion of microtubule stabilisation.

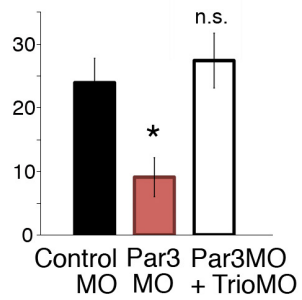
Altogether, these results demonstrate that Par3 controls cell protrusion collapse and reversal of cell polarity during CIL by promoting microtubule catastrophe via an inhibition of Trio/Rac1 activity.

Figure 10.11. TrioMO rescues Par3MO-induced decrease in microtubule catastrophe at cell-cell contacts

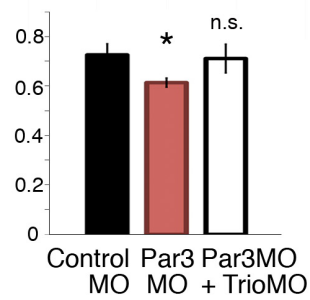
(A-D) EB3GFP-labelled cells were manually analysed for microtubule catastrophe. (A-C) Low magnification images of ControlMO-injected (A), Par3MO-injected (B) and Par3MO+TrioMO-injected (C) cells during collision, along with high magnification consecutive frames of time-lapse movies highlighting one microtubule of interest (green). White box: area of high magnification. Arrowhead: highlighted microtubule. White line: cell outlines. Red line: cell-cell contact. Arrow: microtubule contacts the membrane. Circle: site and time point of microtubule collapse. (D) A greater percentage of microtubules at the cell-cell contact collapse in ControlMO-injected cells (n=5 cells) than in Par3MO-injected cells (n=5 cells; $p=0.026$). This can be rescued by coinjection of Par3MO with TrioMO (n=5; $p=0.307$). (E) Analysis of EB3GFP-labelled cells using plusTipTracker showed that Par3MO-injected cells exhibited a lower catastrophe rate compared to ControlMO-injected cells ($p=0.020$) but that Par3MO+TrioMO-injected cells had an equivalent catastrophe rate to ControlMO-injected cells ($p=0.421$).



D Manual analysis:
microtubule catastrophe (%)



E plusTipTracker:
Catastrophes/ μm



IV Discussion and conclusions

11. Discussion

11.1. Model overview

Effective neural crest migration is dependent on CIL. Here, I show that Par3 is required for neural crest migration because it controls CIL both *in vivo* and *in vitro*. I demonstrate that Par3 controls CIL by inhibiting the Rac-GEF Trio to prevent Trio-mediated activation of Rac1 at cell-cell contacts. I propose the following model for neural crest cell migration (Fig. 11.1). In single migrating cells, microtubule stability in the lamellipodia is promoted by Rac1, which is activated by Trio (Fig. 11.1B). Upon collision with another neural crest cell, Par3 is recruited to the cell-cell contact, sequestering Trio and preventing it from activating Rac1. This local inhibition of Rac1 activity triggers microtubule catastrophe at the cell-cell contact, resulting in the reversal of cell polarity (Fig. 11.1C), formation of a new protrusion and a change in the direction of migration (Fig. 11.1D).

11.2. Par3 at the cell-cell contact

Although Par3 is consistently associated with cell polarity, its localisation varies depending on cell type and situation. As Par3 is routinely localised at cell-cell contacts in epithelial cells and some motile cells such as wound-edge fibroblasts (Izumi et al., 1998; Kuchinke et al., 1998; Schmoranzer et al., 2009), but also found at the leading edge in both wounded epithelial layers and single migrating cells (Du et al., 2010; Pegtel et al., 2007), it was not obvious where Par3 would be found in a collectively

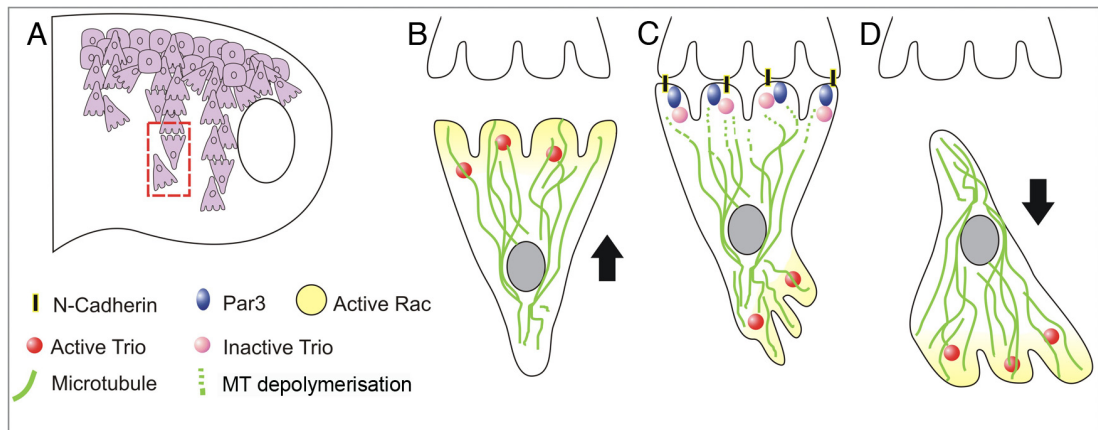


Figure 11.1. Par3 controls contact inhibition of locomotion by inhibiting Trio and thus promoting microtubule catastrophe at cell-cell contacts

(A) CIL is required during neural crest development for directional and collective migration. (B) Trio activates Rac1 in the lamellipodia of migrating cells, promoting microtubule stability. (C) Upon cell-cell contact, Par3 and other proteins such as N-cadherin are localised to the cell-cell contact. Par3 sequestration of Trio works with other pathways activated at the cell-cell contact to inhibit Rac1. This prevents Rac1 stabilisation of microtubules and so promotes microtubule catastrophe. Rac1 activation is biased towards another part of the cell, promoting microtubule stability and lamellipodia extension there. (D) The change in Rho-GTPase activity alters cell polarity. A new lamellipodium is developed and the cell changes its direction of migration to move away from the contact, completing the process of CIL.

migrating cell type such as the neural crest. Here, I show that Par3 also localises to cell-cell contacts between neural crest cells.

11. 2. 1. Par3 and adhesion proteins

I did not directly investigate the colocalisation of Par3 with adhesion proteins such as cadherins or catenins at the cell-cell contact, but Par3 was consistently localised to the contact as is typical for adhesion proteins (for example, Theveneau et al., 2010). In contrast to epithelial cells and to the migrating cells in which Par3 has been found at the cell-cell contact (Izumi et al., 1998; Kuchinke et al., 1998; Schmoranzner et al., 2009), analysis of cell adhesion molecules and cell sorting does not support the notion that Par3 regulates cell adhesion at neural crest cell contacts.

It is true that no individual assay used here to analyse the role of Par3 in neural crest cell adhesion can definitively demonstrate this conclusion. For example, it was possible that Par3 is required to inhibit the localisation or promote the turnover of adhesion proteins at the cell-cell contact in neural crest cells, rather than to define their localisation as has been reported in the cell types discussed above. If this were the case, Par3MO-injection would lead to stronger or more stable cell-cell adhesions, which could explain the lack of dispersion observed in explants taken from Par3MO-injected embryos. Differences in β -catenin or p120-catenin levels between ControlMO- and Par3MO-injected cells may not necessarily be detected by immunostaining, or Par3 may affect a different group of adhesion proteins. Stronger or more stable cell-cell adhesions could also explain the lack of neural crest migration *in vivo* as shown by *in situ* hybridisation. However, if this were the case, one would expect the cell reaggregation assay to show cell sorting between ControlMO- and Par3MO-injected neural crest populations as was observed in the positive control (ControlMO- and N-cadherinMO-injected neural crest populations), which was not

the case. This hypothesis is also not supported by the observed increased overlap of both explants and protrusions following Par3MO injection, which one would expect to be inhibited if cell-cell adhesion was promoted.

As such, although I cannot rule out the possibility that Par3 modulates subtle cell adhesion changes that are undetected by the assays used in this thesis, the combination of results presented in this thesis provides no evidence that Par3 affects cell-cell adhesion in the neural crest. However, this could be investigated further using techniques such as FRAP of adhesion molecules.

Instead, the hypothesis that Par3 is required for CIL fits with the results of each assay. A lack of CIL would not be expected to affect cell reaggregation, which is indeed not affected by Par3MO injection. However, disrupting CIL would be expected to disturb the dispersion of cultured explants and the repolarisation of individual cells following cell-cell collision, as well as to promote the overlap of explants and protrusions between cells, as observed. A lack of CIL can also explain the loss of neural crest migration in embryos following Par3MO injection. Thus, the combination of assays performed to investigate the role of Par3 at the cell-cell contact in neural crest cells clearly demonstrate a role for Par3 in CIL.

It is probable that the requirement for Par3 changes during neural crest development as the neural crest changes from an epithelial to a mesenchymal cell population. Considering that Par3 inhibition had no effect on neural crest induction it seems unlikely that it has an important role in the pre-migratory neural crest; however, it is possible that no effect was observed due to a low level of residual Par3 protein following incomplete knockdown. It would also be interesting to look at the role of Par3 later in neural crest migration, perhaps when the population has dispersed somewhat. It is tempting to speculate that Par3 localisation may change from the cell-

cell contact to the lamellipodia, as has been observed in singly migrating cells (Pegtel et al., 2007), as migration changes from a collective to a looser or more individual mode.

11. 2. 2. Control of Par3 localisation

Even in cells in which it is found at the leading edge, Par3 localisation can be controlled by other adhesion complex molecules (Du et al., 2010). Although Par3 appears not to influence other cell adhesion molecules in the migrating neural crest, my results suggest that cell adhesion molecules such as N-cadherin or cadherin-11 are likely regulators of Par3 localisation or activity at the cell-cell contact. These cadherins are both found at the contact and are required for neural crest migration (Borchers et al., 2001; Kashef et al., 2009; Theveneau et al., 2010). This hypothesis is further supported by the fact that N-cadherin-dependent contacts have been shown to affect microtubule organisation and cell polarity in other cell types including glial cells, endothelial cells and epithelial cells (Camand et al., 2012; Chausovsky et al., 2000; Dupin et al., 2009).

It would be interesting to investigate the effect of N-cadherin or cadherin-11 knockdown on Par3 localisation and activity as well as on microtubule dynamics in neural crest cells.

11. 2. 3. Control of CIL at the cell-cell contact

A number of molecules are required for neural crest migration and are localised to the cell-cell contact. Many have also been identified as being important in CIL. These include Dsh and other members of the Wnt PCP pathway, and N-cadherin and other typical adhesion proteins. These were discussed in detail in the Introduction to this thesis. We can now also add Par3 to this group.

An important question is whether these molecules act together and, if so, in what manner. One common factor in their control of CIL is the convergence on the control of RhoGTPases. Considering the important role of small GTPases in neural crest migration, it is not surprising to find many pathways controlling their activation.

These pathways thus appear to have a similar “output”; that is, they seem to exercise their control on cell polarity and/or CIL in a similar way. Despite this they do not appear to be redundant, in that disturbance of any of one will perturb RhoA and/or Rac1 activity as well as CIL. However, further investigation of the effect of the knockdown of each protein on the others will need to take place before their relationship (or lack thereof) is definitively demonstrated. It would also be interesting to assess microtubule dynamics in this respect. As RhoGTPases can influence microtubule dynamics, it would be surprising if N-cadherin and the PCP pathway did not also affect microtubule dynamics in some way.

There is currently no evidence of any link between N-cadherin and the PCP pathway, suggesting that they may be two parallel pathways. It is possible that N-cadherin plays a role in the localisation of Par3, as discussed above. However, it is important to note that there are differences in the knockdown of N-cadherin, Dsh and Par3, again suggesting that they all have at least some discrete roles. For example, knockdown of either N-cadherin or components of the PCP pathway leads to protrusions that overlap neighbouring cells in all directions, both in the middle and at the edge of the explant (Carmona-Fontaine et al., 2008; Theveneau et al., 2010). In contrast, Par3MO injection seems to lead to the overlap of protrusions over each other (see Fig. 8.8F, page 149), but fewer - or perhaps less obvious - protrusions between cells within an explant (not shown). Considering this difference, I would suggest that these molecules are not entirely dependent on each other. However, it is

certainly possible that, for example, N-cadherin recruits Par3 to the contact where it activates a pathway separate to others that N-cadherin more directly regulates.

Throughout the literature – and, indeed, throughout this thesis - it is generally assumed that all contacts between neural crest cells are equivalent. That is, it is considered that the molecules and pathways set in motion when two individual cells collide is the same as those that are involved in contacts between cells in the middle of a group of cells. This may well be the case; however, whether or not this is true has not actually been determined. The differences in timing of these two examples of cell-cell contact, albeit at two ends of the extreme, suggest that this is a question worth investigating. Considering the potential differences in the phenotype of overlapping cells following knockdown of Par3, Dsh and N-cadherin, Par3 could well differentiate between various “types” of cell-cell contact.

11. 3. Par3 interacts with the RacGEF Trio

Here, I report a novel interaction between Par3 and the Rac-GEF Trio, which can be added to the previously characterised interaction between Par3 and the Rac GEFs Tiam1 and Tiam2 (Mertens et al., 2005; Nakayama et al., 2008). In neural crest cells, Trio interacts with cadherin-11 and β -catenin, which are localised to cell protrusions as well as to cell-cell contacts (Kashef et al., 2009). Trio can act as a GEF for both RhoA and Rac1 (Bellanger et al., 1998). Trio inhibition or Rac1 inhibition compensated for a lack of Par3, suggesting that Par3 inhibits Trio activity at cell-cell contacts during neural crest migration. Rac1 inhibition can also rescue phenotypes induced by Par3 inhibition, suggesting that Par3 reduces Rac1 activity during neural crest migration. This could be confirmed in the future by directly investigating differences in Rac1 activity using FRET.

Interestingly, Par3 is able to activate or inhibit other Rac-GEFs in a context-dependent manner. For example, Par3 appears to sequester Tiam1 at cell-cell contacts in epithelial cells (Chen and Macara, 2005) but to activate Tiam1 at the tip of axons (Nishimura et al., 2005). As such, it will be interesting to investigate whether Par3 activates rather than inhibits Trio in other situations. This could be another mechanism by which the role of Par3 could change during development, from the epithelial pre-migratory neural crest to the collectively migrating group and finally to the more loosely migrating and differentiating cells.

11. 3. 1. A potential interaction with RhoA/ROCK

RhoA is active at cell-cell contacts between neural crest cells (Carmona-Fontaine et al., 2008; Matthews et al., 2008), and ROCK phosphorylation of Par3 has been shown to disrupt Par3-aPKC-Par6 complex formation (Nakayama et al., 2008). However, if ROCK disrupted Par3 activity in the neural crest, one would not expect Par3 to have an effect at the cell-cell contact as has been shown in this thesis. Results presented here also show that aPKC is not required for Par3 activity in neural crest migration, suggesting that Par3 does not act as a component of the Par3-aPKC-Par6 complex and thus making it even more unlikely that ROCK is affecting Par3 by disrupting complex integrity in this context.

Interestingly, ROCK phosphorylation of Par3 does not affect the actual interaction between Par3 and Tiam1/2; rather, it disrupts the ability of the Par3-aPKC-Par6 complex from activating Rac1 through these GEFs (Nakayama et al., 2008). As such, ROCK phosphorylation of Par3 and so inhibition of Par complex formation may be a pathway through which RhoA inhibits Rac1 in situations where Par3 has been shown to activate Rac1 (Nakayama et al., 2008). ROCK can also phosphorylate Tiam2, but this does not affect the GEF activity of Tiam2 and also does not interfere with Par3-Tiam2 interaction (Takefuji et al., 2007). This may have more relevance for the

neural crest: ROCK could theoretically disrupt Par3-aPKC-Par6 complex formation to simultaneously inhibit Rac1 activation and promote independence of Par3, thus allowing Par3 to sequester Trio and so further inhibit Rac1 at the cell-cell contact.

11. 4. Par3 and the microtubule array

Par3 has been shown to influence cell polarity and migration by controlling centrosome position (Schmoranzner et al., 2009). In this situation, Par3 and dynein appear to tether microtubules to the cortex to pull the centrosome into place. In contrast, I find that Par3 is not required for appropriate positioning of the centrosome and that inhibiting dynein has no effect on neural crest migration. Instead, actin filaments are required to polarise the centrosome towards the cell-cell contact and both the microtubule and actin arrays are required to pull the centrosome away from the cell centroid. Along with a more precise description of the mechanism behind centrosome polarisation, the role of rearward centrosome positioning in migratory neural crest cells is a possible future avenue of research. Indeed, the function of rearward orientation of the centrosome has not been explored thoroughly, as many migrating cells orient their centrosome anteriorly (Luxton and Gundersen, 2011). Fibroblasts change centrosome orientation depending on the substrate (Schutze et al., 1991), so it would be interesting to assess whether centrosome position is altered in neural crest cells *in vivo* compared to *in vitro*. Indeed, considering the varying roles of microtubules and actin filaments in migration on different substrates, it may also be that centrosome position is not even controlled by the cytoskeleton in the same way *in vivo* as *in vitro*.

The requirement of a correctly polarised microtubule array for CIL has been demonstrated previously (Stramer et al., 2010), and microtubule stabilisation at the

cell-cell contact has been shown to inhibit CIL (Kadir et al., 2011). Par3 stabilises microtubules and promotes directional migration at the leading edge of single migrating cells through Rac1 activation (Pegtel et al., 2007). However, illustrating the context-dependent flexibility of Par3 interactions and roles, the model proposed in this thesis predicts that Par3 inhibits Rac1 activation at the cell-cell contact to promote microtubule catastrophe. Localised microtubule catastrophe leads to an asymmetry in stability across the microtubule array. Works by others show that this can result in a change in cellular polarity as well as migration of the cell away from the cell-cell contact as part of a CIL response both *in vivo* and *in vitro* (Kadir et al., 2011; Stramer et al., 2005). My results suggest Par3 as a likely candidate to link the cell-cell contact signal to the microtubule catastrophe response in this process.

11. 4. 1. Control of the microtubule array over cell polarity

It is important to note that, in this model, Par3 simply induces a change in direction rather than dictating the direction in which the new protrusion should be extended. As such, it is interesting to note that microtubule depolymerisation, Rac1 inhibition or Trio inhibition - all of which presumably affect the entire cell - are able to rescue the effect of Par3 inhibition. Work presented in this thesis also illustrates that microtubule dynamics are important for neural crest cell migration and essential for CIL. It may be that these results, showing a defect in CIL following neural crest culture in taxol and high concentrations of nocodazole, are partly due to the lack of asymmetry across the microtubule array. I suggest that microtubule depolymerisation, Rac1 inhibition or Trio inhibition were able to rescue Par3 deficiency because only low levels of inhibition were used, allowing the redefinition of some aspects of this asymmetry. It may be that residual Par3 expression or other, uninterrupted pathways were able to provide a basis for the reassertion of asymmetry.

If asymmetry were the key to regulating cell polarity as hypothesised, one would also expect the overexpression of Par3 to affect CIL. No phenotype was observed following exogenous expression of Par3GFP or Par3RFP in this thesis; however, this technique was used to assess protein localisation rather than to undertake a gain-of-function experiment. Injection of higher concentrations of either of these constructs could be used to address this question.

My results could also support a hypothesis put forward by Kadir and colleagues (2011) that increasing microtubule dynamics (including increasing microtubule catastrophe) at the cell-cell contact beyond a certain threshold is sufficient to induce CIL. In order to determine between these two possibilities (asymmetry of microtubule dynamics versus a threshold of microtubule dynamics), one would need to assess whether CIL occurs with a variety of combinations of microtubule dynamics at the front and back of the cell (or cell-cell contact and free edge). If crossing a threshold of microtubule dynamics at the cell-cell contact is sufficient to induce CIL, CIL should be possible irrelevant as to the microtubule dynamics in the other half of the cell. Conversely, CIL should not occur if this threshold is not reached. Alternatively, if asymmetry is required, a cell may be able to undergo CIL even if microtubule dynamics are very low in both halves of the cell, as long as it is higher at the site of cell-cell contact. This question is highly relevant for the topic of this thesis and for the investigation of cellular migration. The development of further tools to control microtubule dynamics will help to address this question.

Interestingly, microtubule polymerisation has been proposed to promote Rac1 activity (Best et al., 1996; Waterman-Storer et al., 1999) and depolymerisation to activate RhoA (Bershadsky et al., 1996; Enomoto, 1996). This may help maintain directionality once polarity has been established and further underscores the importance of controlling microtubule dynamics in directional cell migration.

12. Conclusions

Although cell-cell adhesion proteins are essential for epithelial cell polarity and thus tissue integrity, the role of polarity proteins such as Par3 in collectively migrating mesenchymal cells has not been closely investigated before now. In this thesis, I demonstrate that Par3 is located to the cell-cell contact during CIL of neural crest cells. I report a novel interaction between Par3 and the Rac-GEF Trio and show that Par3 sequesters Trio at the cell-cell contact, illustrating a new pathway for Rac1 inhibition. This work also characterises the microtubule array and its dynamics in neural crest cells during CIL and demonstrates that Par3 inhibition of Trio at the cell-cell contact promotes microtubule catastrophe. This is required for CIL between neural crest cells and as such is essential for collective and directional neural crest migration.

Further work is required to tease out the relationships between the various molecular pathways involved in CIL between neural crest cells and their distinct roles, if any. Future studies should also provide further information regarding the intricacies of microtubule dynamics in cell polarity, particularly during a change of direction. As well as contributing to the answering of these questions, this work also leads to a deeper understanding of neural crest migration, of the potential mechanisms behind neurocristopathies and of the migration of other cellular collectives during development and disease.

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